RESEARCH Summary

Pro00078468

NCT03089047

PI: Ian Welsby

1. Protocol Title: A Pilot Study on the Impact of Rejuvenated Autologous Blood Transfusion on VO_2max in Healthy Subjects

2. Purpose of the Study: To compare the physiologic benefits of rejuvenated RBCs (rejRBCs) to standards RBCs (PRBCs), we will emulate critical conditions by safely maximizing stress on the cardiovascular system, in an elective, feasible volunteer study. Maximal oxygen uptake (VO_2max) will be measured in an anemic, post-donation, pre-transfusion state, and also after transfusing 14-day-old, autologous blood randomized to standard storage or standard storage with rejuvenation. We hypothesize that transfusion of standard PRBCs is less effective at increasing oxygen delivery (measured by VO_2max) compared with transfusion of rejRBCs*.*

3. Background & Significance: Transfusion is one of the most commonly performed medical procedures, designed to improve oxygen delivery to tissues throughout the body, yet there are few data to support the efficacy of this therapy in terms of its proposed purpose – oxygen delivery.

Previous studies have shown that a reduction in arterial oxygen content, by reducing hemoglobin, results in reproducible decreases in individuals' maximal oxygen uptake or consumption (VO2 max),[1] a measure that reflects the body's capacity to both deliver and utilize oxygen. While an increase in VO2 max has been demonstrated after transfusing autologous, cryopreserved, RBCs, these data cannot be extrapolated to the standard, liquid stored RBCs used clinically[2-6] and previous testing of liquid stored units did not demonstrate a benefit. The most likely explanation for poor oxygen delivery with transfused RBCs is the lowering of p50 (increased affinity for oxygen with poor off-loading in the tissues) related to the loss of 2,3 DPG that happens over the first 7-10 days of storage.[7]

This proposal addresses two fundamental questions: does standard RBC transfusion even improve oxygen delivery and can rejuvenated RBCs (with a high/normal p50) maximize the oxygen delivery capacity of transfused RBCs. Oxygen delivery is considered to be dependent upon hemoglobin concentration, cardiac output and hemoglobin saturation. Transfusion of blood with a lower than normal p50 (such as stored RBCs) is expected to be less effective at delivering oxygen as supported by tissue pO2 (tpO2) being dependent on modulating p50 in an animal exchange transfusion model.[8] Modulation of p50 is possible in animal models and increases exercise capacity in mice with heart failure[9] and ischemic hearts,[10,11] consistent with a physiological benefit to increased O2 off-loading in the tissues. One unit of optimized, rejuvenated RBCs (rejRBCs), could be as valuable as multiple units of standard RBCs, especially for patients unable to further compensate for reduced oxygen delivery with increased cardiac output, such as the burgeoning heart failure population. Decreasing RBC exposures, costs, and adverse effects in high-risk populations could improve patient safety and revolutionize transfusion medicine. Evaluating volunteers stressed by the demands of exercise is an essential, proof-of-principle, pilot step. We will test the hypothesis that: Repurposing an FDA approved rejuvenation process to increase the p50 of stored RBCs and increase their ability to off-load oxygen at the tissue level, will translate into better oxygen delivery, as measured by exercise capacity (VO2max).

Red blood cells are rejuvenated according to the procedure in the labeling of the Rejuvesol product (FDA approved).

4. Design & Procedures:

Study type: Prospective randomized "healthy volunteer" study

Site(s):
1) Duke University where consenting, screening, exercise testing, and transfusion of RBCs will take place.

