From Development to Implementation: Adjusting the Hematocrit of Deglycerolized Red Cell Concentrates to Meet Regulatory Standards

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Keywords

Red blood cells · Hematocrit · COBE 2991 ·
Glycerolization · Deglycerolization

Summary

Background: Before transfusion, thawed frozen red cell concentrates (RCCs) must be deglycerolized. In order to ensure that these products meet regulatory standards for hematocrit, an approach to manipulate hematocrit post deglycerolization was developed and implemented. **Methods:** Glycerolized and frozen RCCs were thawed and deglycerolized using the COBE 2991 cell processor, and the final product's hematocrit was adjusted by addition of various volumes of 0.9% saline / 0.2% dextrose. The in vitro quality of RCCs (hematocrit, hemolysis, hemoglobin content, volume, recovery, ATP, supernatant potassium, and others) were compared to Canadian Standards Association (CSA) and other standards for deglycerolized RCCs. **Results:** Addition of saline/dextrose re-suspension solution in a range of 65–90 g post deglycerolization led to acceptable hematocrits. In the pilot study, this approach resulted in RCCs meeting all CSA standards for deglycerolized RCCs, with stimulation of RBC metabolism demonstrated by increased ATP concentration. In the validation phase, results were similar, although the CSA hemolysis standard was not met. Pre- and post-implementation data confirmed that manipulated RCCs met CSA hematocrit standards. **Conclusion:** This process was implemented at Canadian Blood Services to provide deglycerolized RCCs that meet the CSA hematocrit standard. However, pre- and post-implementation data reveal that this deglycerolization process is not sufficient to have RCCs consistently meet hemolysis standards.

Introduction

Red cell concentrates (RCCs) that are stored refrigerated for up to 42 days are used routinely in most red blood cell (RBC) transfusions. Cryopreservation (freezing) can extend RCC storage time to 10 years and beyond [1, 2], and is predominantly used to preserve stocks of rare blood phenotypes, for storage for autologous and allogeneic use, and for military applications [3]. Cryopreservation is possible through the addition of high concentrations of glycerol, which acts as a cryoprotectant, to the RCC before freezing [3]. In North America, RCCs are commonly cryopreserved in 40% w/v glycerol concentrations with slow cooling rates ($\sim 1^\circ\text{C}/\text{min}$) prior to storage at $\leq -65^\circ\text{C}$, while in Europe lower glycerol concentrations and rapid freezing are also used [4]. After thawing cryopreserved RCCs, the glycerol must be removed. The process of deglycerolization is complicated and time-consuming, but is necessary before the unit can be transfused to avoid intravascular hemolysis of the RBCs and subsequent post-transfusion adverse events.

The current method used by Canadian Blood Services to deglycerolize RCCs employs the COBE 2991 cell processor (TerumoBCT), an open system that produces products with an outdate of 24 h. Deglycerolization is achieved using multiple washes with saline solutions of decreasing concentrations. Each wash is followed by centrifugation and removal of the supernatant, a process that is automatically stopped when RBCs are detected in the exit tubing to the waste bag. This results in an RCC that is more concentrated and has a lower supernatant volume and thus a higher hematocrit (Hct) than a standard RCC processed from whole blood (WB) that has not undergone deglycerolization. Past quality control (QC) data at Canadian Blood Services indicate that the average Hct of deglycerolized RBC units is $0.81 \pm 0.04 \text{ l/l}$ (range 0.74–0.93 l/l, $n = 75$). Thus, 53% of deglycerolized RBC units tested for QC do not meet regulatory requirements for Hct (Canadian Standards Association (CSA) standard states that Hct must be $\leq 0.8 \text{ l/l}$ in 90% of units tested [5]). In other jurisdictions, the Council of Eu-

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rope recommends that deglycerolized RCCs have a Hct of 0.35–0.70 l/l [6], while the AABB (2009) guidelines do not state a specific Hct recommendation for deglycerolized RCCs but generally recommend a Hct of ≤ 0.80 l/l for RBC units [7].

In this study, we developed and investigated an approach to manipulate RCCs post processing on the COBE 2991 in order to ensure that these products meet the Hct regulatory standard. The approach involved addition of saline/dextrose to the deglycerolized RCC and was designed to have as little impact on the standard operating procedure as possible. The hypothesis was that addition of solution would reduce the RCCs' Hct and result in products with Hct within the acceptable range. Here we report the results of the study to investigate and validate this approach and determine its impact 1-year post implementation.

