Published in final edited form as:

Transfusion. 2020 November ; 60(11): 2633–2646. doi:10.1111/trf.16017.

Donor-dependent aging of young and old red blood cell subpopulations: Metabolic and functional heterogeneity

Olga Mykhailova¹, Carly Olafson¹, Tracey R. Turner¹, Angelo D'Alessandro², Jason P. Acker^{1,3}

¹Centre for Innovation, Canadian Blood Services, Edmonton, Alberta, Canada

²Department of Biochemistry and Molecular Genetics, University of Colorado Denver–Anschutz Medical Campus, Aurora, Colorado

³Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta, Canada

Abstract

Background: Characteristics of red blood cells (RBCs) are influenced by donor variability. This study assessed quality and metabolomic variables of RBC subpopulations of varied biologic age in red blood cell concentrates (RCCs) from male and female donors to evaluate their contribution to the storage lesion.

Study Design and Methods: Red blood cell concentrates from healthy male (n = 6) and female (n = 4) donors were Percoll separated into less dense (“young”, Y-RCCs) and dense (“old”, O-RCCs) subpopulations, which were assessed weekly for 28 days for changes in hemolysis, mean cell volume (MCV), hemoglobin concentration (MCHC), hemoglobin autofluorescence (HGB), morphology index (MI), oxygen affinity (p50), rigidity, intracellular reactive oxygen species (ROS), calcium ([Ca²⁺]), and mass spectrometry–based metabolomics.

Results: Young RCCs having disc-to-discoid morphology showed higher MCV and MI, but lower MCHC, HGB, and rigidity than O-RCCs, having discoid-to-spheroid shape. By Day 14, Y-RCCs retained lower hemolysis and rigidity and higher p50 compared to O-RCCs. Donor sex analyses indicated that females had higher MCV, HGB, ROS, and [Ca²⁺] and lower hemolysis than male RBCs, in addition to having a decreased rate of change in hemolysis by Day 28. Metabolic profiling indicated a significant sex-related signature across all groups with increased markers of high membrane lipid remodeling and antioxidant capacity in Y-RCCs, whereas O-RCCs had increased markers of oxidative stress and decreased coping capability.

Correspondence: Jason P. Acker, 8249-114 Street, Edmonton, AB, Canada. jason.acker@blood.ca.

CONFLICT OF INTEREST

A.D. is a founder of Omix Technologies Inc and Altis Biosciences LLC, and a consultant for Hemanext Inc. The other authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

Conclusion: The structural, functional, and metabolic dissimilarities of Y-RCCs and O-RCCs from female and male donors demonstrate RCC heterogeneity, where RBCs from females contribute less to the storage lesion and age slower than males.

Keywords

RBC metabolomics; RBC morphology; RBC senescence; RBC storage lesion; young and old RBCs

Red blood cells (RBCs) are the most frequently transfused blood component with approximately 113 million units transfused globally each year.¹ However, like any medical therapy, blood transfusion has risks. Recent studies have linked morbidity and mortality of patients transfused with red blood cell concentrates (RCCs)^{2–5} to the RBC storage lesion. The storage lesion is associated with oxidation of cellular structures,^{6–8} depletion of key metabolites such as ATP and hemoglobin (Hb) regulator 2,3-diphosphoglycerate,⁹ and microvesicle formation.^{10,11} Microvesicle formation is thought to be a main cause of posttransfusion complications due to their role in: (a) the pathogenesis of thrombosis,¹² inflammation,^{13–15} and responses to pathogens;¹⁶ (b) platelet activation;¹⁷ (c) deformability changes that affect RBC rheology and blood flow;¹⁸ and (d) decreased oxygen delivery due to microvesicle-entrapped Hb,¹⁹ among others. The extent of this storage lesion is highly variable depending on factors including, but not limited to, blood processing methods,^{20–24} storage additive solutions,^{25–27} and donor-related characteristics.^{22,28}

At the time of donation, RCCs consist of a population of RBCs with varying biologic ages, from recently matured (young) to senescent (old). RCCs containing a greater proportion of old RBCs may have increased lysis products contributing to an overall decrease in cellular recovery, oxygen delivery, and metabolic stability of RCCs during in vitro hypothermic storage.²⁹ The concept of RCCs as a heterogeneous population of RBCs of varying age that exhibit different structural, functional, and metabolic properties is increasingly being described.^{30–34}

This study proposes that donor characteristics such as age, sex, and number of previous blood donations have implications on the distribution of young and old RBC subpopulations. This maturity-related RBC heterogeneity may in turn impact the RCC storage lesion and therefore the quality of transfused products. The aim of this study is to analyze RBC subpopulations, comparing those from female and male donors, to assess how dissimilarities (structural, metabolic, and functional) may contribute to the storage lesion. A better understanding of donor-related differences in RBC subpopulations may lead to improved RBC manufacturing processes and/or optimal selection of RCC units for safe and effective transfusion.

1 | MATERIALS AND METHODS

1.1 | RCC source and storage

Ten CPD/SAGM leukoreduced RCCs from healthy male (n = 6) and female (n = 4) donors (Table S1) were provided by Canadian Blood Services' Blood for Research Facility (Centre for Innovation, Vancouver, BC, Canada). All RCCs were shipped within 24 hours of

leukoreduction and were stored between 1°C and 6°C in a monitored refrigerator before processing.

1.2 | Sample preparation of young and old RBCs by density separation

On Day 3 postcollection, 50 mL of each RCC unit was sampled using aseptic technique as previously described³⁵ for fractionation into young (less dense) and old (denser) RBC subpopulations using a Percoll-density centrifugation method.

1.2.1 | Estimation of the Percoll density for each RCC unit—To achieve an approximate 1:1 ratio of young and old RBCs, a preliminary separation of the RBC sample was performed using a range of Percoll densities (Percoll GE Healthcare, Sigma-Aldrich, St Louis, MO). Briefly, 3 mL of six different Percoll solution densities (1.080, 1.088, 1.092, 1.095, 1.098, and 1.100 g/L) were placed in separate 5-mL tubes (BD Falcon, BD Biosciences, San Jose, CA) and a 1-mL aliquot of the RCC was layered on each Percoll solution and centrifuged (3200g, room temperature [RT], 10 minutes; Eppendorf 5810R, Eppendorf AG, Hamburg, Germany). The Percoll density that achieved the appropriate separation ratio by visual inspection was used for subsequent separations with larger volumes.

1.2.2 | Fractionation of RCCs (young and old)—Using the previously determined Percoll density for each RCC unit, 40 mL of RBCs was fractionated by gently layering, to prevent mixing, 8 × 5-mL RBC aliquots onto 8 mL of Percoll in separate 15-mL conical tubes. Tubes were centrifuged (3200g, 10 minutes, RT) with low acceleration and deceleration speeds, and the top layer of less dense (young) RBCs was isolated from the bottom layer of denser (old) RBCs. Both fractions were washed three times in 1× PBS (1100g, 10 minutes, RT) to remove residual Percoll solution, mixed with SAGM in 3:2 ratio (RBCs:SAGM), and transferred to a small 150-mL container for storage (4R2001, Fenwal, Lake Zurich, IL).

