ORIGINAL REPORT

Effect of cryopreservation on a rare McLeod donor red blood cell concentrate

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Units of red blood cell (RBC) concentrates with rare phenotypes are typically not included in method validation studies for cryopreservation processes; rather, they are reserved for patients with rare blood needs. Some rare RBC phenotypes may demonstrate membrane abnormalities, like acanthocytosis as observed for RBCs with the McLeod phenotype, and are specifically banked for these rare attributes; however, the impact that rare RBC phenotypes have on post-thaw quality has not been well studied. To evaluate how a rare RBC phenotype is affected by the cryopreservation process, 4 RBC units, cryopreserved in 1993 using manual methods, were selected for evaluation. These RBCs included one with the McLeod phenotype and three with phenotypes not known to cause significant membrane changes. Post-thaw, an altered deglycerolization protocol, implemented to reduce supernatant glycerol after cryopreservation, was used before processing RBCs on an automated closed system (ACP 215; Haemonetics, Boston, MA) to accommodate the use of a closed system cell processor not available when the RBC units were previously cryopreserved. RBC quality was tested at 24 hours, 7 days, and 14 days post-deglycerolization. Before deglycerolization, an extracted sample from the thawed glycerolized RBC unit was used to obtain genetic material for phenotype confirmation. Genotyping confirmed the McLeod phenotype. When comparing McLeod with non-McLeod units, RBCs from the McLeod donor exhibited acanthocytosis, higher rigidity, and lower morphology scores than RBCs from the non-McLeod units post-deglycerolization. Hemolysis, however, was comparable across all 4 units, meeting regulatory standards. Therefore, McLeod RBCs can withstand cryopreservation, suggesting that units from these donors, glycerolized using older methods, can be deglycerolized using the ACP 215 and stored hypothermically for 14 days. It was also determined that genotyping can be performed on non-leukocyte–reduced cryopreserved RBCs, allowing for confirmation of genetic profiles of donor units banked before the implementation of molecular methods. ***Immunohematology*** **2021;37:78–83. DOI:21307/immunohematology-2021-012.**

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Cryopreservation of red blood cell (RBC) concentrates is used for long-term storage of rare phenotyped RBCs to provide life-saving therapies to patients. To produce these components in Canada, blood manufacturers must demonstrate that the production method they use yields RBC units that meet regulatory guidelines for hemolysis, hemoglobin (Hgb) content, and RBC recovery.[1] The Canadian Blood Services (CBS) Rare Blood Program maintains a carefully selected inventory of cryopreserved RBCs with rare phenotypes, although these units are not represented in method validation or quality control data sets presently used to establish standard cryopreservation methods. This finding is problematic, since rare donor RBCs may have abnormal morphology and membrane structure that can affect component quality associated with the mechanical stresses of cryoprotectant addition/removal, freezing, thawing, and cell processing. Considering there is little evidence of the effect that rare phenotypes associated with membrane alterations have on deglycerolized RBC component quality, investigations to evaluate the downstream implications to patient care and cell banking practices would be useful.

An example of a rare RBC phenotype that should be cryogenically banked is the McLeod phenotype. This phenotype results from the absence of the Kx protein on the RBC membrane due to various missense and small to major nucleotide deletions of the *XK* gene.[2,3] This information is important because Kx, a 10-pass transmembrane protein disulfide linked to the Kell glycoprotein, contributes to the normal structure of biconcave-shaped RBCs, and the lack thereof could affect the ability of the RBCs to withstand the stresses associated with cryopreservation.[2–6] These membrane changes produce RBCs exhibiting various degrees of acanthocytosis, which can lead to compensated hemolytic anemia in patients with the McLeod phenotype, resulting in the need for transfusion and the necessity for cryopreserved components to meet future autologous and allogeneic use.[3–5,7,8] However, component quality post-deglycerolization for this phenotype has not been previously described.

