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Functionality of Red Blood Cells

After Cryo-Preservation

By

Charles Elder

Submitted in partial fulfillment of the requirements

for Graduation summa cum laude

and

for Graduation with Honors from the Department of Biology

University of Louisville

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Abstract

One of the most common medical procedures performed in US hospitals is blood transfusions. Unfortunately, the red blood cells (RBCs) for transfusion have a limited shelf life after donation due to detrimental storage effects on morphological and biochemical properties. Inspired by nature, I am developing a biomimetics approach to preserve RBCs for long-term storage using compounds that occur in animals that have developed a natural propensity to survive in a frozen or desiccated state for decades. Trehalose was employed as a cryoprotective agent when added to the extracellular freezing solution. The highest percent of RBCs with intact membranes after freezing and thawing was obtained using a cryopreservation solution comprised of 250 million RBCs/mL, 300 mM trehalose, 100 mM NaCl, in 20 mM HEPES buffer, pH 7.1. Under these conditions RBCs with intact membranes were recovered at 86 ± 12%. I have demonstrated the effectiveness and feasibility of using trehalose as a cryoprotective agent, and morphological intact RBCs were recovered after freezing and thawing with low cellular loss.

Introduction

One of the most common medical procedures performed in US hospitals is blood transfusions[1]. Unfortunately, the red blood cells (RBCs) that are transfused have a limited shelf life after donation due to detrimental storage effects on morphological and biochemical properties[2]. Unused RBCs must be stored at 1–6 °C and discarded after only 42 days in the United States[3]. This short lifespan and temperature dependent storage of transfusable RBCs, also known as packed RBCs (pRBCs), can lead to detrimental shortages in resource-constrained environments[4]. Despite the fact that blood transfusions are one of the most essential part of hospital-based health care, methods to increase their shelf life or allowing for long-term storage of RBCs are either cumbersome, non-practical, or entirely lacking. Currently, the only method to preserve RBCs for years is in a frozen state that utilizes glycerol, a compound that permeates human cells relatively easily, but requires to confer protection a very high intracellular concentrations (~3-4 mol/L) which leads to a time-consuming process of compound-unloading after thawing of the cells (45 min–2 hrs)[5]. This limits the utility of RBCs preserved with glycerol in emergency situations that depend on readily transfusable cells. Furthermore, glycerol is not applicable for alternative preservation approaches such as lyophilization of RBCs for dry storage. Inspired by nature, I am developing a biomimetics approach to preserve RBCs for long-term storage using compounds that occur in animals that have developed a natural propensity to survive in a frozen or desiccated state for decades[6-8]. The most promising compound currently under investigation is trehalose, a non-toxic sugar, which is predicted to only require intracellular concentrations of ~0.05–0.15 M to confer protection during freezing or drying [9]. Trehalose has also been shown to be safe to administer intravenously, unlike glycerol[10]. Unfortunately, human cells lack trehalose transporters in their membranes, which would allow for loading of the necessary concentrations

of the compound into RBCs. The only known cellular mode of transport for this compound across the plasma membrane into nucleated cells is by endocytosis, a slow cellular process that can deliver small amount of extracellular material into the cell through invagination, but unfortunately RBCs do not undergo endocytosis[9]. It has been a goal of my lab at UofL to develop a process that would allow for freezing of RBCs at high concentrations and then removing the frozen water from the solution (lyophilization) in order to store RBCs in a dry state for long periods of time and rehydrate them when needed. Theoretically, this mechanism should be feasible in presence of high trehalose concentrations as is postulated by the water replacement hypothesis[11]. The hypothesis states that when water is stripped from molecular structures, such as proteins and membranes, disaccharides like trehalose are thermodynamically driven, because of the arrangement of their hydroxyl groups, to replace the water bound to these structures. This can help to stabilize them in the desiccated state.

For RBCs to be lyophilized or freeze-dried, they must be able to survive the freezing process to be dried and rehydrated successfully. This has been the main focus of my undergraduate research career. It has been my goal to determine if trehalose can assist in protecting RBCs from the cellular damage that occurs during the freeze-thaw process and if different concentration of trehalose and other cryoprotectants have an effect on preventing this damage. After much trial and error, I also came to realize that not only was the storage solution affecting the membrane integrity of the RBCs, but the thawing temperature and the concentration of the RBCs also played a significant role in the number of cells that could withstand the process. Furthermore, RBCs judged as being intact when viewed under a hematocytometer do not necessarily function properly to deliver oxygen throughout the body. The protein hemoglobin must also maintain its quaternary structure to bind and release oxygen at the appropriate tissues in an organism[12].

