Supplement 1.

Tissue oxygenation by transfusion in severe anemia with lactic acidosis (TOTAL): a prospective, randomized, non-inferiority trial of blood storage duration

Summary of the Protocol and Statistical Analysis

1. Protocol Summary

1. Specific aim: To determine, in a cohort of children ages 6 months to 60 months with severe anemia (Hgb ≤ 5 g/dL) and lactic acidosis (≥ 5 mM), whether transfusion of RBCs stored for 25 days or more (longer-storage) is not inferior to RBCs stored for 10 days or less (shorter-storage) for global tissue oxygenation as judged by the reduction of elevated blood lactate levels.

Hypothesis: It is known that children with severe anemia develop lactic acidosis that is corrected by transfusion. We test the hypothesis that RBCs with a longer storage time are not inferior to RBCs with a shorter storage time for correction of lactic acidosis.

2. Design: The study was designed as a prospective, randomized, non-inferiority clinical trial with 1 to 1 allocation.

Non-inferiority margin: Balancing the value of rapid reduction of elevated blood lactate levels in patients with severe anemia versus the value of longer blood storage for maintenance of adequate inventories of blood for transfusion, we selected a non-inferiority margin of 25%. To be non-inferior, longer-storage RBCs needed to be at least 75% “as good as” shorter-storage RBCs for reduction in blood lactate levels.

3. Subject selection:

a. Inclusion criteria

* Children (6 months – 60 months)
* Severe anemia (Hemoglobin ≤ 5 g/dl)
* Lactic Acidosis (Blood lactate ≥ 5 mM)

b. Exclusion criteria

* Children undergoing transfusion with blood products other than packed cells.
* Children with known or suspected concurrent cardiac disease.
* Children with severe acute malnutrition.

Rationale for age group to be studied: Study enrollment was focused on children aged 6 months to 60 months because the prevalence of severe anemia is highest in this age group. Cardiac output is not impaired in most patients of this age group.

Protocol change to the inclusion criteria during the course of the study:

During year one of this two-year study, children with a hemoglobin ≤5 g/dL, a blood lactate ≥5mM, and evidence of malaria were enrolled. Evidence of malaria consisted of either a positive blood smear for red cell parasites as detected by a trained laboratory observer or a positive bedside rapid diagnostic test (SD Bioline Malaria Antigen P.f/Pan). During year two, we continued to enroll patients with a hemoglobin ≤5 g/dL and a blood lactate ≥5mM, and we continued to test for malaria, but we changed the protocol to no longer require evidence for malaria as a criterion for enrollment. This change was made in order to broaden the underlying causes for anemia and thus increase the generalizability of the results. All oversight committees approved the change in protocol.

c. Source of subjects.

Research subjects were those presenting to the Mulago Hospital Pediatric Acute Care Unit (ACU) for treatment. The Mulago Hospital is a 1,500 bed government-owned and operated national referral center, as well as the nation’s largest teaching hospital, affiliated with Makerere University College of Health Sciences in Kampala, Uganda. The Department of Pediatrics and Child Health admits over 20,000 children annually through the ACU from the over 100 emergency patients assessed daily. In addition to the inpatient care areas, the department operates several general and specialized outpatient clinics throughout the week. There are 34 staff pediatricians with other healthcare
workers, including post-graduate medical officers, nurses, and laboratory staff. All the clinical services provided by the department are free of charge to the patients.

4. Subject enrollment:
   a. Prior to randomization.
   Patients presenting with suspected anemia underwent a screening hemoglobin or hematocrit test by ACU staff. For patients with a hemoglobin value ≤5 g/dL (hematocrit < 15%), verbal consent was obtained for measurement of the blood lactate. Children with a blood lactate ≥5mM who had no exclusion criteria were then eligible for full consent, enrollment, and randomization.

   b. Consent:
   Consent was obtained from the accompanying parent or guardian of the child. The nature of the study, its purpose, the anticipated risks, the study duration and planned enrollment, patient safeguards, and opportunity for withdrawal were explained. Information was provided both verbally and in writing in either English or native Luganda. Consent was documented in writing.

