Proven and potential clinical benefits of washing red blood cells before transfusion: current perspectives

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Abstract: Red blood cells (RBCs) are washed for a variety of reasons such as to remove excess potassium, cytokines, and other allergen proteins from the supernatant and/or to mitigate the effects of the storage lesion. The storage lesion is a product of RBC aging and include leakage of potassium and chloride from the RBCs, depletion of 2,3-diphosphoglycerate and adenosine triphosphate, loss of phospholipids and cholesterol, exposure of phosphatidylserine, elaboration of lipid mediators, loss of glutathione, autoxidation of hemoglobin to methemoglobin contributing to decreased blood flow viscosity and adherence to endothelial cells, increased microparticle formation, and disruption of NO-mediated vasodilation. A storage lesion is thought to be caused in part by oxidative stress, which is characterized by functional and structural changes to the RBCs. The effects of the RBC storage lesion on patient morbidity and mortality have been studied intensively with mixed results. Here, we will summarize the potential benefits of RBC washing. Notably, all patient-based studies on washed RBCs are single-center, small randomized studies or observational data, which await replication and tests of generalizability. Some of the most promising preliminary data suggest that washed transfusions of red cells and platelets reduce mortality in low risk, younger patients with acute myeloid leukemia, mitigate lung injury, and substantially reduce mortality in cardiac surgery. Larger randomized trials to replicate or refute these findings are urgently needed and, most importantly, have the potential to strikingly improve clinical outcomes following transfusion.

Keywords: washed blood, transfusion, immunomodulation, red blood cell

Introduction
Blood transfusions are among the most common procedures performed in hospitals.1 They entail risks such as transfusion-related acute lung injury (TRALI), transfusion-associated graft versus host disease, transfusion-associated circulatory overload, immunomodulation, inflammation, infection, and thrombosis.2–13 The effect of the storage age of red blood cells (RBCs) on patient morbidity and mortality has been the subject of numerous studies with contradictory findings.14–27 As blood ages, the RBCs develop a “storage lesion” that includes leakage of potassium and chloride from the RBCs, depletion of 2,3-diphosphoglycerate and adenosine triphosphate, loss of phospholipids and cholesterol, exposure of phosphatidylserine, elaboration of lipid mediators, loss of glutathione, autoxidation of hemoglobin to methemoglobin, decreased blood flow viscosity and adherence to endothelial cells, increased microparticle formation, and disruption of nitric oxide (NO)-mediated vasodilation.28–34 Free hemoglobin, heme and iron, some within microparticles, may be significant mediators of toxicity. The...
storage lesion is hypothesized to be due, at least in part, to oxidative stress, which causes functional and structural changes to the RBCs.35-38

One of the primary causes of febrile nonhemolytic transfusion reactions (FNHTRs) are cytokines produced by white blood cells (WBCs).39 The rate of FNHTRs has substantially decreased following widespread implementation of leukoreduction; however, there are still some WBCs remaining in the blood products and WBC-derived cytokines increase as the blood product ages.40 Some of the cytokines and chemokines that have been shown to increase during storage of RBCs and platelets are interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α.40 Notably, these cytokines and chemokines have been implicated in FNHTRs. Other cytokines and chemokines have also been shown to accumulate such as RANTES (regulated on activation, normal T cell expressed and secreted; CCL5), which is associated with allergic transfusion reactions;41 soluble CD40 ligand (sCD40L), which induces pro-inflammatory mediators such as IL-6, IL-8, and MCP-1 and may be associated with TRALI;10,12 and Fas ligand and TGF-β, which may contribute to transfusion-related immune modulation.42,43 A recent study by Muszynski et al44 showed that soluble mediators in the RBC supernatant from 30-day RBC units induced monocyte suppression in transfusion recipients. This group has previously observed that critically ill children have a suppressed monocyte function following transfusion of RBC units stored for long periods of time.45,46 Thus, longer stored RBC units have been shown to accumulate various chemokines and cytokines that contribute to a variety of different types of transfusion reactions.

