Temperature-dependent haemolytic propensity of CPDA-1 stored red blood cells *vs* whole blood - Red cell fragility as donor signature on blood units

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Background. To preserve cellular integrity and avoid bacterial growth, storage and transfer of blood and blood products follow strict guidelines in terms of temperature control. We evaluated the impact of ineligible warming of whole blood donations on the quality of blood components.

Materials and methods. One-hundred and twenty units of whole blood (WB) from eligible blood donors were collected in CPDA-1 and stored at 4±2 °C. During shipment to the blood processing centre, a gradual warming up to 17 °C was recorded within a period of less than eight hours. The warmed units were processed to packed red blood cells (PRBCs) or stored as WB units at 4±2 °C. In-bag haemolysis, osmotic fragility (mean corpuscular fragility, MCF) and bacterial growth were assessed in blood and blood components throughout the storage period.

Results. Normal basal and early storage levels of haemolysis were recorded in both PRBC and WB units. Thereafter, PRBCs exhibited higher average in-bag haemolysis and MCF index compared to the WB units throughout the storage. Moreover, 14.3 and 52.4% of the PRBC units exceeded the upper permissible limit of 0.8% haemolysis at the middle (1.220±0.269%) or late (1.754±0.866%) storage period, respectively. MCF index was similar in all PRBCs at the middle of storage but significantly lower in the non-haemolysed compared to the haemolysed units of PRBCs on the last days. The fragility of stored RBCs was proportional to the donor-related values of day 2 samples ($r=0.861$, $p<10^{-32}$). In the qualified PRBCs, MCF was correlated with haemolysis at every time point of the storage period ($r=0.332$, $p<0.050$). Bacterial growth was detected by blood culture in two units of PRBCs.

Discussion. Transient, gradient warming of whole blood from 4 to 17 °C led to increased incidence of in-bag haemolysis in PRBC but not in WB units. Haemolysis is a multi-parametric phenotype of stored blood, and MCF is a donor-related and highly dynamic measure that can, in part, predict the storage lesion.

Keywords: haemolysis, red blood cell storage lesion, temperature control, stored whole blood, osmotic fragility.

Introduction

Whole blood (WB) is not usually collected close to the processing site since laboratory conditions and facilities at the place of blood donation may not allow separation of blood to components. Hold and shipment of donated blood to processing centres need to follow strict conditions of temperature control to avoid deterioration in the biochemical and functional features of blood components as per World Health Organization 2005 guidelines[1]. For subsequent platelet preparation, WB can be held/transferred from 8 to 24 hours at ambient temperature, after which the units are cooled. Like the units of cold packed red blood cells (PRBC), the cold units of WB, however, should be transferred by using appropriate cooler systems at low temperature, similar to that of storage, which in any case should not exceed +10 °C, to keep the growth of any bacterial contamination to a minimum and the oxygen-carrying capacity to a maximum. Whole blood can be then used for preparation of PRBCs or it can be stored for up to 35 days if collected in CPDA-1[2].

Haemolysis of RBCs during storage in the cold is the most obvious manifestation of storage system failure. The rate of in-bag haemolysis in units of PRBCs depends on the individual donor, the preservative solution, and the duration of storage[3]. It can also occur as a result of mishandling of blood during shipping (e.g. heating or freezing under transfer with solid ice, etc.) and storage,

or as a result of bacterial contamination, resulting from donor-dependent (e.g. skin, blood, etc.) and donor-independent (disposables, environment, etc.) sources[4]. Low temperature during shipment and storage reduces the risk of skin or blood-borne bacterial growth in units of blood or blood components, although cold-tolerant contaminating bacteria can survive and grow slowly during storage[5].

In the current study, we evaluated the RBC integrity and bacterial growth in stored units of WB or PRBCs prepared from blood donations that had been accidentally warmed up to 17 °C during transportation to the blood processing centre. Variation in the osmotic fragility of stored RBCs was also studied as a function of donor, storage strategy, time and haemolysis parameters.

Materials and methods

Blood collection and component processing

Whole blood from 120 eligible, regular male blood donors (18-25 years old) was collected in CPDA-1 bags and stored at 4±2 °C shortly after withdrawal. During shipment to the blood processing centre, an accidental gradual increase in their temperature up to 17 °C was recorded within a period of less than eight hours. More specifically, upon arrival of shipments at the processing centre, the temperature of the very bottom, middle and very upper blood units was recorded in each shipment and found to be in the range of 15-17 °C. According to the guidelines, the units of whole blood were considered unsuitable for processing to component preparation and transfusion. Following that, 105 units were processed to packed red blood cells (PRBCs) without prior leucoreduction and 15 units were kept as whole blood (WB) units, before storage at 4±2 °C for research purposes. Samples were collected aseptically using a sterile sampling device at the beginning (day 2), the middle (day 17), and the end (day 35) of the storage period. The study has been submitted and approved by the Research Bioethics and BioSecure Committee of the Faculty of Biology of the National and Kapodistrian University of Athens, Greece. Investigations were carried out in accordance with the principles of the Declaration of Helsinki.

