Transfusion Medicine Reviews 33 (2019) 125–130

Contents lists available at ScienceDirect

Transfusion Medicine Reviews

journal homepage:
https://www.journals.elsevier.com/transfusion-medicine-reviews/

Extracellular Vesicles in Red Blood Cell Concentrates: An Overview

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ARTICLE INFO

Available online 23 February 2019

Keywords:
Extracellular vesicles
Blood transfusions
Red blood cell concentrate
Microparticles

ABSTRACT

Red blood cell (RBC) concentrates may be stored for up to 42 days before transfusion to a patient. During storage extracellular vesicles (EVs) develop and can be detected in significant amounts in RBC concentrates. The concentration of EVs is affected by component preparation methods, storage solutions, and inter-donor variation. Laboratory investigations have focused on the effect of EVs on in vitro assays of thrombin generation and immune responses. Assays for EVs in RBC concentrates are not standardized. The aims of this review are to describe the factors that determine the presence of erythrocyte-EVs in RBC concentrates, the current techniques used to characterize them, and the potential role of EV analysis as a quality control maker for RBC storage.

Contents

According to the World Health Organization there are approximately 112 million blood donations every year. Red blood cell (RBC) concentrates are the most requested blood product [1]. In Europe, the United States, Canada, and other nations, RBC concentrates are generally stored for up to 42 days at 4°C in additive solutions. The duration of acceptable RBC storage is defined in part by the in vivo recovery of a small radiolabeled aliquot of RBCs which (following maximal storage) should average >75% when measured 24 hours after autologous transfusion, and on the percentage of hemolysis which should be <1% at the end of storage. The quality of RBC concentrates is also dependent on the preservation solution used [2,3]. Although some studies report adverse events or poor clinical outcomes for patients receiving RBC concentrates

Abbreviations: ATP, adenosine triphosphate; CPDA, citrate–phosphate-dextrose-adenine; CPD-SAGM, citrate–phosphate-dextrose-saline-adenine-glucose-mannitol; CXCL-8, chemokine C-X-C motif ligand 8; EV, extracellular vesicle; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; FAS, apoptosis stimulating fragment; IL, interleukin; NADPH, nicotinamide adenine dinucleotide phosphate with bonded hydrogen; PAC-1, platelet activator complex; PS, phosphatidylserine; RBC, red blood cell; REV, red blood cell-derived extracellular vesicle; RSL, red blood cell storage lesion; SAGM, saline-adenine-glucose-mannitol; TEM, transmission electron microscopy; TF, tissue factor; TNFα, tumor necrosis factor alpha; TRALI, transfusion related acute lung injury.

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https://doi.org/10.1016/j.tmrv.2019.02.002
0887-7963/

stored for a longer period [4-6], high-quality randomized clinical trials and their meta-analyses have failed to demonstrate any clinically important effect of blood storage on patient morbidity or mortality [7-10]. In fact, some studies have suggested a higher incidence of adverse events from receipt of short-storage RBCs. Thus, the topic of blood storage age remains an area of active investigation.

Regardless of clinical effects, storage conditions may affect laboratory measures of RBC parameters including measures of key metabolites or measures of oxidative stress during storage. Extracellular vesicles (EVs) can be released during the storage period, and may be related to storage conditions [11-15]. The development of RBC-derived EV (REV) may also be related to donor variability or the manufacturing process used (eg, the additive solutions used and the method and timing of leucocyte removal).

Extracellular vesicles (EVs) are sub-micron vesicles derived from a parent cell [16]. They can have different names depending on their size (ranging from 30 nm to 1000 nm), or on their production mode (eg, exocytose, membrane budding or apoptosis).In the literature these vesicles are variously called exosomes, microvesicles, microparticles, or apoptotic bodies [17,18]. In this review, the term 'EV' is used to characterize all types of EV, and the term 'vesiculation' to describe EV generation, regardless of the size or the production pathway. Indeed, the level of our current knowledge and the tools currently at our disposal do not permit an accurate distinction between these entities [17-19].

REVs carry bioactive compounds which could potentially have a clinical impact. Even in the absence of any meaningful clinical effect, the role of REVs on procoagulant activity and immunomodulation is a matter for laboratory investigation. A better understanding of REVs and their relationship to storage conditions and manufacturing processes may provide an opportunity in the future to use REVs to monitor the quality RBC concentrates. In this review we describe REVs found in RBC concentrates, discuss the factors influencing the vesiculation process inside the concentrates, and provide an overview of proposed REV detection methods.