   c. Enrollment: unique patient number:
   Each enrolled patient was assigned a unique patient number (UPN). Pre-printed UPN labels were used in place of patient identifiers for case report forms, laboratory requisitions, archived specimens, and reports.

   d. Randomization, treatment assignment, and blinding:
   Patients were randomized in a 1:1 ratio to receive RBCs stored 1-10 days versus 25-35 days. Randomization was done using consecutively numbered, otherwise identical, opaque, sealed envelopes that contained a slip of paper indicating the treatment assignment. There were equal numbers of envelopes for each treatment group. Prior to the start of the study, the sealed envelopes were extensively mixed, numbered consecutively on the outside, and then stored in a locked cabinet. Responsibility for preparation of the envelopes used for randomization was by an investigator who was not responsible for patient selection, enrollment or treatment allocation. Nurses responsible for clinical observations during the study did not participate in the preparation of randomization envelopes. Envelopes were opened in consecutive order with the envelope number matching the unique patient number. Only one envelope was opened per subject and only at the time of enrollment. Physicians were aware of the treatment assignment. Nurses performing bedside clinical observations were not aware of the treatment assignment.

   Protocol change to blinding during the course of the study: To further ensure blinding, during the second year of this two-year study, the expiration date on the blood unit was obscured with an overlying label.

5. Study Procedures:
   a. Visits:
   The period of enrollment was 24 hours and was completed during one visit to the hospital. Patients underwent testing according to the schedule in Table 1. All demographic, clinical, and laboratory data were collected on a standardized case report form.

   b. Procedures / interventions:
   The intervention was a blood transfusion with leukoreduced packed RBCs stored for either ≥ 25 days or stored ≤ 10 days. All RBC units were in-date (not expired).

   c. Blood transfusion products:
   All products for transfusion were provided by the Uganda Blood Transfusion service from volunteer donors. Blood donors were tested for exposure to hepatitis B, hepatitis C, syphilis, and HIV according to Uganda national standards using a commercial assay (Architech, Abbott Diagnostics, Abbott Park, IL). Units were collected using commercially-available blood collection sets equipped with an in-line leukoreduction filter and designed to prepare packed Red Cells suspended in AS-3 additive solution. Leukocyte reduction by filtration was done by trained blood center staff according to the manufacturer’s direction and prior to release of blood to the hospital. Quality control of the leukocyte reduction process was monitored with a CBC on at least every 10th unit processed. Units were refrigerated on the day of collection to maintain 2,3 diphosphoglycerate levels. Blood released for transfusion met all regular testing requirements of the Uganda Blood Transfusion Service. To reduce the risk of an ABO incompatible unit being given, all units were collected from donors known to be Group O. Use of all group O units
also facilitated the ability to maintain at least one short-storage and one longer-storage unit at the ACU at all times. Units awaiting transfusion were stored at the hospital in a dedicated, locked refrigerator under the control of the study investigators. Refrigeration temperatures were monitored daily and were always in-range throughout the study. At the time of enrollment, the oldest unit within the category assigned by randomization was selected for transfusion. At the time of transfusion, a well-mixed aliquot of each unit was tested for hemoglobin (Hemocue) and an aliquot of the plasma supernatant of the unit was frozen and stored in a monitored study freezer.

**d. RBC Transfusion**

Blood was transfused by peripheral vein using an electromechanical infusion pump. Vital signs and finger-stick blood testing were taken at regular intervals according to Table 1. Basic blood chemistries and prohormone B-type natriuretic peptide (pro-BNP) were measured on a small volume of peripheral blood (< 100 microliters) using an Abbott I-stat device (see Table 1).

**Dose of transfusion:**

Children in either arm of the study received the same volume of blood at the same rate: 10 mL/kg over 120 minutes.

