ANESTHESIOLOGY

Bedside Allogeneic Erythrocyte Washing with a Cell Saver to Remove Cytokines, Chemokines, and Cell-derived Microvesicles

A Clinical Feasibility Study

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Allogeneic erythrocyte transfusions occur frequently in cardiac surgical patients.
- Allergic and febrile reactions and transfusion-associated circulatory volume overload can occur in the setting of allogeneic erythrocyte transfusion.
- Cytokines, chemokines, and cell-derived particles (biologic response modifiers) in the supernatant of allogeneic erythrocytes may cause a proinflammatory response of the recipient's immune system.
- Feasibility of bedside allogeneic erythrocyte washing and the differences in biologic response modifiers pre- to posterythrocyte washing are not established.

What This Article Tells Us That Is New

- This study found that bedside point-of-care washing of allogeneic erythrocytes was feasible for 99% of elective cardiac surgical patients included in the study, with 93% of allogeneic erythrocyte units washed per study protocol.
- Biologic response modifiers were significantly decreased in supernatant after allogeneic bedside erythrocyte washing when compared with before washing.
- Cell-free hemoglobin was significantly increased after allogeneic erythrocyte washing when compared to before washing. Percent hemolysis was assessed in a five-unit subset of washed erythrocyte units and was less than 0.8%.
- Future reporting of the larger randomized control trial results will help to determine impact of these findings on clinical outcomes after cardiac surgery.
- Cell-free hemoglobin and percent hemolysis in washed cells should be further assessed in relation to clinical outcomes and findings should be validated in other cardiac surgical cohorts.

ABSTRACT

Background: Removal of cytokines, chemokines, and microvesicles from the supernatant of allogeneic erythrocytes may help mitigate adverse transfusion reactions. Blood bank–based washing procedures present logistical difficulties; therefore, we tested the hypothesis that on-demand bedside washing of allogeneic erythrocyte units is capable of removing soluble factors and is feasible in a clinical setting.

Methods: There were *in vitro* and prospective, observation cohort components to this *a priori* planned substudy evaluating bedside allogeneic erythrocyte washing, with a cell saver, during cardiac surgery. Laboratory data were collected from the first 75 washed units given to a subset of patients nested in the intervention arm of a parent clinical trial. Paired pre- and postwash samples from the blood unit bags were centrifuged. The supernatant was aspirated and frozen at –70°C, then batch-tested for cell-derived microvesicles, soluble CD40 ligand, chemokine ligand 5, and neutral lipids (all previously associated with transfusion reactions) and cell-free hemoglobin (possibly increased by washing). From the entire cohort randomized to the intervention arm of the trial, bedside washing was defined as feasible if at least 75% of prescribed units were washed per protocol.

Results: Paired data were available for 74 units. Washing reduced soluble CD40 ligand (median [interquartile range]; from 143 [1 to 338] ng/ml to zero), chemokine ligand 5 (from 1,314 [715 to 2,551] to 305 [179 to 488] ng/ml), and microvesicle numbers (from 6.90 [4.10 to 20.0] to 0.83 [0.33 to 2.80] $\times 10^6$), while cell-free hemoglobin concentration increased from 72.6 (53.6 to 171.6) mg/dl to 210.5 (126.6 to 479.6) mg/dl ($P < 0.0001$ for each). There was no effect on neutral lipids. Bedside washing was determined as feasible for 80 of 81 patients (99%); overall, 293 of 314 (93%) units were washed per protocol.

Conclusions: Bedside erythrocyte washing was clinically feasible and greatly reduced concentrations of soluble factors thought to be associated with transfusion-related adverse reactions, increasing concentrations of cell-free hemoglobin while maintaining acceptable (less than 0.8%) hemolysis.

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Until recently, transfusion-related acute lung injury was the leading cause of transfusion-related death. Fortunately, recent mitigation strategies have substantially reduced the frequency of this life-threatening transfusion complication.[1] However, their impact is primarily limited to the high plasma volume products (plasma and platelets). In contrast, little has been done to address the rate of erythrocyte-mediated adverse transfusion reactions. While the donor antibody-recipient antigen mechanism is classically ascribed as the mechanism underlying transfusion-related acute lung injury resulting from the transfusion of high plasma volume products,[2] an alternate mechanism has been proposed after erythrocyte units.[3] Here, it has been postulated that infused chemokines, cytokines, or microvesicles (plasma membrane–derived vesicular structures 0.1 to 1.0 μm in size) in the infused donor erythrocyte unit supernatant (collectively referred to as soluble biologic response modifiers), especially soluble CD40 ligand, may activate the recipient's immune

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system, resulting in adverse transfusion reactions,[4] including allergic or febrile, nonhemolytic reactions.