Haemolysis

In-bag haemolysis was calculated by measuring free haemoglobin (Hb) levels in the supernatant of the blood units. Both Harboe's method[6] and Drabkin's reagent (SIGMA)[7] were used. For Harboe's method, each sample was centrifuged at 1,000 *g* for 10 minutes (min) and the collected supernatant was processed again under the same conditions. After dilution of the supernatants in distilled water and incubation at 20 °C for 30 min, Hb absorbance was measured *vs* blank at 380, 415 and 450 nm. The final optical density (OD) was calculated using an Allen correction as follows:

$$\text{Hb (mg/dL)} = (167.2 \times A_{415} - 83.6 \times A_{380} - 83.6 \times A_{450}) \times 1/1{,}000 \times \text{dilution factor} \times 100$$

The twice centrifuged supernatants were also used for free Hb determination using Drabkin's reagent. Briefly, supernatants were mixed with the reagent in a 1:25 ratio, respectively, and incubated at 20 °C for 45 min. Absorbance was measured at 540 nm and plotted against a standard curve for Hb concentration estimation. In both methods, percentage of haemolysis was calculated using the following formula:

$$\%\ \text{haemolysis} = \frac{[\text{supernatant Hb (mg/dL)} \times (100 - \%\ \text{Hct})]}{\text{total Hb (mg/dL)}}$$

where Hct stands for haematocrit.

Osmotic fragility

To test the osmotic behaviour of RBCs, solutions of increasingly saline concentration were used (0.0-0.9% w/v NaCl, SIGMA)[8]. Blood samples were added in each saline solution (1:100 ratio, respectively) and incubated for 15 min at 20 °C. After centrifugation at 200 *g* for 5 min, Hb released in the supernatant was measured at 540 nm and plotted against saline concentration to produce an osmotic fragility curve for each sample. The mean corpuscular fragility (MCF), which corresponds to the saline concentration causing 50% of haemolysis, was calculated from the curves.

Bacterial contamination

The microbiological analysis of WB and PRBC units was performed according to the Technical Manual of the American Association of Blood Banks[9]. The units were divided into 12 pools of ten; 1 mL of each pool sample was inoculated into MacConkey and Blood agars and incubated at 37 °C for 24 hours (h). Presence of colonies (colony forming units, CFU/mL) in both types of agar and additional haemolysis in blood agar were considered as a positive result. For positive pools, individual examination of units was followed. Representative colonies from both agars were chosen for biochemical identification (API STAPH-IDENT system and Gram stain) and for antimicrobial sensitivity testing (amikacin, tobramycin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, norfloxacin, ofloxacin, pefloxacin, clarythromycin, erythromycin, clindamycin, linezolid, teicoplanin, vancomycin, doxycycline, minocycline, tetracycline, tigecycline, fosfomycin, fucidic acid, rifampicin, trimethoprime, and trimethoprime/sulfamethoxazole) (Vitek, BioMérieux, Combourg, France).

Statistical analysis

All measurements were run in triplicate to rule out experimental bias. For statistical analysis, statistical software was used (Statistical Package for Social Sciences, SPSS v.22.0; IBM Corp., Chicago, IL, USA). All variables were tested for normal distribution profile using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Between groups differences were evaluated by one-way ANOVA and Mann-Whitney analysis, as appropriate. A Bonferroni correction for multiple comparisons was used where needed. Pearson's and Spearman's correlation tests were performed to assess the relationship between parameters. $p<0.05$ was considered significant.

Results

Haemolytic propensity

All donors demonstrated normal levels of free plasma Hb (on average 4.36±3.72 mg/dL) before processing and storage. Likewise, percentage of haemolysis was steadily low at the first days of the storage period in both PRBC and WB units (0.018±0.014% and 0.017±0.008%, respectively) (Figure 1A). However, at the middle of the storage period, 14.3% of PRBCs exhibited haemolysis levels above 0.8% (Figure 1B). Despite the fact that 85.7% of the PRBCs units exhibited acceptable, low haemolysis on day 17 of storage, their haemolyis was significantly higher than that of WB units (0.166±0.136% *vs* 0.091±0.071%, respectively; $p<0.050$) (Figure 1A). At the end of the storage period, more than half (52.4%) of the PRBC units exceeded the acceptable quality limit of in-bag haemolysis (1.754±0.866%) (Figure 1A and 1B). As at the previous time point, haemolysis levels in the non-haemolysed PRBC units were statistically higher compared to that of WB units (0.523±0.160% *vs* 0.193±0.167%, respectively; $p<0.010$) (Figure 1A).

The osmotic fragility factor

The osmotic fragility of stored RBCs increased constitutively during storage but it was significantly lower ($p<0.01$) in units of WB than in PRBCs (irrespectively of their haemolysis levels), throughout the storage period (Figure 2A). In PRBCs, similar MCF index values were recorded between units of low and high haemolysis until the middle of storage (0.530±0.024% and 0.523±0.024% NaCl, respectively), but toward the last days, the fragility of haemolysed PRBC units exceeded that of non-haemolysed ones (0.574±0.021% *vs* 0.558±0.022% NaCl; $p<0.01$) (Figure 2A). In addition, and in similarity with previous findings in leucoreduced PRBC units stored in CPD-SAGM[10], the upward fluctuation in MCF index during storage in CPDA-1 was proportional to the donor-related values of day 2 samples in all units under examination (n=120), while strong and statistically significant correlations between MCF values corresponding to the three time points tested were present in WB and PRBC units (Figure 2B), irrespectively of the haemolysis levels (<0.8% or >0.8%) (Table I).