Material and Methods

Pilot Study

Estimation of Re-Suspension Solution Volumes

For standard RCCs produced by Canadian Blood Services that are stored refrigerated and do not undergo freezing/deglycerolization, the Canadian Blood Services Circular of Information (2011) indicates Hct should be 0.5–0.7 l/l in at least 90% of units [8]. Therefore, an ideal Hct value for deglycerolized RCCs post processing of 0.60 l/l was chosen. However, as manual addition of re-suspension solution was chosen to manipulate deglycerolized RCCs post processing, it was decided a range of target Hct values would best accommodate this process. These values were used to calculate the volume of re-suspension solution needed to adjust the Hct to a range of acceptable values (0.50 l/l, 0.60 l/l, and 0.70 l/l) as follows: $(0.81 \text{ l/l} \times 224 \text{ ml})$ divided by the desired Hct. The average QC volume was then subtracted from the result to determine the volume of re-suspension fluid needed to adjust to the desired Hct. Re-suspension solution was added by weight; therefore, the volumes calculated were converted to gram. An estimated addition of 65 g, 75 g and 90 g of re-suspension solution would result in a theoretical average Hct of 0.70 l/l, 0.60 l/l and 0.50 l/l, respectively.

RCC Collection and Processing

This was a pool-and-split study using RCCs processed by both methods used at Canadian Blood Services. Briefly, WB ($n = 48$) was collected from healthy volunteers into either MacoPharma (Tourcoing, France) or Fenwal (Lake Zurich, IL, USA) collection sets with citrate-phosphate-dextrose (CPD) anticoagulant. 24 of the units were processed using the buffy coat method, as described previously [9]. The remaining 24 were processed by the WB filtration method, also as described previously [9]. From these, 12 groups of 4 units each, matched for processing method, collection date and ABO group, were pooled into large 2 l Fenwal bags. Pools were mixed and split into equal volumes (± 10 g of each other) back into their original bags. RCCs were weighed using a GSE 574 scale (GSE Scale Systems, Livonia, MI, USA) and stored in a refrigerator at $1-6^\circ\text{C}$ until glycerolization and freezing.

RCC Glycerolization and Freezing

The RCC, at 14 days post collection, and glycerol solution (Glycerolyte 57 Solution – 4A7831; Baxter Health Care Corporation, Deerfield, IL, USA) were warmed in a 37°C water bath for 20–30 min and then attached to a 2 l freezing kit (4R2422, Fenwal, Lake Zurich, IL, USA). The glycerol solution was added using a two-step method: 100 ml was added with gentle mixing followed by an equilibration period of 5–15 min. An additional 300 ml was added to achieve an approximately 40% glycerol concentration in the RCC. The glycerolized RCC was placed into a metal freezing cassette and stored in a -80°C freezer for 24–48 h.

Thawing of Frozen RCCs and Deglycerolization

Frozen RCCs were placed in a 37°C water bath for 7–15 min until thawed. The RCC was attached to a COBE 2991 Blood Cell Processing Set (REF90819, TerumoBCT, Lakewood, CO, USA) and loaded onto the COBE 2991 Blood Cell Processor following the manufacturer's instructions. Before automated washing, 150 ml of 12% sodium chloride processing solution (4B7874Q, Baxter Health Care Corporation) was manually added to the thawed RCC over 60 s with constant mixing. The RCC was allowed to equilibrate for 3–5 min before the addition, with constant mixing, of approximately 650 ml of 1.6% sodium chloride processing solution (4B7871, Baxter Health Care Corporation). The RCC was then processed using the automated settings on the COBE 2991 which involved one additional wash with 1.6% sodium chloride followed by two washes with 0.9% saline / 0.2% dextrose.

During the final centrifugation of the washed RCC, a visual supernatant hemoglobin (Hb) reading was obtained using a color comparator (#18501C; Haemonetics, Braintree, MA, USA). If the visual supernatant Hb level was graded > 4 (on a scale of 1–8), an additional wash with 1,000 ml of 0.9% sodium chloride (JB1324, Baxter Health Care Corporation) was performed to reduce the supernatant Hb concentration. A new bag of 0.9% saline / 0.2% dextrose was then attached and used as the re-suspension fluid for the RBCs. The tubing between the RCC and the re-suspension solution was clamped using a hemostat, and the processing kit was removed from the processor (including the lid of the centrifuge).

Addition of Re-Suspension Solution

The RCC and the COBE 2991 centrifuge lid were placed onto a GSE 574 scale (GSE Scale Systems), and the scale was tarred. The 0.9% saline / 0.2% dextrose re-suspension solution was hung at least 90 cm above the RCC. Each of the four units in an RCC pool received a different mass of re-suspension solution: 0 g (Group A; control), 65 g (Group B), 75 g (Group C) or 90 g (Group D), all ± 5 g. All RCCs were stored in a $1-6^\circ\text{C}$ refrigerator.

RCC Sampling

RCCs were gently mixed and inverted five times. Samples were obtained by inserting an 18-gauge needle attached to a syringe through a sampling site coupler (4C2405, Fenwal) inserted into one of the unit ports. The needle was removed, and the sample was dispensed drop-wise into aliquot tubes for testing. RCCs were sampled at three different time points during the study; 3 ml prior to glycerolization, 1 ml immediately post deglycerolization for spun hct, and 10 ml at 20–24 h after re-suspension post deglycerolization for measurement of RBC parameters.