With transfusion-related acute lung injury mitigation efforts, transfusion-associated circulatory overload has now surpassed transfusion-related acute lung injury as the leading cause of transfusion-related death in many countries.[5] The only mitigation strategy suggested has been diuretic therapy.[6] Accumulating evidence suggests a potential role for soluble mediators in the erythrocyte supernatant in this and other serious transfusion complications,[7,8] which may be particularly relevant in recipients with a significant risk at baseline for volume overload or congestive heart failure, such as in those with cardiac or renal disease. Importantly, soluble mediators are present in the supernatant of erythrocyte products, which may directly cause proinflammatory changes in the recipients' innate immune system.

The persistence of erythrocyte-associated reactions presents a significant unmet challenge in transfusion medicine. If the etiological factors underlying erythrocyte-mediated respiratory complications reside within the donor unit supernatant, cell washing before transfusion may negate the potential pathophysiologic effects of mediators in the supernatant. Specifically, provision of over 28,000 erythrocyte units washed in the blood bank was associated with a zero incidence of adverse pulmonary reactions,[9] albeit in a retrospective analysis with associated limitations, such as underreporting of adverse outcomes. Unfortunately, the logistical challenges of providing washed erythrocyte units are particularly relevant in the dynamic and time-sensitive cardiothoracic surgery environment, while the risk for transfusion-related respiratory complications is likely the greatest.[10] To address these knowledge gaps and feasibility concerns, we hypothesize that on-demand, bedside erythrocyte washing is clinically feasible and effectively removes cytokines, chemokines, and erythrocyte-derived microparticles from the supernatant of erythrocyte units.

Materials and Methods

Study Design

This was a planned substudy of the parent clinical trial, completed before the end of subject enrollment, that addressed the specific question of the feasibility and effectiveness of bedside erythrocyte washing in removing potentially injurious soluble biologic response modifiers from allogeneic erythrocyte units. This is distinct from the comparison of patient-level biomarkers and recipient clinical outcomes, powering the parent clinical trial, that we plan to report in a separate manuscript once the data and laboratory analyses are complete.

There were *in vitro* and prospective, observation cohort components. First, laboratory data were collected from the first 75 washed units given to a subset of patients in the intervention arm of a clinical trial evaluating bedside erythrocyte washing, with a cell saver, during cardiac surgery (NCT02094118).[11] Paired pre- and postwash samples from these blood unit bags were centrifuged. The supernatant was aspirated and frozen at –70°C, then subsequently batch-tested for cell-derived microvesicles, soluble CD40 ligand, chemokine ligand 5, or regulated on activation, normal T expressed and secreted (often referred to as CCL5/RANTES) and neutral lipids (previously associated with transfusion reactions) and cell-free hemoglobin (possibly increased by washing). Second, from the entire cohort randomized to the washing arm of the trial, bedside washing was defined as feasible if at least 75% of prescribed units were able to be washed per protocol. Institutional review board approval for this study protocol was obtained from both participating institutions before enrolling the first study subject in the parent trial.

Study Population

All adult patients scheduled for cardiac surgery *via* sternotomy at one of two tertiary referral, academic heart centers (Duke University Medical Center, Durham, North Carolina; or Mayo Clinic, Rochester, Minnesota) between May 2015 and June 2019 were screened for study eligibility. Those projected to require four or more units of allogeneic erythrocytes, based on a published scoring system (supplemented by the opinion of experienced cardiac anesthesiologists, I.J.W. and W.J.M.), were further assessed for trial eligibility.[11] Those meeting inclusion criteria and lacking all exclusions were approached for consent to participate. Exclusion criteria were emergency surgery, history of severe recurrent transfusion reaction, refusal to receive allogeneic erythrocytes or provide informed consent, prevalent acute lung injury or congestive heart failure before randomization, expected hospital stay less than 48 h, not anticipated to survive more than 48 h, or a reoperation in a patient previously enrolled in this trial. After written informed consent, patients were randomized to either the intervention arm or the control arm for the entire day of surgery. Our laboratory and feasibility data all came from the intervention arm. A Data Safety Monitoring Board (Englewood, New Jersey) met every 6 months to determine the suitability of continuing the study for reasons of safety or futility.

Study Intervention

For transfusions deemed necessary by the clinical team, units were handed to the study team from the four erythrocyte

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Submitted for publication April 13, 2020. Accepted for publication December 28, 2020. From the Department of Anesthesiology, Duke University Medical Center, Durham, North Carolina (I.J.W., M.V.P., T.C.); Vitalant Research Institute and the Departments of Laboratory Medicine and Medicine, University of California, San Francisco, San Francisco, California (P.J.N., S.M.K.); Department of Anesthesiology and Perioperative Medicine (W.J.M., L.M., D.J.K.), Transfusion Medicine Services (C.M.C.), and Division of Biomedical Statistics and Informatics (P.J.S.), Mayo Clinic, Rochester, Minnesota; and Vitalant Research Institute and the Departments of Pediatrics and Surgery, School of Medicine, University of Colorado, Aurora, Colorado (C.C.S., M.K.).