Inter-parameter correlations

Negligible variation in haemolysis or osmotic fragility was observed among blood units as a function of ABO or Rhesus blood group classification (*data not shown*). In contrast, moderate but statistically significant correlations between haemolysis and MCF levels were found for the group of PRBC units with haemolysis below 0.8% throughout the storage period (Figure 3A). Based on R^2 value assessment, osmotic fragility variation is an intrinsic part of haemolysis variation in the low haemolysis PRBCs consecutively throughout storage, at a percentage of approximately 11%. On the contrary, there was no correlation between the two variables tested in the PRBC units exceeding in-bag haemolysis levels of 0.8% (Figure 3B) or in units of WB (Figure 3C).

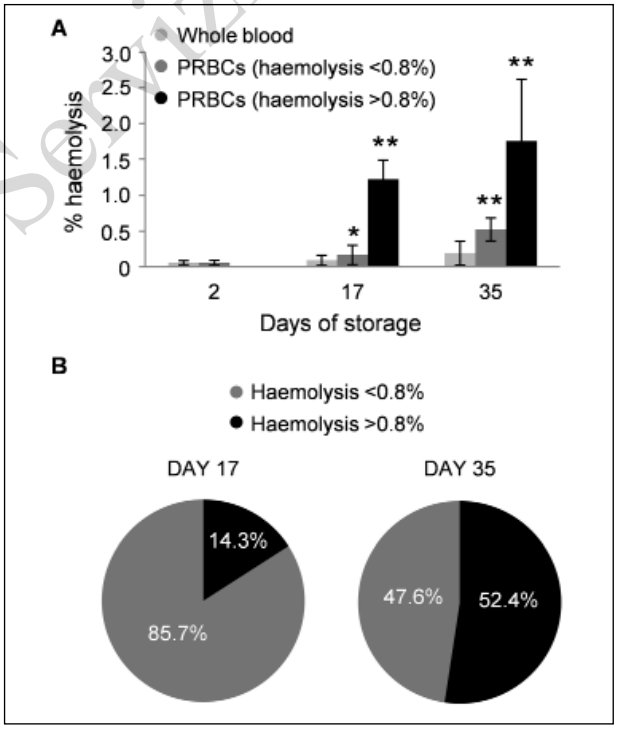

Figure 1 - Profile of haemolysis in CPDA-1-preserved packed red blood cell (PRBC) and whole blood (WB) units throughout the storage period.
(A) In-bag haemolysis levels at the beginning (day 2), middle (day 17) and end of the storage period (day 35) for WB and PRBC units following or not following the quality control criterion of less than 0.8%. Data are shown as mean±SD (**p<0.01, *p<0.05 PRBC *vs* WB units). (B) Percentage of CPDA-1-preserved PRBC units with or without aberrant levels of haemolysis (n=105). SD: standard deviation.

Bacterial contamination

Two Gram-positive isolates (*Staphylococcus aureus* and *Staphylococcus epidermidis*) were recovered from 2 PRBC out of 120 PRBC and WB units (1.7% of total) which were sensitive to all clinically relevant antibiotics tested. Moreover, they were associated with negligible haemolysis in blood agar. Both contaminated PRBC units exhibited very low in-bag haemolysis until the middle of storage (0.17 and 0.14%) that increased, however, to more than 0.8% on the last day of storage (0.82 and 2.19%, respectively).