1.2.3 | Control unseparated RBCs—To control for effects of processing by Percoll separation and storage in small-volume containers, 20 mL of each RCC was sampled and washed three times in 1× PBS (1100g, 10 minutes, RT), mixed with SAGM in 3:2 ratio (RBCs:SAGM), and transferred to a small 150-mL container for storage and assessment alongside the fractionated RBCs.

1.3 | Processed sample storage and assessment

Prepared young (Y-RCC) and old (O-RCC) RCCs, the washed unseparated RBC concentrate (control; W-RCC), and the original parent RCC unit (control; P-RCC) were stored at 1°C to 6°C for 28 days postprocessing. Samples were drawn for quality assessment on Days 7, 14, and 28 of hypothermic storage.

1.4 | RBC quality assessment

Red blood cell quality was assessed for all units for the variables described here. RBC count and indices, including mean cell volume (MCV), mean cell Hb (MCH), and mean cell Hb concentration (MCHC), were analyzed using a hematology analyzer (poch-100i, Sysmex

Corporation, Kobe, Japan). RBC deformability (the maximum theoretical elongation index $[EI_{\max}]$ and the shear stress required to achieve half of the $EI_{\max}$; LORRCA, RR Mechtronics, Zwagg, the Netherlands), RBC hemolysis, spun hematocrit, and oxygen affinity (p50; except for W-RCC samples; Hemox, Model B, TSC Scientific, New Hope, PA) were all performed as previously described.^{36,37}

1.5 | Metabolomics assay

At each time point the Y-RCC, O-RCC, and W-RCC samples were centrifuged (2500g, 10 minutes, 4°C), separated into supernatant and RBCs, stored at -80°C, and shipped on dry ice to Dr. D'Alessandro's lab at the University of Colorado Denver-Anschutz Medical Campus. A 50-μL volume of the frozen RBC aliquots was extracted 1:10 in ice-cold extraction solution (methanol: acetonitrile:water, 5:3:2 vol/vol).³⁸ Samples were vigorously mixed and any insoluble material was pelleted, as previously described.^{39,40} For lipidomics evaluation, supernatants were diluted 1:1 (vol/vol) with 10 mM ammonium acetate for analysis by ultrahigh-pressure liquid chromatography coupled to mass spectrometry (Thermo Fisher Scientific, San Jose, CA). Lipidomic and metabolomic analyses were performed as previously reported.^{41,42}

1.6 | Imaging flow cytometry assay

Samples were prepared as described below and analyzed using an imaging flow cytometer (IFC; Amnis ImageStreamX Mark II, EMD Millipore, Seattle, WA), four-laser two-camera system (ASSIST calibrated) with eliminated debris and speed beads. At least 20 000 bright-field singlet RBC images were captured using the low-speed/high-sensitivity settings at 60× image magnification. Each file containing 20 000 raw bright-field images for each sample was processed on the IDEAS software platform (Version 6.2, EMD Millipore).

1.6.1 | IFC RBC morphology index—For RBC morphology index (MI) assessment, 5 μL of each RBC sample was suspended in 200 μL of 1× Dulbecco's PBS (DPBS; Sigma-Aldrich). A sequentially numbered set of individually captured RBC bright-field images (195 ± 34 images per sample) was manually assigned by a human operator to six morphology subclasses: smooth discs (SDCs), crenated discs (CDCs), crenated discoids (CDDs), crenated spheroids (CSDs), crenated spheres (CSEs), smooth spheres (SSEs), and multiplied by fractional weights^{43,44}:

$$MI, \% = ((SDCs \times 1.0) + (CDCs \times 0.8) + (CDDs \times 0.6) + (CSDs \times 0.4) + (CSEs \times 0.2) + (SSEs \times 0)) \times 100 / (SDCs + CDCs + CDDs + CSDs + CSEs + SSEs).$$

1.6.2| Hb autofluorescence—The ability of the label-free IFC method to detect differences in intracellular Hb concentration for various RBC subpopulations was assessed using median fluorescence intensity (MFI) for intracellular Hb autofluorescence (HGB) after RBC excitation at 488 nm (180 mW laser intensity). Emission was registered in Channel 2 (480–560 nm) and resulted in approximately 10-to 13-fold increase of MFI of RBCs. Samples were prepared as described above and 100 000 bright-field singlet RBC images were captured and analyzed.

1.6.3 | Intracellular Ca²⁺ and reactive oxygen species levels—Red blood cell samples (5 µL) were suspended in 200 µL of 1× DPBS and stained with 2',7'-dichlorofluoresceindiacetate (35845, Sigma-Aldrich) for 30 minutes at 37°C, washed (5 minutes, 1200g, RT) and stained with Cal-500 AM (20 412, AAT Bioquest, Sunnyvale, CA) for 60 minutes at 37°C and analyzed by IFC.

1.7 | Statistical analysis

Statistical analysis was performed using computer software (GraphPad Prism v. 8.3.1, GraphPad Software Inc., La Jolla, CA). Significance was tested on nonnormalized data using paired *t* tests when data showed a normal distribution (D'Agostino-Pearson normality test) and with the Wilcoxon signed-rank test for nonnormal distribution. Significance between RBCs from female (fRBCs) and male donors (mRBCs) was analyzed using the Mann-Whitney test. Mixed-model analysis for repeated measurements with multiple comparisons (with Tukey's adjustment) was performed with computer software (SAS/STAT 9.4, SAS Institute Inc., Cary, NC) to estimate the effects of sex (female or male), group (P-RCC, W-RCC, Y-RCC, O-RCC), and storage time (7, 14, 28 days) and their interaction on the tested variables. A *P*-value less than 0.05 was considered significant.

2 | RESULTS

2.1 | Structural characteristics of RBCs during storage

Fractionation of RCC units into different subpopulations demonstrated a significant difference in structural characteristics of young and old RBCs. Young RBC fractions had higher MCV (Figure 1A) and lower MCHC (Figure 1B) and HGB (Figure 1C) compared to old RBC fractions and both control samples: P-RCCs and W-RCCs. Significant differences for MCV and MCHC between young and old RBCs were retained at Day 14 of hypothermic storage. By Day 28 of storage, MCV and HGB were comparable between the RBC groups, but MCHC remained significantly lower in Y-RCCs compared to O-RCCs and both control groups (P-RCCs, W-RCCs).

To assess the effect of donor sex on different RCC groups (P-RCCs, W-RCCs, Y-RCCs, O-RCCs) throughout storage (7, 14, 28 days) a mixed-model analysis was used (Table 1). It showed that all three variables (sex, group, and time) had a significant effect on MCV and HGB of RBCs. Of note, fRBCs had higher MCV (*P* = 0.0283) and HGB (*P* = 0.0211). For MCHC, only RBC group had significant effect on results, while sex and time were not significant (Table 1). The mixed-model analysis also showed a significant interaction between group and time for MCV, MCHC, and HGB (Table 1).