Therefore, it will be important to follow up with studies on the impact of storage on hemoglobin structure and function for frozen or dried RBCs to be judged on their usefulness in emergency situations.

Materials and Methods

Chemicals

Low endotoxin α,α-trehalose dihydrate was obtained from Pfanstiehl Inc. (Waukegan, IL). All other compounds were obtained from VWR (Radnor, PA) or MilliporeSigma (Burlington, MA) and were of the highest purity commercially available. Water for solution preparation was purified with a Milli-Q Reagent Water System (Burlington, MA).

Porcine RBC Collection

Porcine whole blood was acquired from a slaughterhouse (JBS USA, Louisville, KY) and collected in 50–100 units heparin per mL blood to inhibit coagulation. The whole blood solution was quickly transported back to the laboratory on the University of Louisville's Belknap campus, where blood cells were pelleted using a Centrifuge 5804 R from Eppendorf (Hamburg, Germany) at 600*g* for 10 min. After centrifugation, the supernatant of the solution was decanted and the pellet was resuspended in modified PBS (–calcium, –MgCl) from Cytiva (Marlborough, MA) and then the pellet was resuspended. This centrifugation-based RBC washing process was performed two more times but with the final resuspension (50–60% hematocrit) in an FDA-approved RBC storage solution known as Additive Solution-3 (AS-3). These washed pRBCs were stored for no longer then 14 days at 4 °C before use.

RBC Experimental Preparation

RBCs were prepped for freezing and thawing by determining their concentration using a hematocytometer, and then adding the volume of pRBCs needed to achieve concentrations of 50 million, 250 million, 500 million, 1 billion, or 2.5 billion RBCs/mL in a solution containing 200 mM, 250 mM, 300 mM, 400 mM, 500 mM, or 600 mM of trehalose, 75 mM, 100 mM, or 150

mM NaCl, in 20 mM HEPES buffer, pH 7.1. These different RBC and compound concentrations were compared to identify conditions that conferred optimal membrane integrity after freezing and thawing. Serial dilutions were used for concentration less than 1 billion RBCs/mL. Two counts using a hematocytometer were performed and averaged to determine a precise RBC concentration in the preprocessed samples.

RBC Freezing

All RBC solutions were frozen in an identical fashion. Rapid freezing of RBC was performed by placing 1 mL of sample into a cryovial and dropping it into a Dewar of liquid N_2. The sample remained in liquid N_2. for at least 10 min before it was thawed for analysis.

RBC Thawing

RBCs were thawed by removing the frozen sample from liquid nitrogen and quickly placing it into a water bath at 30 °C, 40 °C, 50 °C, 55 °C, 60 °C, or 70 °C then whirling the sample around in a circle with plyers, performing 180 circles per minute. Each rotation had a diameter of about 15 cm. The sample was pulled out of the water bath when only a small piece of sample remained frozen.

Membrane Integrity Analysis

Membrane integrity was determined using a hematocytometer to enumerate RBC concentration. After thawing, a sample was held at room temperature for 10 min. An aliquot of the sample was diluted using modified PBS, and red blood cells were quantified using a hematocytometer. The determined concentration was then compared to the preprocessed RBC concentration to determine the percentage of cells that maintain membrane integrity after freezing and thawing.

Hemolysis Analysis

The percentage of hemolysis of RBCs was determined by comparing the amount of hemoglobin in the supernatant to the amount in the pellet after centrifugation at 600*g* for 10 min using a Hemoglobin Assay Kit from MilliporeSigma (Burlington, MA). Hemolysis was determined for samples of 250 million and 1.25 billion RBCs/mL in a solution containing 300 mM trehalose, 100 mM NaCl, in 20 mM HEPES buffer, pH 7.1 with thawing at 55 °C and for control samples that did not undergo freezing and thawing. After thawing, a sample was held at room temperature for 10 min. The sample was then centrifuged at 600*g* for 10 min. An aliquot of the supernatant was collected to quantify the hemoglobin concentrations. The supernatant was then removed, and the pellet was resuspended in 1 mL of purified water with 0.1% Triton-X to ensure complete cell lysis. A portion of the lysed pellet was collected, and hemoglobin concentrations were determined. The total amount of hemoglobin within the sample was determined, and the amount in the supernatant was divided by the total amount to determine percent hemolysis.

