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Microfluidic deformability analysis of the red cell storage lesion

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ABSTRACT

A key challenge in transfusion medicine research and clinical hematology is to develop a simple and non-destructive method to measure the quality of each blood unit prior to use. RBC deformability has long been proposed as an indicator of blood quality. We measured RBC deformability using the pressure required for single cells to transit through a micrometer scale constriction to examine longitudinal changes in RBC deformability, as well as the variability in blood quality and storage capacity across donors. We used a microfluidic device to monitor deformability changes in RBCs stored in plastic tubes and in blood bags over 14 and 56 days respectively. We found consistent storage based degradation of RBC deformability with statistically significant variability in both the initial RBC deformability and storage capacity among donors. Furthermore, all samples exhibited a transient recovery phenomenon. Deformability profiling of stored RBCs using transiting pressure showed significant donor variability in initial quality and storage capacity. This measurement approach shows promise as a rapid method to individually assess the quality of stored RBC units.

1. Introduction

The deformability of red blood cells (RBCs) is essential to their movement through the smallest capillaries of the microvasculature. The loss of deformability results in the sequestration of these cells in the microvasculature and removal by the endosplenic system (Chen and Weiss, 1973). Cold storage of RBC units for use in blood transfusions leads to the development of the storage lesion (Alessandro et al., 2010) and results in their decreased deformability. Specifically, characteristics of the storage lesion include structural, metabolic, and morphologic transformations (Gifford et al., 2006) that all result in the increased clearance of transfused RBCs from the circulation (Deplaine et al., 2011). Consequently, the circulatory lifespan of transfused RBCs decreases from the expected lifetime of 120 days to, in some cases, hours or minutes (Weed, 1970).

RBC units collected and processed by blood banks around the world are currently stored for up to 42 days. This 42-day storage window was established based on early studies that found on average, at least 75% of donor RBCs remain in circulation in the recipient at 24 h post-transfusion (Dumont et al., 2008). While this guideline is likely observed for blood transfusions on a population average, there is significant variability in the circulatory half-life and rates of degradation of RBCs across different donors (Dumont et al., 2008). Additionally, there is increasing evidence that transfusion of older RBCs is less beneficial than fresh RBCs, which further suggests that not all blood units provide an equal amount of health benefit to the recipient (Marik and Corwin, 2008; Zallen et al., 1999). This opinion is well-supported by correlation between blood storage time and clinical complications, such as mortality (Basran et al., 2006; Leal-Noval et al., 2001; Purdy et al., 1997), pneumonia (Leal-Noval et al., 2001; Vamvakas and Carven, 1999), serious infections (Leal-Noval et al., 2001; Offner et al., 2002), multi-organ failure (Basran et al., 2006; Zallen et al., 1999) and length of hospital stay (Keller et al., 2002; Leal-Noval et al., 2001; Martin et al., 1994).

Therefore, a key challenge in transfusion medicine research and clinical hematology is developing a simple method to measure blood quality and to discriminate low quality units. This capability would enable real-time assessment of blood quality in order to most efficiently utilize available supply while delivering maximum

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health benefit to recipients. Given the inherent need for RBCs to deform, RBC deformability has long been proposed as an indicator of blood quality (Guo et al., 2014, 2011; Kwan et al., 2013). Specifically, blood bags with greater mean rigidity or have a greater fraction of rigidified RBCs can be deemed as low quality as these RBCs are more rapidly removed from circulation. Consequently, rapid characterization of RBC deformability in each bag could provide optimized methods to use each bag, as well as a metric for establishing improved production and storage practices.

Existing methods for measuring RBC deformability include bulk flow methods, such as micropore filtration (Berezina et al., 2002) and ektacytometry (Bessis et al., 1980; Kuypers et al., 1990), which measure the average rigidity of a RBC sample, but cannot capture the heterogeneous nature of RBC aging during cold storage. Single cell techniques, such as micropipette aspiration (Singh and Nagaprasad, 1998), optical tweezers (Suresh et al., 2005), and the Cell Transit Analyzer (Scott et al., 1990), can overcome this heterogeneity and estimate the fraction of rigid RBCs that are unlikely to transit through the microvasculature, but typically involve delicate low throughput experiments performed by skilled technicians using specialized equipment.

To address these issues, we recently developed the multiplexed fluidic plunger (MFP) mechanism that measured the deformability of individual RBCs based on the pressure required for each cell to transit through a micrometer scale constriction (Guo et al., 2014, 2012; Kwan et al., 2013) (Myrand-Lapierre et al., 2015). In this study, we demonstrate the potential to use this technology for rapid evaluation of the RBC deformability lesion in stored blood. We show consistent storage based degradation of RBC deformability with notable variability in both the initial RBC deformability and storage capacity among donors. Furthermore, we show that all samples showed the ability to temporarily recover their deformability during the storage period.