Additional assessment of differences between female and male samples showed that fRBCs appear to have higher MCV than mRBCs with a significant difference in P-RCCs on Day 14 and in O-RCCs on Days 14 and 28 (Figure S1A). For HGB, the same elevations for fRBCs were observed with a significant difference in O-RCCs on Day 7 and P-RCCs on Day 28 (Figure S1C). The opposite effect was shown for MCHC data: mRBCs had higher MCHC results than fRBCs with a significant difference in O-RCCs on Day 7 and in the P-RCCs on Day 28 (Figure S1C).

2.2 | RBC morphology changes during storage

Morphology index is a robust assay for revealing structural changes of RBCs associated with storage lesion or chronological senescence. Assessment of morphology subclasses of RBCs (Figure 2A) show that washing of RBCs alone (W-RCCs) led to a shift from discoid-shaped RBCs to spheroid-shaped RBCs that resulted in a significant decrease in mean MI (Figure 2B) compared to P-RCCs. However, by Day 28 of storage, the MI of W-RCCs increased to the control level in P-RCC samples with a prevalence of discoid-shaped RBCs. It was expected that fractionation of the RCC into young and old RBCs by Percoll-density centrifugation would affect RBC morphology to a greater extent than washing alone. Nevertheless, young RBCs had a higher MI after separation than both W-RCCs and O-RCCs (Figure 2B) with a prevalence of crenated discoids in the samples. By Day 28 of storage, Y-RCCs showed a slight shift to a crenated spheroid shape but MI was not significantly different when compared to Day 7. Old RBCs did not change their morphology distribution and MI throughout 28-day hypothermic storage but, compared to both control groups (P-RCCs and W-RCCs), retained a significantly lower MI (Figure 2B).

2.3 | Metabolomics of RBCs after separation

Metabolomics analysis of RBC subpopulations after separation (Figure 3) indicated a significant sex-dependent signature across all three assessed groups. Subpopulations showed unique metabolic signatures with respect to amino acid levels, acyl-carnitines and pentose phosphate pathway (PPP) metabolites highest in young RBCs, and glycolysis/purine oxidation⁴⁵ and oxidant stress markers highest in old RBCs (Figure 3A,B). Of note, both markers of increased oxidant stress (glutathione disulfide) and decreased capacity to cope with oxidant stress through repair mechanisms of isoaspartyl damage to proteins³⁸ (*S*-adenosylmethionine) were detected in the O-RCCs (Figure 3C). Of note, sex- and age-dependent changes in these pathways have been recently reported.²⁵ In particular, increased purine oxidation or impaired activation of the PPP have been negatively associated with the capacity of stored RBCs to circulate upon transfusion.⁴⁶ Here we expand on these observations by describing an impact of the age of RBC subpopulations on these pathways.

2.4 | Qualitative and functional characteristics of RBCs during storage

Red blood cell quality assessment demonstrated increased hemolysis (Figure 4A) in all treated groups (W-RCCs, Y-RCCs, O-RCCs) when compared to the control P-RCCs after 14 days of storage. However, hemolysis of Y-RCCs was lower than that for O-RCCs and W-RCCs. By Day 28 of storage, no statistical differences were detected between treatment groups and only O-RCCs exceeded the Canadian Standards Association guideline for allowable hemolysis (<0.8%).⁴⁷

Estimation of functional activity of different RBC groups by their Hb-p50 showed higher Hb affinity for both Y-RCCs and O-RCCs compared to the P-RCCs (Figure 4B). Of note, on Day 14 of RBC storage, a significant decrease of p50 values for O-RCCs was observed compared to Y-RCCs, which indicates increased p50 of old RBCs and thus reduced availability to the tissues. However, by Day 28 of storage, this difference was not observed.

The mixed-model analysis showed a significant effect of group and time on both hemolysis and p50 results as well as their interaction (Table 1). Based on the final model with Tukey's adjustment, fRBCs had lower hemolysis than mRBCs ($P = 0.02$). Moreover, a significant interaction between sex and time for hemolysis data was observed and between all three variables: sex, group, and time. However, the effect of storage time on the hemolysis level was stronger in mRBCs than in fRBCs, indicating a faster increase of hemolysis in mRBC samples.

Assessing the difference between female and male data for p50 by paired comparison did not show any significant differences (Figure S2B). For hemolysis, female samples on Day 7 were higher than male samples with a significant difference detected only in the O-RCC group. By Day 28, hemolysis in mRBCs increased and appeared to be slightly higher than fRBCs; however, no statistical difference was detected ($P = 0.0667$) in the RBC group (Figure S2A).

Hematocrit levels of different RCC groups after processing did not differ between fRBCs and mRBCs (Figure S2A,B) or between young and old RBC groups without donor sex consideration (Figure S2C). Additionally, there were no differences between fRBCs and mRBCs for pH measurements (Figure S3A,B). However, despite the similarity in RCC processing, O-RCCs had higher pH levels (Figure S3C) than Y-RCCs on Days 7, 14, and 28 ($P = 0.0202$, $P = 0.0117$, and $P = 0.0378$, respectively). The lower acidity of O-RCCs might have resulted in improved preservation of quality and metabolic characteristics (elevated glutathione potential) of the O-RCCs compared to more acidic Y-RCCs that have not been confirmed by other results.

2.5 | Deformability of RBCs during hypothermic storage

Deformability characteristics of RBCs, namely rigidity and elongation capability ($EI_{\max}$), were used to estimate the ability of RBCs to change shape under applied stress (Figure 5). It was shown that washing alone (W-RCCs) affected deformability parameters of the RBCs compared to the P-RCCs and resulted in increased rigidity (Figure 5A) and decreased $EI_{\max}$ of the RBCs (Figure 5B).

Despite increased rigidity in W-RCC samples caused by washing alone, the rigidity of Y-RCCs right after Percoll-density centrifugation did not differ from P-RCCs and was significantly lower compared to the W-RCC and O-RCC samples (Figure 5A). By day 14 of storage, Y-RCCs still had a lower rigidity compared to P-RCCs and O-RCCs. The rigidity of O-RCCs was significantly higher compared to other RBC groups during 14 days of storage and this difference was retained compared to P-RCCs by Day 28. Processing of RBCs (washing or Percoll separation) induced a decrease in $EI_{\max}$ of all RBC groups compared to P-RCCs (Figure 5B). However, by day 28 only O-RCCs retained a significant difference compared to P-RCC.