Statistical analyses

Data were analyzed with one-way ANOVA tests using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA) using a Holm-Sidak post hoc analysis.

Results

Cryoprotective Solution

RBC membrane integrity was analyzed after freezing and thawing the cells in different cryoprotective solutions. Initially the impact of changing the trehalose concentration was analyzed. A concentration of 50 million RBCs/mL was used in different cryoprotective solutions containing trehalose concentrations ranging from 200 mM to 600 mM in 20 mM HEPES buffer, pH 7.1. Thawing was performed at 40 °C. The percentage of RBCs with intact membranes after freezing and thawing was determined for each solution. A cryoprotective solution containing 300 mM trehalose in 20 mM HEPES buffer, pH 7.1 showed numerically the highest recovery of cells with a membrane integrity of 87 ± 4% (n = 3, 3 nested replicates), but this value was not statistically significant different from results obtained at 250 mM and 400 mM trehalose (Fig. 1). From prior experiments, we noticed that the addition of sodium chloride to the

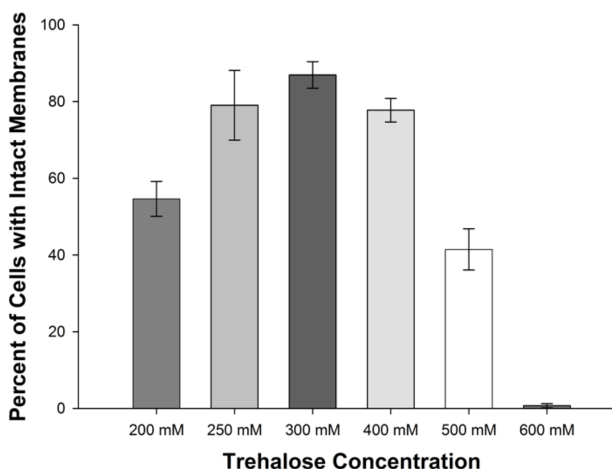

Fig. 1. Percent of RBCs with intact membranes after freezing in a cryoprotective solution containing different concentrations of trehalose with 50 million RBCs/mL in 20mM HEPES buffer, pH 7.1. Thawing was performed at 40 °C (n = 3, 3 nested replicates).

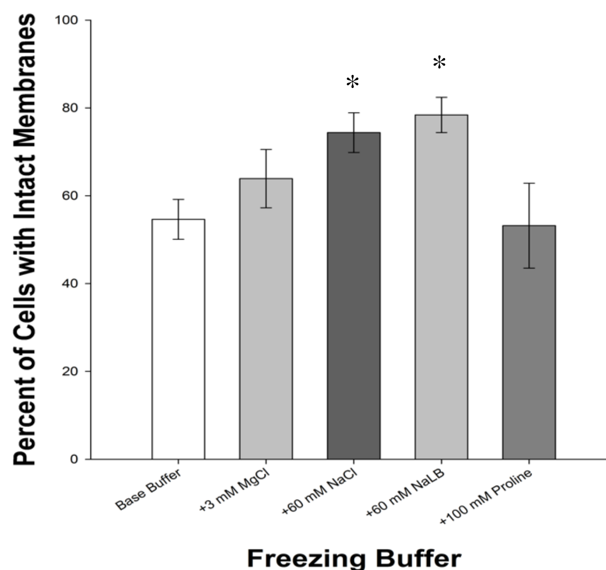

Fig. 2. Percent of RBCs with intact membranes after freezing in a cryoprotective solution containing different cryoprotective compounds, 200 mM trehalose, in 20 mM HEPES buffer, pH 7.1. Thawing was performed at 40 °C. * denotes statistically significant difference from base buffer ($p<0.05$, n = 3, 3 nested replicates).

cryoprotective solution increased membrane integrity (Fig. 2). I then wanted to determine an optimal NaCl concentration for the cryopreservation solution. I hypothesized that the overall increase in osmolarity by adding excess NaCl to the solution could have a negative effect on the RBCs freeze-thaw membrane integrity. An experiment was designed and performed to determine if osmolarity has an effect on RBCs membrane integrity and to acquire an optimal NaCl concertation for the freezing solution. Therefore, RBC concentration of 250 million cells/mL were processed in solutions containing 200 mM, 250 mM, or 300 mM trehalose, and 75 mM, 100 mM, or 150 mM NaCl, in 20 mM HEPES buffer, pH 7.1, and the percent membrane integrity was determined after thawing at 40 °C. There was no statistically significant difference in recovery of RBCs among solutions with different NaCl concentrations. The highest percentage of cells with intact membrane after freezing and thawing was a solution of 250 million RBCs/mL, 300 mM trehalose, 100 mM NaCl, in 20 mM HEPES buffer, pH 7.1 with a membrane integrity of 86 ± 12% (n = 3, 3 nested replicates) (Fig. 3).