2) The Blood Connection (TBC) is located in Piedmont, SC with a satellite in Raleigh, NC and was established in 1962. The Blood Connection is registered and licensed by the US FDA and accredited by the American Association of Blood Banks. TBC has two blood mobiles, which travel throughout the Triangle and one fixed site where blood is collected and products are produced. Blood collection is performed by single whole blood donation and automated apheresis for the collection of red cell, plasma and platelets. TBC (Raleigh) processes approximately 18,000 donations per year, manufactures red blood cell products, frozen plasma products, and platelet products; in North Carolina TBC is the primary supplier to Wake Med and Rex Hospitals in Raleigh. They also supplyDuke University Medical Center, as inventory and need allows, especially for autologous units. The VP Business Development/Chief Technical Officer of TBC (Tracy Bridges) and the medical director (Robert Rainer,MD) has agreed to allow the blood collections and processing for this study to take place at TBC using their standard procedures for autologous blood collection by apheresis. A fee is being charged to Duke for these services as if this were a standard autologous donation.

Study size:
In this pilot study, we will plan to enroll 20 subjects.

Eligibility Criteria:

Healthy, habitually exercising, non-smoking, non-diabetic males and females (as described in the inclusion/exclusion criteria below):

Qualified volunteers will be enrolled at a single center (Duke University Medical Center). After subjects provide written informed consent, eligibility will be confirmed with 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 (the American College of Sports Medicine Guidelines for Exercise Testing defines this age group with no or no more than one 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 (< 30 mL/kg/min) will be excluded (screen failure). Based on our

previous experience, we anticipate that up to 25% of the subjects will fall into this category; therefore, 20 subjects will be screened to obtain 10 participants who go on to donate their blood.

iv. Calculated total blood volume of at least 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]

v. Has access to transportation to visit the blood collection facility and to return to Duke for all study visits
vi. Weighs at least 130 pounds

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
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
   c. Plasma donation by apheresis within the past 4 weeks
   d. Any self-reported blood donation within a year
xii. Inadequate red blood cell mass evidenced by total blood volume <4500 mL (above) or screening hemoglobin <13.3 g/dL
xiii. Known hypersensitivity to lithium compounds. And known taking lithium salts for treatment of other medical/psychiataric conditions

xiv. Students and employees who are under direct supervision of the investigators

Study structure: study visits (A-E).

A	Screen	Screen, consent, H&P, ECG, screening labs, and VO_2max
B	Day 0	Donate 2 units of PRBCs by autologous apheresis
C	Day 14	8am Measure pre-transfusion, "anemic" VO_2max
		9 am Transfuse 2 units of standard or rejuvenated PRBCs
		3 pm Measure post-transfusion VO_2max (end of study)

listed in this study.

VISIT A (– Screening) Written informed consent will be obtained from subjects who respond to an IRB-approved advertisement/flyer and fulfill eligibility requirements. The following video will help explain the study: vimeo.com/167291799. Individuals who respond to an IRB approved advertisement/flyer (uploaded to eIRB) will be given information about the study and asked to read an IRB approved consent form. An investigator or designee will obtain written informed consent from each individual participating in this study, after adequate explanation of the methods, objectives, and potential hazards of the study. The investigator or designee will also explain to the subjects that they are completely free to refuse to enter the study and free to withdraw from it at any time. A copy of the signed consent form will be given to the subject and will be shared with The Blood Connection to ensure proper labeling of autologous units.

After subjects have provided written informed consent, they will be asked to answer several questions in 2 written questionnaires (uploaded to eIRB) in order to confirm that they are eligible to donate blood. These questions are similar to those routinely asked to people prior to a blood donation. These are focused upon keeping the risks of blood donation to the lowest possible level. Subject will undergo screening lab tests for baseline hemoglobin, sickle cell trait, serum pregnancy tests only for women of child-bearing potential, ECG, exercise test. Subject will receive a phone confirmation from the investigator or delegated research staff after review of the screening lab results. Subject will bring a copy of the signed consent form to The Blood Connection if qualified.

VISIT B (Day 0 – Blood donation) The Blood Connection (TBC), Raleigh, NC, is registered and licensed by the US FDA and accredited by the American Association of Blood Banks (AABB) to manufacture RBCs and other blood products. Two units of blood will be withdrawn from each subject according to standards for double, AS-3, apheresis units, and will be marked for autologous donation, and purchased by Duke Transfusion Services, as arranged with the medical director of TBC (Robert Rainer, MD). RBC handling and storage will meet FDA and AABB standards; units will be stored with Duke Transfusion Services c/o Dr. Poisson.

