Rapid bedside rejuvenation of red blood cell with an autologous cell salvage device

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Background and Objectives During storage, red blood cells (RBCs) undergo physicochemical changes which affect the quality, function, and in vivo survival of transfused packed RBCs (pRBC). Changes include decreased 2,3-diphosphoglycerate (2,3-DPG) levels, decreased ATP, changes in mechanical properties and oxidative injury. RBC rejuvenation is a method used to increase levels of 2,3-DPG and ATP in pRBCs. This process requires incubating the pRBCs with a rejuvenation solution and subsequent washing. Standard blood bank protocols using the COBE 2991 Cell Processor require several hours of preparation. The objective of this study was to verify if a bedside protocol for rejuvenating pRBC and washing with the Sorin Xtra autologous cell salvage system could be used.

Materials and Methods Outdated pRBC units were obtained and rejuvenated in a model operating suite using a dry air incubator for 1 h at 37°C. Six units of pRBCs were pre-diluted with saline (1000 ml) and six units were not pre-diluted with saline. All units were washed with normal saline (1000 ml) using an apheresis-design cell salvage device in manual mode and wash volume set to 3000 ml. Samples were collected and analyzed for standard RBC quality parameters at baseline and post-wash.

Results Total pRBC wash efficiency was 94%–12% at a final hematocrit of 67.7–5.9% while maintaining post-wash hemolysis 0.24–0.12%. Pre-dilution prior to washing did not confer statistically significant differences in final RBC quality parameters with the notable exceptions of calculated hemolysis and supernatant potassium levels (P < 0.05). The washing process can be completed within 10 min. The post-wash RBC parameters are appropriate for immediate transfusion to patients.

Key words: blood processing, quality management, transfusion medicine (in general), transfusion strategy.

Introduction

The goal of red blood cell (RBC) transfusion is to treat anaemia and improve oxygen delivery to tissues [1, 2]. However, the direct benefits of RBC transfusion have yet to be confirmed by a prospective controlled clinical trial [1, 3]. Alternatively, there is a large body of research relating to the risks, morbidities and mortality associated with RBC transfusion [1–4]. Regardless of the fact that it is difficult to separate these risks from the co-morbidities and mortality associated with anaemia, transfusion practices have become increasingly conservative over the years [1, 5]. Under this lens of a conservative approach to transfusion, the storage age of blood transfusion products has been scrutinized [3, 5, 6]. While the age of blood products was not previously found to be an indicator of
morbidity and mortality by large randomized unblinded studies such as The Age of Blood Evaluation (ABLE) trial and more recently by a double blinded prospective trial, the Standard Issue Transfusion vs. Fresher Red-Cell Use in Intensive Care (TRANSFUSE) trial [7–10], it is clear that over time storage of blood causes morphological, metabolic and biochemical changes in RBCs [11]. The effects of storage on blood products and the resulting physiological, biochemical and metabolic changes are called storage lesions (SLs)[6, 11].

After prolonged liquid storage, visible morphological changes can be observed where RBCs change from their typical discoid shape to a spiked echinoid shape [11]. This reduces cell deformability and increases osmotic fragility, making those cells more prone to lysis under the strain of in-vivo fluidic stressors [11, 12]. Haemolysis that does occur during storage releases proteins, nucleosides, microvesicles and ions such as ubiquitin and potassium that are harmful in high concentrations [7, 12–14]. While it has been shown that these undesirable extracellular components can be reduced to tolerable levels using a simple saline wash, a saline wash does not affect any RBC metabolic changes [15].

Metabolic changes during liquid storage increase the affinity of haemoglobin for oxygen by depletion of 2, 3-diphosphoglycerate (2,3-DPG). Reduced levels of 2, 3-DPG result in a decrease in the partial pressure of O₂ at 50% haemoglobin saturation or p50. This change may bring into question the efficiency of peripheral oxygen unloading of ≥14-day-old liquid stored RBCs following transfusion [16, 17]. While it is unknown whether oxygen delivery is significantly affected by transfusion of stored RBCs, immediate and significant 2,3-DPG concentration deficit in-vivo in patients receiving blood transfusions after surgery do occur[18]. Furthermore, homeostatic restoration of these levels in-vivo is incomplete for up to 72 h post-surgery [18].