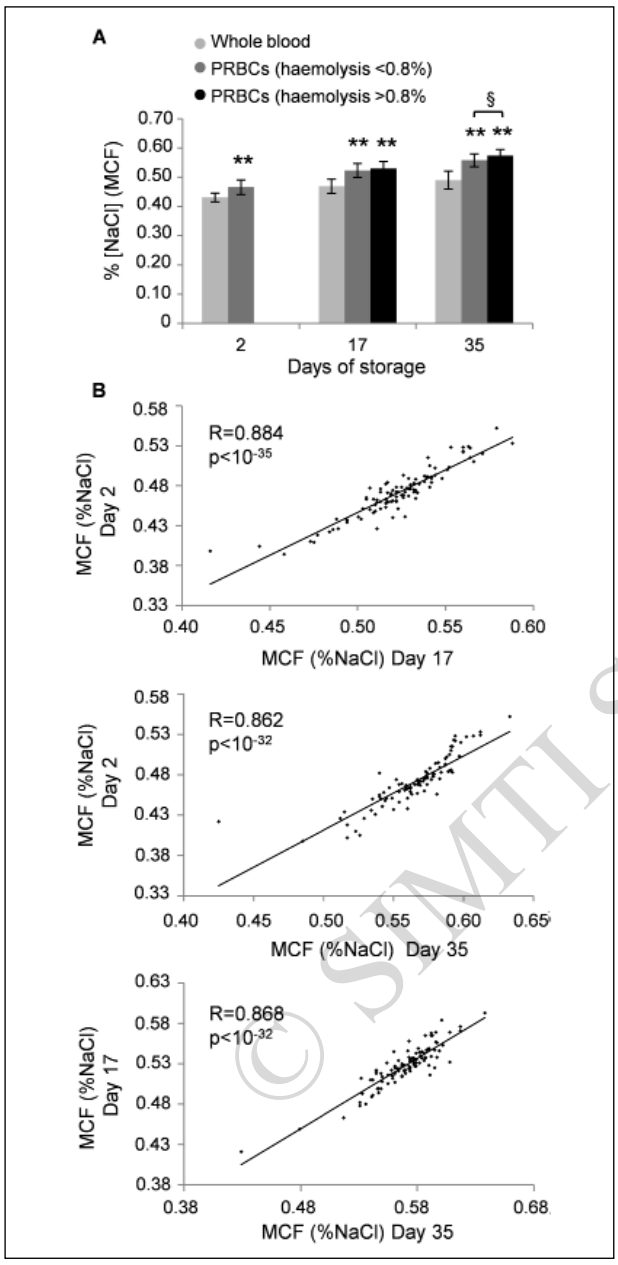

Figure 2 - Time course evaluation of osmotic fragility in stored red blood cells (RBC).
(A) Mean corpuscular fragility (MCF) levels of whole blood (WB) and packed red blood cell (PRBC) units during storage. Data are shown as mean±SD (**p<0.01 PRBCs *vs* WB units; §p<0.01 haemolysed *vs* non-haemolysed PRBC units). (B) Scatter plots showing the strong and statistically significant correlations between the MCF levels of PRBCs stored for any storage period, regardless of their haemolysis levels (n=120). SD: standard deviation.

Discussion

In the present study, we evaluated the impact of ineligible warming of whole blood donations up to 17 °C during transportation for less than eight hours on the quality of WB and blood components (PRBC) prepared thereafter. We have measured supernatant Hb as an indication of haemolysis, and red cell osmotic fragility as an indication of more subtle effects on the RBC membrane and geometry. The units of WB were shipped under the same conditions, and the two groups of PRBCs and WB units were also stored under identical conditions.

Pre-processing warming of whole blood up to 17 °C for less than eight hours was related to negligible bacterial contamination

Bacterial contamination in blood products may originate from donor skin flora, from donor asymptomatic bacteremia, or from contamination during blood processing. The Gram-positive bacteria that were detected in 2 PRBC units are normally present in skin, in hospital environments, etc., and their optimal growth temperature is between 20 and 37 °C[11,12]. They were sensitive to all clinically relevant antibiotics tested and seemed to be non-haemolytic *in vitro* (in agar tests). The respective contaminated PRBC units exceeded the acceptable levels of in-bag haemolysis only toward the end of the storage period, along with the majority of the non-contaminated PRBC units. According to recent studies, although bacterial growth is slightly promoted during 5-h RBC exposure to room temperature, it does not reach clinically significant levels[13]. Bacterial growth is usually reported in similar percentage (1.0-1.3%) in randomly selected units of non-leucoreduced PRBCs under standard conditions of shipment and storage by using conventional test systems such as BacT/Alert and confirmation by culture[14]. Consequently, its presence in our samples seems to be incidental and not a result of the ineligible warming of blood during shipment.

Pre-processing warming of whole blood caused haemolysis in stored packed red blood cells

A transient increase in temperature up to 17 °C is not expected to cause an immediate haemolytic effect on RBCs. Studies of thermal stress on cold stored WB or RBCs in CPD, CPDA-1 or AS-1 in settings of

Table I - Intra-parameter correlations of red blood cell osmotic fragility in packed red blood cells and whole blood units throughout storage.

	Day 2	Day 17	Day 35
PRBC units with haemolysis <0.8% (n=50-90)			
Day 2	-	0.885**	0.864**
Day 17	-	-	0.842**
PRBC units with haemolysis >0.8% (n=15-55)			
Day 2	-	0.891**	0.830**
Day 17	-	-	0.852**
Whole blood units (n=15)			
Day 2	-	0.727**	0,804**
Day 17	-	-	0,949**

