Rejuvesol® Washed RBC in Sickle Cell Patients Requiring Frequent Transfusions

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Research Summary

Protocol Title: Rejuvesol® Washed RBC in Sickle Cell Patients Requiring Frequent Transfusions

Purpose of the Study – The long-term goal of reducing morbidity and increasing treatment intervals for chronically transfused patients, the objective of this study is to test the feasibility of the novel application of red blood cell (RBC) rejuvenation to chronic transfusion in sickle cell disease (SCD) and the potential benefit in terms of RBC longevity, prothrombotic microparticle (MP) formation, and oxygen (O2) delivery capacity of transfused RBCs.

The purpose of this study is to:

1. To demonstrate that Rejuvenation improves *in vivo* RBC survival.

We hypothesize that the loss of HbA over a transfusion interval will be reduced after a transfusion treatment with rejuvenated compared to standard RBCs.
This will allow prolonging transfusion intervals to maintain the same %HbA target.

2. To determine whether Rejuvenation reduces *in vivo*, RBC microparticles.

We hypothesize that *in vivo* RBC-MP counts and RBC/RBC-MP-mediated thrombin generation before transfusion therapy will be lower after a prior treatment with rejuvenated compared to standard RBCs ("trough level"). Similarly, RBC-MP counts and RBC/RBC-MP-mediated thrombin generation will be lower immediately after transfusion therapy with rejuvenated compared to standard RBCs ("peak level"). This will minimize the contribution of transfusion therapy to the pre-exisiting, prothrombotic MP burden.

3. To demonstrate optimal *in vivo* O2 delivery capacity after Rejuvenation

We hypothesize that, compared to standard RBCs, transfusion of rejuvenated blood will not reduce the p50 of patients' blood. A high p50 is a compensatory mechanism to maintain tissue O2 delivery in SCD, since standard RBCs have a low p50 from 2,3-DPG loss.

Background & Significance –SCD is a group of inherited RBC disorders that affects approximately 100,000 SCD patients in the United States and millions worldwide, making it a major national and international health concern. Patients with SCD that require RBC therapy carry the burden of approximately monthly transfusions to maintain their health due to clearance of transfused cells. In contrast to other medical treatments for SCD complications, repeated RBC therapy via RBC-ST or RBC-XT is considered for those with the most severe disease manifestations. These patients have often suffered or have an elevated risk of developing SCD complications such as stroke, transient ischemic attack, acute chest syndrome, priapism, or persistent chronic pain. While beneficial, transfusion is also associated with potential risks and adverse effects, including immune hemolysis, RBC alloimmunization, iron overload, transfusion reactions, and infection with bacteria, viruses, and parasites are all potential risks of transfusion; therefore maximizing the benefit and minimizing the risk of each transfusion is necessary.
RBC transfusion was approved on the basis of short-term quantitative parameters, including <1% hemolysis in the unit at time of infusion and an *in vivo* recovery >75% of

transfused cells 24 hours after infusion. There was no regard for the RBC quality, longer-term *in vivo* survival kinetics, burden of RBC byproducts with repeated transfused doses, or tissue oxygenation capacity. Subsequent research has demonstrated that RBCs exhausted by refrigerated storage are structurally and functionally abnormal. These cells are more rigid, less tolerant of stress, form increased pathogenic *ex vivo* RBC-MPs, and abnormally adhere to the vascular endothelium.1,2,6,20 Intracellular ATP levels decrease during storage and can impair the ability of RBCs to effect local vasodilation in hypoxic microcirculatory environments to promote O2 delivery.24,25 Furthermore, ATP losses decrease the function of vital intracellular ATP-dependent enzymes, such as the flippases that maintain normal membrane phospholipid asymmetry.26 Stored RBCs are also depleted of 2,3-DPG and have lower p50, thus are less effectively able to transport and release O2 where it is needed in the tissues.3 Once transfused, these altered RBCs are cleared from circulation and produce additional burdens of RBC byproducts and RBC-MPs already present in SCD patients.4-6 Rejuvenation of stored RBCs in the Blood Bank is a FDA-approved process that improves several cell parameters that are directly relevant to SCD pathophysiology, including RBC recovery, RBC-MP burden, *in vivo* RBC-MP formation, ATP and 2,3-DPG levels, p50, microcirculatory oxygenation, membrane deformability, and vascular endothelial adhesion.2,3,14-20 Surprisingly, benefits of RBC rejuvenation for transfusion in the SCD population specifically have not been explored. We postulate that RBC rejuvenation increases the *in vivo* survival of transfused RBCs, decreases pathogenic RBC-M formation *in vivo*, and can improve *in vivo* tissue oxygenation via normalization of p50.

