Rheologic changes of hypothermic preserved red blood cells
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Chapter 3

Red blood cell aggregation; an important phenomenon in damage control resuscitation?

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Abstract
To improve the survival of severely bleeding trauma patients, a damage control resuscitation strategy has been recommended. This strategy includes the early infusion of fresh frozen plasma (FFP), platelets and RBCs in a 1:1:1 unit ratio to control the bleeding and favor survival of these patients. Although lower FFP to RBC ratios have been linked to higher rates of mortality, these high ratios have been associated with adverse outcomes as well. The formation of RBC aggregates in regions with low shear rate could play a key role in these findings. Administration of FFP and thus fibrinogen is essential for coagulation. Yet, fibrinogen also promotes RBC aggregation. Although physiological levels of RBC aggregation support the hemostasis, promotion of aggregation could be disadvantageous in patients in which the RBC rheology is already compromised, as was observed in certain trauma states. Notably, enhanced RBC aggregation may hinder tissue perfusion and contribute to the occlusion of micro-vessels. We consider that RBC aggregation could play an important role in damage control resuscitation of severely injured trauma patients.
Uncontrolled bleeding is the leading cause of death in trauma patients. It was recognized that approximately 25% of severely injured trauma patients are coagulopathic upon admission and that these patients are three times more likely to die than those without it.¹ This acute coagulopathy of trauma has been attributed to multiple factors such as loss, dilution and consumption of coagulation factors and platelets as well as to fibrinolysis, hypothermia and metabolic acidosis.² To improve the survival of severely bleeding trauma patients, an early damage control resuscitation strategy has been recommended. This resuscitation approach primarily advocates limited crystalloid use, prevention and treatment of acidosis and hypothermia, as well as an early administration of fresh frozen plasma (FFP), platelets and RBCs in a 1:1:1 unit ratio.³ Although the optimal blood component ratio is still a matter of debate, the general consensus is that an early resuscitation approach with this high FFP to RBC ratio, controls the bleeding and potentially favors survival of severely bleeding trauma patients.⁴ Limited attention has been addressed, to the mechanism by which a high FFP to RBC ratio influences survival in these trauma patients. Recently, the improved survival has been linked to inhibition of vascular endothelial permeability and subsequently diminished interstitial edema.⁵ Yet the ability of RBCs to form aggregates in the presence of plasma proteins, especially fibrinogen, could also play a pivotal role in damage control resuscitation.

The formation of RBC aggregates in regions with low shear rate is a physiological phenomenon that has been studied for decades. Although RBC aggregation is a major determinant of the whole blood viscosity at low shear rate, the physiological role of this process is still elusive. Under normal physiological conditions, RBC aggregates are easily dispersed by the rise in blood flow rate. However, under pathological conditions stronger and/or larger RBC aggregates are formed which are more resistant to dispersion by shear forces. Enhanced RBC aggregation may impair the blood flow in the microcirculation and contribute to the occlusion of micro-vessels, which may induce local hypoxia and damage to endothelial cells.⁶,⁷ In this regard, the LORCA is a useful device, which allows RBC aggregation to be studied ex vivo.

In cases of massive bleeding, fibrinogen is the first coagulation factor that reaches critically low levels. Administration of blood components in an 1:1:1 unit ratio will replenish depleted coagulation factors and platelets and minimize dilutional coagulopathy, as is the case when only RBCs or volume expanders will be administrated.⁸ Yet, in a trauma setting...
the use of FFP in massive bleedings has also been questioned. Adverse outcome such as increased incidence of nosocomial infections, multiple organ failure, lung injury and death have been linked to the usage of FFP.\textsuperscript{9} Studies have shown mixed results, but in general, high FFP to RBC ratios have been associated with adverse outcomes whereas low FFP to RBC ratios have been linked to increased rates of mortality.\textsuperscript{1,10}

For many years, it has been recognized that RBCs can actively participate in clot formation by enhancing platelet de-granulation and by recruitment of additional platelets into the forming clot.\textsuperscript{11} The role of RBC aggregation in hemostasis has been given less attention. RBCs migrate from the endothelial wall into the center of the blood vessel where they form aggregates. RBC aggregates exclude leukocytes and possibly platelets from the axial core and direct them towards the vascular wall.\textsuperscript{12,13} This process is essential since leukocytes and platelets need to get into close contact with the damaged endothelium, in order to exert their function.