VISIT C (Day 14 – "Anemic" VO2 max, Transfusion (Tx) and "post-Tx" VO_2max))
Small amount of blood [3 lavender topped tubes] will be collected from subject to test for hemoglobin, type & screen and 1 cross match to verify the donated blood received from

The Blood Connection belongs to the same subject who arrives at the appointed date at Duke facility

Anemic VO2max

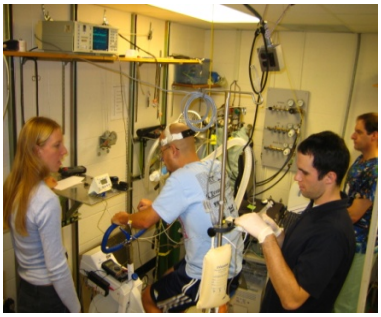
Three minutes of resting baseline measures will be recorded before the start of exercise. The subject will then begin to pedal the cycle ergometer at a cadence of 75 rpm. Resistance will be manually set according to a standardized, progressive protocol. A rating of perceived exertion (RPE) will be obtained at the end of each exercise stage. Vital signs will be recorded every 3 minutes, and ECG monitoring will be continuous. Exercise will be terminated when the subject reaches volitional fatigue. VO_2max will be reported as the highest oxygen consumption averaged over two 30-second periods, which typically occurs in the last stage of the progressive maximal exercise test. Maximum heart rate will be the heart rate at or near VO_2max. To minimize variability in VO_2max testing procedures, the same 2 facilitators (an engineer/technician and a research staff) will be present at every test. Radial arterial access will be obtained for cardiac output monitoring (LiDCO pulse contour analysis) at this visit.

Tx and post-Tx VO2max

Duke Transfusion Services will randomize each subject to receive 2 units of standard PRBCs or rejRBCs over 120 minutes using a standard infusion pump. All units will be washed to equalize the transfusion volumes; rejuvenation includes a mandatory washing step. Rejuvenation of donated blood will be done according to the procedures in the labeling of the Rejuvesol product. Vital signs will be monitored and recorded every 15 minutes. After a total 4-hour rest after completing the first VO_2max testing, and a light snack, the exercise test will be repeated.

Laboratory testing: The p50 will be measured in one of the blood units transfused and in the subject before the exercise test. Radial arterial access will be obtained for serial cardiac output monitoring (LiDCO Pulse Contour Analysis, LiDCO Products, London, UK), arterial blood gas analysis and lactate measurement before and after transfusion, and after exercise testing. Final blood collected for a hemoglobin level will be done at the conclusion of blood transfusion. This is the final visit and participation is completed.

5. Study Interventions:



Detailed Description of VO_2 max Testing

We have significant experience performing VO_2 max testing (e.g. DUHS IRB Pro00011747). 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 the Center for Hyperbaric Medicine and Environmental Physiology, which is part of Duke University Medical Center. Dr. Moon (Co-Investigator) is medical director of this center,

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The photo (right) shows a VO_2 max test in progress in our laboratory.

Each subject will be instrumented with a three-lead ECG (Spacelabs, Model 511) to monitor cardiac rhythms and a pulse oximeter (Ohmeda, model 3740) 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 (Consentius Technologies, ParvoMedics TrueMax 2400). Data will be recorded every 30 seconds as 30-second averages. Subjects will wear a nose-clip and breathe air through a two-way non-rebreathing valve (Hans Rudolf, model 2700) 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 (Monark, model 818E). 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 and recorded on a computer (Macintosh Quadra/MacLab data acquisition).

Three minutes of initial resting baseline measures will be collected prior to 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 (above). A rating of perceived exertion (RPE) is obtained at the end of each exercise stage. The VO_2 max test is terminated when the subject reaches volitional fatigue, or achieves one of the other test termination criteria as defined in the Table above.