**2nd Dose of transfusion:**

All patients underwent an assessment at the 4 hour mark (2 hours after completion of the first dose of blood) to determine if the child should receive a second dose of the same unit of blood. This assessment was based on the vital signs and the hemoglobin level. Children with hemoglobin levels <5 g/dL received a second dose. Those with hemoglobin levels 5-6 g/dL accompanied by persistent tachycardia >15% above the upper limit of normal for age without worsening respiratory status also received a second dose of blood. See figure at right. All data obtained at hours 0, 2, 4, 6, 8 and 24 was the same for patients receiving one dose or two doses of RBCs.

**e. Data collection schedule:**

<table>
<thead>
<tr>
<th>0 hr</th>
<th>2 hr</th>
<th>4 hr</th>
<th>6 hr</th>
<th>8 hr</th>
<th>24 hr</th>
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</tbody>
</table>

**f. Supplemental oxygen**

Supplemental oxygen was administered via nasal prongs at 1-2 liters/min in the following settings:

* Arterial oxygen saturation (pulse oximetry) values <95%.
* Suspected severe transfusion reaction, e.g., transfusion-associated acute lung injury
* Unconscious child
* Patients with shock evidenced by severe hypotension, cold periphery, or delayed capillary refill.

**g. Additional treatment**

The physician in attendance could treat with other agents, such as antibiotics, supplemental glucose, anti-malarials, or other treatments according to best judgment. Fluid boluses were not used. Any intravenous fluid, if given, was recorded on the CRF.
6. Assay Methods:

Patient hemoglobin concentration was measured from either finger stick samples or peripheral venous samples using a point-of-care device (Hemocue, Angelholm, Sweden). Lactate was measured from either finger stick or peripheral venous samples using the Lactate Pro device (Arkray Corp, Kyoto, Japan) which included quality control and calibration strips provided by the manufacturer. Additional quality control testing in a subset of patients on study demonstrated no significant difference in lactate results obtained simultaneously from finger-stick and venous blood samples. Finger pulse oximetry was measured using a commercial instrument (Radical 7, Massimo Corp, Irvine, CA). Glucose, hemoglobin, hematocrit, electrolytes, BUN, and creatinine measurements were tested on peripheral venous blood samples using a point-of-care device (I-stat, Abbott, Abbott Park, IL) and commercially-available cartridges (Chem8, and EC8+) according to the manufacturer’s directions. Complete blood count measurements were done from peripheral blood samples collected in EDTA tubes, using an automated analyzer (Sysmex NX, Kobe, Japan). The hematology laboratory of the Uganda Cancer Institute performed hemoglobin electrophoresis. Malaria parasites were detected by microscopy in peripheral blood smears by qualified laboratory personnel. Quantitative parasite levels were determined by the laboratory of Dr Samuel Nsobya (Makerere University-UCSF Molecular Laboratory, Kampala, UG). Patients with a negative blood smear for malaria were further tested using a rapid diagnostic test for histidine-rich protein II and LDH antigen (SD Bioline Malaria Antigen P.f/Pan, Standard Diagnostics, Inc., Gyeonggi-do, Korea). ABO grouping was done by direct hemagglutination using commercially-available monoclonal reagents.

Protocol change to assays during the course of the study:
The use of non-invasive tissue oxygenation sensors was added at the mid-point of the two-year study to obtain additional information on tissue oxygenation. Cerebral tissue O2 saturation was measured with a commercial device (EQUANOX™, Nonin Medical Inc, Plymouth, MN) using non-invasive pediatric probes placed on the child’s left forehead according to the manufacturer’s directions. Cerebral readings were taken every four seconds during the 120 minutes of transfusion. At each five-minute interval, the data from one minute (15 readings) were averaged and used to prepare a graph of tissue oxygen saturation over time. The net area under the oxygen saturation curve was determined using Graphpad Prism v6.4 (GraphPad Software Inc, La Jolla, CA) using the saturation at time zero as the baseline.