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units typically provided in a cooler, under ice, for all cardiac cases. These were washed on-demand using a continuous automated transfusion system (CATS, Fresenius Kabi USA LLC, USA) with the Food and Drug Administration (Silver Spring, Maryland)–approved 0.9% saline wash solution, after a 4:1 dilution with 0.9% saline in the cell saver reservoir, as previously described.[12] Predilution was included to reduce the hematocrit of packed erythrocytes to better approximate that of the salvaged blood mixed with heparin anticoagulant that cell savers were designed to process. This automated washing procedure has been shown to more efficiently remove contaminants from the supernatant.[12,13] This cell saver system was chosen as the *g* force applied to the cells is less for this apheresis belt system (approximately 800*g*) than for the Latham bowl design favored by most cell saver devices (approximately 2,000*g*),[14] and has been reported to induce less hemolysis when compared to a standard blood bank–based cell washing device (Cobe 2991, Terumo BCT, USA).[15] Staff trained to use this cell saver included research personnel, anesthesia technicians and perfusionists (Duke University Medical Center), and transfusion medicine personnel with specific expertise in cellular washing techniques (Mayo Clinic).

Laboratory Testing

From the group randomized to receive allogeneic units washed with the cell saver, 6-ml samples were taken from each unit of blood before and after the washing procedures. Samples were acquired from the first 75 washed units (no more than four units from the same individual were included for the logistics of sample processing), as defined *a priori* in the study protocol.[11] These paired samples contained erythrocytes and supernatant (plasma, additive solution, and anticoagulant prewash, and mostly saline postwash). Samples were stored on ice for less than 2 h and centrifuged at 2,000*g* in refrigerated centrifuges with supernatant removed for a second 2,000*g* centrifugation before dividing into aliquots for frozen storage (–70°C). Subsequent batch analysis tested the removal of the following biologic response modifiers: chemokine ligand 5, a T cell–derived chemokine that recruits leukocytes (other than neutrophils) to sites of inflammation; soluble CD40 ligand, a platelet and T cell–derived protein that has proaggregatory effects on platelets[16]; the neutrophil priming activity of neutral lipids; and cell-derived microparticles present in the unit. The mediators have been shown to accumulate in stored blood products and have previously been associated with lung injury.[10,17–22]

Assays performed included Luminex Assays (USA) and flow cytometry (Professor Philip Norris, Vitalant Research Institute, San Francisco, California). Flow cytometry characterized the number and cellular origin of microparticles in pre- and postwash samples, with the following pairing: CD62p (P-selectin), activated endothelial cell– or platelet-derived; CD 235a (glycophorin A), erythrocyte-derived; CD108 (semaphorin-7A), activated lymphocytes or erythrocytes; and CD41a (glycoprotein IIb), megakaryocyte/platelet-derived. Due to the potential for inducing hemolysis with manipulation of the units,[23] cell-free hemoglobin was also measured in pre- and postwash samples by ELISA (Bethyl Laboratories, Inc., USA). Specific lipid priming assays were performed as previously described.[24,25] All measurements were performed in triplicate.

Due to concerns about excess sample volume being taken from the 75 patient units, additional data required to calculate percent hemolysis (pre- and postwash hematocrits, product and supernatant volumes and measures of the total amount of cell-free hemoglobin present in the supernatants) were only obtained in a *post hoc* quality control study using five test units that had been stored for 18 days. For percent hemolysis calculations, cell-free hemoglobin was measured by a point-of-care analyzer (Hemocue Plasma/Low Hb system, HemoCue America, USA) after immediate point-of-care processing to account for the potential for the storage on ice and the processing necessary for microparticle separation inducing hemolysis in the sample from the bags (especially compounding the stress of blood washing). The ratio of the amounts of cell-free hemoglobin to total units hemoglobin (in milligrams) was used to derive percent hemolysis.

Feasibility of Providing Washed Units

Provision was made for the clinical teams to administer "off-protocol," standard-issue units should the bedside washing process be expected to or be incurring an unacceptable delay based on the clinical situation. Washing erythrocyte units according to protocol determined feasibility; if an individual patient received 75% of prescribed units on protocol, then bedside washing for that patient was considered feasible.

