Effects of storage time and leucocyte burden of packed and buffy-coat depleted red blood cell units on red cell storage lesion

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**Background.** The red cell storage lesion (RCSL) comprises the biochemical and biomechanical changes that take place during red blood cell (RBC) storage, reducing the survival and function of these cells. Contaminating white blood cells have been major contributors to the RCSL. Markers of RCSL, such as CD47 and phosphatidylserine (PS), on RBC are attracting more attention. The aim of this study was to elucidate the effects of storage time and buffy-coat removal on CD47 and PS expression on RBC. Potassium and free haemoglobin levels in the supernatant plasma were also assessed.

**Materials and methods.** Forty-three red cell concentrates were divided into two groups [Group 1: packed red cells (n=22); Group 2: red cell units from which the buffy-coat had been removed (n=21)] and samples were collected on days 1, 14 and 28. Flow cytometry was used to monitor changes of CD47 and PS expression on RBC over times. Supernatant potassium was measured and percent of haemolysis calculated.

**Results.** A significant, progressive decrease in RBC CD47 expression during storage was observed in both groups. The decrease in RBC CD47 expression was significantly less in the buffy-coat-removed group of units than in the other group. The percentage of annexin V-positive cells increased significantly in both groups. Buffy-coat depleted components showed less expression of PS only in the early samples. There were significant, progressive increases in percentage of haemolysis and supernatant potassium during storage in both groups.

**Conclusion.** RBC stored for more than 14 days exhibited reduced CD47 and increased PS. Buffy coat removal reduced the loss of CD47, but had no impact on plasma haemoglobin, potassium or RBC PS exposure.

**Keywords:** CD47; phosphatidylserine; red cell storage lesion; leucocyte burden.

**Introduction**

Red cell storage lesion (RCSL) can be defined as a series of biochemical and biomechanical changes in red blood cells that occur during the storage of this blood product and that reduce the subsequent in vivo survival and function of the red blood cells. Ho and colleagues recorded some changes that occur to the red blood cell during storage. These changes include a reduction in red blood cell deformability, altered red blood cell adhesiveness and aggregability, and a reduction in 2,3-diphosphoglycerate and ATP. Bioactive substances, including histamine, lipids, cytokines (interleukin-1, interleukin-8, and tumour necrosis factor), fragments of cellular membranes and soluble human leucocyte class I antigens—many of which are at least in part white blood cell (WBC)-derived and with pro-inflammatory effects, also accumulate in the storage medium. These changes reduce the post-transfusion viability of red blood cells. Evidence suggests that the storage lesion may reduce tissue oxygen availability, have pro-inflammatory and immunomodulatory effects and influence morbidity and mortality. Also, they found that leucoreduction improves the quality of stored red blood cells.
The markers of RCSL and its in vitro and in vivo manifestations are receiving more attention, and the changes in CD47 and phosphatidylserine (PS) during storage have been well documented. It has been suggested that CD47 plays a role as a marker of self on RBC. CD47 is a 50-kDa plasma membrane protein with an extracellular immunoglobulin-like domain, five transmembrane domains, and a short cytoplasmic tail. CD47 on RBC is recognised by a signal regulatory protein α (SIRP α) on macrophages. The interaction between the CD47 molecules on normal RBC and SIRP α receptors on macrophages sends a negative signal to macrophages which protects RBC from phagocytosis.

Another marker that is both indicative of RCSL and capable of influencing the function of transfused RBC is PS. PS is normally retained on the inner side of almost all body cell membranes by means of energy-dependent transfer. When nucleated cells enter apoptosis, the asymmetric distribution of the cell membrane is randomised. This results in PS being present on the membrane surface and is a signal for phagocytosis. Increased expression of PS on the surface of the cell could, therefore, play a major role in the clearance of the transfused RBC, as evidenced by removal of senescent cells from the circulation and rapid clearance of PS-expressing RBC by macrophages. Finally, PS exposure could up-regulate cellular apoptosis and/or necrosis during storage.

Annexin V is a calcium-dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS.

White blood cell contamination contributes to red cell storage lesion. However, leucoreduction before storage improves RBC morphology and decreases hemolysis, microvesiculation, and potassium leakage.

This study was designed to elucidate, separately, the effects of storage duration and buffy-coat removal of red cell units on the level of expression of CD47 and PS on RBC, and the concentration of potassium and free haemoglobin in the supernatant plasma. This might provide important information for aiding the selection of appropriate blood components for critically ill and severely anaemic patients.