RCC and RBC Parameters

Mean cell volume (MCV), Hb, and Hct were measured on an automated Advia 120 Hematology Analyzer (Siemens, Mississauga, ON, Canada). Spun Hct, ATP concentration, supernatant potassium, Drabkin's total and supernatant Hb, and RBC hemolysis were determined as described previously [9].

Due to the differences in dilutions between treatment groups, supernatant potassium was corrected using the following calculation as described previously [11]:

$$\text{Supernatant potassium } (\mu\text{mol/gHb}) = (\text{supernatant potassium (mmol/l)} / (\text{hemoglobin (g/l)} \times 1,000) \quad (1).$$

Total Hb and supernatant Hb per unit were determined using the following calculations:

$$\text{Hb (g/unit)} = \text{volume (l)} \times \text{Hb (g/l)} \quad (2).$$

$$\text{Supernatant Hb (g/unit)} = \text{volume (l)} \times (1 - \text{Hct (l)}) \times \text{supernatant Hb (g/l)} \quad (3).$$

Pre-glycerolization and post-deglycerolization mass were measured for each unit using a GSE 574 scale (GSE Scale Systems). RBC recovery was calculated using these masses and the Hct results obtained from the Advia 120 (Siemens) using the following calculation:

Table 1. Pilot study: 20- to 24-hour post-deglycerolization RCC results compared to CSA [5], Council of Europe [6], and AABB [7] guidelines for deglycerolized RCCs^a

	Group A (n = 12)	Group B (n = 12)	Group C (n=12)	Group D (n=12)
<i>Hematocrit, l/l^b</i>	0.78 ± 0.03	0.64 ± 0.01*	0.62 ± 0.02*	0.60 ± 0.02* ^{^††}
CSA	fail	pass	pass	pass
90% of units ≤ 0.8 l/l (pass/fail)	(75% pass)	(100% pass)	(100% pass)	(100% pass)
Council of Europe	fail	pass	pass	pass
100% of units 0.35–0.70 l/l (pass/fail)	(0% pass)	(100% pass)	(100% pass)	(100% pass)
<i>Hemoglobin, g/U</i>	48.7 ± 5.9	50.8 ± 5.1	51.2 ± 4.5	50.7 ± 4.7
CSA	pass	pass	pass	pass
90% of units ≥ 35 g/unit (pass/fail)	(100% pass)	(100% pass)	(100% pass)	(100% pass)
Council of Europe	pass	pass	pass	pass
100% of units 36 g/unit (pass/fail)	(100% pass)	(100% pass)	(100% pass)	(100% pass)
<i>Volume, ml</i>	209 ± 31	275 ± 26*	286 ± 22*	297 ± 24*
Council of Europe	fail	pass	pass	pass
100% of units > 185 ml (pass/fail)	(67% pass)	(100% pass)	(100% pass)	(100% pass)
<i>RBC hemolysis, %</i>	0.57 ± 0.15	0.53 ± 0.07	0.48 ± 0.06	0.52 ± 0.06
CSA	pass	pass	pass	pass
90% of units < 0.8% (pass/fail)	(92% pass)	(100% pass)	(100% pass)	(100% pass)
<i>Supernatant hemoglobin, g/U</i>	0.289 ± 0.102	0.267 ± 0.041	0.248 ± 0.032	0.265 ± 0.041
Council of Europe	fail	fail	fail	fail
100% of units < 0.2 g/U (pass/fail)	(25% pass)	(0% pass)	(0% pass)	(0% pass)
<i>RBC recovery, %</i>	85.8 ± 5.4	90.7 ± 3.3**	91.8 ± 3.7**	91.4 ± 3.9**
CSA and AABB	pass	pass	pass	pass
Mean RBC recovery in units tested ≥ 80% (pass/fail)				
^a All results shown as mean ± 1 SD.				
^b Automated hematocrit is displayed.				
*Statistically significant difference when compared to Group A (p < 0.001).				
**Statistically significant difference when compared to Group A (p < 0.05).				
[^] Statistically significant difference when compared to Group B (p < 0.001).				
^{††} Statistically significant difference when compared to Group C (p < 0.05).				

$$\text{RBC recovery (\%)} = ((\text{post-deglycerolization mass (g)} \times \text{post-deglycerolization Hct (l/l)}) / (\text{pre-glycerolization mass (g)} \times \text{pre-glycerolization Hct (l/l)})) \times 100 \quad (4).$$

Volume post deglycerolization and post re-suspension was calculated by dividing the mass obtained by a specific gravity of 1.08 for control units with no re-suspension solution added (high Hct – Group A) and by a specific gravity of 1.06 [12] for units with re-suspension solution added (lower Hct – Group B, C and D).