Ex-vivo rejuvenation of allogeneic RBCs, with a solution containing sodium pyruvate, inosine, adenine and sodium phosphates, increases the levels of ATP and 2, 3-DPG and increases the p50 of stored RBCs by right-shifting the Oxyhaemoglobin Dissociation Curve towards a state where disassociation of oxygen from haemoglobin is more likely to occur [16, 17]. This is represented by a higher RBC Oxygen Release Capacity (ORC), or the per cent of oxygen removed from haemoglobin across the arterial (100 mmHg O₂)–venous (40 mmHg O₂) pressure gradient [17, 19].

RBC rejuvenation is an FDA-approved method used to increase levels of 2,3-DPG and ATP in packed red blood cells (pRBCs). The rejuvenation process requires incubating pRBCs with a rejuvenation solution for 1 h at 37°C followed by subsequent washing. Standard blood bank protocols using the COBE 2991 Cell Processor (Terumo BCT, Lakewood, CO) or Haemonetics ACP 215 (Haemonetics Corp, Braintree, MA) require several hours of preparation due to logistical constraints such as supply chain management and on-site blood bank management [20].

Intraoperative cell salvage devices are widely used in complex surgical cases to perform autotransfusion of collected shed blood [21]. In vitro characterization of the wash efficiency has demonstrated appropriate reductions of supernatant potassium, proteins and plasma-free haemoglobin can be achieved [22]. Current literature suggests that use of cell salvage systems that produce washed RBCs with potassium and haemolysis at tolerable levels would allow for rapid preparation of rejuvenated pRBCs, thereby making the use of rejuvenated blood products more accessible. The objective of this study was to assess whether a bedside protocol, for rejuvenating pRBC and washing with the Sorin Xtra autologous cell salvage system, could be used with similar results.

Methods

Blood selection criteria

A unit of pRBCs is considered acceptable for transfusion purposes as long as 75% of the cells transfused are circulating 24 h after transfusion. The American Association of Blood Banks (AABB) guidelines place the expiration of blood at 42 days of liquid storage at 1–6°C, as cell viability decreases dramatically after the 42-day mark [5]. To verify our rejuvenation and washing procedure, recently expired leukoreduced CP2D/AS-3 pRBC units were individually sourced from our hospital blood bank. Briefly, each blood product was prepared from 500 ml ± 10% donor whole blood. After collection, blood was soft spun with a Roto Silenta 630 RS (Hettich Benelux, Geldermalsen, NL) at a relative centrifugal force (RCF) of 2498g at 22°C. Leukoreduction of whole blood was achieved using a Leukotrap RC System with a RC2D filter (Haemonetics Corp, Braintree, MA). Anti-coagulation was maintained with a solution of 70 ml citrate phosphate double dextrose (CP2D) and 110 ml of AS-3 (Nutricel® Solution). Prior to the rejuvenation and washing procedure, the pRBC unit was weighed and dated to determine wash efficiency and mean sample age, respectively.

Blood rejuvenation and washing procedure

The pRBCs were weighed and sampled before and after rejuvenation and after washing to determine the RBCs quality and the quantity of RBCs that were recovered after the rejuvenation and washing process; the in vitro recovery of RBCs as a percentage of the original sample.
was then calculated. A total of 12 pRBC units were rejuvenated using rejuvesol® Red blood cell processing solution (Citra Labs, Braintree, MA) in a model operating suite using a dry air incubator (Sahara III, Sarstedt Inc., Newton, NC) for 1 h at 37°C. As autotransfusion devices were originally designed for processing wound blood with a haematocrit (Hct) level under 25%, the effect of a pre-dilution of the banked blood was also tested. Following incubation, six rejuvenated pRBC units were pre-diluted with saline (1000 ml) and six units were not pre-diluted with saline. All units were washed with normal saline (1000 ml) using an apheresis-design cell salvage device (Sorin Xtra, Arvada, CO) in manual mode with the wash volume set to 3000 ml.

