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Impact of technical and assay variation on reporting of hemolysis in stored red blood cell products

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ABSTRACT

Background: Hemolysis of RBCs is an important measure of product quality and is influenced by donor factors, blood component manufacturing and storage. Percent hemolysis is determined using hematocrit (Hct), supernatant Hb (SHb) and total Hb (THb), each of which can be measured using a variety of methods.
Methods: Sixteen members of an international collaborative were surveyed to understand equipment and procedural variation in hemolysis testing. In a laboratory-based evaluation, we examined how hemolysis was impacted by: measurement of Hct, SHb, THb and number and force of centrifugations for SHb preparation. The number and size of extracellular vesicles (EVs) was also examined.
Results: There was no consensus in equipment or procedures used by international laboratories to measure hemolysis. The centrifugation force used to prepare samples influenced SHb concentration when a single or double ($p = 0.0001$) centrifugation step was used. The number and force of centrifugation related directly to the ability to remove EVs and EV-bound Hb from samples. Hemolysis varied significantly from 0.16% to 0.32% (mean of 0.22%) depending on the combination of methods or centrifugation conditions used to test expired samples.
Conclusion: Method and preparative procedures have a critical impact on measurement of hemolysis in RCC.

1. Introduction

Hemolysis measurement is one of the most important and useful determinants of blood product quality [1,2]. Hemolysis signifies the disruption of the intact red blood cell (RBC) membrane which results in the release of hemoglobin (Hb). Hemolysis of RBCs takes place during ex vivo storage, and the levels of supernatant Hb (SHb) can be influenced by donor and manufacturing specific factors during collecting and processing of the blood products [3–5]. Even with proper processing and storage of RBCs, hemolysis still occurs in stored RBC units [6]. To prevent or minimize the adverse post-transfusion consequences of RBC hemolysis, stored red cell concentrates (RCC) should meet acceptable levels of hemolysis according to the regulatory agency guidelines (<1% in the U.S. or <0.8% in Europe and Canada) [1,7,8]. Using current storage systems, RCC do meet these acceptable levels of hemolysis. However, different laboratories perform RBC hemolysis assays using a variety of means and methods, [1–4,6,9–11] making it very difficult to compare published data relating to this fundamental RCC quality indicator across laboratories or blood operators.

Hemolysis is calculated by the hematocrit (Hct), the amount of SHb and total Hb (THb) in a sample using the following equation [2]:

$$\%\text{hemolysis} = [(100-\text{Hct}) \times \text{SHb}]/\text{THb} \quad (1)$$

A variety of manual and automated methods exist to determine Hct and Hb concentration, resulting in many combinations of possible methods for determining the hemolysis of RCCs [1–4,9]. For instance, while the reference method to determine Hb concentration is the Drabkin's method, several laboratories use other assays such as the HemoCue and Harboe methods [12]. In addition, Hct can be measured by 2 different methods: directly by the spun (manual) method - the reference standard [13] - or indirectly using RBC count and mean cell volume (MCV) obtained from automated hematology analyzers. Both soluble and extracellular vesicle (EV)-bound Hb are present in RCC [14]. The contribution of soluble and EV-bound Hb to the calculated hemolysis levels is a direct function of the methods used (sampling,

Abbreviations: BEST, Biomedical Excellence for Safer Transfusion; CBS, Canadian Blood Services; HiCN, cyanmetHb; EV, extracellular vesicle; FITC, fluorescein isothiocyanate; Hb, hemoglobin; Hct, hematocrit; RMPs, RBC microparticles; RBC, red blood cell; RCC, red cell concentrate; SHb, supernatant hemoglobin; THb, total hemoglobin.

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centrifugation, Hb and Hct assays) to perform the hemolysis measurement [15]. Therefore, the measurement of hemolysis is not simply a function of the THb, SHb and Hct of the RCC, but will depend on the characteristics of the EVs present in the product.

The aim of this study was to determine the variability in hemolysis measurement that exists in RBC quality testing labs and assess the impact of this variability on reported hemolysis values. Survey data from an international research collaborative was used to understand equipment and procedural variation in hemolysis testing. From these survey results, leading practices for performing hemolysis were used to guide a laboratory-based systematic evaluation of the potential consequences of the different approaches and procedures on the measurement of hemolysis in RCCs. In addition, the influence of EVs on measured SHb was specifically examined.

2. Materials and methods

2.1. Survey of hemolysis methods

A literature review revealed that many details of hemolysis methods are often not reported, preventing a meta-analysis of the subtleties among hemolysis methods (i.e. number and force of centrifugation, dilution factor, and assay temperature). We therefore collected detailed information on hemolysis methodologies in routine use from international blood operators via a survey of members of the Biomedical Excellence for Safer Transfusion (BEST) Collaborative. Survey results guided the selection of variables in the laboratory study.

2.2. Blood collection

Blood was collected and handled according to standard operating procedures at Canadian Blood Services (CBS). Briefly, whole blood units were collected from eligible donors and processed using the buffy coat (top/bottom) method [16]. Leukoreduced RCC (n = 5) with saline-adenine-glucose-mannitol (SAGM) preservative were obtained from CBS Network Centre for Applied Development program. Units were stored at 1–6 °C for up to 44 days. To reduce donor-to-donor variability and to have enough volume for all assays at different testing points, five ABO/RhD-matched units were pooled by sterilely connecting units to a large pooling bag, gently mixed well and returned to the original bags to generate five equivalent RCC products. Baseline testing on the split units included hematologic indices using an automated cell counter (Coulter AcT, Beckman Coulter) and hemolysis using the Drabkin's method (see below).