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

VO_2 max is determined as the highest oxygen consumption averaged over two 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.

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 (ie, 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.

To minimize variability in VO_2 max testing procedures, whenever possible, the same 2 facilitators will be present at every test (Michael Natoli - engineer/technician for VO_2 max equipment; a nurse Research Coordinator who will collect data and coach each subject).

Additional Measurements During VO_2 max Testing
On the Day 14 visit for VO_2 max testing , the following additional study procedures will be performed:

- A radial arterial catheter will be inserted under local anesthetic prior to exercise testing, consistent with previous studies on healthy volunteers done in our laboratory.
- Arterial blood samples (6 mL each) will be obtained from the radial arterial catheter immediately before exercise, twice during exercise, and after exercise completion (total 24 mL). Blood samples will be analyzed for arterial lactate, PO_2, PCO_2, and pH. We know that arterial lactate increases during VO_2 max testing, and we will conduct exploratory analyses to determine whether there are differences in peak lactate between study arms (Standard vs Rejuvenated RBCs).
- Cardiac output, cardiac index, and stroke volume will be determined during VO_2 max testing using the validated and FDA-approved LiDCO monitor, which includes a lithium dilution determination of cardiac output, with subsequent measurements from radial arterial pulse contour analysis. These data will be used to confirm that cardiac output is similar for subjects in the two study arms, ie, no difference in VO_2 max is attributable to differences in cardiac output between study arms. We do not expect to see any differences in cardiac output between groups since the study arms will receive an identical volume of fluid (2 RBC units) over the 2-hour transfusion period.

Detailed Description of Rejuvenation Process: Rejuvenation refers to the process of adding a mix of solutes (Rejuvesol®, Citra Labs, Braintree, MA; consists of sodium pyruvate, inosine, adenine, mono- and dibasic sodium phosphate) to older, stored (i.e., 2-3 DPG-depleted) blood to immediately restore 2,3-DPG and ATP levels in the stored red blood cells[15]. Rejuvenation was originally developed to prolong the storage life of rare-phenotype RBC units. It is FDA-approved for use in RBC units stored in CPD, CPDA-1, and AS-1. The major contraindication for the use of Rejuvesol® is in RBC units stored for fewer than 6 days due to high baseline 2,3-DPG and ATP levels.[16] Rejuvenated blood should not be used in patients with PaO_2 < 40 mmHg due to the impaired pulmonary oxygen loading at low arterial oxygen tensions seen with high-p50 hemoglobin; we will appropriately exclude these patients at the first screening visit.

Rejuvenated RBC units will be prepared according to manufacturer's protocols, which include a mandatory washing step on an approved cell-washing device.[16] The manufacturer reports no adverse effects resulting from use of Rejuvesol® per manufacturer's instructions, and further notes that the risk of toxicity due to Rejuvesol® solution components is low since all solution components are common endogenous chemicals that are normally metabolized *in vivo*.[16]

6. Risk/Benefit Assessment:

1) Risks associated with venipuncture: Venipuncture results in momentary discomfort. Infection, excess bleeding, clotting, or fainting is also possible, although unlikely. Phlebotomy performed for blood donation has complications including hematomas, nerve injury, thrombophlebitis, arterial puncture, and compartment syndrome. Excluding the blood collected at The Blood Connection an additional 110 ml of blood will be withdrawn from the subject over the screening period through Study Day 14.

2) Risks associated with <u>blood donation</u>[5]: 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 citrate toxicity (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 TBC procedures, all volunteers will be offered some food and liquid after donation and will be encouraged to sit for 30 minutes after donating. Additionally, fewer donor adverse events are seen with double red cell collection due to the saline blood volume replacement they receive. In addition, consistent with The Blood Connection normal routine procedures, blood will only be collected from subjects with a hemoglobin of at least 12.5 g/dL.