7. Data management

Each patient was assigned a unique patient number (UPN). Patient names were not used on any records transferred outside the hospital. Signed consent forms and the log linking each UPN to a specific patient were kept securely locked at the study site. Observations were recorded on paper using an approved case report form (CRF). Data recorded on the CRF were transferred at the study site to a digital CRF (FileMaker Pro12.0v3, Filemaker Inc, Santa Clara, CA) that explicitly matched the paper CRF. The digital CRF was professionally designed to contain features that reduce transcription errors including the inability to re-use a UPN; inability to leave required fields unfilled; inability to record duplicate data for single value responses; radio buttons; calendars; range limits on data fields; and required digital signatures. The validity of data transfer from the paper CRF to the digital CRF was tested after every 25th CRF. Digital CRF data were exported to Excel (Excel 2007, Microsoft Corp, Redmond, WA) and GraphPad Prism for analysis. At the completion of the study and prior to analysis, the database underwent substantial integrity testing by investigators different from those who originally recorded the data in order to identify potential transcription errors. All suspected errors were resolved by comparison to the original paper hardcopy CRF.

8. Proposed Sample size:

Sample size was based on determining the number needed to enroll to test the hypothesis that longer-storage RBCs were not inferior to shorter-storage RBCs for reduction of elevated blood lactate levels. Our primary hypothesis was that RBCs stored for >25 days were at least 75% as effective at reducing lactate levels as RBCs stored for <10 days. Preliminary data provided a point estimate that 0.75 of patients achieve a blood lactate level <5mM at two hours. If longer storage RBCs were 75% as effective as shorter-storage RBCs, then blood lactate levels would need to reach <5mM in 75% of 0.75 patients, or 0.56 of patients. If equal numbers of patients are randomized to the two study arms and
with $\alpha=0.05$ and $\beta=0.2$, then in order to observe that a proportion of 0.56 of patients achieve a lactate <5mM in the test arm compared with 0.75 in the control arm would require 90 subjects in each arm, Fishers test. See middle line in Figure. In order to ensure adequate statistical power, the study was approved for enrollment of up to 150 subjects in each arm. See top line in Figure. The study was voluntarily closed by agreement of all investigators after enrollment of 145 patients in each arm.

9. Monitoring and Quality Assurance:

a. Safety and Outcomes Monitoring:

An independent Data and Safety Monitoring Committee reviewed progress of the study. The Committee was composed of a pediatrician, a hematologist and a statistician from Mulago Hospital. The Committee monitored subject safety and reported any concerns to the principal investigator and local lead investigator. The Committee reviewed study outcomes after every 50 patients and prepared a written report of meetings.

Outcomes reviewed included:

- Rate of enrollment
- Rate of informed consent
- Adverse events attributed to transfusion in each study arm (blinded)
- Proportion of children in each study arm who reduced blood lactate to <5mM at hour 4 (blinded)
- Number and nature of adverse events in each study arm (blinded)
- Number of fatal cases in each study arm (blinded)

Guidance for stopping the study:

Because there are potential risks associated with RBCs stored either for a short-duration or for a long-duration, guidelines for suspending the study were blinded to the treatment arm. The Committee could recommend suspending the study until specific safety concerns were resolved to their satisfaction. In addition, the Committee specifically monitored both mortality and the proportion of subjects whose lactic acidosis improved (<5mM at hour 4). An imbalance in outcomes between the two study groups was grounds for recommending suspension or stopping of the study. The following two conditions were specific triggers for further action: a) an imbalance in mortality outcomes according to the table below; or, b) the proportion of subjects who achieved a blood lactate <5mM at 4 hours was significantly different ($p < 0.01$) in one group compared with the other (Fisher’s test).

If either of the above two conditions were met, then the interim results were to be un-blinded to the treatment assignments and the Committee would reconvene after an interval of 25 additional patients. If either of the above imbalances persisted or recurred in the same treatment arm as the original imbalance, then the Committee could recommend that the study be stopped.