Statistical Analysis

A power and sample size calculation for the overall "Point-of-Care Washing of Allogeneic Red Blood Cells for the Prevention of Transfusion-related Respiratory Complications" (WAR-PRC) trial was reported previously,[11] which was based on biomarker outcomes in patients randomized to control/intervention groups. A power calculation was not performed for this specific aim *a priori*, but a sample size of 75 washed bags with pre- and postwash samples was thought to be feasible and within budget, based on the clearance of mediators in preliminary data. A total 75 bags with pre- and postwash paired samples provides 90% power to detect an effect size (location shift divided by the SD of the paired differences) of 0.39 using a Wilcoxon signed-rank test with a two-sided alpha level of 0.05.

For the primary prespecified analysis, pre- and postwash bag samples were compared for each of the primary outcomes using a paired Wilcoxon signed-rank test. Change

in hematocrit was analyzed similarly. In secondary, *post hoc* analyses, we assess the association between erythrocyte storage additive solutions and outcomes. We calculated estimates that reflect the estimated multiplicative increase in geometric mean of each mediator, microvesicle subtype, and total count, as associated with a 1-day increase in storage duration. We also assessed whether there was a nonlinear relationship between storage duration and outcomes. Prewash outcomes are compared across additive solutions using Kruskal–Wallis tests. Postwash outcomes are analyzed using linear regression, adjusted for prewash values. Log transformations were applied to satisfy assumptions for normality of residuals and results transformed to report the association between additive solutions and multiplicative change in the geometric mean outcome. The association between storage age and outcomes was assessed similarly.

Applying Bonferroni correction to our nine total *a priori* outcomes analyzed would specify $P < 0.0055$ for significance. *Post hoc* analyses are identified as such. Statistical tests were two-tailed, and data were analyzed in using R software (version 3.6.1; http://archive.linux.duke.edu/cran/; accessed January 15, 2021).

Results

Of 314 erythrocyte transfusions prescribed by the clinical team on the operative day among 81 patients in the washed arm, we were able to complete the bedside washing procedures per protocol on 293 units (93%). Feasibility was defined at the patient level, with patients considered feasible if at least 75% of prescribed units were washed per protocol; all transfused patients were evaluated for the feasibility of providing units washed at the bedside with a delay acceptable to the clinical team. By this definition, of 81 patients receiving a transfusion on the operative day, 80 (99%) were considered feasible. A total of 21 nonwashed units were provided off-protocol to 11 patients randomized to the washing arm of the trial. The time from decision to transfuse to start of transfusion (median [25th to 75th] percentiles) for all units transfused on the day of surgery was 5 (3 to 12) min for the standard and 31 (23 to 50) min for the washed arms. This suggests the washing process approximately incurs a 25-min delay in transfusion initiation, which was acceptable to the clinical team for 99% of patients.

Complete data from paired pre- and postwash samples, with sufficient volume to test all proposed biologic response modifiers and cell-free hemoglobin, were available from 74 of the 75 units tested (39 from the Duke University Medical Center site and 35 from Mayo Clinic). The distribution of storage ages of transfused units (standard blood bank issue) is illustrated in figure 1 with a median storage duration (median [25th to 75th] percentile) of 15.5 (12 to 23) days (maximum, 41 days); the majority of units (65%) had been stored for 20 days or less. While storage age duration was unrelated to any biomarker or cell-free hemoglobin concentrations, there were additive solution–dependent differences. Most units (n = 41, 55%) were stored in additive solution-3, with the remainder in the mannitol-containing additive solution-1 (n = 24, 32%) or additive solution-5 (n = 8, 11%); there was one citrate/phosphate/dextrose/adenine-1 unit (not analyzed). All units from the Mayo Clinic were stored in additive solution-3. After adjusting for prewash cell-free hemoglobin, there was still evidence of postwash differences by additive solution group (overall $P = 0.021$). Specifically, in *post hoc* pairwise comparisons, there is evidence that for postwash, additive solution-3 was associated with 53% higher cell-free hemoglobin compared to additive solution-1 units (estimated ratio [95% CI], 1.53 [1.06 to 2.22], $P = 0.027$) and 91% higher concentrations compared to additive solution-5 units (1.91 [1.11 to 3.28], $P = 0.022$). There were also significantly more platelet derived microvesicles postwash in the additive solution-3 units (11.19 [2.8 to 44.67], $P = 0.001$ and 2.08 [1.34 to 3.25], $P = 0.002$ respectively) and overall more microparticles in the additive solution-3 units (1.66 [1.03 to 2.69], $P = 0.043$) compared to additive solution-1 units.