Materials and methods

Materials

Approximately 450 mL of whole blood were collected, from each of 43 qualified donors, into double and triple blood bags containing 63 mL CPDA-1. The donors met the standards of blood donation criteria for allogeneic blood transfusion (Standards of American Association of Blood Banks, 1999). All blood units were stored under standard blood bank conditions at 4 ± 2°C for 5 weeks. Initial leucocyte counts were done on an automated cell counter (Sysmex KX-21 cell counter, Kobe, Japan) for all units before separation. The collected blood units were further divided into two groups.

Group 1 consisted of 22 units of packed red cell units: hard-spun red cell concentrates were prepared. The blood bags were centrifuged in a Rotanta 460R centrifuge (Hettich, Tuttlingen, Germany) at 5000 g for 6 min with no brake (within 4 hours of donation). The plasma supernatant was extracted and separated into the empty satellite bag using a manual separator, while keeping the packed RBC to a haematocrit level of approximately 70% in the primary bag.

Group 2 consisted of 21 red cell units from which the buffy-coat had been removed: whole blood (within 4 hours of collection) was centrifuged at 3600 g for 11 min with no brake. The supernatants (plasma and buffy-coat) were transferred to the other satellite bag using a manual separator, while keeping the remaining RBC concentrates in the primary bag.

After gentle mixing under a laminar airflow cabinet, 10 mL samples were collected from each unit on days 1, 14, and 28. The effect of storage was studied up to day 28 only since most RBC units get issued within this period. On day 1, plasma haemoglobin, haematocrit and leucocyte counts were measured by an automated cell counter (Sysmex KX-21 cell counter, Kobe, Japan). On days 1, 14 and 28, 4 mL of the samples were analysed for CD47 and PS. Five millilitre aliquots of the samples were centrifuged at 480 g for 10 min. Supernatant was aspirated and centrifuged again at 3400 g for 15 min to be used for the analysis of potassium and free haemoglobin. All samples were analysed on the day of collection.

Methods

Flow assisted cell sorting (FACS) was used to measure and monitor both the CD47 and the PS levels on the RBC. RBC were labelled with phycoerythrin-conjugated anti-glycophorin-A (BD Biosciences) in order to isolate the RBC population on the flow
To test cells for CD47, the cells were labelled with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibody (clone B6H12 from BD Biosciences). Appropriate isotype negative controls and fresh RBC as positive controls were used. Aliquots of RBC were washed twice in Tris-buffered saline (TBS) and 25 µL were added to 250 µL of TBS for the CD47 test cells and isotype controls. Five microlitres of the RBC preparation were added to appropriately labelled tubes, followed by 5 µL of the appropriate FITC-conjugated antibody. The tubes were gently mixed and incubated in the dark at room temperature for 15 minutes. One millilitre of TBS was added to each tube. The analysis was done by FACS within 1 hour.

Annexin V-FITC (BD Biosciences) was used to test cells with exposed PS according to the method described by Kuypers and colleagues. Positive controls for PS testing were prepared by treating fresh RBC with N-ethylmaleimide (NEM) and calcium ionophore. Negative controls for PS testing were obtained by resuspending positive control cells 1 x annexin V binding buffer containing EDTA to a final concentration of 5 mmol/L to chelate the calcium required for annexin V binding.

Prior to flow cytometric analysis, samples were passed through a 25 gauge needle in order to ensure uniform cell dispersion. Samples were analysed on a FACSCalibur flow cytometer (BD Immunocytometry Systems) with a 488 nm argon laser and standard filter set. Data were acquired and analysed using Cell Quest software (version 3.1). A forward scatter versus side scatter plot with logarithmic scales was used to set a region around the RBC population, which excluded debris and agglutinates. Mean channel fluorescence (MCF) was determined from 20,000 gated events in the FL1 channel. The instrument was aligned and calibrated using CaliBRITE beads and FACSComp software (BD Biosciences).

Potassium levels in supernatant plasma were determined using the ion-selective electrode methodology (AVL 983-S, Graz, Austria).