RBC aggregation increases proportionally with fibrinogen levels.\textsuperscript{14} Administration of FFP, platelets and RBCs in a 1:1:1 unit ratio, a composition that approximates whole blood, could promote RBC aggregation. On the one hand promotion of aggregation would be beneficial for supporting hemostasis in severely injured trauma patients. Especially, since it has been recognized that people with leukocyte adherence deficiency suffer from recurrent bacterial infections and impaired wound healing and because it has been recognized that infections remain a concerning complication of combat-related injuries.\textsuperscript{15,16} On the other hand, promotion of aggregation could be detrimental to patients in which the RBC rheology is already compromised, as was observed in certain trauma states.\textsuperscript{17-19} In this regard, enhanced RBC aggregation could subsequently hamper tissue perfusion and contribute to the occlusion of micro-vessels. Enhanced RBC aggregation, which was also evident during long-term storage of non-leukoreduced RBC units, could furthermore explain the finding that blood component infusion was less effective than fresh whole blood in supporting hemostasis of trauma patients.\textsuperscript{3,20,21} The above mentioned data underline the potential importance of RBC aggregation in damage control resuscitation.

Most studies regarding resuscitation practices are retrospective. Although these studies are limited inherently to their retrospective design, they do provide interesting hypotheses. Early FFP infusion is considered lifesaving in severely bleeding trauma patients. Yet, to determine the influence of high FFP to RBC ratios on promoting RBC aggregation in these patients, ex vivo aggregation testing will be necessary. In this regard, the LORCA could be
useful to demonstrate RBC aggregation tendencies after damage control resuscitation of severely bleeding trauma patients.

References


Chapter 4

Is red blood cell rheology preserved during routine blood bank storage?

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Abstract

RBCs refrigerated stored for more than 2 weeks at 4°C are currently considered of impaired quality. This opinion has among others been based on altered RBC rheologic properties (i.e. enhanced aggregability, reduced deformability and elevated EC interaction) observed during storage of non-leukoreduced RBC units. Nevertheless, with the implementation of leukoreduction the storage-induced lesions have considerably diminished. In this study, the aggregability and deformability of leukoreduced RBCs during routine blood bank storage were investigated. At the blood bank, ten leukoreduced RBC units were refrigerated stored in SAGM preservation solution for up to 7 weeks. RBCs were weekly tested for aggregability, deformability and other hematologic variables. The RBC aggregability was significantly reduced after the first week of storage but recovered during the following weeks. After 7 weeks of storage the aggregability was slightly reduced (from 46.9 to 44.3 AI; p < 0.05). During storage the osmotic fragility was not significantly enhanced and the deformability at a shear stress of 3.9 Pa was not significantly reduced. The deformability at a shear stress of 50 Pa was reduced (from 0.58 to 0.54 EI; p < 0.05) but remained within physiological values (0.53 ± 0.04). During 5 weeks of storage the ATP content was reduced by 54% whereas the MCV, pH and MCHC were minimally affected. We conclude that the rheologic properties of leukoreduced RBC units were well preserved during routine blood bank storage.
4.1. Introduction

During refrigerated storage at 4°C, RBCs undergo physical and biochemical alterations collectively referred to as the storage lesion. Recent publications suggest that transfusion of long-term refrigerated stored RBCs are associated with adverse clinical outcome in critical ill, cardiac surgery, and trauma patients.

The RBC rheology, that is, the ability to aggregate, deform and adhere to ECs, are important determinants of the blood flow and hence the oxygen delivery to the tissues. Aggregation takes place in the venous system where RBCs form linear stacks of cells or multi-cellular aggregates at low shear rates. Normally the increasing blood flow is sufficient to disperse these aggregates. However, under pathologic conditions stronger and larger aggregates may form, which are more resistant to dispersion by the blood flow. The RBC ability to deform due to applied forces makes these cells capable of passing the capillaries. High RBC deformability and a rapid recovery to the normal shape are therefore essential factors for maintaining tissue perfusion. The RBC deformability is also a major determinant of the posttransfusion survival, since less deformable cells will be sequestered and destroyed in the spleen. Elevated adherence of RBCs to ECs can reduce the blood flow in the microcirculation and activate ECs, contributing to the occlusion of micro-vessels. Alterations in RBC rheology have been observed in a variety of diseases such as cardiovascular disease, hypertension, diabetes mellitus, renal failure, malaria, thalassemia and sickle cell disease. Transfusion of rheologic impaired RBCs may hinder or obstruct the blood flow in micro-vessels leading to impaired tissue perfusion, ischemia or infarction. Therefore, rheologic impaired RBCs may form a hemodynamic risk particularly in recipients with circulatory and/or cardiovascular disorders. Long-term refrigerated storage may alter the RBC rheologic properties and adversely influence transfusion outcome. In vitro studies with non-leukoreduced RBCs demonstrated enhanced aggregability, reduced RBC deformability and elevated adherence to ECs already after the second week of storage.