3) <u>Decreased hemoglobin level</u>: Volunteers should not exhibit significant anemia during the study since a hemoglobin of at least 12.5 g/dL is required at The Blood Connection prior to donation of blood. 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 and have decreased exercise performance until their hemoglobin level is increased on Day 14(Study visit C).

4) Risks of <u>blood transfusion</u>:

   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. Welsby. The subject may be excluded from continued participation in the study

b. Additional transfusion reactions, such as chills, fever, drop in blood pressure, shortness of breath, and (very rarely) 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 this 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 standard Duke Medical Center transfusion procedures
iii) an extended crossmatch will be performed between the subject's stored donated unit and a fresh sample of the subject's blood
iv) only one subject per day will be transfused to minimize risks
v) the research subject will be asked to review the autologous RBC unit label including their own name, and date of birth to confirm they are receiving their own blood

e. Additional risk of rejuvenated blood: If lung function is abnormal, use of rejuvenated blood may lower the subject's oxygen levels making him/her short of breath.

5) Risks associated with <u>exercise testing:</u> 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 non-fatal 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).*[14]

6) Risks associated with <u>arterial catheter placement</u>: Arterial catheter insertion in the wrist can cause occlusion of the radial artery, although most instances occur after many hours or days of a catheter in place. Radial artery catheterization has been used extensively in our laboratory, with more than 200 being performed in healthy volunteer studies with no significant complications. The arterial line insertion (standard 20 g) will be performed by an experienced anesthesiologist under sterile technique with local anesthetic.

7) Risks associated with <u>LiDCO cardiac output monitoring:</u>

The LiDCO cardiac output monitor is an FDA cleared monitor, which is used routinely in critically ill patients (e.g. ICU) and other high risk patients, e.g. during surgery. It has been used extensively in patients in the U.S. for over 10 years. Cardiac output is measured by injecting a tiny, non-therapeutic dose (0.15-0.3 mM) of lithium intravenously (through an IV) and then approximately 5 ml of blood is sampled from the arterial catheter over the next few seconds. A dilution curve is calculated from which the cardiac output is determined. This lithium calibration will be done a total of 2 times. We are not aware of any risks associated with injection of this dose of lithium for the LiDCO monitor except individuals with lithium hypersensitivity. The device cannot be used in persons taking lithium salts for treatment of other medical/psychiatric conditions, persons who weigh less than 40kg. According to the manufacturer's instructions the maximum dose is 20 ml of the lithium solution (0.15 mM/ml). and we will be using approximately 2 two-ml doses on any given study day (Day 14).

8) We are not aware of any risks associated with the ECG and spirometry done for screening.
9) The subject will not be able to donate platelets or RBCs for 1 year following this study.
10) 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.
11) Risks associated 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 standard risk management procedures will be used to minimize this risk.

7. Subject Identification, Recruitment, and Compensation: Healthy volunteers who respond either to our advertisement or by word of mouth will be shown the eligibility criteria and asked if they believe they are likely to be eligible. Volunteers who responded to advertisement and left their phone numbers in voicemail will be returned call by investigator/delegated key personnel. Investigator/ designee will use IRB-approved phone script as reference to discuss the study activities over the phone and at the same time ask permission to PHI use prior to written consent form. Volunteers who ended up enrolling in the study but are screen failures (after lab results out) will be compensated $100. Participants will received up to total of $1000 by completion of all study activities, inclusive of screening visit and blood donation . Volunteers who has donated blood and missed subsequent visits will be compensated $100 for that specific visit. In addition, subjects will be provided with supplemental oral fluids (juices) and a light snack on day 14 during the break after the autologous blood transfusion and before the second exercise testing .

A member of the study team will conduct the consent process in an office at Duke Medical Center. He/she will describe the study and ask the volunteer to read the consent form and ask any questions they may have. As much time as the subject needs will be provided for conducting the consent process, and the potential subject will have as much time as they need to consider whether or not to participate.

8. Costs to the Subject: Subjects will incur no direct costs as a result of this study. Subjects will only be responsible for indirect costs such as parking fees.

