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Review

Extracellular vesicle characteristics in stored red blood cell concentrates are influenced by the method of detection

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ABSTRACT

Extracellular vesicles (EVs), including microvesicles and exosomes, are small phospholipid vesicles (≤1 μm in diameter) that are present in blood products, accumulate during storage, and have a potential transfusion-related immunomodulatory role. Knowledge of EVs in stored blood products is limited due to the challenges and difficulties in detecting these heterogeneous submicron-sized vesicles. The aim of this study was to assess the impact of different approaches to characterize EVs in stored RBC products. Quantification and size-profiling of EVs in leukoreduced red cell concentrates (RCCs) were examined on day 3, 7, 21, and 42 of storage using tunable resistive plus sensing (TRPS), flow cytometer (FC), and dynamic light scatting (DLS) methods. Using the TRPS method, the concentration of EVs < 200 nm significantly increased throughout storage ($p < 0.05$). This change in exosome concentration was not detectable with FC or DLS due to limitations in their ability to resolve particles <200 nm and/or accurately determine EV concentration. Both the TRPS and FC demonstrate that the concentration of EVs ≥ 200 nm significantly increases in RCCs by day 42/43 compared to EVs present on day 3 ($p < 0.001$). As the DLS measures the average size of particles in suspension, only an increase in the zeta-average size was observed during storage. EV size and concentration in RBC products is significantly influenced by the length of storage. Overall, this study shows that combining technologies may be important to improve the characterization and study of EVs in stored RCCs.

Contents

Abbreviations: EVs, extracellular vesicles; CPD, citrate–phosphate–dextrose; DLS, dynamic light scatting; FC, flow cytometer; MPs, microparticles; MVs, microvesicles; NP, nanopores; RBC, red blood cell; RMP, RMP red cell microparticles; RCCs, red blood cell concentrates; SAGM, saline–adenine–glucose–mannitol; TRPS, tunable resistive plus sensing.

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1. Introduction

Heterogeneous populations of extracellular vesicles (EVs) have been shown to be present in and accumulate in red blood cell concentrates (RCCs) throughout storage and can be an indicator of red blood cell damage [1–5]. Extracellular vesicles, including exosome and microvesicles/microparticles (MVs/MPs), are submicron-sized vesicles (50–1000 nm in diameter) released in vitro and in vivo from many types of cells [6–9]. Extracellular vesicles have recently gained considerable attention due to their roles in numerous biological processes [10–13]. Extracellular vesicles contain various bioactive molecules (proteins, lipids, and nucleic acids such microRNAs) [12,14–18]. Recently, it has been shown that EVs, both MVs and exosome, are key mediators of intercellular signaling and communication [14,16,18–20]. The biological complexity of EVs including the variation in morphology, size, composition, cellular source and the biogenesis, create excessive challenges and technical difficulties in detecting, quantifying and size profiling EVs [6,21–23]. It has been shown that different EV characteristics can be observed when a sample is prepared or analyzed with different procedures or techniques [6,24,25]. Of note, most studies examining the biological implications of RBC EVs do not take into account the heterogeneity of EVs in the RCC products in terms of concentration, content, size, and phenotype.

Even though there are a wide variety of methods and techniques being used to detect and characterize EVs in blood products, there are several limitations associated with these methods [6,24–26]. For instance, flow cytometry (FC), is the most common optical method used to identify, quantify and characterize the EVs as it is readily available to research groups [11,13,26,27]. Nonetheless, one of the major limitations of standard FC is the lower limit of detection as most flow cytometers are unable to detect EVs less than 300 nm [7,11,13,26]. Notably, several studies have shown considerable attention to the importance of small EVs (exosomes) in cell–cell communication, cell signaling by participating in antigen presentation, and their potential roles in immunomodulation [15,28,29]. Therefore, improved instrumentation and techniques have become available to allow the characterization of small EVs [6,11,30,31].

Nanosizing instruments can be used to determine a wide size range of nano-sized particles by measuring the Brownian motion of particles in a sample using the dynamic light scattering (DLS). Dynamic light scattering is simple to apply and it can be used to accurately determine the size distribution of monodisperse populations of particles ranging from 1 nm to 6 μm [26]. This technique enables the detection of the small particle that cannot be detected by FC. However, measuring polydisperse or heterogeneous populations, such as EVs from body fluids, becomes problematic because there is a tendency to bias results toward the detection of larger particles [7,26]. Furthermore, absolute concentrations of EVs cannot be determined by DLS techniques [26]. A tunable resistive pulse sensing (TRPS) technology can be used to determine the size and concentration of small particles [6,22]. The TRPS technology uses nanopore electrical impedance to achieve single-molecule detection that can be used to determine the concentration and size profile of a wide range of particles in a sample [22]. DLS and TRPS techniques provide the opportunity to resolve nanoscale EVs in blood products.

The aim of this study was to evaluate the advantages and disadvantages of using three different techniques (TRPS, flow cytometer, and dynamic light scatting) to characterize EVs in stored RCCs.

2. Materials and methods

2.1. Blood collection

Whole blood was collected and RCCs were produced according to standard operating procedures at Canadian Blood Services. Briefly, whole blood units were collected from eligible donors into Top-and-Bottom blood collection packs *(LCRD quadruple T/B CPD/SAGM 500 mL Bactivam ITL, LQT7291LX, MacoPharma, Mouvaux, France)* and processed using the Top-Bottom filtration system ($n = 3$). As previously explained [33,34], approximately 480 mL of whole blood was collected in 70 mL of citrate–phosphate–dextrose (CPD)-anticoagulant. Units were held overnight and centrifuged to separate the blood components. The extracted red blood cells were suspended in about 110 mL of saline–adenine–glucose–mannitol (SAGM) within 24 h of stop-bleeding time, and the RCC units were luekoreduced by filtration at room temperature. All units were then stored at 1–6 °C for up to 42 days.

2.2. Sampling and study design

Sampling was performed using a validated technique as previously described [35,36]. At each testing point (day 3, 7, 21, and 42) units were gently massaged and thoroughly mixed by inversion and 12 mL of RBCs was aseptically drawn from each bag into 15 mL conical tubes using a sampling site coupler and an 18-gauge needle that attached to a 25 mL syringe. Samples were centrifuged at 2200 × *g* for 10 min at 4 °C (Eppendorf 5810R). The supernatant of each sample was equally distributed into three 1.5 mL microtubes for triplicate measurements. The microparticle size and concentration by the TRPS were measured on day 3, 7, 21, and 42. The microparticle count by flow cytometry and sizing by DLS were completed on day 4, 8, 22, 43.

2.3. Tunable resistive pulse sensing assessment

Quantification and size characterization of EVs were measured using a tunable resistive pulse sensing instrument (qNano system; IZON Science Ltd, Christchurch, New Zealand). Two different nanopores (NP200 and NP400, IZON Science Ltd) were used in this study to target EVs in the size range of 100–1000 nm using a standard stretch range (43–47 mm). Carboxylate polystyrene calibration particles (CPC200 and CPC 500; IZON Science Ltd.) were used with the NP 200 and NP400 nanopores respectively for optimization and to insure an accurate size and concentration measurements. The size profile of EVs in RCC supernatant samples was performed by comparing the resistive pulses (blockades) of the EVs (unknown diameter) with the resistive pulses resulting from measurement of calibration particles (known diameter). Both CPC200 and CPC400 were suspended in Solution A (Fluid Cell Electrolyte, IZON Reagent kit, RK1, IZON Science Ltd) according