Since 2002, leukoreduction of RBC units is a standard procedure in many European countries. Pre-storage leukofiltration reduces RBC damage caused by cytokines and enzymes derived from activated leukocytes. As a result leukoreduced RBC units demonstrate a lower degree of hemolysis, potassium leakage, osmotic fragility, and free radical production during storage. Interestingly, it was also demonstrated that activated...
leukocytes induce cellular changes that adversely affect the rheologic properties of the RBCs. The in vitro aggregability and deformability of leukoreduced RBCs during routine blood bank storage remains to be determined and may contribute to the ongoing discussion of safety of blood transfusions. In this study the aggregability, deformability and other hematologic variables of leukoreduced refrigerated stored RBC were investigated during a 7-week period.

4.2. Materials and Methods

Preparation and sampling of RBC units

Blood (500ml ± 10%) was collected from ten volunteers donors at the Sanquin blood bank in a quadruple top and -bottom bag system (Composelect, Fresenius Hemocare, the Netherlands) containing 70 ml of CPD anticoagulant. After cooling under butandiol plates for at least 4 hours, whole blood was separated into plasma, buffycoat and RBCs using an automated blood processing device (Compomat G4, Fresenius HemoCare, the Netherlands). SAGM solution (110 ml) was transferred to the RBCs and in-line filtration was carried out to remove residual leukocytes. The resulting RBC suspension had a Hct of 45-60% and contained less than 10^6 leukocytes per unit. RBCs were refrigerated stored at 4 ± 2 °C for 7 weeks. RBCs were released for use on day 3 after donation, hereafter referred to as Time 0, which were the freshest RBCs routinely available for transfusion. Weekly, samples were aseptically withdrawn from the RBC units for analysis after gentle mixing by inversion.

Rheologic features

RBC aggregability and deformability were monitored in vitro by the LORCA (R&R Mechatronics, Zwaag the Netherlands). Aggregation was induced by the addition of 10% HES (MW 200-kDa). Briefly, RBCs suspensions were centrifuged for 1 minute at 3500 x g and the supernatant was discarded. RBCs were resuspended in 10% HES 200-kDa solution (Fresenius, Bad Homburg, Germany). The Hct in all the samples was corrected to a constant value of 45%. Aggregability was tested with 1 ml of the RBC suspension. Aggregation of RBCs was monitored after disaggregating under increased shear stress. Both the aggregation measuring procedure and the subsequent analyses were computer
controlled. Aggregability of RBC was expressed by the AI, where a larger AI reflects an increased ability to aggregate.

The deformability of stored RBCs was determined with RBC suspension diluted 1:100 in PBS, (pH 6.5), containing 5% polyvinylpyrrolidone (PVP; MW 360 kDa, Sigma-Aldrich, Germany) and with a viscosity of 30 mPa.s. One ml of the latter RBC suspension was inserted into the LORCA and the RBC diffraction pattern was recorded at various shear stresses at 36.8 ± 0.2°C. The deformability of the RBCs, which is expressed by the EI, was determined by the LORCA from the size of the vertical (L) and horizontal (W) axes of the diffraction pattern according to the formula: 

\[ EI = \frac{(L-W)}{(L+W)} \]

An increased EI at a given shear stress indicates greater RBC deformability. A deformability curve was obtained by plotting the calculated values for EI versus the corresponding shear stress. The deformability at two shear stress values were examined more closely; the deformability at a shear stress of 3.9 Pa, which reflects the rigidity of the cell membrane, and the maximal deformability at shear stress of 50 Pa. Since the freshest available RBCs for transfusion were already 3 days old, the deformability was also performed with RBCs that were obtained within 2 hours after donation from healthy donors.

