Cryopreserved Red Blood Cells Maintain Allosteric Control of Oxygen Binding When Utilizing Trehalose as a Cryoprotectant

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Abbreviations

2,3 BPG, 2,3-bisphosphoglycerate

CPA, Cryoprotective Agent

DPBS, Dulbecco's Phosphate-Buffered Saline

LN_2, Liquid Nitrogen

p50, Pressure of Oxygen at which Hemoglobin is 50 % Saturated

RBCs, Red Blood Cells

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Abstract

One of the most common life-saving medical procedures is a red blood cell (RBC) transfusion. Unfortunately, RBCs for transfusion have a limited shelf life after donation due to detrimental storage effects on their morphological and biochemical properties. Inspired by nature, a biomimetics approach was developed to preserve RBCs for long-term storage using compounds found in animals with a natural propensity to survive in a frozen or desiccated state for decades. Trehalose was employed as a cryoprotective agent and added to the extracellular freezing solution of porcine RBCs. Slow cooling (-1 °C min^{-1}) resulted in almost complete hemolysis (1 ± 1 % RBC recovery), and rapid cooling rates had to be used to achieve satisfactory cryopreservation outcomes. After rapid cooling, the highest percentage of RBC recovery was obtained by plunging in liquid nitrogen and thawing at 55 °C, using a cryopreservation solution containing 300 mM trehalose. Under these conditions, 88 ± 8 % of processed RBCs were recovered and retained hemoglobin (14 ± 2 % hemolysis). Hemoglobin's oxygen-binding properties of cryopreserved RBCs were not significantly different to unfrozen controls and was allosterically regulated by 2,3-bisphosphoglycerate. These data indicate the feasibility of using trehalose instead of glycerol as a cryoprotective compound for RBCs. In contrast to glycerol, trehalose-preserved RBCs can potentially be transfused without time-consuming washing steps, which significantly facilitates the usage of cryopreserved transfusible units in trauma situations when time is of the essence.

1. Introduction

In 2019, approximately 11 million red blood cell units were transfused to patients in the United States [1]. An elaborate blood collection and distribution system has been established in developed countries to ensure a sufficient supply for transfusions. After donation, RBCs must be stored between 1 to 6 °C and discarded after 35 – 49 days [2]. The short lifespan of transfusable RBCs can lead to life-threatening shortages in resource-constrained environments and during natural disasters [3]. Although RBC transfusions are essential to hospital-based health care, methods to increase their shelf life or allow for long-term storage are cumbersome but do allow storage for up to 30 years. Currently, the only method to preserve human RBCs for extensive periods of time utilizes ultra-low temperatures (-80 °C to -196 °C) and glycerol as a cryoprotective agent (CPA). Glycerol is added at very high concentrations (40 % v/v) to confer protection to RBCs during a relatively slow cooling process of -1-3 °C min^{-1}, and requires a time-consuming procedure of compound-unloading after warming the cells (45 min – 2 h). This RBC preservation method is referred to as the high-glycerol method and is currently preferred over the low-glycerol method (17 – 20 % v/v) [4; 5; 6; 7]. The long loading times and extensive washing limit the utility of RBCs preserved with glycerol in emergencies that depend on readily available transfusable cells. Inspired by nature, biomimetic approaches to preserve RBCs utilize compounds that accumulate in animals before they can survive in a frozen or desiccated state for decades [8; 9; 10]. One of the most promising compounds under investigation is trehalose, a non-reducing sugar that, unlike glycerol, is safe to administer intravenously [11]. The challenge with using trehalose to protect mammalian cells during preservation is overcoming the plasma membrane's inherent impermeability for trehalose.

The most apparent damage to RBCs during storage is the degradation or loss of hemoglobin [12]. Hemoglobin and related oxygen-transport proteins are essential to the physiology of nearly all vertebrates because they increase the transport maximum of oxygen to tissues [13]. During RBC storage, hemoglobin can be lost to the extracellular environment due to cell lysis, the formation of extracellular vesicles, or membrane deformation, which can occur during osmotic stress [14; 15]. Extracellular hemoglobin can cause platelet aggregation and excessive scavenging of nitric oxide, which can eventually lead to thrombosis, causing a restriction in blood flow [16; 17]. Furthermore, intracellular hemoglobin may become oxidized during storage, changing the oxidation state of its bound iron from Fe^{2+} to Fe^{3+}, but this mechanism of damage is only prevalent during hypothermic and dry preservation [12; 18]. Hemoglobin oxidized to methemoglobin (Fe^{3+}) cannot bind oxygen and significantly reduces the utility of RBC transfusions [19; 20]. Both these damage mechanisms, hemolysis and methemoglobin formation, must be avoided during processing and storage to provide functional RBCs in transfusible units.