Combining the data from both sites, we identified a wide range of concentrations of soluble mediators in the individual units before washing varying over 1,000-fold for chemokine ligand 5, over 10,000-fold for soluble CD40 ligand, and almost 100-fold for total microvesicle counts between units. These data are detailed in table 1. As shown in figures 2 and 3, there were significant reductions in the amount of each marker after washing with a clearance of 93%, 76%, and 86% for soluble CD40 ligand, chemokine ligand 5, and total microvesicles, respectively (significant P value after Bonferroni correction < 0.0055). Only 1%

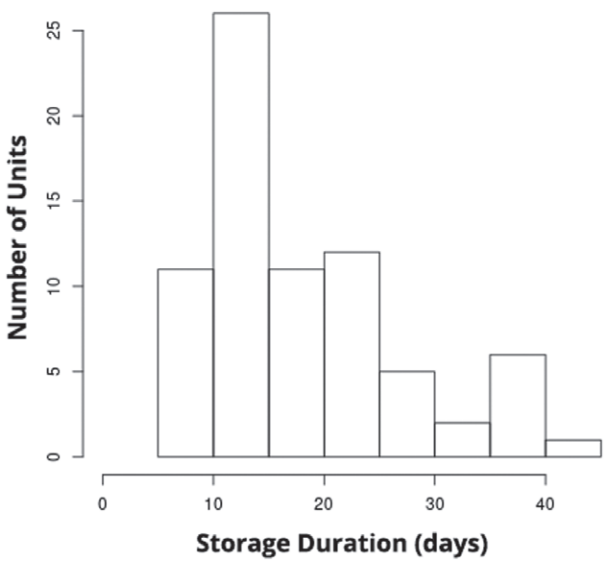

Fig. 1. Histogram of erythrocyte storage duration with storage age ranges on the x-axis and number of units in those categories on the y-axis.

Table 1. Microvesicle Counts and Details of the Microvesicle Cellular Origin from the 74 Paired Erythrocyte Units Pre- and Postwashing in a Cell Saver

	Prewash Median (Interquartile Range)	Postwash Median (Interquartile Range)	*P* Value
Total count per microliter	6,900,000 (4,100,000–20,000,000)	830,000 (330,000–2,800,000)	< 0.0001
Cell markers			
P-selectin, activated endothelial cell or platelet-derived microvesicle	99 (0–350)	20 (0–56)	< 0.0001
Glycophorin A, erythrocyte-derived microvesicle	13,000 (5,400–32,000)	4,600 (2,400–15,000)	0.004
Semaphorin-7A, activated lymphocytes or erythrocyte-derived microvesicle	730 (300–1,800)	130 (43–310)	< 0.0001
Glycoprotein IIb, megakaryocyte/platelet-derived microvesicle	33,000 (21,000–48,000)	6,900 (3,200–17,000)	< 0.0001

Most are of indeterminate cellular origin. After Bonferroni correction, a significant P value is < 0.0055.

of the median counts of microvesicles were identified by a cell-specific marker, but microvesicles from all cell lines were significantly reduced ($P < 0.004$ for all), as shown in table 1. In terms of neutral lipids, there was no difference in the neutrophil priming ability of the erythrocyte supernatants before or after washing ($P = 0.694$).

From a safety standpoint, there was an overall increase in median cell-free hemoglobin concentration in the supernatant from approximately 70 mg/dl to approximately 210 mg/dl as measured by ELISA after two 20-min, 2,000*g* centrifugations. In a *post hoc* study of purchased additive solution-3 units (n = 20), we measured a median (IQR) increase in hematocrit from 62 (58 to 64) to 66 (60 to 70) percent ($P = 0.004$) after the washing procedure, and an expected decrease in product volume from 330 ml to 262 ml (n = 5 test units, not compared); after a 800*g* centrifugation to separate cells and supernatant, the average dose of cell-free hemoglobin from the supernatant of these five test units was less postwash than prewash (85 mg *vs.* 148 mg), mostly due to hemoconcentration. The percent hemolysis of these five test units ranged from 0.1 to 0.3% prewash to 0.1 to 0.2% postwash, all less than the acceptable upper limit of 0.8%. The percent hemolysis was not calculated for all 75 washed patient units, as hematocrit and volume pre- and postwash were not able to be measured with the time pressure of the clinical workflow.

Discussion

The reported data support the feasibility of bedside washing of allogeneic erythrocyte units in the setting of elective major cardiac surgery both in the operating room and in the early intensive care unit course, with nearly all of the units assigned to the washed group being washed. Additionally, bedside washing of prediluted, allogeneic erythrocyte units was able to eliminate the vast majority of cytokines, chemokines, and microvesicles. This study suggests bedside washing with this cell saver is feasible, is safe, and greatly reduces the concentrations of potentially injurious factors in the erythrocyte supernatant.