Factors influencing vesiculation in red blood cell concentrates

REVs have a heterogeneous morphology, both round or filament shapes [20-22]. As with all EVs, REVs have a phospholipid bilayer. The factors influencing REV release inside RBC concentrates are discussed below.

The storage conditions

RBC concentrates dedicated to transfusion may be stored for several weeks at 4°C in additive solutions [2,3,23]. During this time, observed changes may be biochemical (eg, levels of adenosine triphosphate (ATP), 2,3-diphosphoglycerate (2,3 DPG), nitric oxide, and oxidative stress), biomechanical (eg, deformability, osmotic fragility) or immunologic [24-28]. Some of these changes, commonly referred to as the "RBC storage lesion (RSL)", are reversible in vivo following transfusion, but other changes, including those that affect the RBC membrane are not [27,28].

During the storage period, the adenosine triphosphate (ATP) concentrations inside RBC concentrates decrease [27,29], and this depletion may be one cause of vesiculation. Indeed, ATP is essential, most notably for spectrin junction integrity. In conditions with decreased ATP, the membrane susceptibility to budding is increased due to a destabilization of the RBC membrane skeleton [30-33]. Moreover, a loss of ATP leads to an increase of intracellular calcium, another cause of vesiculation [29,34].

The development of REV in RBC concentrates could also be favored by the temperature of storage. At 4°C, the RBC membrane permeability can be decreased, promoting an increase of calcium inside the cell, increasing the potential for vesiculation of RBCs [35]. This effect is amplified by the fact that the Na^+/K^+ cationic pumps are ATP-dependent, and their activity are inhibited at 4°C. Increased intracellular Na^+ concentrations promote Ca^{++}entry into the cell [29,34].

RBCs also undergo oxidative stress during storage. This phenomenon is strengthened by loss of endogenous antioxidants (eg, decreased glutathione levels and inhibition of peroxiredoxin-2 recycling, an antioxidant that protects RBC from H_2O_2) [27,36-38]. Under these conditions, some RBC elements may undergo oxidative degradations. Band 4.1, band 4.2, band 3, band 4.9, spectrin, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are the proteins most rapidly affected by this phenomenon. Ankyrin can also be affected, but later during the storage time [38,39]. Most of these proteins are cytoskeletal components (eg, band 4.1, spectrin, β-actin) or participate in cell membrane integrity by linking other molecules (eg, band-3, band 4.2, band 4.9, ankyrin), explaining why the presence of reactive oxygen species can facilitate vesiculation processes [37,38]. GAPDH and hemoglobin may also be affected by oxidation, and this may have an impact on RBC metabolism. GAPDH is a key enzyme for glycolysis. It exists in two forms: an active cytosolic form and an inactive membrane bound form (linked to band 3). In vivo, in cases of oxidative stress, the reversible oxidation of GAPDH is responsible for a switch from glycolysis to a pentose phosphate pathway, allowing the cell to defend itself from oxidative stress by NADPH production (to maintain of the oxidation/reduction balance) [40]. Two oxidation mechanisms of GAPDH have been described in RBC concentrates stored in SAGM. The first consists of a reversible oxidation of the cytosolic enzyme. Reversible inhibition or activation of this enzyme may represent a protective mechanism favoring NADPH production in RBC concentrates [41]. The second mechanism consists of an irreversible storage-dependent oxidation of membrane-bound GAPDH, leading to a decrease in enzyme activity [42]. According to Reisz and colleagues, the level of oxidized enzyme increases with storage time, and this oxidized form is removed due to the vesiculation process. In this context, REV release would be a protective mechanism used by stored RBCs to remove a non-functional enzyme. Oxidative modifications have also been observed in functional residues of the hemoglobin β chain (at the level of the proximal histidine 93, cysteine β94 and histidine 144). The oxidation of these residues may have consequences for hemoglobin functions (eg, cysteine 94 is involved in nitric oxide scavenging, and H144 is necessary for stabilization of the deoxygenated states through direct binding to 2,3-DPG) [43]. According to the authors of this research, further structural and functional analyses are required to determine the implication of these oxidative modifications on gas transport [43].