Sample analyses
Samples were collected and analysed for standard RBC quality parameters at baseline (pre-rejuvenation), after rejuvenation, and after washing by collecting aliquot samples at each time point and sending them to Tampa General Hospital’s certified pathology lab for analysis. Haematocrit (Hct), complete blood counts (CBC), total protein, albumin, potassium and plasma-free haemoglobin were determined using the Sysmex XN-10 Analysis Module after processing in an Abbott Architect 3600 Accelerator centrifuge an RCF of 5488g for 5 min. Haemolysis was then calculated from haematocrit, free haemoglobin and total haemoglobin.

Additional blood aliquot samples were shipped on wet ice overnight for deproteinization with perchloric acid (PCA), neutralized with potassium carbonate and frozen at –80°C until analysis. PCA extracts were analysed for residual and inosine using high-performance liquid chromatography. Residual inosine was compared to the total known inosine supplied in one vial of rejuvesol® to determine total inosine removal through rejuvenation and washing. Significance was determined using a student’s paired t-test with P value <0.05.

Results
Outdated leukoreduced CP2D/AS-3 pRBC units (n = 12) were acquired from the hospital Transfusion Services. The mean blood storage age on the day of rejuvenation was 43.5 ± 2 days (range: 42–44 days). Baseline haemolysis (0.24 ± 0.12%, CI 95%: 0.06–0.55%) was within standard AABB limits of 1% (Table 1). Pre-dilution prior to washing did not confer statistically significant differences in the final RBC quality parameters that were assessed except for potassium and calculated haemolysis (Table 2). Pre-dilution further reduced the supernatant post-wash potassium concentration and calculated haemolysis significantly (P = 0.0112 and 0.023, respectively). While these endpoints were found to be statistically significant between non-diluted and pre-diluted units, all 12 units ultimately fell within AABB transfusion guidelines and were analysed collectively.

RBC recovery rates were calculated according to the following formula:

\[
\text{RBC recovery (\%)} = 100 \times \left( \frac{V_f \times \text{Hct}_f}{V_0 \times \text{Hct}_0} \right)
\]

where \(V_f\) and \(\text{Hct}_f\) are the post-wash volume and haematocrit and \(V_0\) and \(\text{Hct}_0\) are the pre-rejuvenation volume.

Table 1 Pre-rejuvenation baseline comparison of non-diluted vs. pre-diluted units

| Parameter              | Pre-rejuvenation | Non-diluted (Mean SD) | Pre-diluted Mean (SD) | P value |
|------------------------|------------------|-----------------------|-----------------------|---------|
| Spun HCT (%)           |                  | 65.1 (1.8)            | 58.9 (3.3)            |         |
| Total Hgb (g/dl)       |                  | 19.33 (1)             | 17.83 (1.32)          |         |
| pH                     |                  | 6.68 (0.04)           | 6.79 (0.04)           |         |
| RBC (×10^6 µl)         |                  | 6.94 (0.68)           | 5.89 (0.49)           |         |
| MCV (fl)               |                  | 94.42 (7.42)          | 100.35 (5.39)         |         |
| MCH (pg)               |                  | 28.13 (3.48)          | 30.35 (1.26)          |         |
| MCHC (g/dl)            |                  | 29.73 (1.88)          | 30.27 (1.02)          |         |
| Calculation of % hemolysis |              | 0.24 (0.12)          | 0.24 (0.14)           |         |
| Supt. total protein (g/dl) |              | 1.78 (0.3)           | 1.73 (0.36)           |         |
| Supt. albumin (g/dl)   |                  | 0.89 (1.16)           | 1.22 (0.42)           |         |
| Supt. K+ (mEq/l)       |                  | >20                   | >20                   |         |
| Suppt. plasma free hemoglobin (mg/dl) | | 130 (62.93) | 98.33 (53.82) |         |