2. Materials and methods

2.1. Healthy control RBCs

Following informed consent, whole blood was collected on 1–5 independent occasions by lancet from healthy adult donors for analysis of baseline RBC deformability [$n=10$, 5 male (M) and 5 female (F)]. For storage in plastic tubes ($n=5$, 1 M/4 F donors), whole blood was collected into sodium-citrate tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged in polypropylene plastic tubes at 1860 rpm for 50 min. Plasma and leukocyte layers were removed before resuspension in SAG-M (from a blood bag unit) and donor plasma according to guidelines obtained from the Canadian Blood Services (CBS) Network Centre for Applied Development (NetCAD). This model system allowed us to avoid the complexities of storage in and withdrawal of blood from blood bags. Prior to MFP analysis, RBC suspensions were washed in Phosphate Buffered Saline (PBS, Gibco, Grand Island, NY) at 1200 rpm for 5 min and resuspended at 30% hematocrit in Assay Buffer, which consisted of PBS with 0.2% Pluronic F-127 solution (Sigma-Aldrich, St. Louis, MO).

2.2. Donor blood bags

Type A Rh-positive packed red blood cell donor blood bags ($n=8$, 4 M/4 F) were acquired from CBS NetCAD and stored upright at 4 °C for 56 days. Over this period, aliquots were collected under sterile conditions by agitation of the blood bag for 1 min and drawn into a syringe with a 27-gauge needle by insertion into one of the two resealable valves on the blood bag. The valves were sealed with sterile parafilm and the bag was immediately returned to the refrigerator. Hematocrit was assessed by capillary tube (Fisher Scientific, Waltham, MA) and Statspin Critspin centrifuge (Beckman Coulter, Fullerton, CA). For each sample, 100 µl was used for measuring RBC deformability and morphology with the MFP device. Prior to MFP analysis, all RBC suspensions were washed in PBS at 1200 rpm for 5 min and resuspended at 30% hematocrit in Assay Buffer. The remainder of the sample was collected by centrifugation (6000 rpm, 1 min) and the plasma was stored for determination of extracellular ATP, lactate dehydrogenase (LDH) and hemoglobin concentrations.

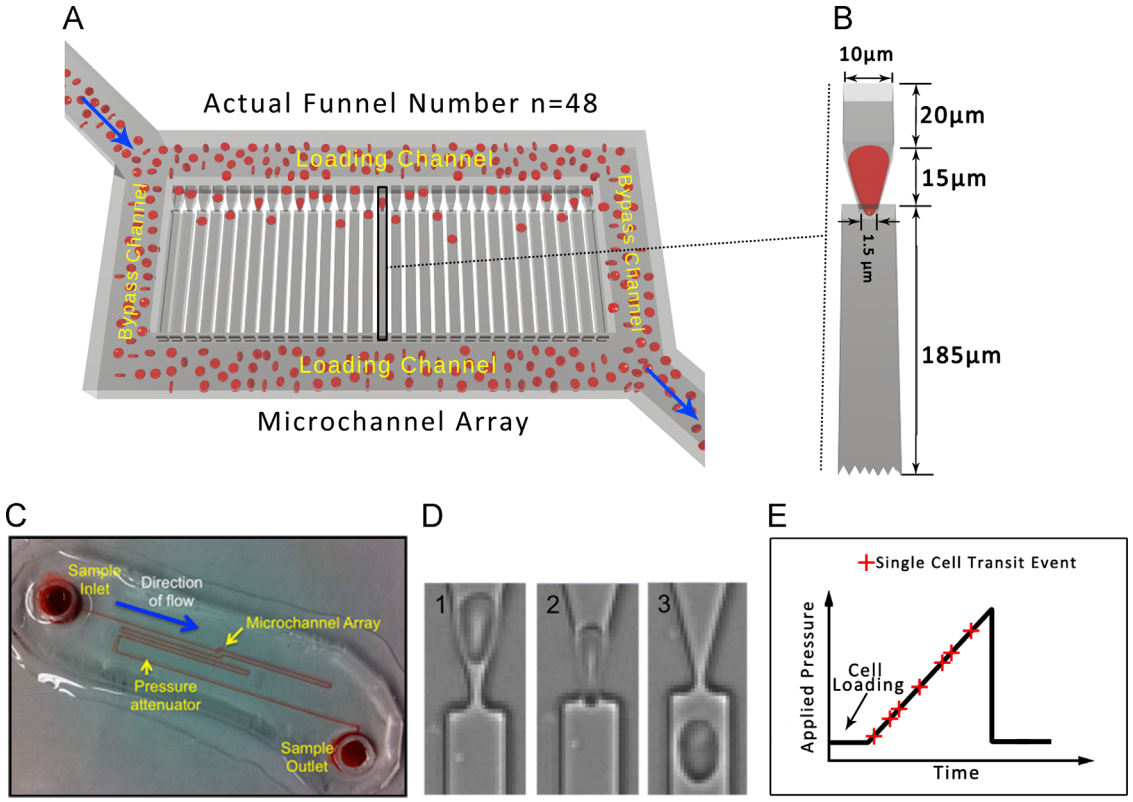

Fig. 1. Overview of the Microfluidic Plunger Device to measure RBC deformability. (A) Magnified 3D model view of the microchannel array showing the loading and bypass channels. (B) Magnified 3D model of one of the deformation channels, showing its dimensions and a RBC in planar configuration. (C) Photograph of the MFP microfluidic device. (D) Micrographs of a RBC in the deformation channel as it transits the microconstriction from 1 to 3. (E) A pressure–time waveform is applied to the inlet while a video of the deformation process is simultaneously recorded.