Three large randomized clinical trials investigated the effects of storage age of blood on recipient morbidity and mortality: Age of Blood Evaluation (ABLE), Red-Cell Storage Duration Study (RECESS), and Age of Red Blood Cells in Premature Infants (ARIPI). The ABLE study conducted at 64 locations in Canada and Europe and evaluated 90-day mortality in critically ill patients receiving fresh (<8 days old) versus longer stored RBCs (mean of 22 days old). No differences in the 90-day mortality between the two groups were detected.47 The RECESS conducted at multiple centers across the US examined clinical outcomes following cardiac surgery in patients (>12 years old) who received fresh RBCs (<10 days old) versus older RBCs (>21 days old). This study did not detect any statistically significant differences in Multiple Organ Dysfunction Scores, 7-day or 28-day mortality.48 The ARIPI trial was conducted at six tertiary neonatal intensive care units in Canada and examined length of stay, nosocomial infections, and organ dysfunction in 377 premature infants weighing <1,250 g who received fresher blood (<7 days old) versus standard issued blood (mean of 14.6 days old). No statistically significant differences were also found in the measured outcomes between both groups.49 Thus, these three large trials support that on average RBC age has little-to-no effect on outcome. However, these studies do not fully address the safety of transfusion of red cells near the end of storage (eg, 35–42 days) because few patients in these trials received transfusion of such longer stored red cells.

Among others, a large multicenter study, the Age of Blood in Children in Pediatric Intensive Care Units (ABC PICU) study, is ongoing. This randomized clinical trial is enrolling 1,538 children and comparing development of new or progressive multiple organ dysfunction syndrome in critically ill children transfused with either RBCs stored ≤7 days or standard issued RBCs (expected mean storage duration of 17–21 days).50 Thus, the question of the effect of RBC age on patient morbidity and mortality is still of concern to many clinicians. Particularly important, none of these studies included patients receiving large doses of much longer stored red cells (eg, >28 days or >35 days). This is relevant because there are robust epidemiologic and observational data indicating that such longer stored RBC may indeed be associated with increased morbidity and mortality, particularly in vulnerable populations such as infants undergoing cardiac surgery.51,52

One potential approach to mitigating the RBC storage lesion is to wash the RBC unit and remove most accumulated mediators, free hemoglobin, microparticles, etc. RBC washing is commonly done for patients with repeated allergic or febrile transfusion reactions; however, it is not routinely available at many medical centers. This review will focus on the potential and hypothesized benefits of washing RBC units prior to transfusion.

**EFFECT of RBC storage lesion on blood transfusion**

As mentioned earlier, the storage lesion refers to molecules and other products that accumulate in stored RBCs as they age. Washing of RBCs removes much of what accumulates in stored RBCs such as microparticles and free hemoglobin. Additionally, washing also removes the RBC storage solution as well as additive solution, plasma proteins, and some of the contaminating WBCs, platelets, and cellular debris. The question is what effects have these substances been shown to have on blood transfusion? Remy et al53 have written an excellent review on the influence of the storage lesion on pediatric transfusion; however, the overall effects on RBC
transfusion will be discussed below. D’Alessandro et al\textsuperscript{16,54} have shown that older RBCs have decreased antioxidant activity and impaired energy metabolism. Proteolytic enzymes and kinases become activated leading to modification of Band 3 and other RBC structural proteins that result in RBC cytoskeletal remodeling. This remodeling was shown to result in increased osmotic fragility, increased shedding of microparticles, and RBC morphologic changes.\textsuperscript{3,54} A recent study looked at RBC deformability before and after transfusion in 16 patients undergoing spinal fusion surgery. RBC deformability was found to be significantly decreased following transfusion in patients receiving \textgtr 5 units of RBCs. Elongation index measured by ektacytometry decreased by 12\%\pm4\% to 20\%\pm6\%, \( P=0.03 \) as compared to patients receiving 0–4 RBC units (3\%\pm1\% to 4\%\pm1\%, \( P=0.68 \)). Notably, these changes did not reverse when measured 3 days postoperatively. Additional studies have shown that as RBCs age, they develop an increasing oxidative environment, which results in fatty acid oxidation and other oxidation metabolites.\textsuperscript{3,54,55} Similarly, after 14 days, the RBCs have impaired energy metabolism.\textsuperscript{3,54,55} A recent study of 34 patients undergoing spinal fusion examined oxidative stress as measured by the presence of fluorescent heme degradation products and methemoglobin as measured by spectrophotometric methods as a function of RBC storage time. In this study, oxidative stress was found to increase as storage time increased (\( R=0.54, P=0.032 \)).\textsuperscript{56} Additionally, oxidative stress was also higher in stored RBCs as compared to fresh RBCs (9.1\pm10.3 vs 7.7\pm0.9 fluorescent arbitrary units; \( P<0.001 \)).\textsuperscript{56} RBC deformability was also found to decrease with increased storage time (\( R=0.60, P=0.009 \)).\textsuperscript{56} Using a proteomics approach, D’Alessandro et al\textsuperscript{57} showed that as RBCs age, free hemoglobin is released into the supernatant as well as glyceraldehyde-3-phosphate dehydrogenase, peroxiredoxin-1, -2, and -6, carbonic anhydrase-1 and -2, selenium binding protein-1, biliverdin reductase, aminolevulinate dehydratase, and catalase. Older RBCs contain a large number of hemolyzed RBCs and free iron. Animal studies have shown that transfusion of old RBCs results in a bolus of free iron, which results in oxidative damage and potentiates bacterial proliferation.\textsuperscript{58,59} Sarachana et al\textsuperscript{60} have shown that several small noncoding RNAs in stored RBCs have levels that correlate with cell death, adenosine triphosphate loss, and changes in RBC indices.