Our proposed, pilot cross-over, single-blind study seeks to demonstrate the feasibility that RBC rejuvenation can improve the longevity and quality of transfused RBCs in chronically transfused SCD patients. Power analysis (Dr. Yi-Ju Li, Duke University) was performed based on the 2015 %HbA results from the monthly transfusion records of 15 UNC SCD patients. Persistence of HbA during the transfusion interval represents survival of transfused RBCs. From these data, the mean (±SD) decrement of %HbA per day (total %HbA decrement per transfusion interval in days) was 0.76% (±0.16). Therefore, the average %HbA decrement of 22.5% over 30 days can be considered a “standard decrement”. In order to increase the interval by 7 days, while maintaining a “standard decrement”, we must reduce the %HbA decrement/day from 0.76% to 0.62% per day (a 19% reduction). This could decrease transfusions visits from 12 to 10 per year, with a projected cost saving of $10,000 per year per patient. Our null hypothesis is that the equivalence limit of mean differences of %HbA decrement per day between two treatments (standard versus rejuvenated) is ±19%.
This study offers to benefit both SCD patients and potentially other massively transfused populations, such as trauma patients. The use of an FDA-approved process permits immediate application from bench to bedside upon completion of the research. RBC-ST and RBC-XT, as detailed in the study timeline. This has the potential to revolutionize RBC therapy and benefit millions of SCD patients worldwide.

We additionally are interested in exploring the effectiveness of rejuvenation at reversing the deleterious biochemical changes that occur in stored RBCs, and have discussed the possibility of collaboration with Dr. Angelo D’Alessandro at the

University of Colorado-Denver (UC-Denver). Dr. D'Alessandro has extensive experience analyzing the metabolic changes that occur during RBC storage[34,35], and is interested in identifying the biochemical differences between RBCs of sickle-cell patients receiving rejuvenated transfusions compared to standard transfusions. As such, we propose an external specimen repository, to be stored securely at UC-Denver under the supervision of Dr. D'Alessandro, which will be used to conduct future research that is unspecified at this time but will generally involve metabolomic analyses of subject RBCs. Investigators at Duke will contribute only leftover blood samples to this repository, and subjects will be given the option to opt-in or opt-out of this repository. Subjects will not receive additional needlesticks for this repository and no additional blood will be drawn. All biological samples will be de-identified and no personnel outside of DUHS will be provided with information that can be used to identify subject samples. All sample transfer will occur within the context of a pending materials transfer agreement, and Dr. D'Alessandro will obtain IRB approval or waiver from his institution for this repository.

Design & Procedures – This is a pilot study. In this prospective, single blind, cross-over clinical trial, patients with sickle cell disease will receive either rejuvenated (R) or standard (S) RBCs with each treatment over a 6-treatment period as follows to maximize detection of any signal: SRR|RSS. Each patient will receive 1 transfusion per treatment. Each transfusion consists of 2 units of RBCs or RBC apheresis exchange consisting of approximately 5 units of RBCs. Treatment 1 after enrollment the patient will receive the standard RBC's (in all units) Treatment 2 and treatment 3 the patient will receive the RBCs treated with Rejuvesol (in the last 4 units). The patient then crosses over to the RSS arm. Treatment 4 the patient receives Rejuvesol treated RBCs (in the last 4 units). Treatments 5 and 6 the patient receives standard RBCs (in all units). For patients receiving RBC-XT, only the final 4 RBC units used in the treatment will be rejuvenated as they are the most likely to persist in circulation following treatment completion.
The Duke site (Dr. Shah) will enroll 4 chronically transfused patients receiving RBC-ST (2 units each month) or RBC-XT (approximately 5 units each month). Total patient enrollment at Duke is expected to be 4 subjects and will be treated as a single site pilot study.
Patients at Duke undergo RBC-ST with 2 units or RBC-XT with approximately 5 units every 4-6 weeks. This established care pattern and current medications will be recorded but not altered. Only the RBC units will be modified.