Cells exhibit an optimal cooling rate resulting in maximal cryosurvival [21]. The cooling rate resulting in maximal cryosurvival is not only cell-specific but also depends on the type of CPA utilized and its concentration. When human RBCs are cryopreserved in the presence of 40 % v/v glycerol, the optimal cooling rate is -1-3 °C min^{-1}, whereas the 'low' glycerol method requires much higher cooling rates (~-60 °C min^{-1}) to obtain maximal cryosurvival [4]. When human RBCs are cryopreserved with trehalose, rapid cooling by plunging in liquid nitrogen (LN_2) produces maximal cryosurvival [22; 23; 24; 25]. Additionally, other non-penetrating and biocompatible compounds such as varying sugars and polymers promote preservation outcomes when rapidly cooling [26]. The ice crystals formed during the rapid cooling ~~rate~~ process tend to be very small and have high curvature compared to those crystallized during slower cooling processes at around -1 °C min^{-1} [27]. The ice crystals' higher curvature and smaller size can reduce physical damage to cells compared to crystals formed during slower cooling rates as long as intracellular ice formation is avoided or minimized [21].

Porcine RBCs were utilized in this study because of their similarities to human RBCs [28]. Not all mammalian RBCs have the same cryopreservation outcomes as human RBCs due to varying membrane permeabilities to cryoprotectants (i.e., canine, bovine, equine, etc.) [29]. Porcine RBCs were frozen in cryovials by direct immersion of the vials in LN_2, which resulted in rapid cooling (~-300 °C min^{-1}) of the sample down to LN_2 temperatures. The scope of this study expands upon previous work that used trehalose to cryopreserve porcine RBCs by determining a trehalose and RBC concentration-dependent relationship for preservation outcomes [22; 23; 24; 25]. We also demonstrate that porcine hemoglobin does not become oxidized and maintains its affinity for oxygen after preservation and that the freezing and thawing process loads RBCs with trehalose intracellularly [22; 24; 25]. Furthermore, storage at -80 °C for 84 days did not negatively affect RBC properties. Finally, we would like to emphasize that none of the reported results are up to current regulatory standards for RBC transfusions (less than 0.8 – 1% hemolysis), but the potential of using trehalose as a transfusible CPA was revealed.

2. **Materials and Methods**

2.1. Chemicals

Low endotoxin α,α-trehalose dihydrate was obtained from Pfanstiehl Inc. (Waukegan, IL). All other compounds were obtained from VWR (Radnor, PA), Thermo Fisher Scientific (Waltham, MA), or Millipore Sigma (Burlington, MA) and were of the highest purity commercially available. Chemicals used for solution preparation include 99.9% sodium chloride (VWR), 99% HEPES (Thermo Fisher Scientific), 97% lactobionic acid (Millipore Sigma), 99.5% citric acid (Millipore Sigma), 99% sodium citrate dihydrate (VWR), 99% adenine (Thermo Fisher Scientific), 99% dextrose (Thermo Fisher Scientific), 99% sodium phosphate monobasic dihydrate (Millipore Sigma), 2,3-diphospho-D-glyceric acid pentasodium salt (Millipore Sigma), TRITON X-100 (VWR), and 8% aqueous glutaraldehyde (VWR). Water for solution preparation was cell culture grade and purchased from VWR.

2.2. Porcine RBC Collection

Porcine whole blood was acquired from an abattoir, JBS USA (Louisville, KY), and collected in a conical tube containing heparin at a final concentration of 50-100 units/mL to inhibit coagulation. RBCs were pelleted at 600 *g* for 10 min using a bench-top centrifuge (5804R, Eppendorf, Hamburg, Germany). After centrifugation, the supernatant was aspirated, and the pellet was resuspended in Ca^{2+}, Mg^{2+} free Dulbecco's phosphate-buffered saline (DPBS) (Cytiva, Marlborough, MA). The samples were washed three times, and the final pellet was resuspended at 50 – 60% hematocrit in the FDA-approved RBC storage solution, Additive Solution-3 (70 mM NaCl, 2 mM citric acid, 23 mM Na_3-citrate, 2 mM adenine, 55 mM dextrose, 23 mM NaH_2PO_4, pH 5.8). RBCs were not leukoreduced and were stored for up to 14 days at 4 °C before use.

2.3. Experimental Preparation

Washed RBCs were diluted in one step with DPBS to approximately 5 million RBCs/mL and enumerated with a hematocytometer. Samples were diluted to final concentrations of 50 million – 2.5 billion RBCs/mL in solutions containing 200 mM – 600 mM trehalose, 60 mM – 150 mM NaCl or 60 mM sodium lactobionate in 20 mM HEPES-NaOH buffer, pH 7.1, and RBC concentrations were verified twice with a hematocytometer and averaged.