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2.3. Microfluidic deformability measurement

The microfluidic devices were fabricated as previously described (Myrand-Lapierre et al., 2015). Once the device (Fig. 1A–C) is filled with Assay Buffer, the blood sample is introduced into the inlet reservoir and a pressure difference sufficient for RBCs to occupy, but not transit, the funnel constrictions is applied to the sample inlet (Cell Loading, Fig. 1E). Once most of the constrictions are occupied, the applied pressure is incrementally increased using the pressure waveform shown in Fig. 1E. The deformation process (Fig. 1D) is simultaneously video recorded for the determination of the threshold deformation pressure from the corresponding pressure–time data (Fig. 1E), using specially designed video analysis software as previously described in more detail (Myrand-Lapierre et al., 2015). Upon completion of the deformability measurement the device is reset to its original state, clearing all RBCs blocking the constrictions, and the same blood sample can be reloaded for subsequent RBC deformability measurements. Each deformability measurement (48 RBCs), including the RBC loading and deformation process, takes approximately 2 min (for fresh RBCs) to approximately 4 min (for older stored RBCs). A minimum of 150 RBCs were tested per sample.

2.4. Assessment of blood plasma

The pH of the blood bags was determined on fresh plasma samples using pH indicator strips with a pH range of 5.5–8.0 (Hydrion, Brooklyn, NY). Blood plasma was stored and batch thawed to determine extracellular ATP, LDH and hemoglobin levels. ATP concentrations were determined using the Molecular Probes ATP Determination kit (Life Technologies, Carlsbad, CA) and LDH was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WA), both according to manufacturer's instructions. The percentage of RBC lysis was measured using the Drabkin's Assay, and normalized to its hematocrit as previously described (Han et al., 2010).

2.5. Statistical analysis

Data were analyzed using Graphpad Prism v5. The D'Agostino and Pearson omnibus and Shapiro–Wilk tests were used to determine the normality of all data. Medians with and without Interquartile Ranges (IQR) were plotted for individual donor and combined deformability results. Student's *t*-test was used to determine statistical differences between results that were normally distributed, while Mann–Whitney, Kruskal–Wallis and Friedman tests were used for results that were not parametric. Correlations between the deformability of RBCs and their corresponding biological analysis were calculated using Pearson *r* with 95% confidence interval.

3. Results

3.1. Healthy control RBC deformability range

To establish the normal RBC deformability range we sampled blood from 10 healthy donors. Each donor was sampled 1–5 times with a minimum of 150 RBCs tested per sample. Normal healthy individuals exhibited RBC deformability with a median transiting pressure of 5.98 Pa and an interquartile range (IQR) from 4.84 to 7.15 Pa (Fig. 2).

3.2. RBC deformability degradation during cold storage in plastic tubes

To validate the utility of the MFP device to measure storage based RBC deformability degradation, we sampled RBC from five different healthy donors stored in plastic tubes. This model system allowed us to avoid the complexities of storage in and withdrawal from blood bags. Expectedly, the median transiting pressure of these RBCs increases with storage time and with a broadening distribution (Fig. 3A). The transiting pressure increased gradually at first and then dramatically accelerated between days 7 and 14 (ANOVA $p < 0.0001$, Fig. 3B). Additionally, the fraction of cells that cannot transit through the constriction at the maximum pressure of 100 Pa also increased with time following a similar trend (Fig. 3B). This value is likely to be reflective of the fraction of rigidified cells that are sequestered by the microvasculature or by the splenic system after transfusion. Interestingly, both the median transit pressure and the fraction of non-transiting cells exhibited a transient recovery during storage, which we will describe in greater detail later.

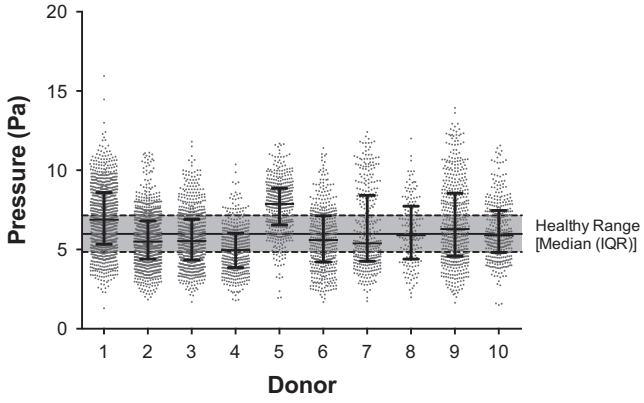

Fig. 2. The RBC deformability distribution of healthy controls is narrow. Each donor was sampled between 1–5 times on different occasions. Each closed circle for each separate donor indicates the median RBC-D from a single experiment or more than 150 individual RBCs. The black lines and error bars for each donor show the median and IQR. The shaded gray area indicates the normal median and IQR range.