REVs released from RBCs during their storage contain Fas (apoptosis stimulating fragment) proteins and their associated caspases 3.8 and Fas-associated death domain protein [44]. They also contain ubiquitinated proteins [45] and are enriched with hemoglobin (including oxidized cysteine 94), acetylcholine esterase (a glycosylphosphatidylinositol-linked enzyme) and stomatin [14,33,43,44,46-48]. Stored-RBCs also express phosphatidylserine (PS), which is partly explained by a decrease in flippase activity induced by ATP depletion and the pH changes in the bags with storage time [14,48-50]. PS expression is also found on the surface of REVs present in RBC concentrates [14].

Donor variability

An intra- and an inter-individual variability in the EV count and in EV-driven procoagulant activity has been described in healthy donors, by Gustafson et al. [51]. In the context of RBC concentrates, the REV release is linked to RSL occurrence, which in turn is influenced by donor specific factors, depending upon the hematological profile of each individual donor [52]. Furthermore, Tzounakas et al. showed that the REV basal amount and the extent of hemolysis during storage may also be donor-related [53], and suggested that other (as yet) unknown donor-related factors could explain a susceptibility of RBCs to vesiculate during the preparation of RBC concentrates. Inter-donor variability in uric acid

levels could also influence RBC adaptation to storage, as uric acid has antioxidant properties which could facilitate resistance to oxidative stress [54].

The leukoreduction method and the additive solution used for storage

The details of leukoreduction methods or the additive solutions used for storage may also influence the development of REV [45]. However, the level of evidence regarding their influence is insufficient to establish any international recommendations. Bicalho et al. showed that the leukoreduction technique used (ie, whole blood filtration versus buffy coat method) may directly influence the size of generated EVs in RBC concentrates during storage. They showed that EV size increased with storage time from 100 nm after 5 days of storage up to 200 nm after 42 days. However, at each storage time point, EVs from RBC concentrates prepared with the whole blood filtration method had a diameter smaller than those found in RBC concentrates prepared with the buffy coat method [15]. Others have shown that RBC concentrates prepared with whole blood filtration method have a higher total REV level and a higher concentration of EVs smaller than 200 nm than those prepared by the buffy coat method [55]. This is important since the size of EVs may modulate their bioactivity, including their procoagulant activity [56]. It has also been reported that RBC concentrates prepared by leukodepletion (ie, buffy coat removal and leukofiltration) are more resistant to osmotic changes during storage, and that ATP concentrations are higher than in units which have undergone buffy coat removal but without leukofiltration [57]. These differences in terms of size, resistance and metabolite levels related to RBC concentrate preparation methods could have an impact on REV emergence. This assumption requires further investigation in order to establish a clear relationship between vesiculation and these parameters.

Several preservation solutions are used worldwide [50]. SAGM, which is used in Europe, has not been licensed by the Food and Drug Administration. In the United States, additive solutions based on citrate–phosphate-dextrose-adenine (CPDA) are used [50,58]. Antonelou et al. compared the senescence markers between non-leukoreduced RBC concentrates stored in SAGM (ie, obtained with citrate–phosphate-dextrose (CPD)-SAGM top-and-bottom bag system) with those stored in CPDA. They found that RBCs stored in SAGM exhibited lower vesiculation, probably due to a lower oxidation status in this type of bag [44,45]. New generation additive solutions, including alkaline additives (eg, phosphate-adenine-glucose-guanosine-gluconate-mannitol, Erythro-Sol 5 and SOLX) are being tested with a goal to improve RBC storage [59]. RBCs stored with these new generation additive solutions had higher concentrations of key metabolites (eg, ATP and 2,3-diphosphoglycerate) and a lower hemolysis compared to RBC stored in SAGM [59-61]. Moreover, D'Alessandro and colleagues performed metabolomics analyses of RBC stored in alkaline additives. They showed that not only did RBCs stored in phosphate-adenine-glucose-guanosine-gluconate-mannitol demonstrated better preservation of ATP and 2,3-diphosphoglycerate levels, but also exhibited benefits in purine and redox metabolism in comparison with RBCs stored in other alkaline solutions (ie, Erythro-Sol or SOLX) [60]. Studies such as these seek to improve on current standard storage solutions and foster development and use of novel additives in the future.

Assessment of red blood cell-derived extracellular vesicles in red blood cell concentrates

The International Society on Thrombosis and Hemostasis and the International Society for Extracellular Vesicles have published guidelines for EV analyses in plasma. However, no specific recommendation has been made for the isolation of EVs inside RBC concentrates [19,62-66]. Table 1 summarizes different centrifugation protocols for REV isolation from RBC concentrates found in the literature, highlighting the diversity of procedures used. Standardization of these protocols would greatly help scientists working with EVs, limiting discrepancies both within and between studies.