9. Data Analysis & Statistical Considerations: The primary outcomes of this study are the change in $VO2_{max}$ and lactate between the "anemic" $VO2_{max}$ and after transfusion of 2 units of either standard PRBCs or rej-PRBCs "post-Tx" $VO2_{max}$. We hypothesize that transfusion of RBCs is less effective at increasing oxygen delivery (measured by $VO2_{max}$ and lactate) compared with transfusion of rej-PRBCs. We expect the changes to be normally distributed and will compare the treatment groups with a 2-group t-test or a rank-sum test if the distribution is non-normal. We will test at a 2-sided significance level of 0.05. The primary analysis will be on an intent-to-treat basis, with follow-up as-treated analysis if necessary. Subjects who withdraw or are excluded before randomization (Day 14) will simply be dropped from analysis. We will further examine the treatment effect in a multivariable regression adjusting for potential effects of baseline $VO2_{max}$, lactate, hemoglobin, and BMI.

Secondary outcomes include the change in "post-Tx" $VO2_{max}$ from baseline and differences in these changes will be compared between groups with t-tests or rank-sum tests. Of note, in contrast to the primary endpoint, this secondary analysis may show larger increases in $VO2_{max}$ attributable to hypervolemia after transfusion. Duration of exercise and peak lactate "post-Tx" will also be compared between study groups.

Based on preliminary data (Richard Moon, personal communication), we know that 2 units of autologous, stored PRBCs increase VO2max on the day of transfusion by 2.4 +/-0.6ml/kg/min. Assuming that rej-PRBCs exceed the oxygen delivery of native fresh blood (due to their p50 of 40mmHg compared to 18mmHg for stored PRBCs and 28mmHg for normal blood), we anticipate that:

- Pre-transfusion/"anemic" $VO2_{max}$ will be decreased by 4.6 ml/kg/min from baseline by day 14.
- 2 units rej-RBC will increase $VO2_{max}$ by > 6ml/kg/min from "anemic" $VO2_{max}$.
- 2 units control PRBC will only increase $VO2_{max}$ by 2.5ml/kg/min from "anemic" $VO2_{max}$.
- Therefore we will set our expected, measured difference (δ) at 3.5 ml/kg/min.

Preliminary data also show that the variability of VO2max has a narrow range, with standard deviation ranging from 0.8-1.9 for a given subject (Richard Moon, personal communication). Combining with our preliminary data (which showed and increase after transfusion of 2.4 +/-0.6 ml/kg/min – see above), we set our standard deviation for the power analysis (σ) at +/-1.5 mL/kg/min.

Using this data, we can expect 80% power to detect a significant ($p<0.05$) intergroup difference in the change in VO_2max from anemic to post-transfusion state with 3 volunteers in each group. More patients will be studied as unexpected variability may occur.

10. Data & Safety Monitoring: 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, and FDA as appropriate. 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.

11. Privacy, Data Storage & Confidentiality: Study records that identify the patient will be kept confidential as required by law. They will be kept in a locked file cabinet in either the Study Coordinator's or Dr. Welsby's office. Data will also be stored in password protected computers. 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 Duke University Health System (DUHS). 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.

As part of the study, Dr. Welsby and the study team will report the results of the study-related laboratory tests named below. These test results will be recorded in the study record and results of tests and studies done solely for this research study will not be included in the volunteer's medical record.

Medical records that identify the subject and the consent form may be inspected by the FDA, DUHS Institutional Review Board (IRB), and Rex Healthcare's regulatory bodies, 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.

The study results will be retained in the study research for at least six years. At that time, either the research information may be destroyed or information identifying the volunteer will be removed from such study results at DUHS. Any research information in the volunteer's medical record will be kept indefinitely.