RBCs provided by the Blood Bank

All patients will receive crossmatch-compatible leukoreduced RBCs that have been phenotypically matched for at least ABO, D(Rh), C, E, and Kell antigens. For sessions in which standard units are used, no additional manipulations will be performed. For sessions in which rejuvenated units are needed, rejuvenation will be performed with Rejuvesol® RBC Processing Solution (Citra Labs, LLC, Braintree, MA) per manufacturer's protocol.30 Briefly, RBC units are incubated with rejuvenating solution for 1 hr at 37 C in the blood bank under the supervision of co-director Dr. Jessica Poisson, washed with saline in a C.A.T.S. (Fresenius Kabi AG; Bad Homburg, Germany), and suspended in 0.9% saline/0.2% dextrose for transfusion. The C.A.T.S. is

an intra-operative cell-salvage device maintained by the Biomedical Engineering Department and routinely used in the ORs. The C.A.T.S. is FDA-approved for the preparation of washed RBC units, and thereby meets Rejuvesol® manufacturer's specifications for the required washing step. Due to logistical challenges, the wash procedure will be performed at the bedside by study personnel specifically trained in the use of the C.A.T.S. apparatus, and the C.A.T.S. apparatus will be maintained using standard blood bank QC procedures. Flowsheets designed in conjunction with the blood bank will be used during these wash procedures to maintain the safety and integrity of the RBC units and wash process. All flowsheets and QC will be maintained in study binder and reviewed monthly by Dr. Poisson of the blood bank. Consistent with AABB regulations, all units washed with the C.A.T.S device will be transfused within 4 hours.

Demographics and standard clinical and laboratory data

Demographic data include age, sex, race, height, and weight. Pre- and post-transfusion session vital signs will be recorded (temperature, heart rate, respiratory rate, blood pressure, and pulse oximetry), complete blood count with differential, peripheral blood film analysis (Advia 120; Siemens Healthcare, Malvern, PA) and HbA/S analysis routinely drawn. ABO type, RBC antibody screen, calculated patient total blood volume, calculated patient total RBC volume, and volume and storage age of RBCs transfused for each session will also be recorded.

Study specific laboratory data

With no additional blood draw time points or venipunctures, an additional 18 ml whole blood will be drawn into 4 citrated tubes and 2 EDTA tubes for RBC-MPs and p50 measurements at the time of routine blood draws pre- and post-transfusion. This means the total monthly volume of study blood is 36ml.

Specific procedures to accomplish each objective

1. Hb A/S determinations. Samples will be whole blood in EDTA. Bio-Rad Variant II HPLC system with Variant II β-Thalassemia Short Program Reorder Pack (Bio-Rad Laboratories, Inc.; Hercules, CA) will be used per manufacturer's instructions. Briefly, samples will be mixed, diluted with specific hemolyzing/wash buffer, and injected into an analytic cartridge. Absorbance will be measured at 415 nm. Background noise will be reduced with use of a second wavelength at 690 nm. Raw data will be integrated by manufacturer's software, and a chromatogram will be generated. Integrated peaks will be

assigned to manufacturer-defined windows derived from retention time of the peaks. The %Hb will be calculated by determining the area of a peak as a fraction of the total area of all Hb peaks on the HPLC chromatogram.