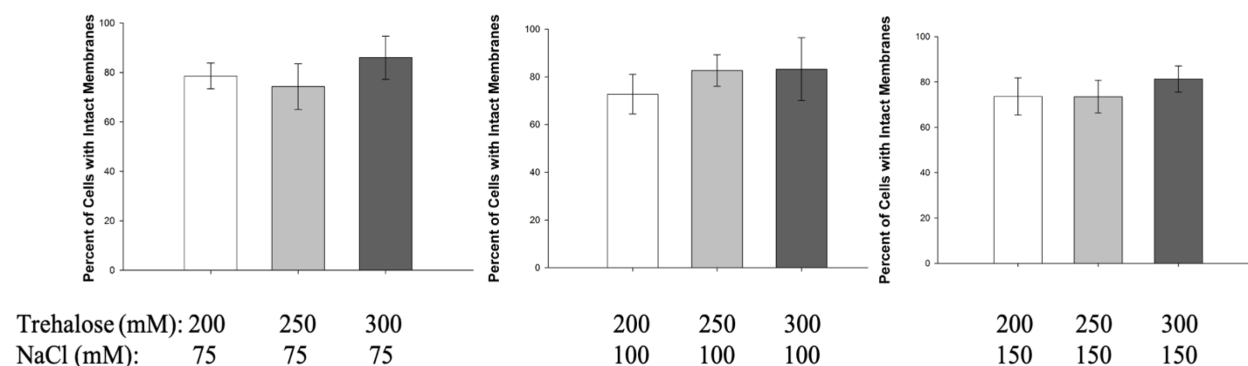

Fig. 3. Percent of RBCs with intact membranes after freezing in a cryoprotective solutions containing different concentrations of trehalose and NaCl, 250 million RBCs/mL, in 20 mM HEPES buffer, pH 7.1. Thawing was performed at 40 °C (n = 3, 3 nested replicates).

RBC Thawing Temperature

RBC membrane integrity was determined at different thawing temperatures. Thawing temperatures ranged from 30 °C to 70 °C using solutions comprised of 50 million RBCs/mL, 200 mM trehalose, in 20 mM HEPES buffer, pH 7.1. The most promising recovery of RBCs was obtained when thawing was being performed at 55 °C (Fig. 4). RBCs thawed at 55 °C had a statistically significant higher membrane integrity of 64 ± 4% when compared against the standard temperature of 40 °C, which had a membrane integrity of 55 ± 5% (*n* = 3, 3 nested replicates).

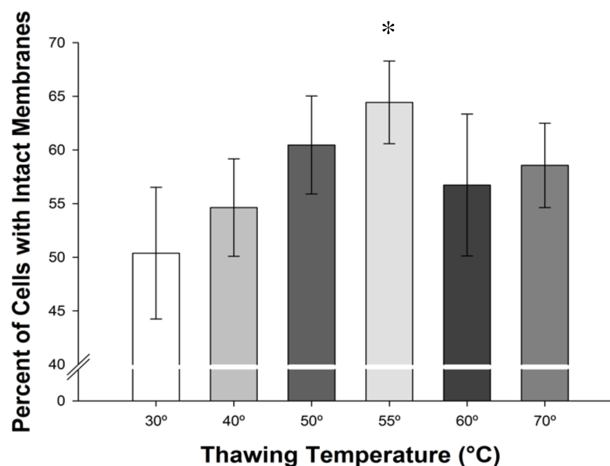

Fig. 4. Percent of RBCs with intact membranes after freezing with 200 mM trehalose in 20 mM HEPES buffer, pH 7.1 while thawing at different temperatures. * denotes statistically significant difference from 40 °C ($p<0.05$, $n = 3$, 3 nested replicates).