In the pilot study, acceptance criteria were CSA standard (Z902–15) values for Hct, Hb, RBC hemolysis, and RBC recovery. Compliance with Council of Europe and AABB guidelines was also assessed. ATP and supernatant potassium were used as indicators of RBC quality in regards to RBC metabolism and membrane integrity.

Validation Study

Blood Collection, Processing, Glycerolization, and Deglycerolization

60 WB units from healthy volunteers were collected into either Maco Pharma or Fenwal collection sets. 40 units were processed using the buffy coat method and 20 using the WB filtration method. All units were glycerolized after 9–14 days of hypothermic storage by the method described above and stored for 4–24 days at ≤ –65 °C before deglycerolization. Units were deglycerolized using a process that incorporated the addition of the re-suspension solution within the

range of 65–90 g. Acceptance criteria were based on CSA criteria and were as follows: Hct ≤ 0.8 l/l in 90% of units tested; Hb content ≥ 35 g/unit in 90% of units tested, RBC hemolysis < 0.8% in 90% of units tested; mean RBC recovery ≥ 80% in tested units, and ‘no growth’ in 100% of units tested for sterility.

RCC and RBC Parameters

In agreement with QC procedures at CBS, at 24–48 h after deglycerolization Hb, Hct, and sterility were measured. Sterility was assessed using an automated bacterial detection system (BacT/ALERT 3D, bioMérieux Canada, Inc., St Laurent, QC, Canada), as described previously [13]. Recovery and RBC hemolysis were calculated as described above. Hemolysis was calculated using total Hb and Hct measurements from an Advia 120 and supernatant Hb measurements using a photometer (HemoCue, Plasma/Low Hb, Ängelholm, Sweden).

Pre- and Post-Implementation QC Analysis

To assess the impact of the validated method change in real-world conditions, data for deglycerolized RBC products were collected from the Canadian Blood Services QC database for 1 year pre implementation and 1 year post implementation from 7 different processing facilities across Canada. RCCs were glycerolized at 2–14 days post collection, stored frozen for 1–1,176 days, and tested at 24–48 h post deglycerolization. Parameters compared were Hct, total Hb per unit, supernatant Hb per unit, volume, RBC hemolysis, and RBC recovery. Measurements for Hct and total Hb were done on the Advia 120, supernatant Hb was obtained using a Plasma/Low Hb photometer (HemoCue), and RBC hemolysis was calculated using these measured values.

Statistical Analysis

Differences between study groups were analyzed using an ANOVA with Tukey HSD post-hoc analysis (SPSS 23, IBM Corporation, Armonk, NY, USA). Results were considered significant when *p* values were less than 0.05.

Results

Pilot Study

CSA and Other Criteria

Prior to glycerolization, no significant differences in volume (300 ± 22 ml, 300 ± 21 ml, 300 ± 22 ml and 299 ± 22 ml in Groups A, B, C and D, respectively) and Hct (0.652 ± 0.021 l/l, 0.655 ± 0.018 l/l, 0.654 ± 0.020 l/l and 0.654 ± 0.018 l/l in Groups A, B, C and D, respectively) were observed, indicating that the pooling and splitting steps taken were effective. Spun Hcts immediately post deglycerolization but prior to addition of the re-suspension solution were 0.80 ± 0.02 l/l, 0.81 ± 0.02 l/l, 0.81 ± 0.02 l/l and 0.81 ± 0.01 l/l in Groups A, B, C and D, respectively. In each group (*n* = 12 RCCs), the mass of re-suspension solution added after deglycerolization was (mean $\pm$ SD): Group A/control – 0 g; Group B (low volume: target 65 ml) – 65 ± 2 g; Group C (medium volume: target 75 ml) – 75 ± 2 g; and Group D (high volume: target 90 ml) – 90 ± 1 g.

Confirming the premise of the study, control RCCs (Group A) failed to meet CSA criteria for Hct (table 1). The addition of re-suspension solution reduced the Hct in a linear manner, and RCCs in Groups B, C, and D had statistically lower Hct compared with Group A and met the CSA Hct standards. RCCs from all experimental groups met the CSA standard for Hb content. All experimental groups met the CSA standard for hemolysis with 100% of units passing in Groups B, C, and D while only 92% passed in Group A. Finally, all groups met the CSA and AABB standard for recovery, with Group A having statistically lower RBC recovery than Groups B, C and D; however, this difference may be related to the recovery calculations used.

Similarly, Group A RCCs failed to meet Council of Europe Hct guidelines for deglycerolized RCCs (table 1), while Group B, C, and D RCCs passed, and RCCs from all experimental groups met the Council of Europe standard for Hb content. Control units failed to meet Council of Europe guidelines for volume, but RCCs that had additional re-suspension solution added passed the standard and, as expected, had statistically higher volumes than control Group A. The Council of Europe standard for supernatant Hb in deglycerolized RCCs (g/unit) was not met by any group, and Group A was the only group in which any units passed the standard with a total of 25% passing the numerical standard.