2: RBC-MP determinations. Samples will be whole blood in citrate. The first blood tube will be discarded to avoid contamination during venipuncture. Blood will be centrifuged at 1,500g for 10 min at 4 C. The supernatant will be carefully removed into microcentrifuge tubes, taking care not to disturb the buffy coat layer. A second centrifugation will be performed at 2,000g for 5 min to obtain platelet free plasma (PFP) to be frozen at -80 C. PFP aliquots (30 µL) will be stained with the following markers/antibodies: 10 µL FITC-labeled AnnexinV mixed 1:1 with Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2) and 2 µL PE-labeled anti-CD235a. All markers/antibodies will be centrifuged at 20,000g x 20 min prior to use.RBCMs will be defined as Annexin+/CD235+ events. Calcium-free samples and

single-stained MP-free plasma samples (supernatant from normal, healthy donor PFP spun at 100,000g x 60min and stored at -80 C until use) will be used as negative controls. BD CompBeads® will be run as compensation controls per manufacturer's instructions. Stained samples will be incubated in the dark for 20 min and then further diluted with 500 µL of Annexin binding buffer or phosphate buffered saline prior to analysis. All buffers will be filtered through a 0.1 µm filter prior to use. CytoCount® beads will be added to each sample in equal volume as PFP (30 µL) immediately prior to analysis to enable MP enumeration. The concentration of MPs in each sample will be calculated as follows: (MPs counted x concentration of beads) / number of beads counted. All samples will be analyzed on a Stratedigm S1000Ex (Stratedigm; San Jose, CA, USA) high-sensitivity flow cytometer equipped with a forward scatter photomultiplier tube (FSC PMT) using a side scatter (SSC) trigger and 0.1 µm filtered sheath fluid. MP size gate for analysis will be set between 200 and 1000 nm utilizing Megamix Plus FSC/SSC® beads, which are a mixture of 900, 500, 300, 240, 200, 160 and 100 nm fluorescent beads. Event rates will be <8000 events/sec for all samples to minimize coincident events.

RBC-mediated thrombin generation determinations. Phosphatidylserine (PS) has been found to directly correlate with rate of production of meizothrombin-antithrombin (mTAT) complexes and thrombin generation on RBCs. Whole blood samples will be collected in EDTA. For positive controls, washed RBCs will be diluted to 20% hematocrit in RBC wash buffer and incubated with 10mM N-ethylmaleimide (NEM) at 37 C for 30 min. NEM-treated cells will then be washed 3 times before incubation with a 4µM concentration of the calcium ionophore A23187 at 37 C for 30 min. Ionophore-treated cells will be washed 3 times and diluted to 2x106 cells/mL in RBC wash buffer. Similar to controls, samples will be incubated and washed but without NEM or ionophore treatment. RBCs will be detected using PE-anti-glycophorin A (CD235a). RBC PS will be measured via binding of FITC-labeled Annexin V on a FACS Canto II flow cytometry (BD Biosciences Franklin Lakes, NJ).

RBC-MP-mediated thrombin generation determinations. Whole blood samples will be collected in citrate. PFP will be prepared as described above and frozen at -80 C. The Zymuphen ELISA (Aniara Inc., West Chester, OH) is a functional assay that measures the procoagulant activity of MPs in PFP. A microtiter plate is pre-coated with annexin V-streptavidin. PS+ MPs bind to the plate and expose their phospholipid surface, allowing

pro-thrombinase complex to cleave prothrombin into thrombin. Phospholipid in the sample is the limiting factor, so there is a direct relationship between MP phospholipid concentration and thrombin generation. Thrombin generation will be measured by a specific chromogenic substrate. Thawed PFP samples and kit controls will be diluted 1:20 in the kit sample diluent and supplemented with calcium, Factor Xa (FXa), and thrombin. A calibrator curve, diluted controls and diluted samples will be added to the ELISA plate and incubated for 1 hr at 37 C. The plate will be washed five times, then 100 µl of Reagent 1 (FXa–FVa) and 50 µl Reagent 2 (prothrombin) will be added and incubated for 10 min at 37 C, then 50 µl of Reagent 3 (thrombin substrate) will be added, and incubated for 3 min at 37 C. Finally, 50 µl of stop solution will be added and absorbance read at 405 nm. Results will be calculated from the calibrator curve.