3.3. RBC Deformability degradation during cold storage in Blood Bags

We assessed a gender-balanced group of 8 anonymous A+ donor blood bags and monitored them for 8 weeks using the MFP device. The microfluidic deformability measurements for all 8 donor blood bags for all weeks of monitoring were combined in one dataset. Here, the initial median transiting pressure of 7.58 Pa measured after 1 day of storage was greater than the median and upper 75% IQR of the healthy RBC deformability profile (Fig. 4). Cold storage based degradation in RBC deformability was measured using the median transiting pressure, which showed a consistent time-dependent decrease in RBC deformability over the entire 8 weeks of monitoring (Freidman test, week 0 vs. week 6, $p=0.0002$ and week 8, $p < 0.0001$). On average, there was a significant increase in the median transiting pressure observed by week 2 (Mann–Whitney, $p < 0.0001$). The fraction of non-transiting cells similarly increased. By week 2, 21.2% RBCs failed to deform through the micro-scale constrictions. By 42 days, this fraction reached 30.1%. Routine hematocrit and pH measurements indicated that each unit had not been compromised during the experiment, as neither hematocrit nor pH deviated significantly during the monitoring period (Table 1). This trend was further supported by the expected trends of supernatant ATP, LDH as well as hemolysis levels measured during the 8 weeks of storage (Table 1). Furthermore, there was a correlation between the proportion of RBCs unable to transit the microconstrictions and the amount of hemolysis measured in each sample (Pearson $r=0.460$, $p=0.0001$, Table 2).

3.4. Identification of blood bags with lower initial quality and storage potential

A key observation from measuring the deformability of RBCs stored in blood bags is the significant variability across donors relative to the comparatively minimal variability in fresh RBCs sampled from healthy donors, which suggest that RBCs from certain donors have lower initial quality and storage potential. The median transiting pressure and fraction of non-transiting cells for measured weekly for each blood bag is shown in Fig. 5. The average values for these two metrics from all 8 bags at day 14 and 42 are shown as a guide. The longitudinal RBC deformability

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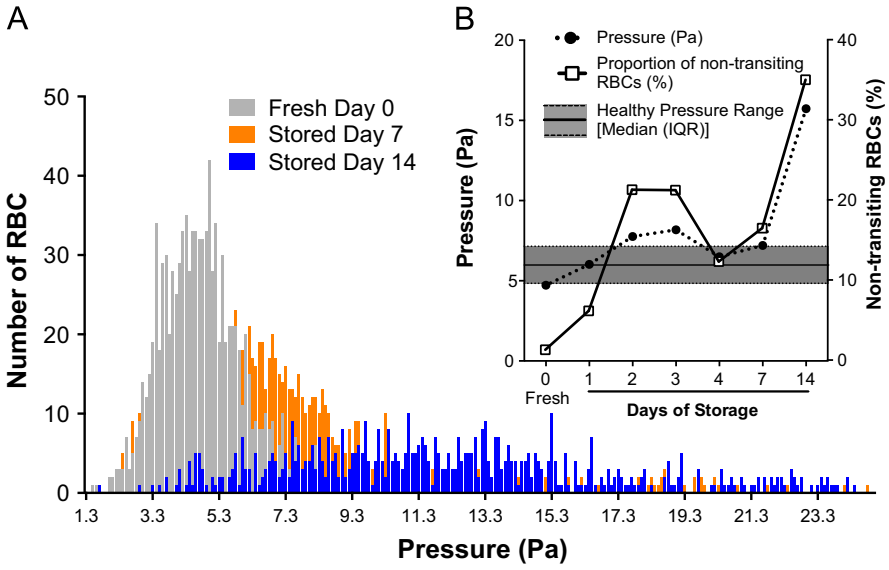

Fig. 3. Degradation of RBC deformability when stored in plastic tubes. (A) Histogram showing clear separation and shift to the right of the RBC deformability distributions during storage in plastic tubes ($n=5$ donors), with the emergence of a peak at day 14 (blue peak). (B) There is an initial loss in deformability during the first 3 days of storage, a slight recovery measured at day 4 and day 7, and significant loss of deformability at 14 days of storage. The same trend is noted for the proportion of non-transiting RBCs. The shaded gray area depicts the median and IQR of healthy pressures required for RBCs to transit the constrictions. The broken line connecting the solid circles denotes the median and IQR of the combined pressure on the left *y*-axis. The solid line connecting the open squares denotes the proportion of non-transiting RBCs on the right *y*-axis.