**Osmotic fragility**

The osmotic fragility of RBCs, which reflects the membrane’s ability to maintain structural integrity, was determined by diluting RBCs in PBS solutions ranging from 0.90% to 0.35%. RBCs with a Hct level of 30 to 35% were diluted 1:100 in each PBS solution, mixed and incubated for 30 minutes at 4°C, followed by centrifugation for 12 minutes at 1100 x g. The free Hb in the supernatant was measured by a spectrophotometer (PowerWave 200 spectrophotometer, Bio-Tek Instruments, USA) The concentration of PBS necessary to induce 50% hemolysis defined the osmotic fragility index of the RBCs. With this method, a larger osmotic fragility index corresponds to more fragile cells.

**Hemolysis**

Hemolysis as measured by the amount of free Hb present in the RBC suspensions was determined according to the method of Harboe.24 Briefly, cell supernatant was obtained by centrifugation of RBC units for 1 minute at 3500 x g. The supernatant was diluted 1:10 in 0.01% sodium carbonate in a flat-bottom 96-well microtiter plate and mixed for 30 minutes.
The Hb concentration in the supernatant was determined with a spectrophotometer by measuring the optical density (OD) at 415 nm and correcting for the OD at 380 and 450 nm according to the formula $\text{OD} = 2 \times (\text{OD}_{415 \text{ nm}}) - (\text{OD}_{380 \text{ nm}}) - (\text{OD}_{450 \text{ nm}})$. The hemolysis was expressed as a percentage of the total amount of Hb present in the RBC lysates.

**Hematologic variables**

To determine the cellular ATP content, RBC samples were incubated for 30 minutes with 8% ice cold trichloroacetic acid in a ratio of 1:3. Samples were centrifuged for 1 minute at 3500 x g and the protein free supernatant was neutralized with 1.5 mol/L sodium carbonate. Aliquots were stored at –80°C for later batch analyses of ATP. The ATP content was determined with a commercially available enzyme assay (Roche Diagnostics, Germany). For detection of ATP, light emission was measured at 560 nm by an illuminometer (Fluostar Optima, BMG Labtech, Germany). The supernatant pH and the RBC MCV were determined with a blood gas analyzer (Rapidlab 860, Siemens, the Netherlands). Total Hb content and Hct were determined with a hematology analyzer (Sysmex K4500, Goffin Meyvis, the Netherlands). The internal viscosity of RBC, as reflected by the MCHC, was determined by dividing the Hb content by the Hct.

**Statistical analysis**

Statistical analysis was performed using statistical software (SPSS, version 16.0, SPSS Inc., Chicago, IL). Data were tested for normality with the Kolmgorov-Smirnov goodness-of-fit test. For each variable a repeated measure analysis of variance was performed to identify subject by time profiles. Post-hoc comparisons were performed to quantify differences between Time 0 and stored RBC values, using paired t-tests. Differences are considered to be significant with a p value of less than 0.05. Results are presented as means ± SD.
4.3. Results

Rheologic features

The RBC ability to aggregate, as represented by the AI, was reduced after the first week of storage (AI from 46.9 ± 2.4 % to 41.9 ± 3.6 %; p < 0.01; Figure 4.1). In the following weeks the aggregability recovered; after week 2, 3, and 4, the RBC AIs were 45.2 ± 4.9 %, 46.0 ± 3.8 % and respectively 45.8 ± 3.5 %, which was not significantly different from Time 0 (46.9 ± 2.4 %). After 5 and 7 weeks of storage the aggregability was significantly reduced (AI 44.4 ± 4.5 % and respectively 44.3 ± 2.2 %) compared to Time 0.