Transfusion-related hyperkalemic cardiac arrest has previously been described in adult and pediatric patients,[26] and bedside washing has successfully reduced hyperkalemia in small case series[27] (useful especially in pediatric cases, recipients with renal failure, or massive transfusions). Washing cellular blood products in pediatric cardiac surgery has also been shown to reduce lactate concentrations[28] and proinflammatory markers[29] in transfusion recipients. The removal of cytokines, chemokines, and microvesicles that have previously been associated with transfusion-associated respiratory complications supports the hypothesis that bedside erythrocyte washing may provide a means for abrogating the effect of injurious mediators in the supernatant, especially in at-risk patients. In a study of adverse reactions to platelet transfusions[4] (the product most commonly associated with reactions), Cognasse *et al.* identified that most patients with an adverse reaction had received a higher concentration of soluble CD40 ligand, and the "pathogenic threshold" they calculated was 6.4 ng/ml. In contrast, the concentrations in our units before washing (albeit using a different assay platform) were higher, with median (IQR) 143 (1 to 338) ng/ml prewash and 0 (0 to 0) ng/ml postwash. Based on this, we believe our washing procedure is capable of removing enough of this cytokine to possibly be able to reduce transfusion reactions with the potential to improve clinical outcomes in a larger, suitably powered study. By effectively removing the vast majority of protein,[14,30] bedside washing offers the possibility of also reducing antibody-mediated transfusion-related acute lung injury. We did not specifically test for immunoglobulins, though, so removal of these antibodies remains speculative.

The elimination of neutral lipids was ineffective with our protocol, which was surprising, as this has previously been demonstrated, albeit with a blood bank washing protocol and using blood stored for 42 days.[8] Whether residual lipids remained after washing the stored units or were released as a result of hemolysis induced by washing is unknown. Regardless, the remaining lipids were capable of neutrophil priming and, therefore, theoretically capable of inducing lung injury or activating endothelium and predisposing to later injury.[10]

As has been previously reported,[31] erythrocyte washing incurred a threefold increase in cell-free hemoglobin concentration in the reduced-volume supernatant, although,

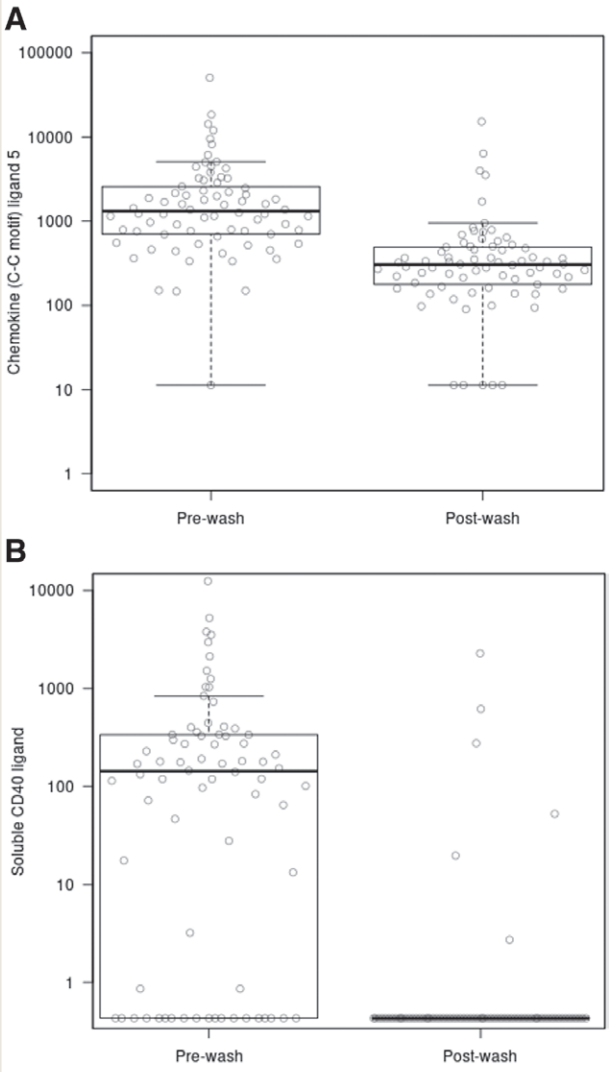

Fig. 2. Concentrations of chemokine ligand 5/regulated on activation, normal T expressed and secreted, a leukocyte derived proinflammatory chemokine (*A*), and soluble CD40 ligand, a leukocyte derived platelet proaggregatory protein (*B*), in blood units pre- and postwash. The axis reflects a logarithmic scale. *Box and whiskers* represent median (interquartile range) and 10th to 90th percentiles in nanograms per milliliter.