12. Bibliography

1. Gledhill N, Warburton D, Jamnik V. Haemoglobin, blood volume, cardiac function, and aerobic power. *Can J Appl Physiol.* Feb 1999;24(1):54-65.
2. Buick FJ, Gledhill N, Froese AB, Spriet L, Meyers EC. Effect of induced erythrocythemia on aerobic work capacity. *J Appl Physiol Respir Environ Exerc Physiol.* Apr 1980;48(4):636-642.
3. Celsing F, Svedenhag J, Pihlstedt P, Ekblom B. Effects of anaemia and stepwise-induced polycythaemia on maximal aerobic power in individuals with high and low haemoglobin concentrations. *Acta Physiol Scand.* Jan 1987;129(1):47-54.
4. Robertson RJ, Gilcher R, Metz KF, et al. Effect of induced erythrocythemia on hypoxia tolerance during physical exercise. *J Appl Physiol Respir Environ Exerc Physiol.* Aug 1982;53(2):490-495.

5. Spriet LL, Gledhill N, Froese AB, Wilkes DL. Effect of graded erythrocythemia on cardiovascular and metabolic responses to exercise. *J Appl Physiol (1985).* Nov 1986;61(5):1942-1948.
6. Thomson JM, Stone JA, Ginsburg AD, Hamilton P. O2 transport during exercise following blood reinfusion. *J Appl Physiol Respir Environ Exerc Physiol.* Nov 1982;53(5):1213-1219.
7. Bennett-Guerrero E, Veldman TH, Doctor A, et al. Evolution of adverse changes in stored RBCs. *Proc Natl Acad Sci U S A.* Oct 23 2007;104(43):17063-17068.
8. Cabrales P, Tsai AG, Intaglietta M. Modulation of perfusion and oxygenation by red blood cell oxygen affinity during acute anemia. *American journal of respiratory cell and molecular biology.* Mar 2008;38(3):354-361.
9. Biolo A, Greferath R, Siwik DA, et al. Enhanced exercise capacity in mice with severe heart failure treated with an allosteric effector of hemoglobin, myo-inositol trispyrophosphate. *Proc Natl Acad Sci U S A.* Feb 10 2009;106(6):1926-1929.
10. Stucker O, Vicaut E, Villereal MC, Ropars C, Teisseire BP, Duvelleroy MA. Coronary response to large decreases of hemoglobin-O2 affinity in isolated rat heart. *Am J Physiol.* Dec 1985;249(6 Pt 2):H1224-1227.
11. Rude RE, Tumas J, Gunst M, Kloner RA, DeBoer LW, Maroko PR. Effects of ortho-iodo sodium benzoate on acute myocardial ischemia, hemodynamic function, and infarct size after coronary artery occlusion in dogs. *Am J Cardiol.* May 1 1983;51(8):1422-1427.
12. Deleuze PH, Bailleul C, Shiiya N, et al. Enhanced O2 transportation during cardiopulmonary bypass in piglets by the use of inositol hexaphosphate loaded red blood cells. *Int J Artif Organs.* 1992;15(4):239-242.
13. Roberson RS, Lockhart E, Shapiro NI, et al. Impact of transfusion of autologous 7- versus 42-day-old AS-3 red blood cells on tissue oxygenation and the microcirculation in healthy volunteers. *Transfusion.* Nov 2012;52(11):2459-2464.
14. Warburton DER, Gledhill N, Jamnik VK, et al. INTERNATIONAL LAUNCH OF THE PAR-Q+ AND ePARmed-X+ The Physical Activity Readiness Questionnaire (PAR-Q+) and Electronic Physical Activity Readiness Medical Examination (ePARmed-X+): Summary of Consensus Panel Recommendations. Health & Fitness Journal of Canada 2011;4:26-37.
15. Raat NJ, Hilarius PM, Johannes T, de Korte D, Ince C, Verhoeven AJ. Rejuvenation of stored human red blood cells reverses the renal microvascular oxygenation deficit in an isovolemic transfusion model in rats. *Transfusion.* Mar 2009;49(3):427-424.
16. Rejuvesol (Red blood cell processing solution) [package insert]. Braintree, MA: Citra Labs, LLC; 2015.