The RBC deformation curve showed a typical s-shape over a shear stress range of 0.6-50 Pa for all different time points (Figure 4.2). The deformation curve of 5 week old RBCs was slightly higher at the low-shear-stress regions and somewhat lower at the high-shear-stress regions compared to Time 0 (Figures 4.2 and 4.3). After 7 weeks of storage (outdated RBCs) the deformability at the high-shear-stress regions was further diminished (Figures 4.2 and 4.3B). The rigidity of the cell membrane, which is reflected by the deformability at a shear stress of 3.9 Pa, was fluctuating during 5 weeks of storage (Figure 4.3A). Ultimately, after 7 weeks of storage the deformability at this low shear stress was not significantly different from Time 0 (from 0.35 ± 0.01 to 0.35 ± 0.02 EI). The deformability at a shear stress of 50 Pa was reduced from week 1 of storage onward (from 0.58 ± 0.01 to 0.54 ± 0.01 EI) as can be seen in Figure 4.3B. This downward trend in deformability (p < 0.01) remained within the physiological range, as determined with fresh RBCs (0.53 ± 0.04 EI).

Osmotic fragility and hemolysis

The osmotic fragility index, represented by the osmolarity at half-maximum hemolysis of the RBCs, was not significantly altered during 7 weeks of storage (0.47 ± 0.02 % PBS; Table 4.1) compared to Time 0.

A gradual increase in hemolysis was observed throughout the storage period (Table 4.1). After 5 weeks of storage, the hemolysis (0.53 ± 0.24%) still remained below allowable levels (i.e., 0.8% in Europe and 1% in the United States), despite the weekly removal of samples. Closer evaluation of individual RBC units demonstrated that after 5 and 7 weeks of storage, respectively, one and four out of ten samples contained hemolysis which...
exceeded 0.8%. After 7 weeks of storage one RBC unit even showed hemolysis exceeding 2%, accounting for the large SD. No bacterial contamination was found in this RBC unit.

**Figure 4.1.** Effect of storage on RBCs ability to aggregate. AI in percentage and corrected for Hct. Values are expressed as mean ± SD of ten RBC units. Significant difference from Time 0 are shown (* p < 0.05 and † p < 0.01).

**Figure 4.2.** Shear stress EI curves for Time 0 (●) and after 5 (-) and 7 weeks (▲) of storage. The shear stress value is plotted on the logarithmic axis. Data represent mean ± SD of ten RBC units.
Figure 4.3. Deformability for two representative shear stress levels as a function of storage time. (A) EI at shear stress of 3.9 Pa. With the exception of week 7, all samples were significantly altered (p < 0.05) compared to Time 0. (B) EI at shear stress of 50 Pa. All samples were significantly reduced compared to Time 0 during 7 weeks of storage (p < 0.04). Data represent mean ± SD of ten RBC units.
Table 4.1. RBC characteristics during 7 weeks of storage

<table>
<thead>
<tr>
<th>Variable</th>
<th>t=0</th>
<th>t=1wk</th>
<th>t=2wk</th>
<th>t=3wk</th>
<th>t=4wk</th>
<th>t=5wk</th>
<th>t=7wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmotic fragility (%)</td>
<td>0.48 ± 0.02</td>
<td>0.48 ± 0.01</td>
<td>0.47 ± 0.02</td>
<td>0.46 ± 0.02</td>
<td>0.48 ± 0.02</td>
<td>0.47 ± 0.03</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>Hemolysis (%)</td>
<td>0.24 ± 0.07</td>
<td>0.24 ± 0.07</td>
<td>0.30 ± 0.09 *</td>
<td>0.36 ± 0.12 †</td>
<td>0.42 ± 0.17 †</td>
<td>0.53 ± 0.24 †</td>
<td>0.89 ± 0.51 †</td>
</tr>
<tr>
<td>ATP (μmol/g Hb)</td>
<td>4.55 ± 1.34</td>
<td>4.14 ± 0.80</td>
<td>4.06 ± 1.02</td>
<td>3.82 ± 1.09</td>
<td>3.80 ± 1.09</td>
<td>2.12 ± 0.37 †</td>
<td>1.52 ± 0.30 †</td>
</tr>
<tr>
<td>pH (22°C)</td>
<td>6.99 ± 0.06</td>
<td>6.80 ± 0.04 †</td>
<td>6.69 ± 0.03 †</td>
<td>6.62 ± 0.03 †</td>
<td>6.55 ± 0.04 †</td>
<td>6.49 ± 0.04 †</td>
<td>6.41 ± 0.04 †</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>89.0 ± 2.4</td>
<td>89.0 ± 2.5 *</td>
<td>91.2 ± 2.7 †</td>
<td>91.1 ± 2.6 †</td>
<td>91.6 ± 2.9 †</td>
<td>92.0 ± 2.8 †</td>
<td>94.0 ± 2.9 †</td>
</tr>
<tr>
<td>MCHC (mmol/L)</td>
<td>20.7 ± 0.6</td>
<td>20.5 ± 0.4</td>
<td>20.5 ± 0.4</td>
<td>20.5 ± 0.6</td>
<td>20.5 ± 0.5</td>
<td>20.2 ± 0.3</td>
<td>19.7 ± 0.4 *</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of ten units. Significant difference from Time 0 are reported (* p<0.05 and † p<0.01).
Hematologic variables