In addition to the challenge of understanding how RBCs having rare blood phenotypes can tolerate the cryopreservation process, blood manufacturers are also faced with the implications of long-term storage of these components. Cryopreserved RBCs can be stored for up to 10 years (30 years in Canada and other jurisdictions with medical approval), ensuring the availability of rare RBCs in acute clinical scenarios. However, given the pace of technological

change, evaluating the impact of manufacturing changes must be ongoing to confirm that components frozen using older methods are compatible with new processing equipment and quality is maintained. The study presented herein evaluated the quality of cryopreserved donor RBC units stored for more than 20 years, including a previously identified McLeod donor unit. The RBC units were originally glycerolized and frozen using a manual method intended for deglycerolization on a semi-automated cell processor (COBE 2991; Terumo BCT, Lakewood, CO). CBS now uses a closed-system automated processor (ACP 215; Haemonetics, Boston, MA) for glycerolization and deglycerolization of RBCs requiring certain adaptations to be made to process the thawed RBCs evaluated in this study. Furthermore, because diagnostic testing has also improved since this rare McLeod donor unit was cryopreserved, this study also aims to evaluate whether samples from non-leukocyte–reduced cryopreserved RBCs can be used for phenotype confirmation and genotyping analysis.[3]

Materials and Methods

Control Unit Selection

To evaluate the component quality of the McLeod donor RBC unit, 3 additional cryopreserved RBC units were selected as controls. These units lacked RBC antigen phenotypes associated with known membrane abnormalities and had been cryopreserved in the same year (1993) and at the same site using the same method (high-glycerol method [40%] in Charter Medical [Winston-Salem, NC] containers) as that for the McLeod donor unit.

Unit Processing

Twenty-four years after cryopreservation, each unit was thawed individually in a circulating 37°C water bath for up to 15 minutes. Before deglycerolization, the contents of the units were transferred to a 1-liter bag (OMAVSE6000XU; Macopharma, Mouvaux, France) followed by supernatant reduction. This reduction was performed by centrifuging the RBCs (HBB-6 Rotor, 2070*g*, ACE 2.88 × 10^7, 18–24°C, Sorvall RC 3BP+; Thermo Fisher, Langenselbold, Germany) to pack the cells followed by supernatant removal using a manual plasma extractor (4R4414; Fenwal, Lake Zurich, IL). The unit was sterile docked (CompoDock; Fresenius Kabi, Bad Homburg, Germany) to a processing set (LN235; Haemonetics), which was pre-loaded into the closed-system cell processor (Haemoentics ACP 215), and automated deglycerolization was initiated using two different concentrations of saline solutions (12% sodium chloride 4B7874Q, and 0.2% dextrose and 0.9% sodium chloride processing solution 4B7878; Baxter, Mississauga, ON, Canada). Finally, the deglycerolized RBCs were suspended in AS-3 (462A; Haemonetics) before storage in a 1–6°C refrigerator for 14 days.

Unit Sampling

Each RBC unit was sampled at five different time points: 10 mL immediately post-thaw; 1 mL immediately post-deglycerolization; and 5 mL at 1, 7, and 14 days post-deglycerolization. Briefly, the RBC unit was mixed by gentle massage while inverting five times. A sampling site coupler (R4R1401; Fenwal) was inserted into one of the ports on the RBC bag. Using aseptic technique, an 18-gauge needle attached to a syringe was inserted through the coupler, and the desired sample volume was drawn slowly into the syringe. After removal of the needle, the sample was aliquoted dropwise into sample tubes for analysis.

Component Quality Testing

Immediately post-thaw and post-deglycerolization, RBCs were analyzed for RBC hemolysis to determine whether there was any noticeable damage due to the length of storage or processing methods. Component quality was tested at 1, 7, and 14 days post-deglycerolization—testing for RBC hemolysis, supernatant potassium, Hgb content, adenosine triphosphate (ATP) concentration, RBC deformability, RBC indices, osmotic fragility, and morphology, all as previously described.[9–11]

Genotyping and Phenotyping

To obtain genomic material for this study, a method to collect samples for analysis from a thawed RBC component was developed. Immediately post-thaw, samples were prepared by centrifuging 9 mL of glycerolized RBCs at 2200*g* for 10 minutes at room temperature. The resulting supernatant was removed and approximately 2 mL of sample from the buffy coat region (cell interface) where white blood cells should be present was extracted. Samples were frozen at less than −65°C until DNA extraction could be completed, which was performed using a commercial kit (QIAamp DNA Blood Mini Kit [51104]; Qiagen, Hilden, Germany). Extracted genomic DNA were shipped frozen to the Diagnostic Laboratories at Versiti BloodCenter of Wisconsin for molecular analysis. Real-time polymerase chain reaction (PCR)-fluorogenic 5′ nuclease TaqMan chemistry was used in a mass-scale nanofluidic open-array format (Smart-Chip Real-Time PCR System; Takara