**p<0.001. PRBC: packed red blood cells.

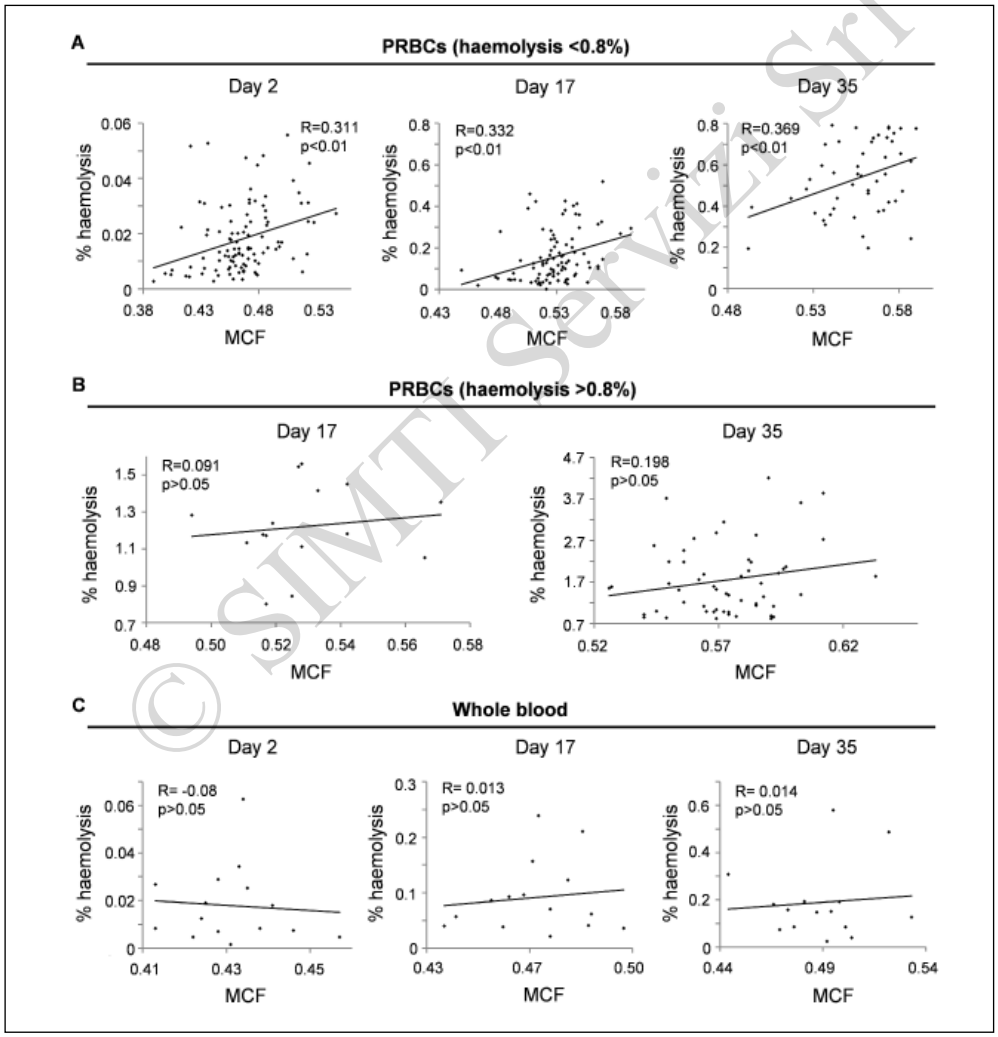

Figure 3 - Correlations between haemolysis and red blood cell (RBC) osmotic fragility variations during storage.
(A) Scatter plots demonstrating the statistically significant correlation between haemolysis and mean corpuscular fragility (MCF) levels in the subgroup of packed red blood cell (PRBC) units exhibiting haemolysis less than 0.8%, throughout the storage period. (B and C) Scatter plots showing the absence of correlation between haemolysis and MCF variations in the subgroup of PRBC units with aberrant haemolysis levels more than 0.8% (B) and in whole blood (WB) units (C).

prolonged (≤0.5-1 h) or rapid incubation at 45 °C have shown no significant haemolysis or osmotic fragility[15-17] and normal survival post transfusion[18]. This corresponds with our findings showing negligible haemolysis before processing and during early storage of both WB and PRBCs units. In contrast, shipment temperature above 4 °C may damage RBCs and predispose them to poor storability, as revealed by the high susceptibility of PRBCs to haemolysis observed for the first time at the middle of the storage period.

Holding WB at temperatures over 4 °C has been shown to affect the classical quality parameters of PRBC components, particularly at the end of the storage period[19-21]. PRBC units prepared from WB after overnight holding at room temperature exhibit lower potassium release, but also lower 2,3-DPG and increased haemolysis, membrane microvesiculation, PS expression, lipid peroxidation and cytokine levels than rapidly cooled RBCs.

Transportation time and temperature might impact RBCs structural integrity, stability and function[22]. RBC metabolic processes, ATPases and kinases, ion pumps and transporter systems are strictly temperature-dependent[23,24]. Ion imbalance may lead to changes in RBC volume and Ca^{2+}-dependent processes. Although CPDA represents a superior preservative medium compared to EDTA or heparin, simulated transportation of blood collected from healthy donors and supplemented with CPDA at 22±2 °C was associated with osmotic fragility and deformability changes compared to transportation at 4 °C[23]. CPDA preserves well cell density, K^+/Na^+ content and the rate of membrane microvesiculation at both transportation temperatures; however, cation gradients are better reserved at higher temperature. Notably the degree of haemolysis was independent of the transportation temperature within 72 h of examination[25].

Warming of WB during shipment is expected to result in increased rates of cellular metabolism. It has been reported that glycolysis and proton accumulation in RBCs occur about twenty times more slowly at 3 °C than at 37 °C[5]. Even at the narrow allowed shipment temperature range (4-10 °C), the rate of RBC metabolism may vary 3-fold with resulting variability in pH[5]. Increased consumption of nutrients, generation of metabolic by products and waste by all the viable cells of the WB units, along with acidification of the supernatant, might exacerbate the development of the storage lesion at cell metabolism level, leading to metabolic failure, oxidative lesions and increased haemolysis. Indeed, a range of proteomic and metabolomic aberrations of stored RBCs show linear correlations with in-bag haemolysis and vesiculation[26,27].

Aberrant values of haemolysis were first observed in PRBC units at the middle of the storage period. Although the exact day preceding that time, when haemolysis of individual units might exceed the threshold of 0.8%, was not specified, this finding was consistent with rapidly accumulating laboratory evidence showing that a significant part of the main biochemical, morphological and omics disturbances of stored RBCs become obvious after approximately 10-14 days of storage[28,29]. In agreement with these data, 14% of the PRBC units exceeded the acceptable levels of in-bag haemolysis already at the middle of storage. Slowing metabolic processes at 4 °C may be beneficial for shipped WB by preventing acidification, gradual depletion of plasma glucose, and changes in the lactate content and, probably, by suppressing ATP production and consumption[25]. Putative lysis of WBCs and PLTs and subsequent enzyme release might also harm RBCs. Finally, since all units were shipped, handled and stored under exactly the same conditions, the wide variation in the percentage of haemolysis among stored PRBCs confirmed that differences between individual donors affect the susceptibility of temperature-stressed units to end-of-storage haemolysis, as previously observed in units stored under established, normal conditions[3,10,30,31]. Nevertheless, it should be recalled that the severity of the storage lesion found in this study may not be indicative of typical storage lesion, because the WB and PRBC units used had been declared unsuitable for transfusion.