The amount of free haemoglobin in the supernatant was determined using a minor modification of Drabkin’s haemoglobin assay for plasma according to Moore et al. on a Humalyzer 2000 spectrophotometer. This method gives linear result from 5 to 2,000 mg/dL. Cyanmethaemoglobin standard, 80 mg/dL, and cyanmethaemoglobin diluent powder from Stanbio (Boerne, USA) were used with fresh pooled, hard spin plasma as plasma controls. The degree of haemolysis is described as the percent of free haemoglobin in relation to the total. The formula used is described below:

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\text{Percent haemolysis} = \left( 100 - \frac{\text{Hct}}{\text{total Hb}} \right) \times \frac{\text{free plasma Hb (g/dL)}}{\text{total Hb (g/dL)}},
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where Hct is haematocrit and Hb is haemoglobin.

Statistical analysis
Statistical analysis was performed with computer software (SigmaPlot, v10.0, Systat Software, Inc., San Jose, CA, USA). Data are presented as mean ± standard deviation (SD). A non-parametric test was used to determine the statistical significance of values. \( p \) values <0.05 were considered statistically significant.

Results
CD47 expression on RBC during storage
The geometric mean fluorescence intensity values of CD47 on the 1st, 14th and 28th days were 184.4, 152.4 and 134.8 in group 1 and 248.4, 184.6 and 160.9 in group 2, respectively. The decrease in CD47 expression along the storage period was statistically significant in both groups (\( p<0.05 \)) (Figure 1). The expression of CD47 was significantly higher in group 2 than group 1 on the 1st, 14th and 28th days (\( p=0.05 \)).

Flow cytometric analysis at time points from day 1 to day 28 of storage showed a significant (\( p<0.05 \)) left shift in MCF (compared to CD47 expression at day 1) of the total RBC population labelled with anti-
CD47-FITC antibody (Figure 2). The left shift in fluorescence intensity suggests a loss of CD47 antigen expression at the RBC external membrane during storage. There was a significant negative correlation (r = -0.574, p < 0.05) between CD47 expression on RBC during storage and number of white blood cells after separation (leucocyte burden) (Figure 3).

**PS expression determined by FITC-annexin V binding**

The mean values of percentage of annexin V-positive cells on days 1, 14 and 28 increased in a statistically significant manner in both groups (Figure 4 and Figure 5). A significantly lower exposure of PS was detected in the group 2 units (buffy-coat removed) compared to the other group only in the samples taken on the first day. There was a statistically significant negative correlation (r = -0.520, p < 0.05) between CD47 expression on RBC during storage and percentage of annexin V-positive cells in both groups at all storage times.

**Haemolysis and potassium levels in supernatant during storage**

There was a statistically significant increase in percentage haemolysis and in the mean values of potassium (p < 0.05) along storage time in both groups (Figure 6). However, haemolysis was much below the permissible level of 0.8% haemolysis in both groups when measured on day 28 of storage. The percentage haemolysis and mean values of potassium were not statistically different between the two groups at the different storage times.

**Discussion**

During the ex vivo storage of blood products, bioreactive substances accumulate in the storage medium and numerous changes occur in the RBC, collectively making up the storage lesion. These changes could have adverse effects on the transfused host, particularly in the presence of pre-existing disease. The impact of the storage lesion on post-transfusion RBC survival and the link between storage-related changes and clinical sequelae remains speculative.

In this study statistically significant decreases in CD47 expression were shown along the storage period in both groups. The decrease in CD47 was...
Percentage haemolysis significantly less in the buffy-coat removed group of units. In agreement with the findings of the present study, Stewart and colleagues found a significant decrease of CD47 on RBC during storage both by a MAIRA (monoclonal antibody immobilisation of red cell antigens) assay and by FACS. The decrease was not linear, with there being a significant drop between day 10 and day 24. The reduction of CD47 was of the order of 52% to 65% by days 24 and 31, respectively. However, the results of these two studies differ considerably from those in the study by Anniss and Sparrow, who reported a significant but only a small reduction of expression of up to 9% with FACS. This difference could be due to the different storage and assay conditions in the studies. Furthermore, the number of red cell concentrates studied by Anniss and Sparrow was small (n=19, six of which were irradiated).

The decrease of CD47 on the RBC is largely due to release of the molecules, because increasing amounts of the latter have been found in the supernatants of RBC units during storage. CD47 may have numerous adverse effects through its binding to ligands on the cells and tissues of the recipient. These effects may include the blocking of receptor sites or alternatively the induction of...

Figure 5 - Representative flow cytometry dot plot showing the increase of the percentage of annexin V-positive cells during the storage of RBC (left, day 1; right, day 28).