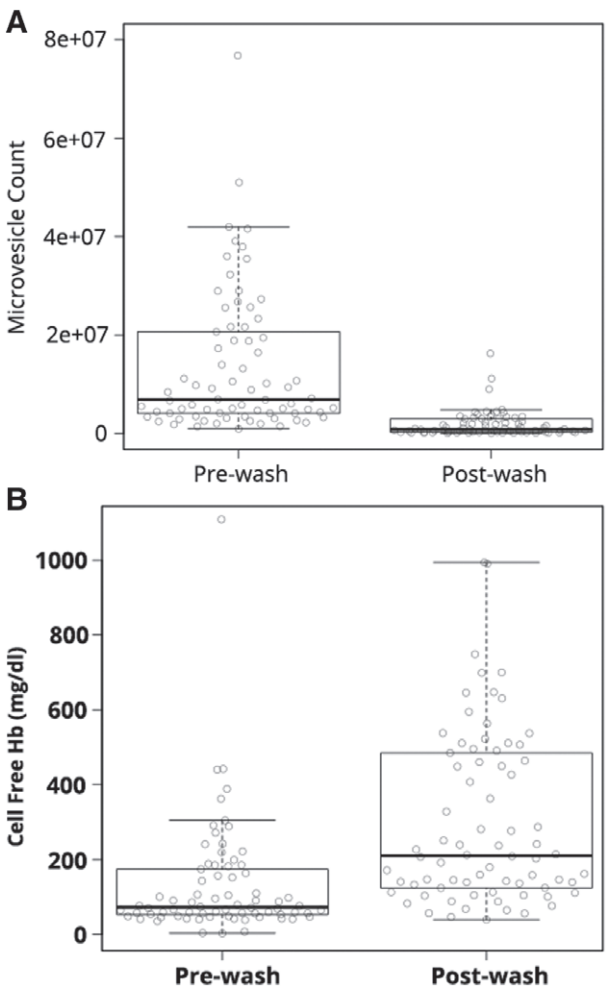

Fig. 3. Total microvesicle count (*A*) and cell-free hemoglobin concentrations (Hb; *B*) in milligrams per deciliter from blood units pre- and postwash. The axis reflects a linear scale.

when calculated, percent hemolysis remained within the acceptable range (less than 0.8%).[32] Our findings regarding additive solutions were unexpected, although the mannitol in additive solution-1 may promote membrane stability[33] and, ideally, reduce hemolysis. They could also reflect variations in erythrocyte processing techniques or interdonor variability associated with the blood centers that favor additive solution-3. As mentioned, the extent of hemolysis, in the small subset we tested, was well below the acceptable level of 0.8%.[32] Similar findings were reported in the "Randomized Trial of Red Blood Cell Washing for the Prevention of Transfusion-associated Organ Injury in Cardiac Surgery" (REDWASH) study in that cell-free hemoglobin in the supernatant of washed units was higher than controls, but there was no increase detected after transfusion *in vivo*, likely due to the avid scavenging of free hemoglobin by haptoglobin in the plasma.[34] There are also some potentially important differences between the REDWASH study and our trial: we prediluted erythrocyte units containing additive solutions-1, -3, and -5 with saline, whereas they used undiluted units with a different additive solution. We also used a more rigorous washing setting on the same cell saver device. The physiologic relevance of the balance between increased cell-free hemoglobin and removal of nitric oxide scavenging microvesicles[35] will await future comparisons of the clinical outcomes between the washed *versus* standard cohorts in the parent study, although there is no reason to expect this to differ from REDWASH.

Similarly, lung injury biomarkers were not included in this manuscript, as measuring the recipients of these 75 units only from the intervention arm of the parent trial was not powered or designed to detect any difference. The results of the clinical biomarkers and outcomes associated with the parent clinical trial have not yet been analyzed or published.

Regarding the microvesicle evaluations, their characterization revealed an identifiable cell lineage in only approximately 1% of detected signals, consistent with previous publications from our group and others.[36,37] Whether they lost the antigens we targeted during storage, the level of antigen per microvesicle was not high enough to detect by flow cytometry, or we should have used a broader panel to identify relevant antigens is not clear. Alternatively, it is possible that the majority of microvesicles detected represent cell debris or vesicles derived from internal multivesicular bodies rather than vesicles that bear cell surface markers, as has been shown previously.[38] Of note, whether the physiologic effect of cell debris *versus* specific cell-derived encapsulated vesicles is equivalent or not is unknown. However, total microvesicle content derived from erythrocyte units has been shown to support prothrombinase activity and thrombin generation,[39,40] so all may be biologically relevant.[41] Regardless, there were substantial and significant decreases in microvesicle counts for those with identifiable cell lineage as well as for those without.