Pre-processing warming did not cause haemolysis in stored units of whole blood

In striking contrast to the stored PRBC units, the temperature stress did not trigger haemolysis in units of WB. Without additional processing, WB units retain all the components of the donation, including donor plasma in higher plasma-to-stored RBCs ratio compared to that of PRBC units. Plasma might exert pleiotropic cytoprotective effects on stored RBCs by providing survival factors, nutrients, amino acids, erythropoietin and ROS-scavengers, such as albumin and uric acid[32,33]. Plasma components such as L-arginine, calcium, insulin, erythropoietin and other proteins might modulate the activity of RBC transport proteins and moreover, they are required for, or affect, NO production by RBCs, thus regulating membrane deformability and the redox state of RBCs[22,34,35]. Moreover, serum albumin has been shown to reverse the echinocytic transformation of stored RBCs[36]. According to data derived from *in vitro* models of transfusion, while the supernatant of long-stored PRBCs induces haemolysis in fresh RBCs, incubation of stored RBCs (short or long storage) with fresh plasma at 37 °C results in reduced haemolysis[37]. The current data suggest that WB storage in CPDA-1 for up to 35 days can "blind" the RBC damage and the accelerated storage lesion resulting from inappropriate warming during shipment.

These results deserve further attention in the light of the recently renewed interest in WB transfusion for the treatment of life-threatening bleeding and haemorrhagic shock[2,38,39]. In that case, WB might be equal or more efficacious than blood components[40]. The US Food and Drug Administration has approved WB leucoreduction filters that are platelet (PLT)-sparing, paving the way for availability of leucoreduced WB as a PLT-containing product[41]. WB provides a biologically balanced amount of RBCs, PLTs and plasma, and recent data strongly suggest that its haemostatic quality is largely unaffected by pathogen reduction technology and, more importantly, that it is preserved beyond the current transfusion practices. *In vitro* studies on the coagulation properties of refrigerated WB stored for 21-31 days have shown preservation of normal coagulation function to a minimum of 11 days of storage[42-44]. In fact, the haemostatic capacity of PLTs in WB stored for 10-15 days in the cold may be improved compared to PLT units stored at 20-24 °C or to blood reconstituted from individual components. Randomised controlled trials indicated that PLT-containing blood products stored at 4 °C have superior haemostatic function compared to PLT-containing blood products at 22 °C[40]. Moreover, cold-stored apheresis PLTs stored at 4 °C better preserve glucose metabolism and haemostatic potential, and release fewer pro-inflammatory mediators than apheresis PLTs stored at 22 °C[45]. Finally, exposure of recipient to 1 donor compared to 3 for reconstituted blood is important to the massively transfused patient. Consequently, storage of WB in the cold might be useful in specific clinical settings.

Osmotic fragility represents a storage time-, medium-, processing- and strategy-independent "signature" of blood donor on stored red blood cells

In-bag haemolysis of stored RBCs exhibit strong donor dependence but its significant variation during storage cannot be predicted by pre-storage values[10]. In contrast, mean osmotic fragility of stored RBCs exhibited a stable variation profile throughout the storage period. In other words, osmotic fragility increases during storage (Figure 2A) in proportion to early storage levels (Figure 2B), which have been shown to be proportional to donor-specific levels in fresh blood[10]. Storage affects cellular fragility by a stable factor of magnitude, preserving inter-donor differences observed in their basal, pre-storage or early-storage levels. This intra-parameter correlation that was previously seen in pre-storage leucoreduced PRBC units from healthy[10] or G6PD-deficient donors[37] stored in SAGM additive solution, is currently detected in non-leucoreduced haemolysed and non-haemolysed units of CPDA-1-preserved RBCs as well as in WB units, suggesting that RBC osmotic fragility represents a clear imprint of each donor in the blood bag that is independent of storage solution (CPDA-1 or SAGM), duration (early to late storage), processing (WB or components preparation) and strategy (with/without leucoreduction).

Osmotic fragility is correlated with in-bag haemolysis in qualified packed red blood cell units

Recent studies have defined MCF of the stored RBCs as a "hub node" in biological networks of "fresh-*vs*-stored" blood, showing strong correlations with many donor-associated variables including HbF, MCHC and RBC shape[10]. Besides, several groups have demonstrated relationships between osmotic fragility and shape distortions in stored RBCs, which might eventually lead to increased haemolytic potential of blood units[46,47]. In the current study, the mean osmotic fragility of stored RBCs correlates with haemolysis at every time point of the storage period in PRBC units showing acceptable haemolysis levels (<0.8%), verifying the physical interaction between these parameters at a cellular level. Our results not only suggest that variation in haemolysis during storage is a function of RBC fragility, but also estimated that osmotic fragility contributes to approximately 11% in the overall haemolysis levels at any time point of storage in PRBCs. Consequently, haemolysis, a donor-associated hallmark and gold quality standard for storage systems, is a multi-parametric phenotype of storage lesion and the cumulative outcome of modifications in many functionally associated factors severely affected by the storage system. In support of this, the functionally important correlation between haemolysis and osmotic fragility was not observed in PRBC units with extreme haemolysis levels (>0.8%) (Figure 3B) or in units of WB (Figure 3C). PRBC units exhibiting haemolysis more than 0.8% represent a severely disturbed biological system in which RBC homeostatic mechanisms fail to preserve physiological equilibrium order and thus, there is a luck of predictability (a common feature of systems characterised by increased entropy), while the units of WB represent a completely different system of biopreservation, compared to that of PRBCs, in which the haemolysis phenotype is more related to physiological parameters other than osmotic fragility. The complexity of the donor- and storage-related factors that contribute to the final outcome of in-bag haemolysis deserves further study through biological data mining and bioinformatics tools.