Groups B, C, and D all fell within the average Hct range stated in the 2015 Canadian Blood Services Circular of Information for standard, liquid-stored RBCs that have not been frozen/deglycerolized (0.54–0.68 l/l) [14].

Other RCC Quality Assessments

RCCs in Groups B, C, and D had significantly higher ATP concentrations compared to Group A (fig. 1). There were lower super-

Fig. 1. ATP concentration and supernatant potassium in RCCs 20–24 h post deglycerolization (pilot study). Target volumes for additional re-suspension solution added after deglycerolization were as follows: Group A (control) – 0 ml; Group B (low volume) – 65 ml; Group C (high volume) – 75 ml; and Group D (medium volume) – 90 ml. ATP concentration was measured 20–24 h later using a commercial kit. *N* = 12 paired pooled and split RCC in each experimental group. Supernatant potassium was measured 20–24 h later using ion selective potentiometry. †Denotes statistically significant difference when compared to Group A (*p* < 0.001). *Denotes statistically significant difference when compared to Group A (*p* < 0.05).

natant potassium levels in the RCCs with extra re-suspension solution added, with Group A having significantly higher levels than all other groups (fig. 1). In all groups, there was a significant increase in MCV (*p* < 0.001; data not shown) after deglycerolization, suggesting that swelling occurs with glycerolization/freezing/deglycerolization. Post deglycerolization, there were no differences among groups A, B, C, and D for MCV.

Validation Study

The actual volume of re-suspension solution added in the validation was 76 ± 4 g (range 68–88 g; *n* = 60). The average age of the units prior to freezing was 13 ± 1 days, and the average length of

Table 2. Validation study: 24- to 48-hour post-deglycerolization RCC results compared to CSA [5], Council of Europe [6], and AABB [7] guidelines for deglycerolized RCCs^a

	All RCCs (n = 60)	Buffy coat processed (n = 40)	Whole blood processed (n = 20)
<i>Hematocrit, l/l</i>	0.61 ± 0.03	0.60 ± 0.03	0.61 ± 0.03
CSA	pass	pass	pass
90% of units ≤ 0.8 l/l (pass/fail)	(100% pass)	(100% pass)	(100% pass)
Council of Europe	pass	pass	pass
100% of units 0.35–0.70 l/l (pass/fail)	(100% pass)	(100% pass)	(100% pass)
<i>Hemoglobin, g/U</i>	48.1 ± 5.2	46.2 ± 4.6	51.9 ± 4.2*
CSA	pass	pass	pass
90% of units ≥ 35 g/unit (pass/fail)	(98% pass)	(98% pass)	(100% pass)
Council of Europe	fail	fail	pass
100% of units ≥ 36 g/unit (pass/fail)	(98% pass)	(98% pass)	(100% pass)
<i>Volume, ml</i>	273 ± 25	262 ± 20	297 ± 16*
Council of Europe	pass	pass	pass
100% of units > 185 ml (pass/fail)	(100% pass)	(100% pass)	(100% pass)
<i>RBC hemolysis, %</i>	0.73 ± 0.28	0.72 ± 0.19	0.76 ± 0.22
CSA	fail	fail	fail
90% of units < 0.8% (pass/fail)	(53% pass)	(58% pass)	(45% pass)
<i>Supernatant hemoglobin, g/U</i>	0.354 ± 0.109	0.335 ± 0.102	0.390 ± 0.114
Council of Europe	fail	fail	fail
100% of units < 0.2 g/U (pass/fail)	(3% pass)	(5% pass)	(0% pass)
<i>Recovery, %</i>	92 ± 5	92 ± 5	93 ± 5
CSA and AABB	pass	pass	pass
Mean RBC recovery in units tested ≥ 80% (pass/fail)			
^a Results are shown as mean ± 1 SD,			
*Statistically significant difference when compared to buffy coat processed RCCs (p<0.001).			

frozen storage was 17 ± 2 days for buffy coat-processed RCCs and 9 ± 4 days for WB filtration-processed RCCs.

All units tested met both CSA and Council of Europe standards for Hct (table 2). All RCCs met the CSA standard for Hb content as well as CSA and AABB recovery standards for deglycerolized units. Contrary to the results of the pilot study, RCCs failed to meet CSA standards for RBC hemolysis. WB filtration-processed units were less likely to meet this standard than buffy coat-processed units. Buffy coat-prepared RCCs that required an additional saline wash during deglycerolization (based on a visual supernatant Hb reading) were even more likely to fail the CSA hemolysis standard. Both WB filtration-processed RCCs that underwent a second wash failed to meet the hemolysis standard.