Our study has important limitations. First, our exclusive use of an apheresis belt system on a high-quality wash program setting precludes generalization of our study findings to other cell washing devices. Regarding the more common Latham bowl design, certain models have been used successfully to wash erythrocyte units,[42] while others appear to induce excessive hemolysis.[43] Second, we were limited to the Food and Drug Administration–approved saline washing solution, whereas the balanced solution Plasmalyte-A (Baxter, USA), appears to be associated with less hemolysis than saline, which has an unphysiologically high chloride level of 154 mM and an acidic pH of 5.5.[44] Future bedside washing protocols to be evaluated include simplifying the workflow by dilution with 1,000 (approximately 1:3) rather than the current 1,200 ml (1:4), using other cell saver devices, optimizing erythrocyte by diluting and washing with Plasmalyte A or other balanced, neutral solutions or even reversing aspects of the erythrocyte storage lesion by replenishing 2,3-diphosphoglycerate or removing toxic phthalates.[45] While our median postwash cell-free hemoglobin value of 210 mg/dl is similar to a mean postwash value of 212 mg/dl previously reported using a spectrophotometric technique,[43] the extreme values seen with our ELISA platform that required serial dilutions for most of our samples present the possibility of error. We only systematically calculated percent hemolysis in a small subset of washed units, but this would be an essential component of a future quality control process, and using spectrophotometric techniques such as that used to measure hemoglobin in our quality control subset may offer a simpler solution. Our reported median value of 210 mg/dl would result in an approximately 3 to 5 mg/dl increase in cell-free hemoglobin (normal value less than approximately 15 mg/dl), assuming a 5,000 ml circulating volume and hematocrit of 0.25, but only if the natural hemoglobin scavenger, haptoglobin, were absent. While an increase in cell-free hemoglobin above the normal limit is not expected, confirmation of the safety of this bedside washing procedure will require sampling recipients' plasma.

A third limitation relates to the external validity of our feasibility findings. The two enrolling institutions leveraged research personnel, anesthesia technicians, perfusionists, and transfusion medicine specialists to oversee the bedside washing procedures for the purposes of the study. However, study procedures can be simplified and plausibly performed by existing personnel in the operating room. Allogeneic units can be washed in the cell saver being routinely used, and the only additional workflow would involve gravity draining the saline and erythrocyte unit into the reservoir of the cell saver using a standard "Y-spike" set. Further processing is automated, and the staff perfusionist (assisted by anesthesia personnel during bypass) could realistically perform this task. This scenario is limited to the operating room environment and our data are limited to the CATS, as we did not test other Latham bowl cell saver designs. Extending the process into the intensive care unit would be more complex and likely to require additional staffing with variable, local, economic implications.

Finally, we did not correlate the current study results with clinical outcomes, as this study was not designed or powered to do so. To be clear, this report is limited to the assessment of our ability to wash allogeneic erythrocyte units in the setting of elective cardiac surgery and to quantify the removal of soluble mediators from the allogeneic erythrocyte units. It is an *a priori* planned substudy of a parent clinical trial that has completed enrollment, but is pending recipient outcome assessments, data analysis, and publication. While this is an important first step in understanding the potential role of bedside allogeneic erythrocyte washing, it is insufficient evidence to support bedside washing for the prevention of transfusion-related respiratory complications. The primary aims of the parent trial, from which the current work arises, will address this question.[11]

In summary, this study demonstrates that bedside washing of allogeneic erythrocytes with a familiar cell saver technology is feasible and effective in removing soluble mediators immediately before transfusion. In acute care environments such as the operative setting, where autologous cell salvage is frequently employed, this technology may offer a practical approach to removing mediators associated with adverse transfusion reactions.

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Competing Interests

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Softly Treading *Digitalis*'s Timeline, Foxglove Has Fingers in Botany and Medicine

Legend has it that fairies fashioned petals of the Foxglove (*Digitalis purpurea, right*) into slippers to assist foxes in sneak(er)ing through the garden. Though etymologists may disagree on how foxglove came by its name, horticulturalists have long brandished the spiring stalks with vibrant flowers as biennial favorites. Ancient healers and patients appreciated the plant's deadly potential, reflected in nicknames like "Dead Men's Bells" and "Witches' Gloves." Less mysteriously, in 1542, botanist Leonhart Fuchs dubbed the plant *Digitalis*, from the Latin *digitus* for "finger." Not until the end of the nineteenth century were foxglove's medicinal properties formally recognized, and its active components, cardiac glycosides digoxin and digitoxin, isolated. Using memorable names and attractive packaging for the blossoming consumer culture, pharmaceutical companies marketed foxglove by the early 1900s as a staple for treating "dropsy," or heart failure. The American boxes from Parke, Davis & Co. (*upper left*) and Murray & Nickell (*upper middle*) date from the 1910s; the one from CIBA (Digifoline, *lower left*) from the 1930s. (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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