Inosine wash out rates

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Table 2 Post-wash outcome comparison of non-diluted vs. pre-diluted units

| Parameter              | Post-wash | Non-diluted (Mean SD) | Pre-diluted Mean (SD) | P value |
|------------------------|-----------|-----------------------|-----------------------|---------|
| Spun Hct (%)           |           | 64.3 (6.53)           | 71.2 (2.3)            | 0.8025  |
| Total Hgb (g/dl)       |           | 19.95 (2.47)          | 22.08 (1.04)          | 0.0802  |
| pH                     |           | 6.68 (0.04)           | 6.69 (0.07)           | 0.7675  |
| RBC (×10^6 µl)         |           | 7.02 (0.58)           | 7.23 (0.43)           | 0.4925  |
| MCV (fl)               |           | 91.65 (6.9)           | 98.72 (5.68)          | 0.0814  |
| MCH (pg)               |           | 28.5 (3.25)           | 30.58 (1.18)          | 0.1714  |
| MCHC (g/dl)            |           | 31 (1.6)              | 31.03 (1.15)          | 0.971   |
| Calculation of % haemolysis |              | 0.3 (0.15)           | 0.13 (0.04)           | 0.023   |
| Supernatant total protein (g/dl) |         | <0.8                | <0.8                 | 0       |
| Supernatant albumin (g/dl) |         | <0.4                | <0.4                 | 0       |
| Supernatant K+ (mEq/l) |           | 3.23 (1.62)          | 1.17 (0.15)           | 0.0112  |
| Supernatant plasma-free hemoglobin (mg/dl) | | 168.33 (90.2) | 100 (31.62) | 0.1105  |
| Inosine wash out rates |           | 97.11% (2.79%)        | 99.29% (0.85%)        | 0.0975  |

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and haematocrit, respectively. Average pRBC in vitro recovery of RBC was 94% ± 12% (CI 95%: 81.2–99.1%) with a final haematocrit of 67.7 ± 5.9% (CI 95%: 34.9–80.7%). All washed pRBC units maintained RBC recovery >80% as required by the AABB. Total haemoglobin concentration paralleled the changes in haematocrit values (Table 3) (Fig. 1). Minor changes were also noted in mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) (Table 3).

Elevated supernatant potassium, albumin and protein levels due to storage were reduced after rapid washing to acceptable transfusion levels (Table 3; Fig. 2). A total of 4996 µmol of inosine was added to each unit with the addition of rejuvesol® which was reduced through the rejuvenation process to 89.9 ± 113.3 µmol/unit after 24 h. This resulted in a calculated washout efficiency of 98.2 ± 2.3%, CI 95%. The washing process was completed within 10 min, pre-dilution on average added an additional 5 min to the washing process. All post-wash RBC quality parameters for pRBCs were appropriate for immediate transfusion to patients.

### Discussion

Given the uncertainty of surgical blood loss, pRBC AABB transfusion requirements, the 24-h dating period applied to rejuvenated pRBCs, and manpower/equipment limitations of current blood bank departments, employing a rejuvenation protocol through the blood bank is difficult to implement [8]. The logistical complications with implementing a rejuvenation program have further been challenged by patient blood management influence on blood bank capabilities and have led to increased consolidation of traditional rejuvenation equipment (i.e. blood washing capability) out of the hospital and into regional blood centres. A rejuvenation and washing protocol based in the operating room could provide clinicians with the ability to deliver rejuvenated pRBCs in a clinically appropriate timeframe. This study demonstrated for the first time appropriate blood banking quality metrics (i.e. haemolysis, potassium and inosine washout) are achievable with routine equipment available within the operating room environment.