The Council of Europe standard for Hb content requires 100% of units to pass, but overall only 98% of the RCCs had Hb content greater than 36 g/unit (table 2). Buffy coat-processed RCCs had significantly lower Hb content than WB filtration-processed units (p < 0.001) and failed to meet Council of Europe guidelines, while WB filtration-processed RCC did. One buffy coat-processed RCC that failed the Council of Europe standard (and those units that fell under 35 g/unit limit in the CSA standards) went through the additional wash process based on a visual supernatant Hb reading. Similar to the results of the pilot study, RCCs in the valida-

tion study also failed the Council of Europe supernatant Hb standard.

Pre- and Post-Implementation Quality Control Data Analysis

Once implemented, an analysis of routine QC data collected on deglycerolized RCCs was undertaken. The new process yielded a deglycerolized RBC product that meets the CSA Hct standard in practice; post-implementation Hct was significantly lower than the pre-implementation Hct values (table 3; p < 0.001). In practice, the pre- and post-implementation Hb content per unit met the CSA standard and the Council of Europe standard. For volume, all units met the standard, but volume was significantly higher after implementation (p < 0.001). As observed in the validation study, in both the pre- and post-implementation groups the CSA standard for RBC hemolysis was not met, nor was the Council of Europe standard for supernatant Hb. The pre-implementation data were aggregated from 10 buffy coat and 65 WB filtration units where the post-implementation data were aggregated from 20 buffy coat and 49 WB filtration units. Pre implementation, 46% of buffy coat-produced units and 72% of WB filtration-produced units met the hemolysis standard. Post implementation, 50% of buffy coat-produced units and 45% of WB filtration-produced units met the hemolysis standard.

Table 3. Pre- and post-implementation study: 24- to 48-hour post-deglycerolization of RCC results compared to CSA [5], Council of Europe [6], and AABB [7] guidelines for deglycerolized RCCs^a

	Pre-implementation			Post-implementation		
	all RCCs (n = 75)	buffy coat processed (n=10)	whole blood processed (n=65)	all RCCs (n = 69)	buffy coat processed (n = 20)	whole blood processed (n = 49)
<i>Hematocrit, l/l</i>	0.82 ± 0.04	0.82 ± 0.04	0.81 ± 0.04	0.59 ± 0.04*	0.60 ± 0.04 [^]	0.59 ± 0.05 [‡]
CSA	fail	fail	fail	pass	pass	pass
90% of units ≤ 0.8 l/l (pass/fail)	(47% pass)	(60% pass)	(45% pass)	(100% pass)	(100% pass)	(100% pass)
Council of Europe	fail	fail	fail	fail	pass	fail
100% of units 0.35–0.70 l/l (pass/fail)	(0% pass)	(0% pass)	(0% pass)	(99% pass)	(100% pass)	(98% pass)
<i>Hemoglobin, g/U</i>	53 ± 6	47 ± 6	54 ± 5 ^{^^}	53 ± 6	48 ± 4	55 ± 5 [†]
CSA	pass	pass	pass	pass	pass	pass
90% of units ≥ 35 g/unit (pass/fail)	(100% pass)	(100% pass)	(100% pass)	(100% pass)	(100% pass)	(100% pass)
Council of Europe	pass	pass	pass	pass	pass	pass
100% of units ≥ 36 g/unit (pass/fail)	(100% pass)	(100% pass)	(100% pass)	(100% pass)	(100% pass)	(100% pass)
<i>Volume, ml</i>	229 ± 24	200 ± 22	233 ± 21 ^{^^}	307 ± 34*	279 ± 28 [^]	318 ± 30 ^{†‡}
Council of Europe	pass	pass	pass	pass	pass	pass
100% of units > 185 ml (pass/fail)	(100% pass)	(100% pass)	(100% pass)	(100% pass)	(100% pass)	(100% pass)
<i>RBC hemolysis, %</i>	0.70 ± 0.38	0.86 ± 0.57	0.68 ± 0.34	0.96 ± 0.52**	1.01 ± 0.78	0.93 ± 0.37 ^{††}
CSA	fail	fail	fail	fail	fail	fail
90% of units < 0.8% (pass/fail)	(71% pass)	(46% pass)	(72% pass)	(46% pass)	(50% pass)	(45% pass)
<i>Supernatant hemoglobin, g/U</i>	0.372 ± 0.199	0.404 ± 0.291	0.367 ± 0.183	0.505 ± 0.264*	0.490 ± 0.364	0.512 ± 0.215 ^{††}
Council of Europe	fail	fail	fail	fail	fail	fail
100% of units < 0.2 g/U (pass/fail)	(13% pass)	(20% pass)	(12% pass)	(7% pass)	(20% pass)	(7% pass)
<i>Recovery, %</i>	93 ± 7	91 ± 5	93 ± 7	95 ± 9	92 ± 8	96 ± 9
CSA and AABB	pass	pass	pass	pass	pass	pass
Mean RBC recovery in units tested ≥ 80% (pass/fail)						

^aResults are shown as mean ± 1 SD.

*Statistically significant difference when compared to pre-implementation values (p < 0.001).