RBC Concentration

RBC membrane integrity was analyzed after freezing and thawing at different RBC concentrations in a cryopreservation solution comprised of 300 mM trehalose, 100 mM NaCl, in 20 mM HEPES buffer, pH 7.1.

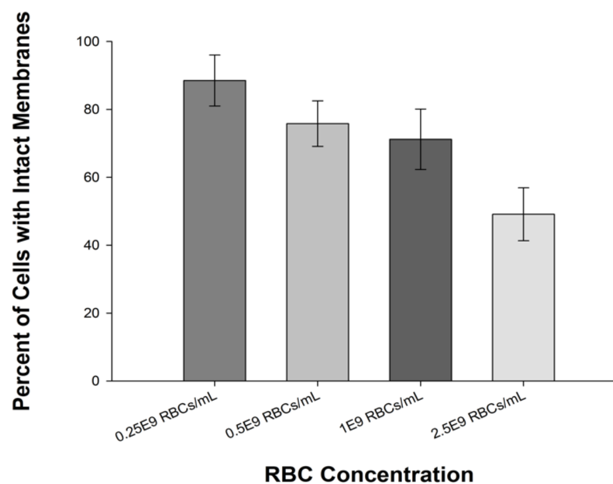

Fig. 5. Percent of RBCs with intact membranes after freezing in a cryoprotective solution containing 300 mM trehalose, 100 mM NaCl, in 20 mM HEPES buffer, pH 7.1 and different RBC concentration. Thawing was performed at 55 °C ($n = 3$, 3 nested replicates).

Thawing was performed at 55 °C, and membrane integrity was determined after freezing and thawing at RBC concentrations of 250 million, 500 million, 1 billion, and 2.5 billion RBCs/mL

(Fig. 5). At a concentration of 2.5 billion RBCs/mL, 49 ± 8% (n = 3, 3 nested replicates) of the cells retained intact membranes after freezing and thawing.

Hemolysis Analysis

The percentage of hemolysis after freezing and thawing was determined for samples of 250 million and 1.25 billion RBCs/mL in a cryopreservation solution comprised of 300 mM trehalose, 100 mM NaCl, in 20 mM HEPES buffer, pH 7.1. Thawing was performed at 55 °C. RBCs at a concentration of 250 million cells/mL had a percent hemolysis of 14 ± 2% (n = 3, 3 nested replicates). In comparison, control samples that did not undergo the freezing and thawing displayed a percent hemolysis of 3 ± 0% (n = 3, 2 nested replicates).

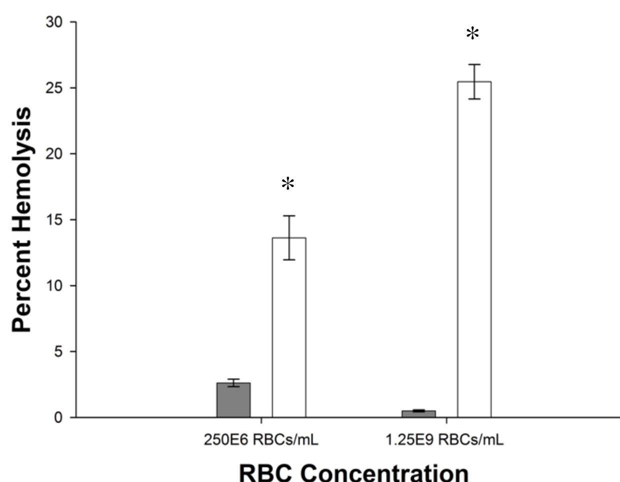

Fig. 6. **Grey Bar**. Percent hemolysis of RBCs that did not undergo freezing and thawing (n = 3, 2 nested replicates). **White Bar**. Percent Hemolysis of cells that did undergo freezing and thawing (n = 3, 3 nested replicates). All RBC samples were placed in a cryoprotective solution composed of different RBC concentrations, 300 mM trehalose, 100 mM NaCl, in 20 mM HEPES buffer, pH 7.1. Thawing was performed at 55°C. * denotes statistically significant difference from control groups ($p<0.05$).

Samples at concentrations of 1.25 billion RBCs/mL after freezing and thawing had a percent hemolysis of 26 ± 1% (n = 3, 3 nested replicates). The control for this concentration had a percent hemolysis of 1 ± 0% (n = 3, 2 nested replicates) (Fig. 6).