The mixed-model analysis showed a significant effect of group and time on both deformability variables (rigidity and elongation), while donor sex did not reach a significant effect (Table 1). Assessment of the difference in deformability between fRBCs and mRBCs showed that in the treated RBC groups (W-RCCs, O-RCCs, and Y-RCCs) rigidity of mRBC

samples was significantly higher than for fRBCs on Day 7 and in the O-RCC group on Day 14 of storage (Figure S5A). By Day 28 of storage, only Y-RCCs retained a significantly higher rigidity for RBCs of male donors. Despite the appearance of mRBCs to have improved $EI_{\max}$ when compared to fRBCs, no statistical differences were observed (Figure S5B).

2.6 | Intracellular reactive oxygen species and Ca^{2+} in RBCs during storage

Intracellular reactive oxygen species (ROS) content was estimated to assess oxidative stress in different RBC groups during hypothermic storage (Figure 6A). It was shown that young RBCs were more susceptible to the Percoll-density centrifugation that resulted in slightly increased fluorescence intensity of the DCF-stained RBCs right after fractionation and by the end of the storage period. Despite low ROS content in old RBC samples right after fractionation, there was a significant increase by Day 28 of storage, indicating the development of oxidative stress.

Intracellular Ca^{2+} level, a crucial component for controlling different biophysical properties,⁴⁸ significantly increased by Day 28 of storage for all experimental groups (Figure 6B). Interestingly, Y-RCCs and O-RCCs did not differ from P-RCC samples, while W-RCCs had the highest intracellular Ca^{2+} level.

Both intracellular ROS and Ca^{2+} changes were significantly associated with all three variables of the experiment: sex, group, and time (Table 1). Both metabolites were higher in fRBCs than in mRBCs ($P = 0.0089$ for intracellular ROS, $P = 0.0211$ for Ca^{2+}). Moreover, interaction between group and time had significant effect on both metabolites, and interaction between sex and group had a significant effect on results for ROS.

Also, higher intracellular content of ROS was shown for fRBC groups after washing (W-RCCs) or fractionation of RBCs (Y-RCCs, O-RCCs) than for mRBCs. However, this difference was no longer detectable by Day 28 of storage (Figure S6A). Similarly, to ROS, fRBCs have higher intracellular Ca^{2+} levels than mRBCs which were significant for O-RCCs on Day 7 and P-RCCs on Day 28 (Figure S6B).

3 | DISCUSSION

This study provides a comprehensive assessment of properties of young and old RBCs based on their morphologic, biochemical, and biomechanical differences throughout hypothermic storage as well as their association with donor sex. Young RBCs from female donors demonstrate less susceptibility to the storage lesion with lower hemolysis rate and changes in rigidity compared to old RBCs and to both subpopulations (young and old) of RBCs from male donors.

According to previously reported data,^{32,34,49} young RBCs lose their unique structural characteristics by Days 21 to 25 of storage and a notable shift to a sphere-shaped morphology occurs after 28 days of hypothermic storage. Therefore, it was anticipated that many of the structural differences between young and old RBCs would be negligible over storage time due to their chronological aging, especially in in vitro conditions where

removal of senescent RBCs through phagocytosis is unavailable. Interestingly, old RBCs retain a lower MI than both control groups even by Day 28 of storage. However, the presence of a high proportion of disc-to-discoid shape RBCs in the young fractions can be beneficial for their posttransfusion survival as a high surface-to-volume ratio may prevent their rapid clearance by the spleen.^{50,51}

Metabolic profiling of young and old RBCs was performed to define “old” and “fresh” blood based on metabolic phenotype.^{31,52–54} Many previous studies have shown that RBC aging shows strong association with the accumulation of metabolites of oxidative stress or lack of metabolites of anti-oxidant defence.^{6,31,45,54,55} One key finding was the metabolic difference in freshly isolated subpopulations of young and old RBCs. Young RBCs have a high content of acyl-carnitines, markers of high membrane lipid remodeling,⁵⁶ and PPP metabolites, which are responsible for glucose-oxidizing pathway for the generation of NADPH. Meanwhile, old RBCs have markers of oxidative stress (glutathione disulfide) and decreased capacity to cope with it (*S*-adenosylmethionine) immediately after separation in Percoll. Interestingly, the widely used method for detection of intracellular ROS was only able to detect the developing oxidative stress in old RBCs on Day 28 of storage, while immediately after separation young cells have higher intracellular ROS compared to old RBCs.

A recent study by Nemkov et al⁵⁵ showed that increases in intracellular Ca^{2+} during hypothermic storage cause metabolic aberrations similar to those seen for RBCs treated with ionomycin (calcium ionophore). The changes included decreased glycolysis and increased purine oxidation,⁴⁵ dysregulated carboxylic acid metabolism,⁵⁹ and increased fatty acid mobilization.^{60,61} Our results show a higher level of accumulation of intracellular Ca^{2+} in old RBCs compared to young RBCs immediately after separation that could be the cause of increased hemolysis in these RCCs on Day 14 of storage as it is known that activation of Ca^{2+} -dependent proteases is able to induce hemolysis.^{57,58}

These progressive morphologic and metabolic changes in RBCs after processing (washing alone or Percoll separation) and throughout storage were accompanied by changes in RBC quality characteristics including increased hemolysis and decreased Hb-p50 and deformability, which is consistent with previously reported results.^{34,62–64} The significantly increased hemolysis and rigidity and decreased elongation in the group of washed but unseparated RCCs show the high sensitivity of RBCs to the secondary effect of washing and storage methods on processed RBCs. Improvements to the methods used to wash RCCs are necessary. Nevertheless, in this study, young RBCs demonstrate lower rigidity and Hb-p50 (on Day 14) as well as lower rate of hemolysis increase (by Day 28) than old RBCs (3.23- and 4.96-fold increase, respectively). This higher rate of decreasing quality in O-RCCs indicates a greater contribution of senescent RBCs to the storage lesion: since no clearance mechanisms are available in closed storage systems, an increased presence of senescent RBCs within a RCC will presumptively have higher accumulation of side products of aging/lysis that undoubtedly affect the quality of the whole RCC unit.

Other studies have discussed the impact of donor age and sex on both the quality characteristics of RCCs during in vitro storage^{22,25,28,65} and the transfusion efficacy,^{66–69}

with an increased risk of mortality due to sex-mismatched transfusions.^{70–74} fRBCs stored in vitro have generally shown superior quality measures, such as less susceptibility to storage-induced hemolysis and mechanical fragility, and better rheologic properties compared to mRBCs^{22,75} that can be related to a larger proportion of biologically younger RBCs in female donors.^{22,76} This study also revealed the effect of donor sex on structural and functional properties of the RBCs by demonstrating the following: fRBCs have greater size and intracellular ROS, Ca^{2+} , and HGB and lower MCHC, hemolysis, and membrane rigidity compared to mRBCs. It should be noted that the rate of change of hemolysis during storage for fRBCs demonstrates a 2.81-fold increase for Y-RCCs and a 2.60-fold increase for O-RCCs, while for mRBCs reaches a 3.56-fold increase for Y-RCCs and an 8.91-fold increase for O-RCCs. Considering the rate of change in hemolysis as one of the main storage lesion factors, this study demonstrates that young RBCs from female donors are less susceptible to the storage lesion and age slower than old RBCs from males, supporting our hypothesis. In addition to structural and functional results, metabolomic analyses show a significant sex-related metabolic signature dependent on the RBC subpopulation, indicating that RBCs from male vs female donors have metabolically different RBCs defined by age.