Bio USA, Mountain View, CA) to determine the following blood group antigens: M, N, S, s, U; C, E, c, e, V, Vs, hr^B, hr^S, Crawford; K, k, Kp(a/b), Js(a/b); Fy(a/b); Jk(a/b); Lu(a/b); Do(a/b), Hy, Jo(a); Di(a/b); Co(a/b); Cr(a); Yt(a); and Vel.[12] To confirm the McLeod phenotype, a custom comparative genomic hybridization array analysis was performed that included overlapping exon hybridization probes in the region of XK (Oxford Gene Technology, Tarrytown, NY). Hybridization was analyzed on a scanner (Innopsys, Carbonne, France) using software (Mapix, version 7.3.0, and CytoSure, version 4.7.13; Oxford Gene Technology, Begbroke, UK). Healthy male and female genomic DNA served as normal controls.

Serology

Deglycerolized RBCs (1 mL) from the McLeod donor was used for serologic phenotyping using direct agglutination testing with monoclonal antisera (Anti-K, Ortho 13129; Ortho Clinical Diagnostics, Raritan, NJ) and the indirect antiglobulin test (IAT) with another antisera (Anti-k [Anti-Cellano], Ortho 721030; Ortho Clinical Diagnostics). The CBS National Immunohematology Reference Laboratory confirmed the weak Kell system reactions using additional commercial antisera and unlicensed polyclonal donor anti-Js^b by the IAT.

Data Analysis

Descriptive statistics were calculated for the control group using spreadsheet software (Excel 2016; Microsoft, Redmond, WA). The *in vitro* results obtained from the McLeod unit were considered either increased or decreased if the result was greater or less than the control group by 2 standard deviations (SDs).

Results

RBC Quality

Immediately post-thaw and before deglycerolization, hemolysis for the McLeod RBC (still in glycerol) was 5.16 percent. RBC hemolysis was within 1 SD of the mean of the control group (5.94 ± 3.87% [1 SD, n = 3]), indicating that the McLeod donor RBC unit was not more susceptible to cellular damage because of mechanical stresses of glycerolization, freezing, and thawing than RBC units with a typical RBC antigen profile. At 24 hours post-deglycerolization, all units, including the McLeod RBC, met the Canadian Standards Association (CSA) standard for hemolysis (<0.8%).[1] Hemolysis for all units tested consistently met the required standard after units underwent extended hypothermic storage up to 14 days, the maximum allowable storage time at CBS for deglycerolized RBCs, indicating that McLeod RBCs withstood the deglycerolization process and post-thaw storage in AS-3 as well as the control group.

The CSA requirements for hematocrit (Hct) (≤80%) and Hgb content (≥35 g/RBC component in 100% of units tested and ≥40 g/RBC component in 90% of units tested) were met by all study RBCs at the accepted 24-hour expiration date for open-system processed units.[1] The McLeod RBC unit had an Hct of 48 percent, which was similar to the control group at 48 ± 0.04 percent (1 SD, n = 3). Total Hgb content (a dose indicating parameter) for the McLeod RBC unit was 54.0 g/RBC component, which was also comparable to the 56.1 ± 5.2 g/RBC component (1 SD, n = 3) achieved for the control group.

Osmotic fragility was represented by the mean corpuscular fragility (MCF) of the RBCs, indicating the saline concentration at which 50 percent RBC hemolysis is achieved. Although the McLeod unit appears to have had increased MCF, the values obtained were within the SD of the control group at all storage time points (Table 1). This result was also in line with data previously reported in the literature by Kuypers et al.,[13] who found that non-cryopreserved McLeod RBCs had similar osmotic fragility to non-McLeod RBCs (although it has been shown that RBCs without Kx do have decreased osmotic water permeability). At 24 hours post-deglycerolization, the McLeod unit demonstrated increased ATP and supernatant K^+, and decreased mean cell volume (MCV), which were all greater than 2 SDs when compared with the control group at that time point (Table 1). However, this observation did not hold true for these parameters for the additional extended storage at days 7 and 14 post-deglycerolization.

RBC deformability (Fig. 1) and morphology (Fig. 2) demonstrate distinct differences between the McLeod unit and the control group, likely due to the observed acanthocytosis in the McLeod unit. The K_{EI}, a measure of RBC rigidity, was increased in the McLeod RBCs more than 2 SDs (21-SD difference) from the control group at 24 hours post-deglycerolization. This result maintains greater than 2-SD differences when compared with the controls at 14 days of hypothermic storage. Figure 2A demonstrates a marked decrease in the morphology index of the McLeod RBCs, which is influenced by the presence of acanthocytes shown in Figure 2B and slightly microcytic RBCs, as indicated by decreased MCV.