The practice of red blood cell rejuvenation is predicated on the idea that restoring RBC 2,3-DPG levels prior to transfusion in an attempt to increase oxygen availability to tissues conveys physiological benefits. Early research

### Table 3 Aggregate rejuvenation outcome

|                         | Pre-rejuvenation | Post-rejuvenation | Post-wash |
|-------------------------|------------------|-------------------|-----------|
| **Mean (SD)**           |                  |                   |           |
| Spun Hct (%)            | 61.98 (4.1)      | 48.85 (5.77)      | 67.73 (5.88) |
| Total Hgb (g/dl)        | 18.58 (1.36)     | 15.13 (1.74)      | 21.02 (2.12) |
| pH                      | 6.73 (0.07)      | 6.72 (0.06)       | 6.68 (0.05) |
| RBC (x10⁹ μl)           | 6.41 (0.79)      | 5.17 (0.63)       | 7.12 (0.5)  |
| MCV (fl)                | 97.38 (6.92)     | 94.68 (7.29)      | 95.18 (7.07) |
| MCH (pg)                | 29.24 (2.75)     | 29.38 (2.71)      | 29.54 (2.57) |
| MCHC (g/dl)             | 30 (1.47)        | 31.04 (1.65)      | 31.02 (1.33) |
| Haemolysis (%)          | 0.24 (0.12)      | 0.24 (0.12)       | 0.21 (0.13)  |
| Supernatant total protein (g/dl) | 1.76 (0.32) | 1.13 (0.24)       | <08 |
| Supernatant albumin (g/dl) | 1.05 (0.35) | 0.7 (0.24)        | <04 |
| Supernatant K+ (mEq/l)  | >20              | >20               | 2.2 (1.53)  |
| Supernatant plasma free haemoglobin (mg/dl) | 114.17 (58.23) | 70.83 (33.97)    | 134.17 (73.66) |
| Inosine wash out rates  | -                | -                 | 98.2% (2.3%) |

Fig. 1 Sample haemoglobin: pre-rejuvenation, post-rejuvenation, post-wash: mean ± 1 SD.

Fig. 2 Supernatant potassium levels in samples: pre-rejuvenation, post-rejuvenation, post-wash: mean ± 1 SD. Instrumentation sensitivity had a maximum of 20 mEq/l.
into 2,3-DPG and p50 manipulation has demonstrated in vivo patient tissue oxygenation could be positively affected [23–25]. However, clinical and health economics evidence of improved tissue oxygenation to support the use of rejuvenated RBC is limited in part because finding a practical method of relating p50 directly to ORC or tissue oxygenation in real time proves difficult and there are practical constraints to implement a blood bank rejuvenation protocol.

Farber et al. showed the benefits of direct infusion of fructose-phosphate and Didronel to stimulate blood 2,3-DPG levels resulting in significant increases in 2,3-DPG levels and p50 (P < 0.01) [23]. Subsequently, physiological changes during intense exercise were also measured. Patients receiving fructose-phosphate infusions showed significant decreases in cardiac index at identical workloads (P < 0.02), implying a strong correlation between 2,3-DPG levels, p50 and ORC [23]

Some physiological benefits have also been demonstrated. In a study by Valeri et al., patients receiving cardiac revascularization surgery showed physiological benefits of rejuvenating and washing blood cryopreserved in citrate phosphate dextrose prior to transfusion [26]. Similar to the benefits presented in the Farber study, elevating levels of 2,3-DPG and ATP prior to infusion were associated with significant improvement in cardiac index relative to typical myocardial depression experienced after cardiopulmonary bypass. Patients receiving washed rejuvenated previously frozen blood also had a significantly higher ORC (P < 0.05) and p50 (P < 0.05) up to 24 h post-bypass when compared to patients receiving previously frozen blood that was not rejuvenated [26].