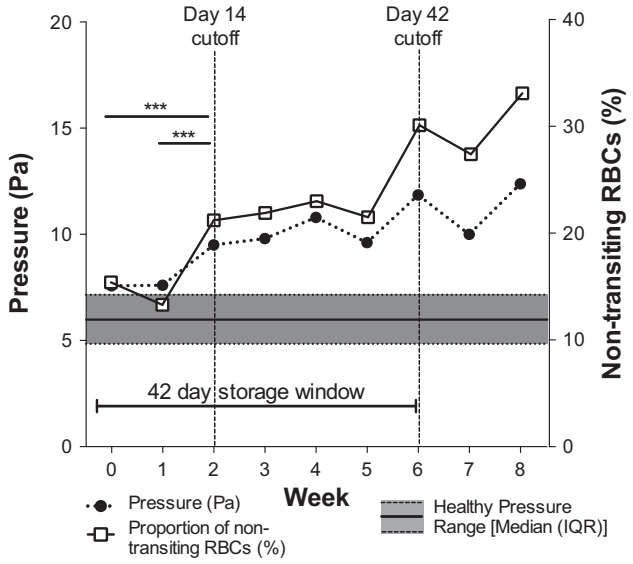

Fig. 4. Degradation of RBC deformability when stored in blood bags ($n=8$). The median pressure required to squeeze RBCs through the microconstrictions increases significantly by week 2 of storage (Mann–Whitney, $p<0.0001$) and continues to increase during the normal 42-day storage window (Friedman test, $p=0.0002$) and beyond to 8 weeks of storage (Friedman test, $p<0.0001$). Correlatory, the proportion of non-transiting RBCs significantly increased to 21.2% by week 2 and 30.1% by week 6. The shaded gray area depicts the median and IQR of healthy pressures required to deform RBCs, offering a reference to the changes that occur during storage. The broken line connecting the solid circles denotes the median of the combined pressure for all 8 blood bag units on the left *y*-axis. ***Mann–Whitney, $p<0.0001$. The solid line connecting the open squares denotes the proportion of non-transiting RBCs on the right *y*-axis.

profile in donors 3 and 4 appears to decrease catastrophically after week 5. Additionally, donors 3 and 4, along with donor 2, also show an accelerated rigidification of RBCs, where they reach the 42 days average transiting pressure after only 14 days of storage. These donors are likely to be considered as donors with poor storage potential. Conversely, RBCs from donors 6, 7 and 8 appear to store better than others and may therefore be considered as donors with good storage potential.

Table 1
Blood bag characteristics and changes in biological analysis from week 0 to 8.

	Gender	Hct (%)	pH	ATP (nM)	LDH (as % of control)	Hemolysis (%)
Donor 1	F	68–51	7.0–6.8	537–0	106–1343	0.23–0.46
Donor 2	M	58–58	7.0–7.2	878–0	723–1658	0.03–1.09
Donor 3	M	59–61	6.8–7.0	1013–0	916–1830	0.07–2.83
Donor 4	F	59–58	6.8–7.2	750–0	916–701	0.14–0.97
Donor 5	M	61–58	7.0–7.0	474–0	933–2162	0.26–2.11
Donor 6	F	60–58	7.0–6.8	250–0	1536–2379	0.37–1.40
Donor 7	F	58–56	7.0–7.0	973–0	1222–1912	0.27–0.60
Donor 8	M	60–60	6.8–7.2	318–0	385–1399	0.03–0.42

3.5. *Gender differences*

As shown in Fig. 6A, it was observed that RBCs from female donors had consistently smaller transiting pressure and fewer non-transiting cells than male donors during the 42-day storage window. Additionally, RBCs from male donors showed increased hemolysis at an earlier stage (Fig. 6B). These results suggest that RBCs from male donors may have lower storage potential than RBCs from female donors, however the sample size is too small for statistical significance.

3.6. *Transient recovery of RBC deformability during storage*

Interestingly, the measured transiting pressures are not necessarily increasing monotonically during cold stored RBCs, but fluctuate over time. In fact, after an initial increase of transiting pressure, there appears a consistent process where RBC deformability recovers temporarily before decreasing again. RBCs stored in plastic tubes showed a consistent transient recovery of deformability between day 4 and day 7 of storage (Fig. 3B). This phenomenon occurred in 4/5 donors sampled and we may have missed this occurrence in the 5th donor as we only sampled at day 1, 7 and again at day 14. These fluctuations were also observed in the combined data of the blood bag monitoring between week 5 and 7 (Fig. 4), as well as in individual blood bags (Fig. 5). Furthermore, these transient changes were observed earlier and more

Table 2
Correlation matrix between Pressure (Pa), % non-transiting RBCs and the biological analysis for individual sample data points of blood bags over 8 weeks of storage.