<table>
<thead>
<tr>
<th>Number of deaths in either arm</th>
<th>Total number of deaths</th>
</tr>
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<tbody>
<tr>
<td>0 deaths</td>
<td>6 – 9</td>
</tr>
<tr>
<td>≤ 1 death</td>
<td>10 – 12</td>
</tr>
<tr>
<td>≤ 2 deaths</td>
<td>13 – 14</td>
</tr>
<tr>
<td>≤ 3 deaths</td>
<td>15 - 17</td>
</tr>
<tr>
<td>≤ 4 deaths</td>
<td>18 – 20</td>
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</tbody>
</table>

c. Adverse event reporting:

Deaths on study and all serious adverse events were reported promptly to the principal investigator and the Makerere University School of Medicine Research and Ethics Committee. All adverse events underwent review at the fortnightly investigators’ conference call.

d. Regular meetings of investigators

Investigators met by conference call every 2 weeks to review pace of enrollment, protocol violations, adverse events, adequacy of equipment and supplies, and integrity of data collection. Site visits by the North American investigators were held at least twice each year. Site visits included both observational audits of protocol compliance and audits of record keeping by an oversight investigator.
II. Statistical analysis:

The statistical analysis plan was finalized at the investigators meeting held in Toronto on June 12, 2014, approximately one year prior to the close of enrollment. It was agreed that only pre-specified comparisons would be presented in the main manuscript. Non-pre-specified exploratory comparisons could be presented in a supplement.

Data Analysis:

Means and standard deviations were reported and groups compared with the t-test. For non-parametric data, medians and IQRs were reported and data compared with a Mann-Whitney test. Proportions were compared with the Fisher’s exact test and presented with accompanying 95% confidence intervals. No corrections were made for multiple comparisons. All comparisons were two sided with \( \alpha = 0.05 \).

Primary outcome:

Designed as a non-inferiority trial, the primary outcome was the proportion of subjects whose blood lactate was \( \leq 3 \text{mM} \) at hour eight of the trial. Although lactate levels in a pilot study at hour two were used for the original sample size calculation, hour 8 was selected for the primary outcome because this was the first time point after equilibration of all transfusions (including those given to patients requiring a second dose) and was prior to the expected restoration of 2,3-diphosphoglycerate levels in longer-storage RBC. Thus, hour 8 maximized the opportunity to observe any disadvantage to recipients of longer-storage units and thus reject the hypothesis of non-inferiority. At the time of study design, we pre-specified a non-inferiority margin of 25% balancing the clinical importance of lactate resolution against the value of adequate blood availability that longer storage permits. When comparing the proportions achieving \( \leq 3 \text{mM} \) at hour eight in the two groups, non-inferiority would depend upon the lower 95% confidence interval of the longer-storage arm being a result greater than the upper 95% confidence interval minus 25% of the shorter-storage arm. The smallest margin of non-inferiority could also be reported.

To further compare the decline in blood lactate following transfusion in the two groups, three additional analyses were used: 1) Mean lactate levels were compared at hour 2, 4, 6, 8 and 24 for the two study arms. 2) Using Graphpad software for non-linear regression, the decline in blood lactate was compared across the 24 hours of observation using a global test of the difference in the slope of lactate decline after fitting the data to a one-phase exponential decay curve. 3) Kaplan-Meier survival analysis was used to estimate the time to achieve a blood lactate of \( \leq 3 \text{mM} \) with the hazard ratio and confidence intervals estimated using the log-rank test.

Secondary outcomes:

Cerebral oxygenation was compared between the two study arms in two ways:
1. For both the shorter storage and longer storage groups, we compared the mean cerebral oxygen saturation pre-transfusion versus post-transfusion.
2. For each study arm, we compared the net area-under-the-curve of cerebral oxygen saturation over time (120 minutes of transfusion) using the saturation at time zero as the baseline. GraphPad provides calculation of the net area under the curve. The net area under the curve integrates the improvement in tissue oxygenation during transfusion.

Clinical and laboratory results:

Analysis compared the shorter-storage versus the longer-storage group for each of the following:
* proportion of patients in coma or stupor, proportion of patients with respiratory distress;
* proportion of patients returning to good health at day 30;
* mean arterial pressure, respiratory rate, heart rate;
* electrolytes (Na, K, Cl, CO2, anion gap), BUN, creatinine.

Subgroup analysis:

The subgroup of patients receiving two doses of RBCs (20 mL/kg total) was analyzed separately. Both primary and secondary outcomes for those receiving shorter-storage versus longer-storage RBCs were compared in the same manner as the main comparisons stated above.