Conclusions

Our results suggest that warming of cold blood supplemented by CPDA-1 up to 17 °C in a period of less than eight hours before component processing is

associated with unacceptable haemolysis in PRBC units but not in units of WB, first observed at the middle of the storage period. Although other parameters of the RBC storage lesion besides haemolysis need to be investigated under similar temperature stress conditions, the current data point to the protective effect of plasma on the biopreservation of RBCs, which might provide a chance for using such a kind of "mishandled" donations to favour the provision of adequate blood supplies. Moreover, our data show that mean osmotic fragility, an easily assessed RBC variable in all laboratory settings, represents a donor-specific signature on stored blood and blood components under a variety of storage conditions. It contributes to the haemolysis phenotype as a part of the RBC storage lesion and might be used for the early assessment of RBC fragility in the donated blood during storage.

Authorship contributions

MHA and AGK are equally last Authors. VLT, ATA, DGK, RAZ and HTG performed the haemolysis and fragility experiments. ODP and OAP performed the microbiological analysis. VLT performed the statistical analysis. VLT and MHA analysed the data. VLT, MHA and AGK wrote the paper. KES provided the units of WB and PRBCs, critically commented on the study design and contributed to the final draft of the paper. ISP critically contributed to the final draft of the paper. All Authors read and approved the final manuscript.

The Authors declare no conflicts of interest.

References

1) World Health Organization. Safe blood and blood products: manual on the management, maintenance and use of blood cold chain equipment; 2005, WH 460. Available at: http://www.who.int/bloodsafety/Manual_on_Management,Maintenance_and_Use_of_Blood_Cold_Chain_Equipment.pdf. Accessed on 13/01/2017.
2) Spinella PC, Pidcoke HF, Strandenes G, et al. Whole blood for hemostatic resuscitation of major bleeding. Transfusion 2016; **56**: S190-202.
3) Hess JR, Sparrow RL, van der Meer PF, et al. Red blood cell hemolysis during blood bank storage: using national quality management data to answer basic scientific questions. Transfusion 2009; **49**: 2599-603.
4) Wagner SJ. Transfusion-transmitted bacterial infection: risks, sources and interventions. Vox Sang 2004; **86**: 157-63.
5) Hess JR. Red cell changes during storage. Transfus Apher Sci 2010; **43**: 51-9.
6) Harboe M. A method for determination of hemoglobin in plasma by near-ultraviolet spectrophotometry. Scand J Clin Lab Invest 1959; **11**: 66-70.
7) Han V, Serrano K, Devine DV. A comparative study of common techniques used to measure haemolysis in stored red cell concentrates. Vox Sang 2010; **98**: 116-23.
8) Kraus A, Roth HP, Kirchgessner M. Supplementation with vitamin C, vitamin E or beta-carotene influences osmotic fragility and oxidative damage of erythrocytes of zinc-deficient rats. J Nutr 1997; **127**: 1290-6.
9) American Association of Blood Banks. *Technical Manual.* 18th ed. Bethesda, MD: AABB; 2014. p. 4.
10) Tzounakas VL, Georgatzakou HT, Kriebardis AG, et al. Donor variation effect on red blood cell storage lesion: a multivariable, yet consistent, story. Transfusion 2016; **56**: 1274-86.
11) Cogen AL, Nizet V, Gallo RL. Skin microbiota: a source of disease or defence? Br J Dermatol 2008; **158**: 442-55.
12) Pastila S, Lonnroth M, Heikkila R, et al. Bacterial skin flora and contamination of blood components: do we defer blood donors wisely? Vox Sang 2012; **103**: 93-8.
13) Ramirez-Arcos S, Mastronardi C, Perkins H, et al. Evaluating the 4-hour and 30-minute rules: effects of room temperature exposure on red blood cell quality and bacterial growth. Transfusion 2013; **53**: 851-9.
14) Soeterboek AM, Welle FH, Marcelis JH, van der Loop CM. Sterility testing of blood products in 1994/1995 by three cooperating blood banks in The Netherlands. Vox Sang 1997; **72**: 61-2.
15) Chalmers C, Russell WJ. When does blood haemolyse? A temperature study. Br J Anaesth 1974; **46**: 742-6.
16) Wilson EB, Knauf MA, Iserson KV. Red cell tolerance of admixture with heated saline. Transfusion 1988; **28**: 170-2.
17) Eastlund T, Van Duren A, Clay ME. Effect of heat on stored red cells during non-flow conditions in a blood-warming device. Vox Sang 1999; **76**: 216-9.
18) Wilson EB, Knauf MA, Donohoe K, Iserson KV. Red blood cell survival following admixture with heated saline: evaluation of a new blood warming method for rapid transfusion. J Trauma 1988; **28**: 1274-7.
19) Eckstein M, Zimmermann R, Roth T, et al. The effects of an overnight holding of whole blood at room temperature on haemoglobin modification and in vitro markers of red blood cell aging. Vox Sang 2015; **108**: 359-67.
20) Wilsher C, Garwood M, Sutherland J, et al. The effect of storing whole blood at 22 degrees C for up to 24 hours with and without rapid cooling on the quality of red cell concentrates and fresh-frozen plasma. Transfusion 2008; **48**: 2338-47.
21) Radwanski K, Garraud O, Cognasse F, et al. The effects of red blood cell preparation method on in vitro markers of red blood cell aging and inflammatory response. Transfusion 2013; **53**: 3128-38.
22) Minetti G, Egee S, Morsdorf D, et al. Red cell investigations: art and artefacts. Blood Rev 2013; **27**: 91-101.
23) Ellory JC, Hall AC. Temperature effects on red cell membrane transport processes. Symp Soc Exp Biol 1987; **41**: 53-66.
24) Grygorczyk R. Temperature dependence of Ca^{2+}-activated K^+ currents in the membrane of human erythrocytes. Biochim Biophys Acta 1987; **902**: 159-68.
25) Makhro A, Huisjes R, Verhagen LP, et al. Red cell properties after different modes of blood transportation. Front Physiol 2016; **7**: 288.
26) D'Alessandro A, Dzieciatkowska M, Hill RC, Hansen KC. Supernatant protein biomarkers of red blood cell storage hemolysis as determined through an absolute quantification proteomics technology. Transfusion 2016; **56**: 1329-39.
27) Reisz JA, Wither MJ, Dzieciatkowska M, et al. Oxidative modifications of glyceraldehyde 3-phosphate dehydrogenase regulate metabolic reprogramming of stored red blood cells. Blood 2016; **128**: e32-42.
28) D'Alessandro A, Kriebardis AG, Rinalducci S, et al. An update on red blood cell storage lesions, as gleaned through biochemistry and omics technologies. Transfusion 2015; **55**: 205-19.
29) Paglia G, D'Alessandro A, Rolfsson O, et al. Biomarkers defining the metabolic age of red blood cells during cold storage. Blood 2016; **128**: e43-50.