2.3. Sampling and study design

Sampling was performed using a validated technique as previously described [17]. Briefly, the RCC was thoroughly mixed by inversion 5–6 times while maintaining a gentle massaging motion. Using sterile technique, 130 ml of RCCs was aseptically drawn. Samples were distributed into eighty-three 1.5 ml microtubes. Three microtubes were used to test the THb, Hct and to analyze RBC microparticles in triplicate. The remaining 80 tubes were used to determine concentration of Hb, microparticle count and microparticle size in the supernatant. To prepare the supernatant, the tubes were divided into five groups of sixteen, with each group labelled according to the force of the first centrifugation to separate cells from the supernatant (S1; 1000, 1500, 2200, 2500, and 3000 ×*g*). Each group was centrifuged for 10 min at 4 °C using a microtube centrifuge (Eppendorf 5810R, Mississauga, Canada). Samples were then further divided into sub-groups based on the second centrifugation (S2) as follows: (i) No S2: samples were subjected to only one centrifugation (S1); (ii) 15,000*g* S2: samples were subjected to second centrifugation of 15,000 ×*g* for 15 min at 4 °C; (iii) 3000*g* S2: samples were subjected to second centrifugation of 3000 ×*g* for 10 min at 4 °C; (iv) 1400*g* S2: samples were subjected to second centrifugation of 1400 ×*g* for 5 min at 4 °C.

All measurements were performed in triplicate using three samples from each subgroup (n = 3) except the microparticle sizing (n = 1). Pre-spin testing, which included measurements on the Sysmex and Coulter, were performed on day 7 (fresh) and day 42 (expired). Microparticle sizing with the Zetasizer, and HemoCue, Harboe and Drabkin's methods were performed on day 8 (fresh) and on day 43 (expired). The microparticle count by the flow cytometer was completed on day 9 (fresh) and day 44 (expired).

2.4. In vitro RBC quality assays

2.4.1. Drabkin's method for percent hemolysis

THb was determined by diluting RBCs 1:200 in Drabkin's reagent (0.61 mmol/l potassium ferricyanide, 0.77 mmol/l potassium cyanide, 1.03 mmol/l potassium dihydrogen phosphate, and 0.1% Triton X-100). SHb was determined by diluting the supernatant 1:12.5 in Drabkin's reagent and incubated in the dark for at least 5 min at room temperature. Two-hundred microliters of each sample was transferred into a flat bottom microplate (Corning Life Science) and the absorbance was measured spectrophotometrically at 540 nm using a microplate reader (SpectraMax 384 Plus, Molecular Devices Corp.).

2.4.2. Harboe method for supernatant hemoglobin

Hb was directly measured by absorbance of oxyhemoglobin at 415 nm with correction for interference at 380 nm and 450 nm [18]. Prepared supernatants were diluted 1:9 in distilled water. Tri-level Hb controls (StanBio Laboratory, Boerne, TX) were used. All samples were analyzed by transferring 200 μl into a flat bottom microplate and reading at 415 nm, 380 nm and 450 nm in a spectrophotometer. Hb concentration was calculated using the following formula [18]:

$$\text{Hb (g/l)} = (167.2 \times A_{415} - 83.6 \times A_{380} - 83.6 \times A_{450}) \times 1/1000 \quad (2)$$

2.4.3. HemoCue - low/plasma Hb for supernatant hemoglobin

The HemoCue system consists of a plasma/low Hb photometer (HemoCue, Inc.) and disposable microcuvettes (HemoCue AB) containing reagent in a dry form. No dilution is required and the reaction is a modified azide-methemoglobin reaction [19]. In order to determine the Hb concentration and compensate for turbidity in the sample, the absorbance is measured at 2 wavelengths (570 and 880 nm).

2.4.4. Total hemoglobin and hematocrit

2.4.4.1. Manual hematocrit. RBCs were aspirated into a plastic clad, plain, self-sealing capillary tube. Samples were read visually after centrifugation for 5 min in a Hct centrifuge (Hettich Haematokrit).

2.4.4.2. Coulter AcT. The RBC sample was mixed thoroughly by inversion and 12 μl was aspirated in open mode by the Coulter AcT 8 Hematology Analyzer (Beckman Coulter). Samples were diluted inside the instrument, as described in the operator's manual (AcT Series Analyzer, 2010). RBC count and MCV was determined based on changes in electrical impedance. THb was quantified by a modified cyanmethemoglobin method. An aliquot of the original dilution was further diluted with Coulter AcT lysing reagent to a final ratio of 1:250 and absorbance was read at 525 nm.

2.4.4.3. Sysmex XE-2100. The RBC sample was mixed well by inversion and approximately 200 μl was aspirated in open mode by the Sysmex XE-2100. RBCs were counted using hydrodynamic focusing as described in the operator's manual (XE-2100 Hematology Analyzer). Hct was calculated using the cumulative pulse height detection method [20]. For THb, 3.0 μl of the aspirated RBC sample was diluted to a ratio of 1:333