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Procoagulant role of microparticles in routine storage of packed red blood cells: potential risk for prothrombotic post-transfusion complications

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Summary

During routine storage, packed red blood cells (PRBC) undergo biochemical and morphological changes including loss of red blood cell (RBC) membrane asymmetry and release of microparticles (MPs) bearing phosphatidylserine (PS), a procoagulant phospholipid. This study investigated the association between PRBC storage duration, MP profile and procoagulant activity. Leukodepleted PRBC-supernatant (PRBC-SN; $n = 13$) was prepared at weekly intervals throughout storage. Phospholipid-dependent procoagulant activity, assessed using a factor X-activated clotting time (XACT) assay, decreased throughout storage ($p < 0.0001$), corresponding with increased procoagulant phospholipid content. As determined by flow cytometry, total numbers of MPs and of PS-bearing MPs increased by Day 28 of storage ($p < 0.01$ and $p < 0.05$, respectively, versus D1), and these MPs were predominantly RBC-derived ($CD235^+$). Depletion of MPs from stored (Day 42) PRBC-SN using 0.22 μm filters reduced the number of PS-bearing MPs ($p < 0.01$) but did not increase XACT clotting times. Furthermore, the reduction in procoagulant activity when lactadherin was used to block PS was not altered pre- or post-filtration of PRBC-SN. In conclusion, routine PRBC storage was associated with accumulation of MPs (particularly RBC-derived PS-bearing MPs) and of procoagulant phospholipids; however, depletion of PS-bearing MPs by 0.22 μm filtration did not reduce phospholipid-dependent procoagulant activity.

Key words: Blood coagulation; blood transfusion; cell-derived microparticles; erythrocyte transfusion; phospholipids.

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INTRODUCTION

Packed red blood cell (PRBC) transfusion is a standard therapy used to increase haemoglobin concentration and to restore the oxygen-carrying capacity of blood in patients with severe anaemia caused by chronic diseases, trauma or complex surgeries.[1] While it is a life-saving therapy, PRBC transfusion is not a risk-free practice. With the risk of transfusion-transmitted infections significantly reduced in recent years, non-infectious complications of transfusion are now the most frequent cause of morbidity and mortality in recipients.[2,3] One risk associated with PRBC transfusion is the development of coagulopathy. As seen in acute coagulopathy, an initial hypocoagulant state occurs due to depletion and consumption of clotting factors or their dilution by transfusion or resuscitation protocols, and this is often followed by the development of a hypercoagulable state which significantly contributes to poor patient outcomes.[4]

The routine *ex vivo* storage of PRBCs at 2–6°C for up to 42 days results in time-dependent degradation in red blood cell (RBC) morphology, metabolism and function, collectively termed the 'storage lesion'.[5] Recent clinical studies have documented that the storage duration of transfused PRBC units is a potential risk factor for increased thrombosis, multi-organ failure and mortality in trauma victims,[6] critically ill patients[7] and cardiac surgery patients.[8] However, there is also contradictory evidence indicating PRBC storage has no impact on post-transfusion outcomes[9,10] and collectively there is currently insufficient evidence regarding the potential association between PRBC storage duration and poor clinical outcomes to prompt changes to clinical practice.[11] Importantly, the possible risk of post-transfusion coagulopathy related to transfusion of stored PRBC necessitates further clinical and *in vitro* investigations to further define the risk, describe the underlying mechanisms and provide an evidence base to inform changes to blood-banking, coagulation testing and clinical transfusion practices in order to manage the potential risk of post-transfusion coagulopathy. The recipient's clinical status and requirement for transfusion may be as imperative as the quality of the blood product being administered.

With increasing PRBC storage times, adenosine-5-triphosphate (ATP) depletion, potassium leakage and oxidative damage contribute to loss of the asymmetric distribution of phospholipids in RBC membranes.[12–14] Consequently, phosphatidylserine (PS), a negatively charged phospholipid, becomes exposed on the outer surface of the membrane. Loss of phospholipid asymmetry and an increase in cytoplasmic Ca^{2+} disrupt the interaction between phospholipids and cytoskeleton, and promote shedding of microparticles (MPs) that bear PS and RBC transmembrane proteins.[15–17] The PS

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on MPs provides a surface for assembly of the tenase and prothrombinase complexes facilitating procoagulant pathways,[18] and it has been demonstrated that MPs from PRBC units contribute to thrombin generation[19–21] and other procoagulant effects.[22,23]

Normally, *in vivo*, the reticuloendothelial system removes damaged RBCs[24] and liver Kupffer cells remove MPs;[25] however, in the static environment of a PRBC unit, damaged RBCs and MPs are not removed and accumulate during routine *ex vivo* storage. Accordingly, several studies have shown the accumulation of MPs during the storage of non-leukoreduced PRBC units.[26,27] While the majority of these MPs are RBC-derived, MPs derived from platelets and leukocytes are also present and accumulate further during storage.[26,27] However, in many countries including Australia, PRBCs undergo pre-storage leukoreduction by filtration.[28] During routine storage of leukoreduced PRBCs, the accumulation of RBC-derived MPs has been demonstrated;[5,29] however, while one study showed an increase in PS-bearing MPs,[29] another showed a decrease in PS-bearing RBC-derived MPs.[5] While leukoreduction of PRBCs reduces the content of platelet and leukocyte-derived factors,[30,31] its impact upon the numbers of MPs derived from platelets and leukocytes remains uncertain. In addition, whether the MPs present in leukoreduced PRBCs are associated with procoagulant activity remains uncertain.