Individual sample data points [$n=64$ (8 donors × 8 weeks)]		Pressure (Pa)	Non-transiting RBCs (%)	ATP (nM)	LDH (as % of control)	Hemolysis (%)
Pressure (Pa)	Pearson $r=$		0.745	0.017	−0.019	0.134
	p-value		<0.0001	0.413	0.878	0.302
Non-transiting RBCs (%)	Pearson $r=$	0.745		0.041	−0.012	0.460
	p-value	<0.0001		0.751	0.328	0.0001

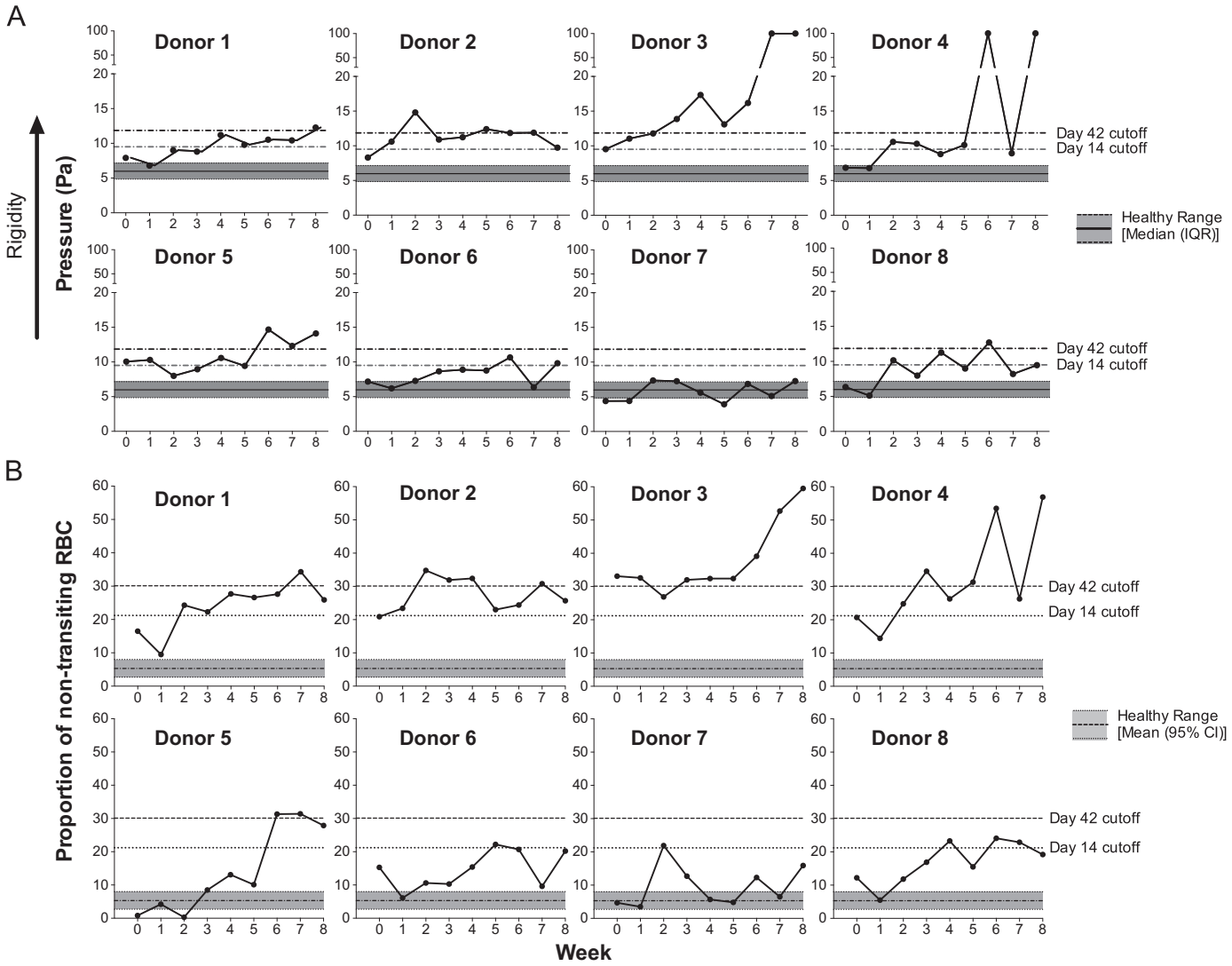

Fig. 5. Median RBC deformability and fraction of rigid RBCs of individual blood bags. (A) The median pressure (Pa) of each separate donor ($n=8$) shows significant variability between time points for each donor as well as between donors over the entire 8 weeks of monitoring. The implementation of cut-off median pressure lines at Day 42 and Day 14 enables the identification of outlier blood bags. Donors whose median RBC deformability crosses the day 42 cutoff line very early during storage, such as donors 2, 3 and 4, may be of a lower quality than the rest. (B) The proportion of non-transiting RBCs may be a better method to distinguish low quality blood bag units. Again, lines at Day 42 and Day 14 as cutoffs show that blood bags from donors 2, 3 and 4 are outliers, and cross these lines early during the storage window. While the other bags do cross this line, it is much later on.

prominently for male donors (at week 5) than female donors (at week 7, Fig. 6).