To date, there have been > 50 observational clinical studies of transfusion in humans, which have been excellently reviewed by Qu and Triulzi.\textsuperscript{14} Notably, some of these studies have shown that RBCs stored for longer periods of time are associated with increased risk of sepsis, multiorgan failure, myocardial infarction, thrombosis, pneumonia, and death. However, others showed no effect of RBC storage age on any of these outcomes, which illustrates uncertainty.

**RBC washing**

Washing process of RBCs is typically performed by normal saline (0.9\% NaCl) in either an open or a closed system. The washing procedure removes ~95\%–99\% of the RBC supernatant, which contains in addition to the additive solution, plasma proteins, electrolytes, some WBCs, platelets, microparticles, and cellular debris.\textsuperscript{13,61} RBCs washed in an open system should be used within 24 hours postwashing due to the theoretical increased risk for bacterial contamination, as well as RBC viability in normal saline.\textsuperscript{62} RBCs washed in a closed system have an expiration time of 14 days.\textsuperscript{62} There have been no observed differences in the safety and efficacy of RBC washed in the closed system with extended expiration versus the open system.\textsuperscript{63} RBC washing is frequently used in neonates and infants undergoing cardiac surgery. One important reason for this practice is the reduction in extracellular potassium that once transfused can cause hyperkalemia in the patients.\textsuperscript{54,64} Notably, Masalunga et al\textsuperscript{65} studied saline washed versus unwashed RBCs and the effect on neonatal extracorporeal membrane oxygenation (ECMO). They found that washing reduced the extracellular potassium but increased the RBC membrane osmotic fragility, which led to increased hemolysis of the washed RBCs within the first 3 days of ECMO.\textsuperscript{65} The benefits of washing RBCs have been attributable to both elimination of toxic accumulations of substances such as free hemoglobin and potassium in the supernatant and removal of the more fragile cells. However, RBC washing may also increase the RBC osmotic fragility, leading to increased hemolysis following transfusion.\textsuperscript{14,65}

Bennett-Guerrero et al\textsuperscript{66} washed 40 units of 40–42-day-old RBCs using two different washing devices, the Cobe 2991 (Terumo BCT, Lakewood, CO, USA) and the Haemonetics Cell Saver Elite (Haemonetics, Braintree, MA, USA). The Cobe 2991 is a device used in a variety of procedures for washing and concentrating cellular components and is typically used in the setting of a blood bank. The Haemonetics Cell Saver Elite is a point-of-care device used for processing autologous shed blood typically used during operating room procedures. Both devices effectively removed the extracellular potassium and other toxic substances from the supernatant and recovered a significant number of starting...
RBCs.\textsuperscript{61} The levels of plasma-free hemoglobin, hemolysis, and RBC microparticle production were found to be higher in RBC units washed using the Haemonetics Cell Saver Elite.\textsuperscript{61} Thus, depending on the washing device used, it is possible that washing RBC units may cause additional damage.