This study aimed to address the limitations in current knowledge regarding the procoagulant potential of leukoreduced PRBCs, their MP content and the contribution of these MPs to the procoagulant potential. It also aimed to investigate how these factors are impacted by routine PRBC storage. This was achieved by enumeration of various subpopulations of MPs in leukoreduced PRBCs during storage and investigation of their association with procoagulant activity as measured by a procoagulant phospholipid dependent assay.

MATERIALS AND METHODS

The Australian Red Cross Blood Service Research Ethics Committee and the Queensland University of Technology Human Research Ethics Committee approved the study.

Preparation of PRBC supernatants

PRBC units (240 ± 20 mL; $n = 13$) prepared according to standard Blood Service protocols based on the Council of Europe Guidelines[32] were obtained from the Australian Red Cross Blood Service (Brisbane, Australia). Citrate phosphate dextrose (CPD) anticoagulant bags with BioR-plus leukoreduction filter were used and RBCs resuspended in saline-adenine-glucose-mannitol (SAGM) (all from Fresenius Kabi, Germany). PRBC were stored at 2–6°C for 42 days and aseptically sampled at Day (D) 1 and then at weekly intervals (D7, 14, 21, 28, 35 and 42; $n = 9$) or at D42 only ($n = 4$). To obtain supernatant (PRBC-SN), 15 mL from each PRBC unit was processed by two sequential centrifugations at 3000 × *g* for 10 min at room temperature. PRBC-SN from each time point was stored in multiple aliquots at −80°C until further analysis.

Assessment of procoagulant activity

Procoagulant phospholipid (PPL) activity was determined using the factor X-activated clotting time (XACT) assay (Haematex, Australia) as previously described.[33,34] Clotting time was measured using an Amelung KC4A microcoagulometer (Trinity Biotech, Ireland). Commercial normal pooled plasma (NPP; Precision Biologic, Canada) was used as a baseline clotting control. PPL concentration was determined from clotting times via interpolation from a PPL reference mixture (provided with assay and is inversely proportional to clotting time).

Flow cytometric method for MP quantitation

Based on previous studies,[17,19] a calibrated-bead strategy was used to optimise the settings of the flow cytometer (Supplementary Methods and Supplementary Fig. 1, Appendix A). TruCount tubes were used to quantitate the number of MPs in PRBC-SN as follows: (events in specific gate/events in TruCount gate) × (number of beads per TruCount Tube/sample volume). A three-laser, FACS Canto II flow cytometer, with FACSDiva software (BD Biosciences, Australia) was used for all data acquisition and FCS Express v3 software (De Novo Software, USA) for data analyses.

Assessment of the cellular sources of MPs in PRBC-SN and their expression of PS

Freshly thawed aliquots of PRBC-SN (50 µL) were incubated with anti-CD235a-fluorescein isothiocyanate (FITC) (1.5 µL), anti-CD41a-allophycocyanin (APC) (1.5 µL) and anti-CD45-peridinin chlorophyll (PerCP) (3 µL) in TruCount tubes [15 min, room temperature (RT), dark]. Subsequently, cold annexin V binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, pH 7.4) was added to a final volume of 240 µL, then annexin V-V450 (10 µL) was added and incubated (15 min, RT, dark) before immediate analysis on the flow cytometer (all reagents from BD Biosciences). Concentration-matched isotype and unstained controls were used (≤2% cut-off) to determine gates and quadrants.

Confirmation of PS expression on MPs using lactadherin

PRBC-SN (50 µL) was incubated with anti-CD235a-BV421 (5 µL), anti-CD41a-APC (1.5 µL) and anti-CD45-PerCP-Cy5.5 (3 µL) in TruCount tubes (all from BD Sciences) (15 min, RT, dark). Following addition of lactadherin-FITC (10 µL) (Haematologic Technologies, USA) and incubation (15 min, RT, dark), PBS was added to a final volume of 250 µL.

Depletion of MPs by filtration

Aliquots of D42 PRBC-SN ($n = 9$) were filtered through a 0.22 µm filter (Millex-GS; Millipore, USA). MPs were quantified pre- and post-filtration via flow cytometry using the staining panels outlined previously and procoagulant activity was determined (XACT assay).

Inhibition of PS-mediated coagulation by lactadherin

The capacity of lactadherin to block the PPL activity of PRBC-SN was tested by modifying the XACT assay. Briefly, 15 µL of freshly thawed D42 PRBC-SN was mixed with 10 µL of 1–150 nmol/L lactadherin and then incubated with 25 µL of PL deficient plasma (37°C, 2 min) and 100 µL of XACT reagent. Clotting times were measured. The negative control consisted of replacing the lactadherin with 10 µL of tris-buffered saline (TBS) (50 mmol/L Tris-Cl, 100 mmol/L NaCl, pH 7.6).

Statistical analyses

Data were analysed using GraphPad Prism 5 (GraphPad Software, USA). Data not normally distributed were presented as median with interquartile ranges (IQRs). Friedman test was used to assess differences across multiple time points for the MP and XACT data and Dunn's multiple comparisons test was used to identify differences between time points (Day 1 as the comparator). The Wilcoxon matched-pairs test was used to compare pre-filtration and post-filtration results. Correlation analyses between MP generation and PPL activity was performed using the Spearman's rank test. Results of inhibition assays were normally distributed and data displayed as mean ± SEM, and analysed by paired t-test. $p < 0.05$ was considered significant.

RESULTS

Procoagulant activity in PRBC-SN increased with storage duration

To assess the procoagulant activity of stored PRBCs during routine storage, the clotting time (XACT assay) induced by PPL present in PRBC-SN, was measured. The median clotting time of NPP, used as a baseline control, was 45 s (IQR 44–48 s). Prolonged storage of PRBC was