Table 1
Centrifugation protocols to obtain sample dedicated to EV analysis from RBC concentrates

Centrifugation protocol	References
400*g* 10′ at 10°C followed by 10 000*g* 5′ at 10°C	[67]
DC 800*g* 10′ at 4°C	[68]
DC 1000*g* 10′	[37]
DC 1500*g* 15′	[69]
1500*g* 10′ followed by 1500*g* 20′	[70]
DC 1550*g* 20′ at 20°C	[71]
DC 1850*g* 10′	[47]
DC 1850*g* 20′	[14]
DC 1850*g* 20′ at 4°C	[13]
1900*g* 1′ followed by 800*g* 10′	[22]
DC 2000*g* 20′ at 4°C	[72]
DC 2000*g* at 4°C	[44]
DC 2200*g* 10′ at 4°C	[15]
DC 2500*g* 20′	[73,74]
DC 2500*g* 15′ at 4°C	[75]
DC 2600*g* 10′ at 4°C	[76]
DC 3000*g* 10′ RT	[77]
DC 4150*g* 10′	[78]
5000*g* 7′ RT followed by 12 500*g* 6′ at 4°C	[79]

Non-exhaustive review of literature to summarize centrifugation protocol to obtain sample dedicated to EV analysis from RBC concentrates. EV, extracellular vesicle; RBC, red blood cell; DC, double centrifugation; RT, room temperature.

Many techniques are available for EV analyses. They can give information about EV size distribution in a sample (eg, dynamic light scattering, nanoparticle tracking analysis, tunable resistive pulse sensing, atomic force microscopy) [15,47,80], about the morphology of vesicles (eg, transmission electron microscopy (TEM, cryo TEM) [30,81], and about the concentration and the phenotype of EVs in a sample (eg, flow cytometry) [13,15,75,82,83].

Flow cytometry is frequently used to quantify and to characterize antigen expression (eg, phosphatidylserine expression) of RBCs at various storage times [11,13-15,55,73]. The flow cytometer needs to be calibrated using standardization beads prior to EV analyses [75,84,85]. These beads allow a standardized EV assessment by establishing a gate dedicated to the analysis [84,86,87]. However, the resolution of the flow cytometer is too low to analyze all EV populations. Those EVs smaller than 300 nm are not detected by the majority of instruments used in clinical laboratories [88].

Gamonet et al. proposed a method, including a pre-analytical step and the calibration of the flow cytometer with beads, to detect REVs inside RBC concentrates [75]. Grisendi et al. also recommend a technique for EV analysis from RBC concentrates [83]. They suggested the use of carboxyfluorescein diacetate succinimidyl ester to stain full EVs and discard potential RBC debris from the sample. This probe, coupled to glycophorin A and annexin V (to highlight PS at EV surface), seems to be efficient for EV measurement [83].

Dynamic light scattering may also be used to analyze REVs [15]. Using this method, Brownian motion and the light scattered by particles allows measurement of the average particle size in a sample and the zeta potential [89]. This technique is accurate for monodisperse samples, but less suitable for polydisperse samples [66]. The technique of nanoparticle tracking analysis is based on the same principle as dynamic light scattering, but this technique also permits visualization of particles (by use of a camera) and tracking of particles [90]. Using this technique the concentration and size distribution of a population of EVs can be determined.

Tunable resistive pulse sensing is an impedance based system. It is based on the premise that when a particle crosses over a pore, it changes the voltage. This method allows determination of the concentration of particles for a particular size-range [65,66]. This technique was used by Almizraq and colleagues to characterize the EV population in whole

blood filtrated RBC concentrates in comparison to leukofiltrated RBC concentrates [55].

Potential impact of red blood cell-derived extracellular vesicles on transfusion recipients

The presence of REVs inside RBC concentrates has been described [11,13-15,55] and these REVs may potentially have clinical effects. Hypothesized, but as yet unproven, effects include those related to coagulation and immunomodulation as summarized in Fig 1. The potential clinical effects of EVs may depend not only on their cell of origin but also on the cargo carried [91].