3: O2-binding curves and P50 determinations. RBCs will be washed and PBS

containing DTPA. A 10% hematocrit suspension will be prepared in Krebs buffer. The RBC suspension will be placed in a specially designed, temperature- and humidity controlled rotating tonometer. RBCs will be progressively exposed to varying gas mixtures in order to produce fixed solution O2%, typically including 2%, 5%, 8%, and 20%. After 10 min of gas mixture at each pO2% sample will be removed from the tonometer for blood gas measurement. An Abbott i-Stat (Abbott Laboratories; Abbott Park, IL) will be used to measure pO2 and directly measures including Hb O2 saturation. The Hb O2 saturation versus pO2 curve will be constructed and p50 extrapolated for each sample. p50 results will be generated and O2-binding curves displayed graphically.
4: **Preparation of samples for external repository.** Any blood remaining after the above analyses are complete will be spun to separate RBCs and plasma at 2000*g* for 10 minutes at 4 degrees Celsius. Once separated, plasma and RBCs will be stored in separate deidentified cryovials at -80 degrees Celsius. These specimens will be batch-shipped to Dr. Angelo D'Alessandro at the University of Colorado-Denver according to the terms of the pending materials transfer agreement.

Selection of Subjects – Inclusion criteria: Adults 18 years of age and older who are undergoing frequent RBC transfusions as part of their routine care for Sickle cell disease will be approached for participation. Exclusion criteria: Patients with acute pain crisis with 2 weeks prior to enrollment. Duke expects to enroll 4 subjects for this pilot study.

Subject Recruitment and Compensation – Subjects will be identified by the Co-investigator Dr. Shah for the Sickle cell patients after review of the sickle cell transfusion schedule. All subjects are Dr. Shah's patients. The consent form will be presented to the prospective subjects/parents by the physician investigator in this study or the study coordinators known to the subject/parents. Participation in the research study will not significantly alter the routine clinical management as currently practiced at Duke University Medical Center. Patients will not be compensated for participation in the research study.

Consent Process – See section 14 of the eIRB submission

Subject's Capacity to Give Legally Effective Consent – All subjects must have the capacity to give legal consent.

Study Interventions – Please refer to item 4

Risk/Benefit Assessment – It is impossible to estimate without conducting this study what the benefit or risk of participation in this study is. There are no apparent risks for subjects participating in this study. Rejuvenation is an FDA approved process and an AABB standardized procedure; furthermore pediatric patients may often receive washed units of packed red blood cells. Risks are no more than receiving a unit of PRBCs as all these products are approved. No subject will receive a transfusion specifically for the

purpose of this study. RBC rejuvenation will only involve units of blood that would have been used for the patient regardless of the study.
No additional blood draws will be done specifically for the study. An additional 36ml of blood (18ml pre-transfusion and 18 ml post transfusion) will be drawn with the standard of care labs..

Costs to the Subject – There is no cost to the subject for participation in this study.

Data Analysis & Statistical Considerations –Routine clinical measurements will be compared with repeated measures analysis of variance. As this is a pilot study and there is no control group, the only relevant changes to analyze will be pre- and post-procedure. Statistical data obtained (noted and described in the Design and Procedures section) will be used for future planning of a larger comparison study.
No interim analysis is planned but adverse events will be reported to the IRB as required.
1: To demonstrate that Rejuvenation improves *in vivo* RBC survival. We hypothesize that the loss of HbA over a transfusion interval will be reduced after a transfusion treatment with rejuvenated compared to standard RBCs. HbA/S determination is routinely performed pre- and post- treatments. The %HbA decrement is the current pre-treatment HbA – previous post treatment HbA in %. Our primary outcome variable will be HbA decrement per day to account for minor variability. A generalized linear model will be applied to determine whether the mean difference in HbA exceeds +/-19%. Secondary outcomes will evaluate actual HbA decrement (g/dl) with and without indexing to calculated circulating blood volume. If %HbA decrement with rejuvenated units exceeds -19% we will reject the null hypothesis and conclude that rejuvenation will permit prolonging transfusion intervals by a week to maintain the same %HbA target. Whether or not we reject the null hypothesis, these data will inform treatment effect size and prospective collection of %HbA variability can validate preliminary data strengthening future sample size estimate calculations.
2: To determine whether Rejuvenation reduces *in vivo*, RBC microparticles. We hypothesize that *in vivo* RBC-MP counts and RBC/RBC-MP-mediated thrombin generation before transfusion therapy will be lower after a prior treatment with rejuvenated compared to standard RBCs ("trough level"). Similarly, RBC-MP counts and RBC/RBC-MP-mediated thrombin generation will be lower immediately after transfusion therapy with rejuvenated compared to standard RBCs ("peak level"). These secondary outcomes will inform on effect size of rejuvenation on the immediate changes in RBC-MP counts after treatment. If reduction in RBC-MPs is augmented by rejuvenation, better efficacy of treatment could be postulated and subsequently tested, as this will minimize the contribution of transfusion therapy to the