**Statistically significant difference when compared to pre-implementation values (p < 0.05).

[^]Statistically significant difference when compared to pre-implementation buffy coat processed (p < 0.001).

^{^^}Statistically significant difference when compared to pre-implementation buffy coat processed (p < 0.05).

[†]Statistically significant difference when compared to post-implementation buffy coat processed (p < 0.001).

[‡]Statistically significant difference when compared to pre-implementation whole blood processed (p < 0.001).

^{††}Statistically significant difference when compared to pre-implementation whole blood processed (p < 0.05).

Discussion

Hct reflects the packed RBC volume of a unit and is related to the RBC dose. CSA guidelines for Hct are broad, stating an upper limit of 0.80 l/l. Beyond simply meeting the standard of less than 0.80 l/l, we developed an approach that would result in deglycerolized RCCs with Hct levels that were comparable to the standard, liquid-stored RCCs produced at Canadian Blood Services (mean ± 2 SD in 2011 Circular of Information (valid when study was initiated): 0.61 ± 0.07 l/l [8]). We also sought an approach that would have as little impact as possible on the already labor-intensive process of thawing and deglycerolizing frozen RCCs. The findings of the pilot study led to the recommendation that a target of 75 g (range of 65–90 g) of 0.9% saline / 0.2% dextrose solution be used to re-suspend RCCs following deglycerolization employing the

COBE 2991. Saline/dextrose was chosen as an appropriate re-suspension solution as the inclusion of dextrose aids metabolism, a point supported by increased ATP levels seen in these units in the current study. However, with a 24-hour outdate, use of a more complex re-suspension fluid (e.g. a RBC additive solution) was deemed unnecessary. This process change was validated and implemented at Canadian Blood Services, allowing the organization to meet the required Hct standard for deglycerolized RBC products.

Although these criteria do not apply in Canada, RCCs were also assessed against Council of Europe and AABB guidelines. Standards differ greatly from jurisdiction to jurisdiction, making comparison challenging. Cryopreservation procedures, which also differ among jurisdictions, affect the level of damage done to RBCs during the freeze/thaw process. For example, initial Hct levels, cryopreservation conditions, glycerol concentrations, and the stor-

age solution after deglycerolization impact RBC recovery, hemolysis, and fragility post cryopreservation [15, 16]. Thus it is unclear how well the results seen in this study with Canadian products and methods would translate to other products produced using different methods or with different instrumentation. It is worth noting that in Europe, many are now using the ACP-215 closed system to wash thawed RCCs, and results would therefore expect to differ from those seen with the COBE 2991.

With the exception of the pilot phase, throughout the study CSA criteria for hemolysis were not met. Hemolysis represents terminal injury of the RBCs and is also a critical measure of RBC quality. Of the standards assessed in this study, only the CSA mandate hemolysis levels are measured. However, the Council of Europe stipulates supernatant Hb levels, and these were also not met at any stage during the study, including the pilot study. Like hemolysis, supernatant Hb levels are indicative of terminal damage to the RBCs. Together, the hemolysis and supernatant Hb levels suggest that the process of freezing and deglycerolization damages the RBCs. This is consistent with reports on the fragility of RBCs that are produced using the COBE 2991 [17]. Washed and deglycerolized RBC products produced on the COBE 2991 have an elevated level of hemolysis compared with unprocessed units, likely due to mechanical damage to the cells. In the original report by Meryman and Hornblower [17] in 1972 on the use of the COBE 2991 for the cryopreservation of RBCs, an almost 10-fold increase in supernatant Hb concentration following deglycerolization was reported, suggesting significant damage to the RBCs. Hemolysis during blood cell processing, including cell washing and deglycerolization has been well described [10, 18, 19]. Hemolysis levels in deglycerolized RBCs have been shown to be affected by many factors including RBC processing factors such as leukoreduction [20], collection and freezing containers [19], the additive solutions used post processing [16], age of the RBC, and the specific methods used for glycerolization, freezing, thawing and cryopreservation [4, 15, 21].

RBC hemolysis results observed in the pilot study were significantly lower ($p < 0.001$) than the results observed in the validation study. The differences in hemolysis results between the pre- and post-implementation study compared to the pilot study show the limitations that can arise when testing moves from a controlled environment to a real-world situation. The pilot study had a small sample size and was a pool and split study, conducted in a completely controlled environment at a single site by two highly trained technicians. Also, different testing methods were used to measure hemolysis in the pilot study and the validation study, which could influence the results [22, 23]. The pre- and post-implementation phase of this study was conducted at multiple production sites across Canada – seven sites in the pre-implementation portion and six in the post-implementation portion. Unlike the pilot study which was a pool and split study, donor factors and the impact of rare RBC phenotypes on RBC fragility may have been at play during the pre- and post-implementation phase. In the post-implementation phase, there may have been a learning curve with the new process and technician-to-technician differences in how the process was performed. Although designed to be simple, the new process

does involve some manual steps where variances could appear, including the speed with which saline/dextrose solution is added post deglycerolization. Most importantly, the length of time before testing occurred may have affected hemolysis, as in both the validation and pre- and post-implementation studies, in line with QC requirements, testing may have occurred up to 48 h after deglycerolization (vs. maximal 24 h in the pilot study). Thus these hemolysis levels may not actually represent the level of hemolysis of the units at the time of transfusion (<24 h post deglycerolization).