Figure 6 - Mean percentage haemolysis during the storage periods in the two groups of RBC units.
However, concomitant internalisation of CD47 molecules during externalisation of PS cannot be ruled out. The extent of reduction of CD47 on stored RBC is critical because RBC become susceptible to phagocytosis by splenic red pulp macrophages when this reduction exceeds 50% and are rapidly cleared from the circulation.

Head and colleagues showed that the ligation of CD47 by monoclonal antibodies mediated PS signalling and expression on red cells and that both mechanisms were responsible for loss of erythrocyte viability and induction of erythrocyte death. Oldenborg et al. demonstrated that the level of IgG opsonisation required to signal phagocytosis in CD47-positive cells was much greater than that required to signal phagocytosis in CD47-deficient cells. This indicates that the IgG signal that initiates phagocytosis is regulated by the CD47–SIRPα signal that protects from phagocytosis.

The results of the current study also showed an increase in PS during storage of both groups of RBC, with a significantly lower exposure in buffy-coat removed red cells compared to the other group only in the samples taken on the first days. These changes are concurrent with changes previously observed in senescent RBC. Our data suggest that RBC continue to age during storage. Moreover, increased PS levels on the cell surface suggest that PS has been moved from the inner monolayer of the plasma membrane to the outer monolayer. The increased levels of PS on the RBC surface could also lead to phagocytic clearance of the cells from the circulation by macrophages. Stewart and colleagues suggest that the movement of membrane phospholipids in this manner is not passive, but rather is the result of scrambling of the plasma membrane, bypassing the normal mechanisms of active transport, and attachment to proteins of the cytoskeleton that keep PS in place. It is thought that this process is a result of a "scramblase" enzyme that moves PS to the outer monolayer of the plasma membrane.

The influence of the age of RBC, within their 120-day life cycle, at the time of blood donation on the RBC storage lesion was investigated by Sparrow et al. who found increased levels of annexin V in the supernatant of RBC stored in the presence of leucocytes, with significantly higher levels found for old RBC than for young RBC.

The present study showed that buffy coat removal did not affect plasma haemoglobin or potassium levels compared to the levels in packed cell units. However, the percentage of haemolysis in all RBC units was below the permissible level of 0.8% on the 28th day of storage. Hess and colleagues reported that donor-to-donor variation, leucoreduction, duration of storage, the amount of mannitol in storage solutions, and storage haematocrit are all important determinants of the degree of end-of-storage haemolysis. Seghatchian et al. observed different patterns of potassium and plasma haemoglobin levels within the same blood components as well as differences in patterns of the storage lesion between different red cell components, suggesting that there may be more than one mechanism of cellular injury. Seghatchian and Krailadsiri compared processing/storage-induced changes in four types of RBC units [whole blood, plasma reduced blood (filtered and unfiltered) and SAGM)] for 35 days, using four markers of red cell storage stability (supernatant potassium, haemoglobin, pH and annexin V). They concluded that supernatant annexin V in combination with potassium levels and plasma haemoglobin is a better indication of global cellular damage. From their study it appears that mechanical trauma and the presence of leucocytes and platelets in the RBC bag have the potential to increase levels of plasma haemoglobin and potassium.

The aetiology of RBC membrane lesions appears multifactorial, with the two main factors perhaps being reactive oxygen species and proteolytic enzyme activity. D'Amici et al. attribute the morphological, biochemical, and metabolic changes in RBC to reactive oxygen species produced during storage, rather than to proteases.

Tinmouth and colleagues concluded that there is strong laboratory evidence suggesting that prolonged RBC storage may be deleterious and that observational studies report a number of associations between prolonged storage and adverse clinical outcomes such as mortality and organ failure. However, in their systematic literature review, Lelubre et al. identified 24 studies that evaluated the effect of RBC age on outcomes in adult patients and demonstrate their contradictory results. They concluded that, from the currently available published data...
in vivo data, it is difficult to determine whether there is a relationship between the age of transfused RBC and outcome in adult patients, except possibly in trauma patients receiving massive transfusion.

The results of the current study indicate that the storage time of RBC concentrates and the leucocyte burden of the concentrate may influence the nature of the RBC storage lesion. Older RBC show lower expression of CD47 at the external membrane of erythrocytes using annexin V-Ferrofluid. Biochem Biosphys Res Commun 1999; 258: 199-203.

References