RBC washing is not widely available. Many smaller and medium-sized hospital blood banks do not have a cell washer available. Additionally, some blood banks do not hold an FDA permit to modify blood products, which would preclude them from washing RBCs. In addition to the widespread lack of availability of RBC washers, the RBC washing process takes ~1.5–2 hours. This delays RBC availability and precludes use of washed RBCs in cases of emergency and in massive transfusion protocols. RBC washing also requires increased technologist time to prepare the RBC unit and widespread use of RBC washing could necessitate increased FTEs in the blood bank. As noted earlier, most RBC washers used are open systems and thus the washed RBC units have an expiration of 24 hours. This shortened life span could generate increased RBC wastage and cost if the washed units are not used prior to expiration.

Reinhart et al\textsuperscript{66} studied the effect of washing RBCs in 1% albumin solution versus normal saline, buffer, or plasma to determine if the RBC echinocyte shape change that develops during storage could be reversed or improved. They found that washing in 1% albumin decreased the proportion of echinocytes and increased the proportion of discocytes. They also observed a decrease in RBC swelling with albumin washing. These improvements were not observed when the RBCs were washed in normal saline, buffer, or plasma. Notably, the RBC shape normalization following washing in 1% albumin did not improve RBC deformability as measured by ektacytometry; however, it did improve the perfusion of an artificial microvascular network.\textsuperscript{66} Thus, washing may be able to improve the shape changes caused by RBC storage for prolonged periods. Further studies are needed evaluating different washing solutions. Normal saline has been reported in preliminary data to increase hemolysis during washing compared with a balanced salt solution (Plasma-Lyte A).\textsuperscript{67}

**Washing RBCs and leukoreduction**

WBCs in blood products are an important cause of transfusion complications, FNHTRs, platelet refractoriness, CMV transmission, and postoperative nosocomial infections. As described previously, the rate of WBC-derived proinflammatory cytokines increases over the storage period.\textsuperscript{68–71} Notably, transfused WBCs may be the target of recipient antibodies that can stimulate the WBCs to release cytokines. Universal leukoreduction has many benefits including reduction of FNHTRs,\textsuperscript{72–74} decreased platelet refractoriness caused by alloimmunization against leukocyte antigens,\textsuperscript{75–77} decreased transmission of CMV,\textsuperscript{78,79} and decreased postoperative infections.\textsuperscript{80,81}

A study by Aston et al\textsuperscript{82} examined whether additional washing of leukoreduced RBC units would reduce production of human leukocyte antigen (HLA)-specific antibodies in children with chronic kidney disease. They studied 106 children and found no difference in HLA sensitization risk between patients receiving nonwashed leukodepleted RBC and patients receiving washed leukodepleted units ($P=0.32$).\textsuperscript{82} They hypothesized that this lack of difference was attributable to the small amount of leukocytes that remained after washing, as washing removed only ~33% of the small number of leukocytes that were present.\textsuperscript{82}

Universal leukoreduction has also been shown to decrease the risk of transfusion-related immunomodulation.\textsuperscript{83} Animal and human studies have shown that allogeneic blood products cause downregulation of cellular immunity and dysregulation of inflammatory innate immunity.\textsuperscript{84} The majority of data support that the storage supernatant is a large contributor to transfusion-related immunomodulation. Several studies have shown that transfused RBCs affect the perioperative release of inflammatory mediators in cardiac surgery patients.\textsuperscript{85} Similarly, studies have shown that washed RBCs are superior to unwashed RBCs when fresh RBC are not available for children undergoing cardiopulmonary bypass.\textsuperscript{86} A randomized trial in adults with leukemia has shown that patients receiving washed, leukoreduced, ABO identical products had substantially improved survival (approximately halving of mortality, $P=0.014$) as compared to patients receiving unwashed, leukoreduced, and ABO identical products.\textsuperscript{11,87} These results have been replicated in our center after implementation of universal washing for younger patients with acute myeloid leukemia (unpublished data). Additionally, Cholette et al\textsuperscript{88} found that children undergoing cardiac surgery who received washed, leukoreduced blood products had lower levels of inflammatory markers when compared to children receiving unwashed, leukoreduced products, and a trend toward reduced mortality.