30) Hess JR. Scientific problems in the regulation of red blood cell products. Transfusion 2012; **52**: 1827-35.
31) Tzounakas VL, Kriebardis AG, Papassideri IS, Antonelou MH. Donor-variation effect on red blood cell storage lesion: a close relationship emerges. Proteomics Clin Appl 2016; **10**: 791-804.
32) Ames BN, Cathcart R, Schwiers E, Hochstein P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. Proc Natl Acad Sci USA 1981; **78**: 6858-62.
33) Vota DM, Maltaneri RE, Wenker SD, et al. Differential erythropoietin action upon cells induced to eryptosis by different agents. Cell Biochem Biophys 2013; **65**: 145-57.
34) Kleinbongard P, Schulz R, Rassaf T, et al. Red blood cells express a functional endothelial nitric oxide synthase. Blood 2006; **107**: 2943-51.
35) Mihov D, Vogel J, Gassmann M, Bogdanova A. Erythropoietin activates nitric oxide synthase in murine erythrocytes. Am J Physiol Cell Physiol 2009; **297**: C378-88.
36) Reinhart SA, Schulzki T, Reinhart WH. Albumin reverses the echinocytic shape transformation of stored erythrocytes. Clin Hemorheol Microcirc 2015; **60**: 437-49.
37) Tzounakas VL, Kriebardis AG, Georgatzakou HT, et al. Glucose 6-phosphate dehydrogenase deficient subjects may be better "storers" than donors of red blood cells. Free Radic Biol Med 2016; **96**: 152-65.
38) Bahr MP, Yazer MH, Triulzi DJ, Collins RA. Whole blood for the acutely haemorrhaging civilian trauma patient: a novel idea or rediscovery? Transfus Med 2016; **26**: 406-14.
39) Karam O, Tucci M. Massive transfusion in children. Transfus Med Rev 2016; **30**: 213-6.
40) Spinella PC, Cap AP. Whole blood: back to the future. Curr Opin Hematol 2016; **23**: 536-42.
41) Yazer MH, Jackson B, Sperry JL, et al. Initial safety and feasibility of cold-stored uncrossmatched whole blood transfusion in civilian trauma patients. J Trauma Acute Care Surg 2016; **81**: 21-6.
42) Jobes D, Wolfe Y, O'Neill D, et al. Toward a definition of "fresh" whole blood: an in vitro characterization of coagulation properties in refrigerated whole blood for transfusion. Transfusion 2011; **51**: 43-51.
43) Pidcoke HF, McFaul SJ, Ramasubramanian AK, et al. Primary hemostatic capacity of whole blood: a comprehensive analysis of pathogen reduction and refrigeration effects over time. Transfusion 2013; **53**: 137S-49S.
44) Strandenes G, Austlid I, Apelseth TO, et al. Coagulation function of stored whole blood is preserved for 14 days in austere conditions: a ROTEM feasibility study during a Norwegian antipiracy mission and comparison to equal ratio reconstituted blood. J Trauma Acute Care Surg 2015; **78**: S31-8.
45) Reddoch KM, Pidcoke HF, Montgomery RK, et al. Hemostatic function of apheresis platelets stored at 4 degrees C and 22 degrees C. Shock 2014; **41**: 54-61.
46) Mustafa I, Al Marwani A, Mamdouh Nasr K, et al. Time dependent assessment of morphological changes: leukodepleted packed red blood cells stored in SAGM. Biomed Res Int 2016; **2016**: 4529434.
47) Blasi B, D'Alessandro A, Ramundo N, Zolla L. Red blood cell storage and cell morphology. Transfus Med 2012; **22**: 90-6.

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