Table 1. *In vitro* parameters for hypothermically stored RBC units post-deglycerolization using the ACP 215 after manual removal of excess glycerol before processing

	Length of hypothermic storage post-deglycerolization					
	McLeod (n = 1)			Control group (n = 3)		
Parameter	1 day	7 days	14 days	1 day	7 days	14 days
RBC hemolysis (%) Mean (SD)	0.23	0.48*	0.55	0.19 (0.02)	0.31 (0.02)	0.52 (0.10)
ATP (µmol/g Hgb) Mean (SD)	4.196*	2.150	1.432	3.735 (0.223)	2.617 (0.512)	1.816 (0.349)
MCF (g/L NaCl)† Mean (SD)	6.104	5.986	5.918	5.541 (0.688)	5.504 (0.656)	5.473 (0.653)
Supernatant K^+ (µmol/L) Mean (SD)	5.2*	15.0	20.6	3.7 (0.5)	13.2 (1.1)	18.8 (1.6)
MCV (fL) Mean (SD)	85.1*	84.9	86.3	104.1 (9.3)	98.4 (8.7)	98.4 (8.4)

*Indicates when the unit of McLeod red blood cells (RBCs) is >2 standard deviations (SDs) from the control group at the same storage time point.

†MCF = mean corpuscular fragility (derived from osmotic fragility); MCF is presented as the concentration of sodium chloride (NaCl) in which 50 percent hemolysis is calculated to be achieved.

ATP = adenosine triphosphate; Hgb = hemoglobin; K^+ = potassium; MCV = mean cell volume.

Phenotyping and Genotyping

Serology results for the McLeod RBCs confirmed the historical phenotype kept on record at CBS for that donor. Typically, McLeod patients' RBCs demonstrate weak reactions for the common Kell blood group system antigens (k, Kp^b, Js^b).[2–5] The donor tested in this study also demonstrated weak phenotype reactions for k (1+), Kp^b (1+), and Js^b (3+) post-deglycerolization. In addition, RBC genotyping confirmed that the donor had the genes to express k, Kp^b, and Js^b. Comparative genomic hybridization array provided genomic evidence that the male donor would have a McLeod phenotype, identifying a 4.69-Mb arr[hg19] Xp21.1p11.1(33,341,040-38,030,042) × 0 hemizygous deletion resulting in the complete loss of *XK* and *CYBB*. This donor was confirmed as having a McLeod phenotype, previously identified at CBS through serology and the presence of corresponding anti-Kx and anti-Km before the donation of at least 6 autologous units for cryopreservation to ensure supply in case of transfusion need.

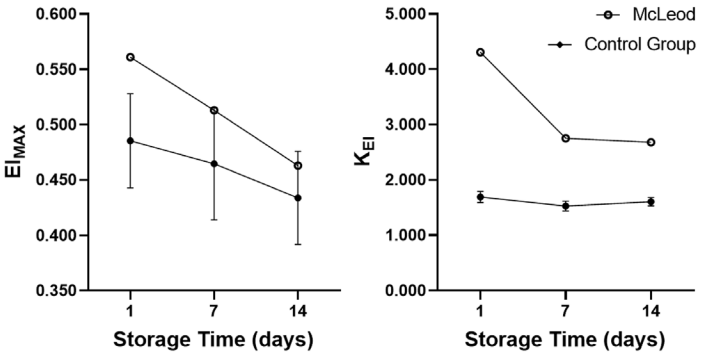

Fig. 1 Red blood cell (RBC) deformability represented by two index parameters: EI_{Max}, the maximum elongation of RBCs under sheer stress, and K_{EI}, a measure of rigidity, represented by the sheer stress required to reach half of the RBC maximum elongation. Data shown as mean ± 1 standard deviation for the control group (n = 3).