The RBC ATP content reduced during 7 weeks of storage (Table 4.1). Notably, after 5 weeks of storage the ATP content had declined with 54% compared to Time 0 (from 4.6 to 2.1 μmol/gHb; p < 0.01). After the first week of storage the PH of the RBC suspension and the MCV were significantly altered (Table 4.1). Ultimately, after 5 weeks of storage the pH was reduced with 7% while the MCV was enhanced by 3%. The MCHC was only significantly reduced after 7 weeks of storage compared to Time 0 (from 20.7 to 19.7 mmol/L).

4.4. Discussion

This study was undertaken to explore the rheologic properties and quality of leukoreduced refrigerated stored RBC units. The RBC aggregability was significantly reduced at the first week of storage but recovered during the following weeks. RBC deformability at shear stress of 50 Pa was significantly reduced during 7 weeks of storage. However, values were still within physiological reference ranges. The osmotic fragility did not change significantly over time. Throughout 5 weeks of storage the ATP content had declined with 54% from initial levels, whereas MCV, PH and MCHC were affected to a lesser degree.

During storage at 4°C RBCs undergo different biochemical and structural alterations that may adversely affect clinical outcome. Currently, RBCs are considered of impaired quality after the second week of storage.11,13 As a result, a more restrictive transfusion strategy is currently favored.26 Nonetheless, pre storage leukofiltration has significantly diminished storage-induced lesions.18,19 Additionally, it has been shown, that activated leukocytes can induce damage which adversely affects the rheologic properties of RBCs.20,21 Increased aggregability and diminished cell deformability during RBC storage have been previously observed. However, in those studies the RBC units had not been leukoreduced prior to storage.11-14,27

In this study, RBC aggregability significantly decreased after 1 week of storage but recovered throughout the following weeks. At the end of storage, aggregability was slightly but significantly reduced. RBC aggregation is primarily dependent on the RBC surface characteristics and the composition of the suspension medium.5 Since the latter was standardized for all the samples, our results suggest that the observed differences are caused
by alterations in cellular properties. In addition, our results show that RBCs are, to some extent, able to adapt to environmental changes during storage.

RBC morphology can affect RBC aggregation. During storage at 4°C, RBC morphology shifts from discocyte towards echinocyte shape, leading to decreased aggregability.\textsuperscript{28,29} Our results show that the aggregability in SAGM solution was minimally reduced at the end of storage. Usage of other additive solutions, which also enhances the MCV during storage, are therefore expected to give a similar aggregation pattern. The clinical relevance of altered RBC aggregability, however, remains an ongoing debate.\textsuperscript{30}

The RBC ability to deform depends mainly on the visco-elastic properties of the cytoskeleton, the intracellular viscosity of the RBC and the overall cell shape.\textsuperscript{31} In this study storage induced minor changes in the deformation curve at low shear stress of 3.9 Pa. The decreasing trend in deformability at a shear stress of 50 Pa has been interpreted as structural changes in the RBC that cannot be corrected by increasing shear force.\textsuperscript{32} However, we suggest that the gradual increase in MCV during storage may be responsible for the observed reduction in deformability, particularly since at high shear stress the cell volume becomes a limiting factor for the ability of RBCs to deform. Our data suggests that this effect plays a role for shear stresses above 5 Pa, as can been seen from the deformation curve. The increased RBC deformation at high shear stress after 5 weeks of storage compared to the preceding week has been observed by others and can only partly be explained by hemolysis of less deformable RBCs.\textsuperscript{11} The occurring deformability changes in this study were not likely biologically relevant because the reduction in deformation observed only at high shear stress was minimal and within physiological reference ranges. Furthermore, shear stresses of 3.9 Pa, which are predominantly found in the microcirculation,\textsuperscript{33} are clinically more relevant than those of 50 Pa.