preexisiting, prothrombotic MP burden. Phosphatidylserine (PS) has been found to directly correlate with rate of production of meizothrombin-antithrombin (mTAT) complexes and thrombin generation on RBCs. The capacity of RBCs and RBC-MPs to generate thrombin could inform evaluation of future anticoagulation needs and targets. Wilcoxon Rank Sum tests will be used to compare RBC-MP counts and thrombin generation.
3: To demonstrate optimal *in vivo* O2 delivery capacity after Rejuvenation. We

hypothesize that, compared to standard RBCs, transfusion of rejuvenated RBCs will not reduce the p50 of the patients' blood. The effect of transfused RBCs on overall, *in vivo* p50should be dose related; this study is uniquely poised to demonstrate this phenomenon by comparing low-dose RBC-ST to high-dose RBC-XT. Determinations of O2-binding curves and, therefore, accurate p50 measurements will inform the potential for rejuvenation to optimize tissue oxygenation. Invasive probes are required to measure the increased tissue O2 and VO2 max of muscle as p50 is increased.31,32 Identifying a dose-dependent modulation of p50 with rejuvenation will support future testing in SCD and other critical populations such as trauma, sepsis, or cardiogenic shock. Wilcoxon Rank Sum tests will be used to compare change in p50 pre- and post-transfusion within groups.
In the future, if the study team wishes to share any data with UNC or any other external researchers an amendment will be submitted.

Data & Safety Monitoring –
Rejuvesol is a blood product additive that is washed out of the unit prior to administration per AABB and FDA standards, it is not given as an iv drug. The effect of Rejuvesol is to increase/normalize the p50 of the RBCs to a level that is seen in normal and anemic individuals. Rejuvenation is a AABB standardized and FDA approved procedure and as such we believe a DSMB is not indicated. AEs and SAEs will be reported to the IRB, per policy,

Specific, product related adverse effect would include be to further decrease SaO2 in hypoxic individuals, especially if the p50 was markedly increased. For this reason the exclusion criterion (per package insert) include not using on units stored for < 10 days (2,3, DPG and p50 would be supranormal) and not enrolling patients with a pO2 <40mmHg or a room air SaO2 <92%.

Data and safety monitoring will be ongoing as part of the standard of care protocol**.** There are no additional procedures or interventions as part of this study. Transfusion SOC monitoring will be done according to guidelines and should there be any type of reaction the transfusion will be stopped and the appropriate actions taken.
Any adverse events will be reported to the IRB as required.

Privacy, Data Storage & Confidentiality – Study records that identify subjects will be kept confidential as required by law. Federal Privacy Regulations provide safeguards for privacy, security, and authorized access. Except when required by law, the subjects 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). For records disclosed outside of DUHS, subjects will be assigned a unique code number. The key to the code will be kept in a locked file in Dr. Shah's office. The study results will be retained in the subject's research record for a minimum of 6 years. Any research information in the medical record will also be kept indefinitely. Some information collected about subjects only for this research study may

be kept in a research study record separate from subject's medical record, and some research information may also be part of the subject's medical record. Subjects will not have access to this research information until the end of the study.

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