3.7. RBC morphology change during storage

We assessed the proportions of RBCs that showed an abnormal morphological shape (Fig. 7B) compared to the normal discoctye shape that RBCs usually exhibit (Fig. 7A). While the proportions of abnormal RBCs increase over the 8 weeks of storage time (Friedman $p=0.0034$), there was a lack of correlation between the proportion of abnormal RBCs and their corresponding median deformability at the same time point ($r=-0.059$, $p=0.644$).

4. Discussion

In this study, we demonstrated the potential to profile the RBC deformability changes during cold storage using the pressure

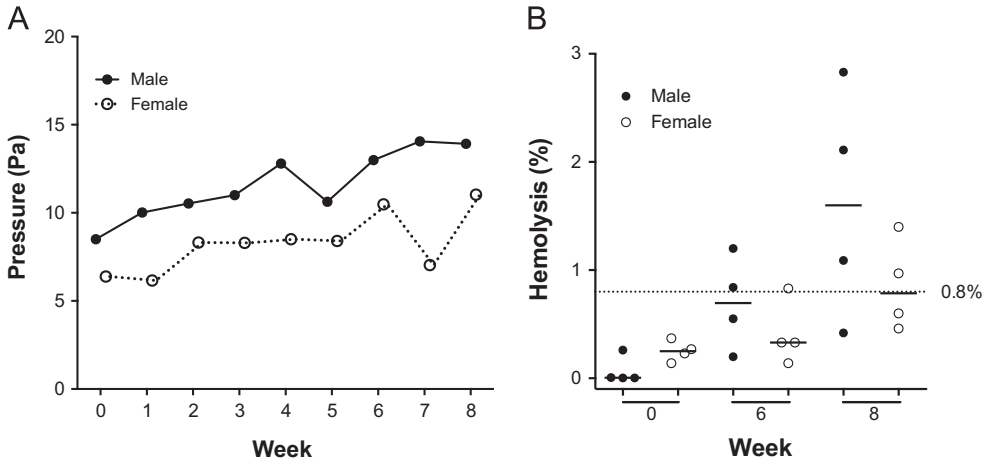

Fig. 6. Gender differences in RBC deformability. (A) RBC deformability as depicted by the median required to squeeze RBCs through the microconstrictions separated by gender shows that RBCs from female donors ($n=4$) tended to have better RBC deformability compared to male donors ($n=4$). (B) Female donor blood bags showed a tendency to have decreased hemolysis compared to males.

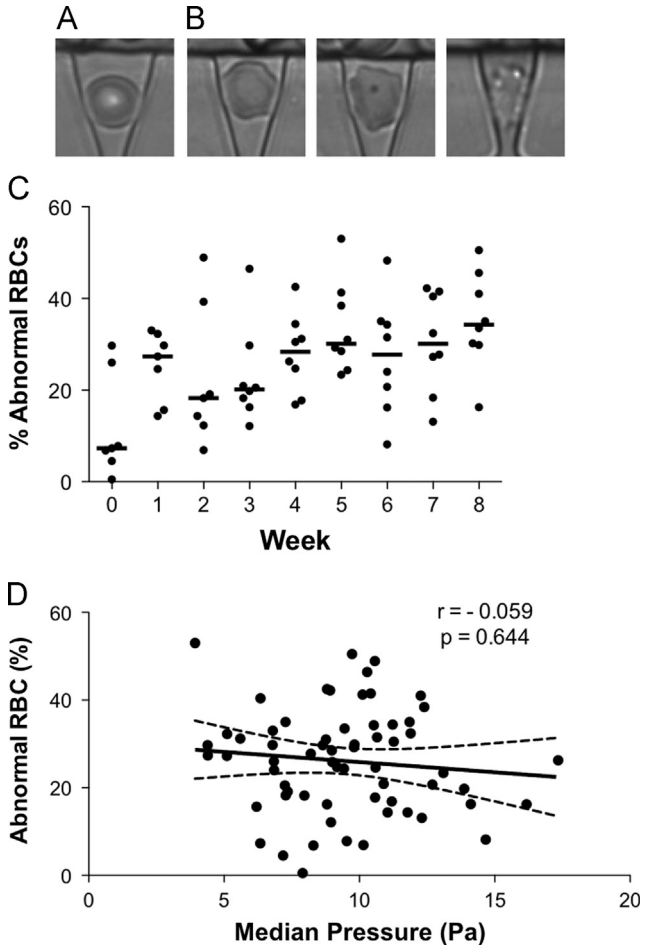

Fig. 7. RBC morphology change during 8 weeks of storage. (A) Normal shaped RBCs in the microchannels. (B) Representative figures of RBCs that were considered and counted as abnormal. (C) The proportions of abnormal RBCs increase over storage time (Friedman test $p=0.0034$), but (D) do not correlate to changes in RBC deformability ($r=-0.059$, $p=0.644$).

required to deform individual cells through micrometer scale constrictions. These microscale constrictions simulate the capillaries of the body and the ability for RBCs to transit through these constrictions may be reflective of the ability of transfused RBCs to transit through the microvasculature and avoid clearance from circulation by transfusion recipients. Using this approach, we monitored 8 A+ blood bags over a period of 8 weeks where we found consistent storage based degradation of RBC deformability with significant variability in both the initial RBC deformability and storage capacity (ability to be stored for long periods of time) across donors.