This study demonstrates the feasibility of obtaining two physiologically distinct subpopulations of young and old RBCs despite limitations associated with: (a) small sample size within groups based on donor sex, (b) reduced storage time (28 vs 42 days postcollection expiry), (c) the impact of Percoll-density centrifugation on RBC quality (as well as washing RBCs in isotonic solution), and (d) separation into young and old RBCs based on one single Percoll density and visually assessed 1:1 ratio.

In conclusion, the extent of the storage lesion depends on donor characteristics such as sex and maturity of RBCs at the time of donation. Results warrant further examination into donor-dependent effects on RBC populations to optimize RBC manufacturing and/or the selection of RBC units for efficient transfusion and/or revealing potential clinical consequences of transfusion of senescent RBCs or sex-mismatched transfusions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors are grateful to Canadian Blood Services' blood donors who made this research possible. The authors acknowledge April Xu (Centre for Innovation, Canadian Blood Services) for her technical support. The authors also acknowledge Dr. Qi-long Yi, Canadian Blood Services biostatistician, for his assistance with the statistical analysis.

Imaging flow cytometry experiments were performed at the University of Alberta Faculty of Medicine & Dentistry Flow Cytometry Facility, which receives financial support from the Faculty of Medicine & Dentistry and Canada Foundation for Innovation (CFI) awards to contributing investigators.

Funding information Canadian Blood Services, Grant/Award Number: IG2018-JA; United States National Heart, Lung and Blood Institutes, Grant/Award Numbers: R01HL146442, R01HL148151, R01HL149714, R21HL150032

Abbreviations:

EL_{max}

maximum theoretical elongation index

fRBC(s)	female red blood cell(s)
HGB	hemoglobin autofluorescence
IFC	imaging flow cytometer
MI	morphology index
mRBC(s)	male red blood cell(s)
p50	oxygen affinity
O-RCC(s)	old red blood cell concentrate(s)
PPP	pentose phosphate pathway
P-RCC(s)	parent red cell concentrate unit(s)
ROS	reactive oxygen species
RT	room temperature
Y-RCC(s)	young red blood cell concentrate(s)
W-RCC(s)	washed unseparated control red blood cell concentrate(s).

REFERENCES

1. World Health Organization. Global Status Report on Blood Safety and Availability. Geneva: World Health Organization; 2016. <http://apps.who.int/bookorders>. Accessed May 1, 2020.
2. Lacroix J, Hébert P, Fergusson D, et al. The Age of Blood Evaluation (ABLE) randomized controlled trial: study design. *Transfus Med Rev*. 2011;25(3):197–205. 10.1016/j.tmr.2011.03.001 [PubMed: 21550205]
3. Aubron C, Syres G, Nichol A, et al. A pilot feasibility trial of allocation of freshest available red blood cells versus standard care in critically ill patients. *Transfusion*. 2012;52(6): 1196–1202. 10.1111/j.1537-2995.2011.03437.x [PubMed: 22082281]
4. Sparrow RL. Red blood cell storage duration and trauma. *Transfus Med Rev*. 2015;29(2):120–126. 10.1016/j.tmr.2014.09.007 [PubMed: 25573415]
5. Spieth PM, Zhang H. Storage injury and blood transfusions in trauma patients. *Curr Opin Anaesthesiol*. 2018;31(2):234–237. 10.1097/ACO.0000000000000565 [PubMed: 29324488]
6. 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(12):e32–e42. 10.1182/blood-2016-05-714816 [PubMed: 27405778]
7. Baryn M, Tissot JD, Prudent M. Oxidative stress and antioxidant defenses during blood processing and storage of erythrocyte concentrates. *Transfus Clin Biol*. 2018;25(1):96–100. 10.1016/j.traccli.2017.08.001 [PubMed: 28888839]
8. Kanas T, Acker JP. Biopreservation of red blood cells—the struggle with hemoglobin oxidation. *FEBS J*. 2010;277(2): 343–356. 10.1111/j.1742-4658.2009.07472.x [PubMed: 19968714]
9. D'Alessandro A, Nemkov T, Reisz J, Dzieciatkowska M, Wither MJ, Hansen KC. Omics markers of the red cell storage lesion and metabolic linkage. *Blood Transfus*. 2017;15(2): 137–144. 10.2450/2017.0341-16 [PubMed: 28263171]
10. Almizraq RJ, Seghatchian J, Holovati JL, Acker JP. Extracellular vesicle characteristics in stored red blood cell concentrates are influenced by the method of detection. *Transfus Apher Sci*. 2017; 56(2):254–260. 10.1016/j.transci.2017.03.007 [PubMed: 28363591]