**RBC washing and neonates**

Premature infants typically suffer from anemia of prematurity due to a multitude of reasons, including decreased levels of plasma erythropoietin, decreased RBC lifespan, blood loss due to phlebotomy, and decreased transport of iron due to premature birth.\textsuperscript{89} Several studies in neonates indicated that
RBC transfusions may be related to necrotizing enterocolitis, chronic lung disease, intraventricular hemorrhage, retinopathy of prematurity, and death.96–99 The ARIPi trial examined whether RBCs stored for <7 days as compared to the standard of care decreased rates of infection or organ dysfunction in infants with birth weights <1,250 g.97 The ARIPi investigators found that the mean age of transfused blood was 5.1 days in the fresh RBC group (n=188) and 14.6 days in the standard group (n=189). The rate of necrotizing enterocolitis, retinopathy of prematurity, bronchopulmonary dysplasia, intraventricular hemorrhage, and death was similar among neonates in the fresh RBC group (n=99, 52.7%) compared with the standard RBC group (n=100, 52.9%). The rate of clinically suspected infection was also similar in the fresh RBC group 77.7% (n=146) compared with 77.2% (n=146) in the standard RBC group.49 The lack of difference observed may have been due to the small difference in the age of the blood studied (5.1 days vs 14.6 days). RBCs typically do not expire until 35 days (CPDA-1) or 42 days (AS), and relatively young RBC units were used in this study. Only a small number of patients received significant amounts of longer stored red cells.

Notably, other transfusion reactions such as TRALI may be underrecognized and underreported in the neonatal population.96,97 One possible explanation for these associations with RBC transfusion is the “two-hit” model of posttransfusion injury proposed by Aiboshi et al.98 In this model, a small number of patients received significant amounts of relatively young RBC units were used in this study. Only a small number of patients received significant amounts of longer stored red cells.

Washing RBCs and transfusion reactions
Washing of RBCs has been shown in several studies to reduce and prevent transfusion reactions. Crews et al100 reported that washing of RBCs was an effective measure in preventing acute hypotensive transfusion reactions in patients unable to metabolize vasodilators present in the donor unit. Similarly, Blumberg et al101 reported that over a 14-year period, the incidence of transfusion-associated circulatory overload and TRALI at a large academic medical center (>800 bed) was eleven out of 319,161 leukoreduced nonwashed and zero out of 97,445 leukoreduced washed RBC and platelet products. Notably, they also reported that the incidence of febrile transfusion reactions to leukoreduced washed platelets...
is close to zero as compared to 1%–2% in the leukoreduced nonwashed platelet transfusions.83

Washing RBCs and trauma
Several observational studies have suggested that transfusion of longer stored RBCs is linked to increased incidences of acute lung injury, acute kidney injury, pneumonia, and mortality in trauma bleeding patients.109–112 The underlying hypothesis is that the older RBCs once transfused, scavenge NO in an iron/heme-dependent manner.30,113,114 Additionally, the older RBCs are believed to be less effective at stimulating endogenous NO formation.113,114 Thus, NO homeostasis is lost.30,113,114 In a canine model, washing of stored canine RBCs has been shown to prevent infectious lung injury in addition to other side effects.115 Similarly, a study by Stapley et al.,114 which evaluated RBC washing in a mouse trauma-hemorrhage model, found that washing 10-day stored red cells protected against its lethality, which was ~90% without washing. Notably, they also found that transfusion with free heme partially restored the toxicity of the older washed RBCs.114 Additionally, they found that inhibition of TLR4 signaling, administration of TAK-242, or hemopexin-dependent sequestration of free heme decreased the storage age-dependent lethality of the RBCs.114 These two animal studies suggest that part of the storage lesion toxicity of RBCs is likely attributable to the disruption of NO homeostasis. Additional studies in humans are needed to further evaluate this phenomenon. Finally, in one negative study and for unclear reason, washing did not abrogate the deleterious effect of red cell transfusion in a mouse model of inflammation.116 The investigators proposed that membrane-encapsulated hemoglobin is required to produce inflammation as there was no inflammatory response to transfusions of either membrane ghosts or stroma-free lysate derived from the stored RBCs. It is possible that the RBCs studied had a low level of bacterial contamination that was not detected despite culturing the cells. However, the investigators did trial injecting the cells with lipopolysaccharides and found a different cytokine profile, making this less likely.116