Our results indicate that significant loss of RBC deformability occurs as early as 2 weeks into storage. Recent studies that have assessed single-cell deformability changes during storage have also indicated that RBC deformability remained fairly constant in the first 15 and 21 days (Czerwinska et al., 2015; Huang et al., 2015) and decreased rapidly after. Another advantage of our MFP device is that we can assess the proportion of individual RBCs that are too rigid to transit the microconstrictions. We speculate that these rigidified RBCs may be the RBCs that are cleared by the spleen after transfusion and we show that by 42 days of storage, 30% of all donor RBCs were too rigid to transit the device. Furthermore, 2 individual donors (Donors 3 and 4) showed that 50–60% of their stored RBCs were too rigid to pass through the microconstrictions. These results are similar to other studies that compared clearance rates of RBCs that were stored for extended periods (Deplaine et al., 2011; Hod et al., 2010; Huang et al., 2015).

The considerable variability between the deformability degradation measured in blood bags is opposed to the relative uniformity of fresh unprocessed RBCs of healthy donors. There are many factors that could account for variability between donors. Tissue hydration (Stuart et al., 1991), metabolism (Handelman and Levin, 2010), diet and exercise (Jenkins et al., 2007; Varlet-Marie et al., 2013), exposure to pharmacologic agents (Clapp et al., 2013; Forsyth et al., 2012), as well as other uncontrolled variables could have a collective effect on donor RBC deformability. The consistent difference between male and female donors in RBC deformability measurements, as well as percentage of hemolysis has been observed in previous studies (Guillet et al., 1998; Nasu et al., 2003), and likely arises from hormonal differences, however the other factors mentioned previously may also play a role. Female sex hormones, estrogen and progesterone, are known to prevent the loss of deformability (DeVenuto et al., 1971; Doucet et al., 2010) by protecting RBCs against both mechanical (Raval et al., 2011) and osmotic stress (DeVenuto and Wilson, 1976). In some cases, where the deformability of RBCs is important, such as in patients requiring chronic transfusions due to sickle cell disease (Steinberg, 1999), it

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may be beneficial to transfuse units from female donors or from male donors that show good initial quality and storage capacity.

Others have previously noted the observed transient recovery of RBC deformability. In one study, it was speculated to be as a result of the reversible changes in RBC morphology (Henkelman et al., 2010) between discoid, echinocyte, and spherocyte shapes (Gifford et al., 2006). Another recent study showed that the normal shaped RBCs show progressive stiffness during storage and that morphology remained constant (Bhaduri et al., 2014), however we did not find that this to be the case in our study. There are other reversible changes that the RBC goes under early on during storage, which may explain the transient recovery of RBC deformability. The inactivation of Na+/K+ pumps (Högman and Meryman, 1999) and depletion of 2,3 diphosphoglyceric acid (DPG) (Beutler and Wood, 1969) add to the storage lesion and can be reversed 24 hours to 4 days post-transfusion and the reversibility is inversely proportional to storage time (Holme, 2005), which may explain the reversal effect seen when blood was stored in the plastic tubes in our study. However, as these transient changes mostly occurred later on during the storage time we suspect that there may be some rejuvenating effect of plasticizer leaching from the plastic blood bag into the sample. Plasticizers are added during manufacture of blood bags to increase their flexibility and durability. There is also evidence that these plasticizers have the unexpected benefit of retarding hemolysis (Draper et al., 2002) and stabilizing the RBC membrane (Lozano and Cid, 2013). We observed this transient recovery process in the RBC deformability measurements in all donors and in multiple storage conditions, which suggest that this result is not an experimental artifact. Further investigations are warranted to study the mechanism for this recovery.

Given the variability and the knowledge that blood from some donors stores better than blood from others (Moroff et al., 1984), the definition of the quality of a blood unit based on an arbitrary storage window may be overly simplistic. Therefore, in place of a ubiquitous storage window, individual assessment of blood units by RBC deformability profiling could potentially help to optimize blood utilization and improve production processes and storage conditions for blood bankers, both of which has also been suggested by another recent study (Huang et al., 2015).

5. Conclusion

We showed that the deformability profiling of individual RBCs of individual blood bags could be used to evaluate the quality of stored RBC units within minutes. This capability could potentially improve clinical outcomes and extend the blood supply by enabling clinicians to optimize use of stored blood in transfusions to reduce complications, as well as a metric for developing improved processes in RBC production and storage.

Conflict of interest statement

None

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