11. Nguyen DB, Thuy Ly TB, Wesseling MC, et al. Characterization of microvesicles released from human red blood cells. *Cell Physiol Biochem*. 2016;38(3):1085–1099. 10.1159/000443059 [PubMed: 26938586]
12. Kim Y, Goodman MD, Jung AD, et al. Microparticles from aged packed red blood cell units stimulate pulmonary micro-thrombus formation via P-selectin. *Thromb Res*. 2020;185: 160–166. 10.1016/j.thromres.2019.11.028 [PubMed: 31821908]
13. Sut C, Tariket S, Chou ML, et al. Duration of red blood cell storage and inflammatory marker generation. *Blood Transfus*. 2017;15(2):145–152. 10.2450/2017.0343-16 [PubMed: 28263172]
14. Tan H, Bi J, Wang Y, Zhang J, Zuo Z. Transfusion of old rbcs induces neuroinflammation and cognitive impairment. *Crit Care Med*. 2015;43(8):e276–e286. 10.1097/CCM.0000000000001023 [PubMed: 25860202]
15. Almizraq RJ, Kipkeu BJ, Acker JP. Platelet vesicles are potent inflammatory mediators in red blood cell products and washing reduces the inflammatory phenotype. *Transfusion*. 2020;60(2):378–390. 10.1111/trf.15590 [PubMed: 31756004]
16. Simak J, Gelderman MP. Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers. *Transfus Med Rev*. 2006;20(1):1–26. 10.1016/j.tmr.2005.08.001 [PubMed: 16373184]
17. Keating FK, Butenas S, Fung MK, Schneider DJ. Platelet-white blood cell (WBC) interaction, WBC apoptosis, and procoagulant activity in stored red blood cells. *Transfusion*. 2011;51(5):1086–1095. 10.1111/j.1537-2995.2010.02950.x [PubMed: 21126256]
18. Waugh RE, Narla M, Jackson CW, Mueller TJ, Suzuki T, Dale GL. Rheologic properties of senescent erythrocytes: loss of surface area and volume with red blood cell age. *Blood*. 1992;79(5):1351–1358. 10.1182/blood.v79.5.1351.bloodjournal7951351 [PubMed: 1536958]
19. Donadee C, Raat NJH, Kanas T, et al. Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. *Circulation*. 2011;124(4):465–476. 10.1161/CIRCULATIONAHA.110.008698 [PubMed: 21747051]
20. Acker JP, Hansen AL, Kurach JDR, Turner TR, Croteau I, Jenkins C. A quality monitoring program for red blood cell components: in vitro quality indicators before and after implementation of semiautomated processing. *Transfusion*. 2014;54(10):2534–2543. 10.1111/trf.12679 [PubMed: 24805193]
21. Almizraq RJ, Norris PJ, Inglis H, et al. Blood manufacturing methods affect red blood cell product characteristics and immunomodulatory activity. *Blood Adv*. 2018;2(18):2296–2306. 10.1182/bloodadvances.2018021931 [PubMed: 30217795]
22. Jordan A, Chen D, Yi QL, Kanas T, Gladwin MT, Acker JP. Assessing the influence of component processing and donor characteristics on quality of red cell concentrates using quality control data. *Vox Sang*. 2016;111(1):8–15. 10.1111/vox.12378 [PubMed: 26991891]
23. Shih AW, Apelseth TO, Cardigan R, et al. Not all red cell concentrate units are equivalent: international survey of processing and in vitro quality data. *Vox Sang*. 2019;114(8): 783–794. 10.1111/vox.12836 [PubMed: 31637738]
24. Hansen AL, Kurach JDR, Turner TR, et al. The effect of processing method on the in vitro characteristics of red blood cell products. *Vox Sang*. 2015;108(4):350–358. 10.1111/vox.12233 [PubMed: 25678039]
25. D'Alessandro A, Fu X, Kanas T, et al. Donor sex, age and ethnicity impact stored red blood cell antioxidant metabolism through mechanisms in part explained by glucose 6-phosphate dehydrogenase levels and activity. *Haematologica*. 2020;In press. 10.3324/haematol.2020.246603
26. D'Alessandro A, Nemkov T, Hansen KC, Szczepiorkowski ZM, Dumont LJ. Red blood cell storage in additive solution-7 preserves energy and redox metabolism: a metabolomics approach. *Transfusion*. 2015;55(12):2955–2966. 10.1111/trf.13253 [PubMed: 26271632]
27. da SilveiraCavalcante L, Acker JP, Holovati JL. Differences in rat and human erythrocytes following blood component manufacturing: the effect of additive solutions. *Transfus Med Hemother*. 2015;42(3):150–157. 10.1159/000371474 [PubMed: 26195928]
28. Kanas T, Lanteri MC, Page GP, et al. Ethnicity, sex, and age are determinants of red blood cell storage and stress hemolysis: results of the REDS-III RBC-Omics study. *Blood Adv*. 2017;1(15): 1132–1141. 10.1182/bloodadvances.2017004820 [PubMed: 29034365]

29. Yoshida T, Prudent M, D'Alessandro A. Red blood cell storage lesion: causes and potential clinical consequences. *Blood Transfus.* 2019;17(1):27–52. 10.2450/2019.0217-18 [PubMed: 30653459]
30. Bosch FH, Werre JM, Roerdinkholder-Stoelwinder B, Huls TH, Willekens FLA, Halie MR. Characteristics of red blood cell populations fractionated with a combination of counterflow centrifugation and Percoll separation. *Blood.* 1992;79(1):254–260. 10.1182/blood.v79.1.254.bloodjournal791254 [PubMed: 1728314]
31. D'Alessandro A, Blasi B, D'Amici GM, Marrocco C, Zolla L. Red blood cell subpopulations in freshly drawn blood: application of proteomics and metabolomics to a decades-long biological issue. *Blood Transfus.* 2013;11(1):75–87. 10.2450/2012.0164-11 [PubMed: 22871816]
32. Tuo WW, Wang D, Liang WJ, Huang YX. How cell number and cellular properties of blood-banked red blood cells of different cell ages decline during storage. *PLoS One.* 2014;9(8):e105692. 10.1371/journal.pone.0105692 [PubMed: 25167052]
33. Bizjak DA, Brinkmann C, Bloch W, Grau M. Increase in red blood cell-nitric oxide synthase dependent nitric oxide production during red blood cell aging in health and disease: a study on age dependent changes of rheologic and enzymatic properties in red blood cells. *PLoS One.* 2015;10(4):e0125206. 10.1371/journal.pone.0125206 [PubMed: 25902315]
34. Hsieh C, Prabhu NCS, Rajashekaraiah V. Age-related modulations in erythrocytes under blood bank conditions. *Transfus Med Hemother.* 2019;46(4):257–266. 10.1159/000501285 [PubMed: 31700508]
35. Turner TR, Lautner L, Hill A, Howell A, Skeate R, Acker JP. Evaluating the quality of red blood cell concentrates irradiated before or after cryopreservation. *Transfusion.* 2020;60(1):26–29. 10.1111/trf.15589 [PubMed: 31724744]
36. Stadnick H, Onell R, Acker JP, Holovati JL. Eadie-Hofstee analysis of red blood cell deformability. *Clin Hemorheol Microcirc.* 2011;47(3):229–239. 10.3233/CH-2010-1384 [PubMed: 21498902]
37. Almizraq R, Tchir JDR, Holovati JL, Acker JP. Storage of red blood cells affects membrane composition, microvesiculation, and in vitro quality. *Transfusion.* 2013;53(10):2258–2267. 10.1111/trf.12080 [PubMed: 23320518]
38. Reisz JA, Nemkov T, Dzieciatkowska M, et al. Methylation of protein aspartates and deamidated asparagines as a function of blood bank storage and oxidative stress in human red blood cells. *Transfusion.* 2018;58(12):2978–2991. 10.1111/trf.14936 [PubMed: 30312994]
39. Nemkov T, Hansen KC, Dumont LJ, D'Alessandro A. Metabolomics in transfusion medicine. *Transfusion.* 2016;56(4): 980–993. 10.1111/trf.13442 [PubMed: 26662506]
40. D'Alessandro A, Nemkov T, Yoshida T, Bordbar A, Palsson BO, Hansen KC. Citrate metabolism in red blood cells stored in additive solution-3. *Transfusion.* 2017;57(2):325–336. 10.1111/trf.13892 [PubMed: 27813142]
41. D'Alessandro A, Nemkov T, Kelher M, et al. Routine storage of red blood cell (RBC) units in additive solution-3: a comprehensive investigation of the RBC metabolome. *Transfusion.* 2015; 55(6):1155–1168. 10.1111/trf.12975 [PubMed: 25556331]
42. Nemkov T, Hansen KC, D'Alessandro A. A three-minute method for high-throughput quantitative metabolomics and quantitative tracing experiments of central carbon and nitrogen pathways. *Rapid Commun Mass Spectrom.* 2017;31(8):663–673. 10.1002/rcm.7834 [PubMed: 28195377]
43. Pinto RN, Sebastian JA, Parsons MJ, et al. Label-free analysis of red blood cell storage lesions using imaging flow cytometry. *Cytometry A.* 2019;95(9):976–984. 10.1002/cyto.a.23846 [PubMed: 31294512]
44. Usry RT, Moore GL, Manalo FW. Morphology of stored, rejuvenated human erythrocytes. *Vox Sang.* 1975;28(3):176–183. 10.1111/j.1423-0410.1975.tb02756.x [PubMed: 1119131]
45. Nemkov T, Sun K, Reisz JA, et al. Hypoxia modulates the purine salvage pathway and decreases red blood cell and supernatant levels of hypoxanthine during refrigerated storage. *Haematologica.* 2018;103(2):361–372. 10.3324/haematol.2017.178608 [PubMed: 29079593]
46. Francis RO, Jhang JS, Pham HP, Hod EA, Zimring JC, Spitalnik SL. Glucose-6-phosphate dehydrogenase deficiency in transfusion medicine: the unknown risks. *Vox Sang.* 2013;105(4):271–282. 10.1111/vox.12068 [PubMed: 23815264]
47. Canadian Standards Association. Z902–18 Blood and Blood Components of Work: CSA Group. Ottawa, Canada: Canadian Standards Association; 2018.