The use of rejuvenated RBCs perioperatively is further supported in specific animal models. Physiological benefits of rejuvenation were recently demonstrated on the microcirculatory level in a hemodiluted rat model [27]. Rats were cannulated and hemodiluted to 30% hematocrit, divided into transfusion groups: 1- to 3-day-old liquid stored RBCs (fresh blood), 5- to 6-week-old liquid stored RBCs (aged blood), rejuvenated 5- to 6-week-old liquid stored RBCs (rejuvenated blood) and a crystalloid infusion (negative control). Rats in the aged blood transfusion group experienced significantly larger decreases in renal microvascular oxygen saturation when compared to the fresh and rejuvenated blood groups (P < 0.05), demonstrating physiological benefits of rejuvenated RBCs under haemodynamic stress when controlling for intravascular volume.

More recently, a significant reduction in inflammatory markers in swine receiving washed or rejuvenated blood in a porcine model of transfusion has also been characterized [28]. When comparing a normal saline control, washed 14-day-old blood, rejuvenated 14-day-old blood and unwashed 14-day-old blood, Woźniak et al. showed statistically significant reductions in IL-6 cytokines and protein levels in bronchoaveolar lavage fluid, indicators of kidney and lung injury, respectively, in swine receiving washed or rejuvenated blood (P < 0.05). Transfusion of rejuvenated RBCs also increased creatinine clearance post-transfusion over 24 h, significantly reduced the incidence of kidney CD14 cells and prevented a rise in serum creatinine levels associated with stored RBCs.

In each animal model, no underlying disease state was present to influence or possibly mask the observed physiological benefits attributed to rejuvenated blood. While these results are promising, adopting these models for human clinical studies will be complicated by the underlying pathophysiology of the patient and the logistic hurdles of providing rejuvenated blood to critically ill patients on demand. Moving the rejuvenation process to the bedside will open up new opportunities for research and allow clinicians to investigate patient populations that cannot wait hours to receive a transfusion.

While RBC rejuvenation is a practice that has been FDA approved as a drug since the late 1990s and existing literature shows physiological benefits associated with rejuvenated blood, research has yet to confirm any clinical significance of this practice in the form of direct health benefits, risk prevention or quality metrics such as morbidity and mortality. Furthermore, the inherent risks of blood transfusion of aged blood products (42 days of storage ≥x ≥14 days of storage) have been found to be clinically insignificant [7, 8, 15, 26, 27]. This, however, does not preclude the fact that storage lesions do accumulate over time during liquid storage. Little is known about the clinical significance of the storage lesion; however, some studies have shown that they may be linked to transfusion-related immune modulation, nitrite transfusion-related toxicity and haemolysis issues in ECMO patients [10–12]. With the clinical implication of the storage lesion so poorly understood, investigating their effects, finding better ways to modulate their propagation, and looking for more efficient means of reversing their effects are all worthwhile pursuits.

**Conclusion**

The use of outdated pRBC units in this study demonstrates the efficiency of this bedside rejuvenation and washing protocol. In spite of the advanced storage age resulting in increased cellular fragility, haemolysis was well controlled during the rejuvenation and washing process and could be further reduced by pre-dilution.
Post-wash haemolysis of 0.21 ± 0.13% was well under the required 1% mandated by AABB transfusion guidelines. Updated pRBCs utilized during normal transfusion would be less fragile and susceptible to lysing. Moreover, the rejuvenation and washing protocol utilized was possible with commonly sourced hospital equipment. Specific blood bank equipment was not required to rejuvenate the pRBC units.

However, this protocol was not performed during the pressures of the surgical environment. While washing with the Sorin XTRA cell salvage device reduced the wash time to less than 10 min for the incubation period of 1 h presents a surgical challenge if the pRBC unit is needed immediately. In complex surgical environments an hour and a half of transfusion, bypassing the costs, complications, and logistics of blood bank product management. Further evaluation within the clinical setting is needed to validate the logistical feasibility of performing bedside rejuvenation while maintaining appropriate quality metrics.

Conflict of interest

AG, KK and ML are employees of Zimmer Biomet. This study was supported in part by funds from Zimmer Biomet to EC.

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