We showed that the osmotic fragility, which is determined by applying deforming stress from inside the RBC, was not significantly enhanced during 7 weeks of storage. In addition, the RBC intracellular viscosity, which is predominantly determined by the MCHC, did not yield significant changes until 7 weeks of storage. Taken together, these findings further substantiate that the observed reduction in deformability was caused primarily by morphology changes of the RBC.

It has previously been shown, that RBC deformability was unaffected at pH values ranging between 6.4 and 7.7.\textsuperscript{34} In our study, the pH was still within these limits after 5 weeks of
storage, suggesting that the reduced RBC deformability was not caused by pH alterations in the storage solution.

ATP as an energy source is important for the overall functioning of the cell. Loss of ATP is associated with more rigid cell membranes, loss of vasodilatation properties, exposure of PS on the outer leaflet of the RBC membrane, microvesiculation and decreased RBC viability.\textsuperscript{35-39} In our study the ATP content gradually reduced during storage. Although loss of ATP stiffens the RBC membrane due to calcium accumulation,\textsuperscript{40} the observed reduction in deformability at high shear stress was within physiological reference values, indicating that ATP loss during storage only marginally affected the RBC ability to deform. As proposed earlier, the RBC ATP content must be at least 2.7 µmol per gram Hb to have a 90 percent chance of acceptable in vivo survival (24-hr in vivo recovery of 75% or higher).\textsuperscript{41,42} The present study showed that after 5 weeks of storage the RBC ATP content was below this limit. Similar findings were observed for leukoreduced RBC that were stored for 6 weeks in SAGM solution.\textsuperscript{43} The in vivo viability of RBCs may be reduced by the low ATP content. However, it is not likely that this results in the proposed adverse clinical outcome, in particular, because the observed alterations in rheologic features were minimal and the hemolysis in the RBC units was still below the allowable limits (i.e. 0.8% in Europe and 1% in the United States).\textsuperscript{15,25}

Leukocytes can affect the RBC deformability even when they are significantly reduced, e.g. during the storage of buffycoat-depleted RBC units.\textsuperscript{44-46} Apparently, removing solely the buffycoat from the RBCs without performing leukofiltration, still resulted in $10^8$ to $10^9$ leukocytes per RBC units,\textsuperscript{15,47} whereas nowadays less than $10^6$ leukocytes per RBC unit are permitted. Our results demonstrate that cell deformability was minimally affected when the RBCs were leukofiltrated prior to storage.

Recently, reduced deformability at shear stress of 3 and 30 Pa during the storage of leukoreduced RBCs has been observed,\textsuperscript{48} whereas others showed no reduction in RBC deformability at these shear stresses.\textsuperscript{49} This reduction in deformability has been linked to loss of Hb-bound nitric oxide, a variable that participates in controlling the RBC deformation.\textsuperscript{50} However, we also suggest that differences in storage procedures could explain this discrepancy in results. Particularly because the reduction in deformability at low and high shear stress was observed in the preservative solution AS-3, while in the SAGM preservation solution\textsuperscript{28} less changes in RBC deformability were observed.
RBC adherence to ECs is mediated by PS exposure on the cell membrane. Additionally, PS expression triggers recognition by macrophages and subsequent clearance of RBCs from the circulation.\textsuperscript{51,52} PS expression was not determined in this study, since recent work demonstrated that leukoreduction significantly lowered the PS expression on the RBCs, resulting in negligible PS exposure after 5 weeks of storage\textsuperscript{48,53} and because leukoreduction reduces the progressive adherence of RBCs to ECs with storage time.\textsuperscript{54,55}

In the Netherlands approximately 37\% of all the transfused RBC units are older than three weeks.\textsuperscript{56} The primary goal of blood transfusion is to deliver oxygen to the microcirculation with high-quality stored RBCs. During storage at 4°C, RBCs undergo different alterations that might influence the patient condition. However, leukoreduction has significantly reduced storage-induced lesions. Based on our findings, we postulate that the observed changes in RBC variables during refrigerated storage minimally affected the RBC ability to aggregate and deform, even after long-term storage. The rheologic properties of leukoreduced RBC units were well preserved during routine blood bank storage and not likely to contribute to adverse clinical outcome after transfusion.

References