48. Cortese-Krott MM, Shiva S. The redox physiology of red blood cells and platelets: Implications for their interactions and potential use as systemic biomarkers. *Curr Opin Physiol.* 2019; 9:56–66. 10.1016/j.cophys.2019.04.016
49. Roussel C, Dussiot M, Marin M, et al. Spherocytic shift of red blood cells during storage provides a quantitative whole cell-based marker of the storage lesion. *Transfusion.* 2017;57(4): 1007–1018. 10.1111/trf.14015 [PubMed: 28150311]
50. Perrotta S, Gallagher PG, Mohandas N. Hereditary spherocytosis. *Lancet.* 2008;372(9647):1411–1426. 10.1016/S0140-6736(08)61588-3 [PubMed: 18940465]
51. Picot J, Ndour PA, Lefevre SD, et al. A biomimetic microfluidic chip to study the circulation and mechanical retention of red blood cells in the spleen. *Am J Hematol.* 2015;90(4):339–345. 10.1002/ajh.23941 [PubMed: 25641515]
52. Bordbar A, Johansson PI, Paglia G, et al. Identified metabolic signature for assessing red blood cell unit quality is associated with endothelial damage markers and clinical outcomes. *Transfusion.* 2016;56(4):852–862. 10.1111/trf.13460 [PubMed: 26749434]
53. Paglia G, D'Alessandro A, Rolfsson Ó, et al. Biomarkers defining the metabolic age of red blood cells during cold storage. *Blood.* 2016;128(13):e43–e50. 10.1182/blood-2016-06-721688 [PubMed: 27554084]
54. Gevi F, D'Alessandro A, Rinalducci S, Zolla L. Alterations of red blood cell metabolome during cold liquid storage of erythrocyte concentrates in CPD-SAGM. *J Proteomics.* 2012;76: 168–180. 10.1016/j.jprot.2012.03.012 [PubMed: 22465715]
55. Nemkov T, Qadri SM, Sheffield WP, D'Alessandro A. Decoding the metabolic landscape of pathophysiological stress-induced cell death in anucleate red blood cells. *Blood Transfus.* 2020;18(2):130–142. 10.2450/2020.0256-19 [PubMed: 32203008]
56. D'Alessandro A, Reisz JA, Zhang Y, et al. Effects of aged stored autologous red blood cells on human plasma metabolome. *Blood Adv.* 2019;3(6):884–896. 10.1182/bloodadvances.2018029629 [PubMed: 30890545]
57. Koshkaryev A, Zelig O, Manny N, Yedgar S, Barshtein G. Rejuvenation treatment of stored red blood cells reverses storage-induced adhesion to vascular endothelial cells. *Transfusion.* 2009;49(10):2136–2143. 10.1111/j.1537-2995.2009.02251.x [PubMed: 19538542]
58. Antonelou MH, Tzounakas VL, Velentzas AD, Stamoulis KE, Kriebardis AG, Papassideri IS. Effects of pre-storage leukoreduction on stored red blood cells signaling: A time-course evaluation from shape to proteome. *J Proteomics.* 2012; 76:220–238. 10.1016/j.jprot.2012.06.032 [PubMed: 22796353]
59. Nemkov T, Sun K, Reisz JA, et al. Metabolism of citrate and other carboxylic acids in erythrocytes as a function of oxygen saturation and refrigerated storage. *Front Med.* 2017;4:175. 10.3389/fmed.2017.00175
60. de Wolski K, Fu X, Dumont LJ, et al. Metabolic pathways that correlate with post-transfusion circulation of stored murine red blood cells. *Haematologica.* 2016;101(5):578–586. 10.3324/haematol.2015.139139 [PubMed: 26921359]
61. Fu X, Felcyn JR, Odem-Davis K, Zimring JC. Bioactive lipids accumulate in stored red blood cells despite leukoreduction: a targeted metabolomics study. *Transfusion.* 2016;56(10): 2560–2570. 10.1111/trf.13748 [PubMed: 27514704]
62. Adams F, Bellairs G, Bird AR, Oguntibeju OO. Biochemical storage lesions occurring in nonirradiated and irradiated red blood cells: a brief review. *Biomed Res Int.* 2015;2015:1–8. 10.1155/2015/968302
63. D'Alessandro A, Zolla L. Biochemistry of red cell aging in vivo and storage lesions. *Haematologica.* 2013;7:389–396.
64. Ugurel E, Kucuksumer Z, Eglenen B, Yalcin O. Blood storage alters mechanical stress responses of erythrocytes. *Clin Hemorheol Microcirc.* 2017;66(2):143–155. 10.3233/CH-160219 [PubMed: 28282803]
65. 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(8): 791–804. 10.1002/prca.201500128 [PubMed: 27095294]