Future directions
As detailed earlier, there have been very few studies looking at the benefits, risks, and effects of washing RBCs. Additionally, there have been even fewer clinical trials to explore washed RBC usage. A search of clinicaltrials.gov in June 2016 showed several studies on washed RBCs to be ongoing and actively recruiting. Thus, hopefully, some answers will soon be available. The Mayo Clinic, Duke University, and Blood Systems Research Institute have an ongoing study (NCT02094118) that is examining point-of-care washing of allogeneic leukoreduced RBCs to see whether this treatment reduces pulmonary complications in cardiac surgery patients. Seoul National University Bundang Hospital is actively engaged in another study (NCT01934907) in which patients with various orthopedic hip problems such as avascular necrosis, etc are receiving washed RBCs and they are examining the hypothesis that washed units can decrease transfusion complications. In another study at Duke University (NCT02485366), researchers are investigating whether rejuvenated, washed RBCs improve outcomes in children undergoing cardiac surgery. Similarly, at Duke University, researchers are investigating the use of Rejuvesol® treated and washed RBCs in sickle cell patients (NCT02731157). Researchers at the University of Miami along with the NHLBI (NCT01185600) are comparing washed RBCs that have microparticles removed to unwashed RBCs with microparticles and examining the differences in endothelial disturbances, inflammatory responses, and procoagulant responses. The last ongoing study of washed RBCs is our own study at the University of Rochester (NCT01976442), which examines the outcomes of adult acute leukemia patients treated with washed RBCs. Thus, in the next few years, hopefully, these studies will yield more detailed answers regarding the benefits, risks, and effects of washing RBCs. It is still quite likely that questions will still remain, and additional studies are needed.

Authors’ point of view
It is our point of view that washed RBCs are beneficial to certain patient populations under certain circumstances. We do not advocate for universal use of washed RBCs as this is cost prohibitive, time consuming, and not feasible under many circumstances such as acute bleeding and MTPs. We have found clinical benefit in using washed RBCs in adult acute leukemia patients <50 years of age. Additionally, we have found clinical benefit in using washed RBCs in patients who have had repeated febrile transfusion reactions. Similarly, patients with IgA deficiency receive washed RBCs. We also provide washed RBCs (first unit) to all pediatric cardiac patients when a bypass pump is being used. We have sufficient staffing to be able to wash the required units in a timely fashion and rarely expire washed products. Thus, we do propose and believe that washed RBC products
have clinical benefit. We do acknowledge that more studies are needed and that little is known as to why washed RBCs provide a benefit to certain patient populations over unwashed RBCs.

**Conclusion**

The previous sections have detailed the numerous studies that have evaluated RBC washing in humans and animals. These studies suggest that washing of RBCs is safe and can sometimes effectively mitigate alloimmune storage lesion effects due to supernatant and perhaps enhance the proportion of normal RBCs. The majority of RBC washing is performed with normal saline and as described previously, additional studies are needed to evaluate other washing solutions such as buffered physiologic salt solutions and 1% albumin, which may be less toxic to both red cells and patients. Finally, the studies described previously suggest the likelihood that washed RBCs can reduce inflammation, morbidity, and mortality in some clinical settings. However, larger randomized clinical trials are needed to replicate and further evaluate these preliminary findings.

**Disclosure**

The authors report no conflicts of interest in this work.

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