Saline/dextrose was used as the re-suspension fluid due to its potential benefits for RBC quality versus saline alone. Addition of extra saline/dextrose led to an increase in ATP levels in the units. This is likely related to dextrose allowing maintenance of RBC metabolism [24], which is associated with better product quality. Lower levels of supernatant potassium were also seen in deglycerolized units to which additional saline/dextrose were added. Overall, despite the outdate being just 24 h, the better maintenance of RBC metabolism and the lower dose of potassium per unit suggests improved RBC quality in the units to which re-suspension fluid was added. RBC quality indices did indicate an increase in MCV post deglycerolization in all groups, suggesting that swelling occurs with glycerolization/freezing/deglycerolization. The swelling observed due to the increased MCV could affect Hct results and create bias in other measurements that require this value (i.e., hemolysis, recovery, Hb/unit).

While all experimental groups passed the CSA guideline for RBC recovery, it is worth noting that the recovery calculation used in this study is limited. This calculation, which is used at Canadian Blood Services for QC purposes, assumes that the density of the pre- and post-deglycerolized products is the same. This assumption is not necessarily true, especially for the control units which have higher Hcts than pre-freeze units. This calculation also assumes that the RBC Hct is not affected pre and post deglycerolization and thus does not take into account swelling of the RBCs, which we clearly observed in this study. This study had several other limitations. Differences in the length of pre-freeze storage time, frozen storage time, and refrigerated storage post deglycerolization may impact comparisons across the pilot, validation and pre- and post-implementation studies. There were also differences in the ratio of buffy coat- and WB filtration-processed RCCs (and different bag set manufacturers) among the various phases of this study, and this may impact some of the results. In the validation study, the additional wash which was applied to some units – an additional manipulation that tends to increase hemolysis at expiry – may be a contributing factor to the levels of post-deglycerolization hemolysis at 24–48 h. Similarly, for the pre- and post-implementation data it is not known whether the units received an additional wash, and thus we cannot conclude whether this was a contributing factor in the higher RBC hemolysis seen in these data.

The method explored in this study produced deglycerolized RCCs with Hct levels that were acceptable. Hct was the focus of this study as this standard was not being met, and it is an important measure that can impact how physicians treat patients with these products. With the original product – deglycerolized RBCs

with a high Hct – physicians could infuse a higher number of RBC units due to the decrease in volume in comparison to a standard unit; thus delivering a higher dosage of RBCs to the recipient while avoiding circulatory overload. The units produced using the new method allow physicians to deliver a possibly better quality product due to the addition of extra dextrose in the supernatant; however, these products will have a lower RBC dosage overall because of the higher volumes of the units lead to fewer units that can be transfused. The difference in in vivo recovery is not known; however, these modified products have been available for use since 2013. Overall, deglycerolization with the COBE 2991 impacts RBC hemolysis, as seen in previous studies [25, 26]. It is also worth noting that this technology produces products in an open system, and the resulting products are limited by a 24-hour outdate. Despite being able to meet the Hct standard, the damage suggested by the hemolysis results in this study indicates that the COBE 2991 is not an ideal system for deglycerolizing RCCs. More modern, closed-system technologies like the ACP-215 from Haemonetics Corporation permit the production of deglycerolized RCCs with an extended outdate time [16, 27–29]. These technologies are in use in many jurisdictions, have been implemented for RBC washing at Canadian Blood Services [30–33], and may provide a means to

produce even higher-quality products with the added advantage of a longer outdate time.

Acknowledgements

The authors thank Caesar DiFranco, Canadian Blood Services QC manager for providing the pre- and post-implementation data; Della Reside, project manager, Michelle Rogerson, director, supply chain, and Terry Milne, validation associate who managed the validation study; Canadian Blood Services staff who performed the validation study in British Columbia and Yukon, Ottawa, New Brunswick, and Edmonton; Dr. Geraldine Walsh, Canadian Blood Services' scientific writer for assistance with manuscript preparation and editing; and all Canadian Blood Services donors.

Disclosure Statement

JPA has received grants from Canadian Blood Services, Canadian Institutes for Health Research, Natural Sciences and Engineering Research Council of Canada, and the Women and Children's Health Research Institute. He has received: honoraria as a member of BioLife Solutions' Scientific Advisory Board and HemoCue America LLC Advisory Board; consulting fees as a faculty member of Biomet Biologics' Education Team, and personal fees from Aquila Diagnostic Systems. TRT, ALH and JDRK have no conflicts of interest to disclose.

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