66. Edgren G, Ullum H, Rostgaard K, et al. Association of donor age and sex with survival of patients receiving transfusions. *JAMA Intern Med.* 2017;177(6):854–860. 10.1001/jamainternmed.2017.0890 [PubMed: 28437543]
67. Bosman GJCGM Werre JM, Willekens FLA Novotný VMJ. Erythrocyte ageing in vivo and in vitro: structural aspects and implications for transfusion. *Transfus Med.* 2008;18(6): 335–347. 10.1111/j.1365-3148.2008.00892.x [PubMed: 19140816]
68. Chassé M, Tinmouth A, English SW, et al. Association of blood donor age and sex with recipient survival after red blood cell transfusion. *JAMA Intern Med.* 2016;176(9):1307–1314. 10.1001/jamainternmed.2016.3324 [PubMed: 27398639]
69. Heddle NM, Cook RJ, Liu Y, et al. The association between blood donor sex and age and transfusion recipient mortality: an exploratory analysis. *Transfusion.* 2019;59(2):482–491. 10.1111/trf.15011 [PubMed: 30414291]
70. Heddle NM, Arnold DM, Acker JP, et al. Red blood cell processing methods and in-hospital mortality: a transfusion registry cohort study. *Lancet Haematol.* 2016;3(5):e246–e254. 10.1016/S2352-3026(16)00020-X [PubMed: 27132699]
71. Chassé M, McIntyre L, English SW, et al. Effect of blood donor characteristics on transfusion outcomes: a systematic review and meta-analysis. *Transfus Med Rev.* 2016;30(2):69–80. 10.1016/j.tmr.2016.01.002 [PubMed: 26920039]
72. Middelburg RA, Briët E, Van der Bom JG. Mortality after transfusions, relation to donor sex. *Vox Sang.* 2011;101(3):221–229. 10.1111/j.1423-0410.2011.01487.x [PubMed: 21477152]
73. Zeller MP, Rochweg B, Jamula E, et al. Sex-mismatched red blood cell transfusions and mortality: a systematic review and meta-analysis. *Vox Sang.* 2019;114(5):505–516. 10.1111/vox.12783 [PubMed: 31124172]
74. Alshalani A, Li W, Juffermans NP, Seghatchian J, Acker JP. Biological mechanisms implicated in adverse outcomes of sex mismatched transfusions. *Transfus Apher Sci.* 2019;58(3): 351–356. 10.1016/j.transci.2019.04.023 [PubMed: 31064732]
75. Raval JS, Waters JH, Seltsam A, et al. Menopausal status affects the susceptibility of stored RBCs to mechanical stress. *Vox Sang.* 2011; 100(4):418–421. 10.1111/j.1423-0410.2010.01439.x [PubMed: 21488881]
76. Acker JP, Marks DC, Sheffield WP. Quality assessment of established and emerging blood components for transfusion. *J Blood Transfus.* 2016;2016:4860284. 10.1155/2016/4860284 [PubMed: 28070448]

FIGURE 1.

Changes in the structural characteristics of the RBCs during hypothermic storage. Changes in, A, MCV, and B, MCHC were assessed using a hematology analyzer. C, HGB was assessed using an IFC assay. Data are shown as means $\pm$ SD. *Significant difference ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$) between groups calculated using paired t test (for normal distribution) and Wilcoxon test (for nonnormal distribution). Symbols ($\odot$, $\circ$, $\square$) represent distribution of donor samples

FIGURE 2.

Classification of RBCs of different morphology and changes in MI of RBCs during hypothermic storage. A, Morphology subclasses of RBCs, and B, RBC MI (means $\pm$ SD) was assessed using bright-field images from an imaging flow cytometer (60 $\times$ magnification). Data are shown as means $\pm$ SD. *Significant difference (* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001) between groups calculated using paired t test (for normal distribution) and Wilcoxon test (for nonnormal distribution). Symbols ($\odot$, $\circ$, $\triangle$, $\square$) represent distribution of donor samples

FIGURE 3.

Metabolomics analysis of RBCs after separation. Multivariate analyses were performed on metabolomics data, including, A, partial least-square discriminant analysis (PLS-DA), and B, hierarchical clustering analysis to identify unique metabolic signature in RBCs from unseparated RCCs of female or male donors (P-RCC, average/middle density) and separated young (Y-RCC) and old (O-RCC) RBCs. C, Changes in *S*-adenosylmethionine and glutathione disulfide, two of the top metabolites sorted by a two-way ANOVA, depending on RBC subpopulation and sex

FIGURE 4.

Qualitative and functional properties of RBCs during hypothermic storage. Changes in, A, hemolysis, and B, p50 of different RCCs. Data are shown as means $\pm$ SD. *Significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$) between groups calculated using paired t test (for normal distribution) and Wilcoxon test (for nonnormal distribution). Symbols ($\odot$, $\circ$, $\triangle$, $\square$) represent distribution of donor samples

FIGURE 5.

Deformability characteristics of RBCs during hypothermic storage. Changes in, A, rigidity, and B, elongation of different RCCs. Data are shown as means $\pm$ SD. *Significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$) between groups calculated using paired t test (for normal distribution) and Wilcoxon test (for nonnormal distribution). Symbols ($\odot$, $\circ$, $\triangle$, $\square$) represent distribution of donor samples. K_{EI} , shear stress required to achieve half of the El_{max}

FIGURE 6.

Intracellular content of ROS and Ca^{2+} in RBCs during hypothermic storage. Changes in, A, intracellular content of ROS, and B, $[\text{Ca}^{2+}]$ of different RCCs. Data are shown as a means $\pm$ SD. *Significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$) between groups calculated using paired t test (for normal distribution) and Wilcoxon test (for nonnormal distribution). Symbols ($\odot$, $\circ$, $\triangle$, $\square$) represent distribution of donor samples

TABLE 1
Effect of sex, RBC group, and storage time on the structural and functional characteristics of RBCs

	Effect of variable		Interaction between variable			
	Sex	Group	Time	Sex vs group	Sex vs time	Group vs time
MCV	F	5	16.44	4.85	0.6	0.33
	P	0.0289	<0.0001	0.011	0.6153	0.7232
MCHC	F	3.05	13.6	2.77	0.43	1.08
	P	0.0855	<0.0001	0.0704	0.7302	0.3470
HGB	F	5.9	14.95	565.38	1.8	3.64
	P	0.0211	<0.0001	<0.0001	0.1679	0.0659
Hemolysis	F	0.03	34.04	230.79	0.93	14.3
	P	0.8661	<0.0001	<0.0001	0.4319	<0.0001
p50	F	0.69	91.16	15.19	0.4	2.39
	P	0.4106	<0.0001	<0.0001	0.6741	0.1058
Elongation	F	1.88	17.14	13.01	0.07	0.64
	P	0.1746	<0.0001	<0.0001	0.9742	0.5305
Rigidity	F	3.6	10.24	3.44	0.66	0.02
	P	0.0624	0.0002	0.0381	0.5780	0.9845
ROS	F	4.31	48.46	52.75	3.21	3.85
	P	0.0461	<0.0001	<0.0001	0.0359	0.0586
[Ca ²⁺]	F	5.9	14.95	565.47	1.8	3.64
	P	0.0211	<0.0001	<0.0001	0.1679	0.0658

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Note: Mixed-model analysis has been used to assess the effects of the sex (female or male), RBC group (P-RCC, W-RCC, Y-RCC, O-RCC), and storage time (7, 14, 28 days) as well as their interaction between each other on tested variables during storage. The numbers in the table represent the *P* and *F*-values for the possibility of effect.