In vitro observations

Thrombin generation

Several studies report an increase in procoagulant activity in RBC concentrates with storage time [13,77,92-94]. This effect is PS-mediated, but tissue factor (TF)-bearing EVs seem to have an increasing role with storage time [92]. Fisher et al. studied the impact of REVs on other cells. They showed that monocytes have a higher TF expression after exposure to REVs by demonstrating an increase of TF messenger RNA in a time and concentration-dependent REV manner. They also observed an increase of TF in plasma. Additionally, they showed that after REV exposure, the platelet phenotype changed with an increase of P-selectin and PAC-1 (platelet activator complex - activated gpIIb/IIIa) expression on the surface of platelets. These observations suggest that REVs in vitro promote procoagulant activity. Whether or not this translates into thrombotic complications following transfusion is not known. However, a recent large dataset study from the Scandinavia found no association between transfusion and thrombosis after controlling for patient co-morbidities [95].

Immunomodulation

The potential for REVs to influence immune responses has been studied in vitro [96]. Some investigators have found that REVs can increase the production of proinflammatory cytokines (interleukin-IL 2, 7 and 15) and tumor necrosis factor alpha (TNFα) by macrophages. They also showed that EVs can increase the proliferation of CD4+ and CD8+ T-cells by an interaction with antigen-presenting cell pathway. This effect was mainly driven by EVs with size of 200 nm or less [96]. The interactions of REVs with monocytes have also been studied [93]. An increase of proinflammatory cytokines (ie, IL-1β and IL-6 and chemokine C-X-C motif ligand 8 (CXCL-8)) in the supernatant of macrophages exposed to REVs has been observed.

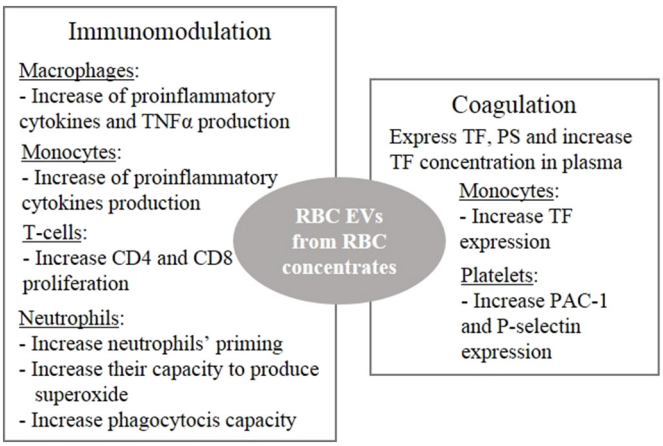

Fig 1. Summary of in vitro laboratory investigations of erythrocyte extracellular vesicles (EVs) obtained from RBC concentrates. TNFα, tumor necrosis factor alpha; TF, tissue factor; PS, phosphatidylserine; PAC-1, platelet ac complex.

The potential contribution of REVs to transfusion related acute lung injury (TRALI) has also been investigated. Cardo et al. have suggested that REVs may be implicated in neutrophil priming [97]. With increased expression of PS expression on the RBC surface, RBCs become more susceptible to the action of phospholipase A2. This enzyme converts phospholipid into arachidonic acid and lysophospholipids, likely explaining why older RBCs and REVs have lysophosphatidylcholine at their surface. These lysophospholipids have a structure similar to platelet activator factor, and so could possibly be related to neutrophil priming. In addition, CD11b (a marker of neutrophil priming) is overexpressed when neutrophils are exposed to REVs [67,97]. Belizaire et al. also showed that neutrophils demonstrated an increased capacity to produce superoxide and undergo phagocytosis upon exposure to REVs [67]. Studies such as these suggest a connection between the presence of REV and laboratory measures of immune response. However, these studies are far from demonstrating any sort of clinical effect on transfusion recipients and much additional work needs to be done before any clinical relevance can be assigned to recipient exposure to REVs at the time of transfusion.

Conclusions

The amount of REVs in RBC concentrates increases with storage time. However, an assessment of REV evolution over time is hindered, not only by limitations in detection methods but also by the fact that REV formation is influenced by manufacturing processes, storage solutions, and donor variability. Despite these limitations, studies of REV formation highlight the potential use of REVs as a future quality control measure for RBC storage. However, at the current time, the lack of standardized protocols dedicated to REV in RBC concentrates limits the reliability and usability of REV as a biomarker in blood transfusion centers. Secondly, the studies presented here show examples of current research investigating the hypothesis that REV may have a clinical effect following transfusion. Future REV research may shed new light on each of these lines of investigation.

Acknowledgments

The authors thank Dr. Ruth Murray for language editing. This work was supported by F.R.S.-FNRS-Télévie Belgium (grant N° 7652916F).

Declarations of Interests

None.

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