Discussion

Trehalose was employed as a cryoprotective agent (CPA) to protect RBCs for biopreservation purposes. I have demonstrated the effectiveness and feasibility of using trehalose as a CPA and morphological intact RBCs were recovered after the freezing and thawing with low loss in cell numbers and hemoglobin content. The cryopreservative solution had an osmolarity of 420 mOsm/kg. This is higher than the intravenous whole blood osmolarity of 280–300 mOsm/kg[13]. The higher osmolarity of the cryoprotective solution likely causes water loss from the RBC cytoplasm[14]. An intracellular dehydration due to the hyperosmotic solution combined with water replacement by trehalose during freezing could be part of the observed cryoprotective effect in this study. Dou et al. (2019) showed similar results using a hypertonic solution comprised of trehalose and L-proline[15].

Several thawing temperatures were tested to determine if the standard 37°C–40°C thawing temperature was the most optimal for the freezing and thawing[16]. The obtained recoveries of RBCs at 55 °C were statistically significant higher when compared to the standard 40 °C. I believe this shows that the most rapid thawing procedure will be optimal for recovering intact RBCs. During the thawing process ice is melting and recrystallizing until the temperature is warm enough to inhibit new ice formation. Ice crystallization is a major cause of cell loss during the freezing and thawing[17]. I hypothesize by rapidly thawing the RBCs the amount of ice recrystallization is reduced due to the decreased time of thawing. Temperatures higher than 55 °C yielded more variable recoveries than compared to other temperatures. This may be due to the outside of the sample becoming too warm before the inside completely melts. This intense heat can cause cell death[18]. Future studies to optimize this process could determine at what exact point in time frozen RBCs should be taken from the water bath at temperatures above 55 °C so

that the walls of the cryogenic vial do not become excessively warm causing cell death. These higher temperatures may improve recovery, but the excessive heat could cause a decrease in oxygen transport functionality since heat can cause proteins to unfold[19]. In future studies, hemoglobin oxygen binding curves should be performed to determine if increased thawing temperatures cause protein denaturation which could change the way oxygen binds to hemoglobin. A possible outcome may be irreversible hemoglobin subunit dissociation[20].

Based on the optimization data of the cryopreservation solution, it may be possible to optimize for even higher concentration of RBCs than tested in this study. The current optimizations were performed at concentrations of 50 million and 250 million RBCs/mL. One characteristic of RBCs that was not taken into consideration for this study was that the RBCs themselves take up volume within the solution. Hematocrit is a measure of RBC concentration and it represents the amount of volume within the solution that the RBCs themselves are taking up[21]. In comparison, 1% hematocrit is equivalent to about 100 million RBCs/mL. This means that at a concentration of 250 million RBCs/mL the cells themselves take up 2.5% of the total volume of solution. This is much lower than used for pRBCs, which has a hematocrit value of 65–80%[22]. When I scale up the RBC concentration, I hypothesize that less total trehalose will be needed since the RBCs take up the majority of the volume of the solution. Utilizing extracellular concentration of 300 mM trehalose will require lower total sugar amounts per sample volume at higher concentration of RBCs which will reduce the sugar load during transfusion.

From the cell recovery studies I was able to determine that the percent hemolysis is very similar to the percent of cell that are not recovered. This indicates that cells that are not lysed during freezing and thawing are able to maintain their native hemoglobin concentration within the cell, and only lysed RBCs lose their hemoglobin to the supernatant. I am unsure if the hemoglobin

that remains in the RBCs after freezing and thawing is still functional for oxygen and CO_2 transport throughout the body which will be the subject of future studies.

Lyophilization will be an important next step to optimize the storage efforts for RBCs. In a lyophilized state RBCs can theoretically remain viable at room temperature for decades. Determining a buffer solution to freeze the RBCs was an important first step but researching the most optimal lyophilization conditions will be the next challenge. Once a method to recover RBCs after lyophilization is determined it will be paramount to make sure the hemoglobin within the cells is still functional, and hemoglobin oxygen-binding curves are needed to address this question. Furthermore, loading of RBCs with trehalose may be required before the cells are frozen and lyophilized since these cells cannot undergo endocytosis[23]. A process using ultrasound, microbubbles, and a microfluidic system to load RBCs with trehalose is currently being developed at UofL[24]. The intracellular concentration of trehalose that can be achieved by this process may assist in maintaining functional hemoglobin during cryo- and lyo-preservation[23].

Acknowledgments

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