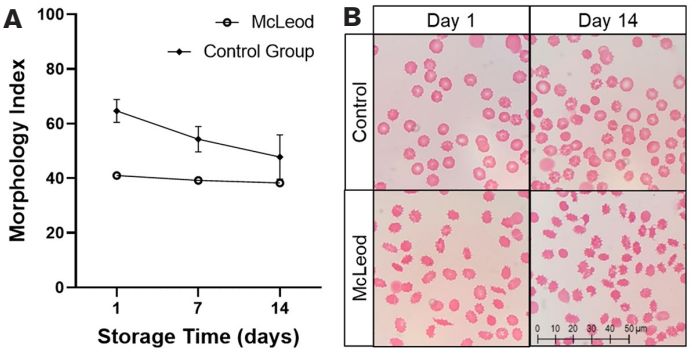

Fig. 2 (A) Morphology index of red blood cells (RBCs) stored for up to 14 days post-deglycerolization at 1–6°C. Data shown as mean ± 1 standard deviation for the control group (n = 3). (B) Microscopic images of RBC morphology at 1 and 14 days of hypothermic storage post-deglycerolization from thin smear slide preparations. The control images are from a representative sample taken from preparations of 1 RBC unit from the control group. The control unit images contain RBCs undergoing various states of crenation due to the hypothermic storage lesion, which should not be confused with pathologic acanthocytosis. RBCs from both the control and McLeod units will naturally undergo crenation, which may obscure or artificially increase the degree of acanthocytosis in the McLeod sample.

Genotyping was also performed on the control units, indicating that the method developed to collect and isolate DNA was successful in producing samples for RBC genotyping for non-leukocyte–reduced cryopreserved RBCs. However, when this method was tested on leukocyte-reduced cryopreserved RBCs ($n = 3$), the insufficient or fragmented DNA collected did not amplify, so no molecular results were obtained.

Discussion

From this investigation, it is apparent that McLeod RBCs can be cryopreserved using a manual glycerolization procedure and an automated deglycerolization method with process adaptations. Like all "open system" unit modifications, RBCs deglycerolized in this manner would have a 24-hour post-deglycerolization expiration date. Nevertheless, this study provides evidence that RBCs of this phenotype can be stored for up to 14 days post-thaw and still meet quality standards for Hgb, Hct, and hemolysis. This finding suggests that RBCs collected from McLeod donors can be cryopreserved using a closed-system cell processor and have the benefit of extended hypothermic storage using current methods at CBS.

Although quality standards were met in this study, there were differences observed between the McLeod RBCs and the control units for the additional quality parameters (osmotic fragility, MCV, RBC deformability, and morphology). These differences are likely attributed to donor factors (McLeod phenotype) and not to cell processing and cryopreservation methods.[14] The most prominent deviation from the control data for the McLeod RBC was detected in RBC rigidity. Typically, as hypothermically stored RBCs age, their morphology demonstrates crenation and eventually becomes more spheroid, but their ability to deform remains unaffected.[15,16] The McLeod donor unit demonstrated a substantial increase in membrane rigidity post-deglycerolization. Because the control units exhibit K_{EI} values lower than typical hypothermically stored RBCs at CBS (indicating that their membranes are less rigid likely due to the effects of cryopreservation), it can be concluded that acanthocytosis typical of McLeod phenotype RBCs causes increased RBC membrane rigidity and reduced deformability.[16] Additionally, it is not surprising that although the McLeod RBCs demonstrate decreases in deformability, they are still stable during hypothermic storage, which agrees with findings previously reported by Ballas et al.,[14] in which McLeod RBCs demonstrated mechanical stability but reduced deformability.

This study was also successful in demonstrating a method to obtain genetic material from non-leukocyte–reduced cryopreserved RBCs for genotyping and molecular investigations. Although the method described requires further development, it shows proof of concept that genomic DNA for molecular analyses on units cryopreserved for 20 years can be obtained. Having this capability allows molecular confirmation of (and potentially further elucidation of) the phenotype of previously banked rare units. Further improvements of this method are required to reduce the potential for bacterial contamination when obtaining samples and to develop an alternative method to obtain genetic material from leukocyte-reduced units. It is unknown whether sufficient DNA can be obtained from leukocyte-reduced units if the sample processing volume was increased or whether leukocyte reduction itself degrades DNA, making sufficient DNA for molecular analyses unattainable.

Finally, it is important to draw attention again to the fact that cryopreserved RBCs with exceedingly rare and unusual phenotypes can be retained in storage for at least 30 years. Management of these units will require a greater understanding of how the quality of these units with rare phenotypes are affected by the cryopreservation process and how changes in manufacturing will influence these characteristics. Future studies should evaluate the quality of cryopreserved RBCs with phenotypes with known RBC membrane abnormalities such as those found in the MNS (MkMk), Diego (southeast Asian ovalocytosis), Gerbich (Yus and Leach), and Gil blood group systems. Not only will this information allow us to put these components to better use, but it will also allow us to improve management policies for this patient population, ensuring that adequate components are available for use.

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