2.4. Freezing

Rapid cooling of RBCs was performed by placing 1 mL of the sample into a 1.2 mL cryogenic vial (Thermo Scientific, Waltham, MA). The cryovial was then placed upright into a Dewar containing LN_2. Cooling rates were measured using a 3 mm hermetically sealed wire RTD probe (Omega Engineering Inc., Norwalk, CT) and were calculated to be ~-300 °C min^{-1}, with the 1 mL sample taking ~40 seconds to complete the phase transition from liquid to solid (Fig. S1). The sample remained in LN_2 for 10 min before thawing for analysis. When samples were studied over time, they were stored at -80 °C after freezing. Slow cooling was accomplished by placing the cryogenic vial into a passive CoolCell container, which yields a cooling rate of -1 °C min^{-1} when placed at -80 °C (Corning Inc., Corning, NY).

2.5. Thawing

RBCs were thawed by removing the frozen sample from LN_2 or -80 °C storage. The samples were then quickly placed into a water bath heated to temperatures between 30 °C – 70 °C, as stated in the figures. Samples were whirled at 180 circles per minute with a rotation diameter of ~15 cm to ensure rapid thawing. Samples were removed from the water bath when only a minor piece of ice remained visible.

2.6. Intact Cell Analysis

The percentage of intact cells was enumerated using a hemocytometer to determine RBC concentration. After thawing, a sample was held at room temperature for 10 min. An aliquot of the sample was diluted in one step into DPBS to roughly 5 million RBCs/mL, and RBC concentrations were enumerated. The determined concentration was then divided by the preprocessed RBC concentration and multiplied by 100% to determine the percentage of intact cells after freezing and thawing (RBC Recovery).

2.7. Hemolysis Assay

RBCs lose intracellular hemoglobin because of damage, degradation, or lysis (hemolysis). The percentage of hemolysis was determined for cryopreserved samples by measuring the amount of hemoglobin in the supernatant and pellet after centrifugation at 600 *g* for 10 min using a hemoglobin assay kit following the instructions by the manufacturer (Millipore Sigma, Burlington, MA). Hemolysis was determined for cryopreserved samples of 250 million and 1.25 billion RBCs/mL in a solution containing 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1 after thawing at 55 °C and for control samples that did not undergo freezing and thawing. After thawing, samples were kept at room temperature for 10 min, followed by centrifugation at 600 *g* for 10 min. An aliquot of the supernatant was collected to quantify the free hemoglobin concentration. The supernatant was next removed, and the pellet was resuspended in 1 mL of purified water with 0.1% TRITON X-100 to ensure complete cell lysis. A portion of the lysed pellet was collected, and hemoglobin concentration was determined. The total amount of hemoglobin within the sample was calculated, and the amount in the supernatant was divided by the total amount and multiplied by 100% to calculate the percentage of hemolysis.

2.8. Oxygen-binding properties of hemoglobin

Oxygen-binding isotherms were recorded using a blood oxygen binding system (BOBS) (Loligo Systems, Viborg, Denmark), and p50-values and Hill coefficients were fitted for control and freeze-thawed samples using the software supplied by the manufacturer by applying the Hill-Langmuir equation [30; 31]. Solutions containing 2.5 billion RBCs/mL were prepared in 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1, and binding isotherms were recorded before and after freezing and thawing. Samples were diluted 10X in H_2O for RBC lysis and then centrifuged at 5000 *g* for 5 min in 1 mL aliquots. After centrifugation, 900 μL of the supernatant was added to 100 μL of 1.0 M NaCl and 1.0 M HEPES-NaOH, pH 7.1. The samples were next diluted 1:1 in the HEPES buffer with or without 20 mM 2,3-bisphosphoglycerate (2,3 BPG), and 10 μL of the solution was loaded on the BOBS sample holder. For samples not containing 2,3 BPG, a protocol was used in which O_2 was ramped up 0.25% v/v every 10 min until a total oxygen content of 3% v/v was reached. For samples containing 2,3 BPG, a protocol was used in which O_2 was ramped up 0.5% v/v every 10 min until a total oxygen content of 5.5% v/v was reached.

2.9. Oxidation During Storage at -80 °C

Samples were prepared at 250 million RBCs/mL in 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1, and frozen in liquid N_2. Samples were either stored at -80 °C for 24 days, 56 days, or 84 days or thawed immediately at 55 °C. After thawing, RBC recovery was determined, followed by preparation for spectral analysis. Samples were prepared as described for the oxygen-binding isotherms above. Spectra were collected from λ = 500 – 650 nm using a UV-1800 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). The occurrence of methemoglobin was judged based on the absorbance at λ = 630 nm.

2.10. Trehalose Loading

Samples were prepared at 250 million RBCs/mL in 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1, and frozen or directly processed for trehalose assays. Samples were washed in DPBS three times to remove residual extracellular trehalose. RBC pellets were lysed in 1 mL water and sonicated to ensure complete lysis. The lysates were heated to 95 °C to denature proteins, and centrifugation was performed for 5 min at 5000 *g* to remove cellular debris. A trehalose assay kit (Megazyme, Bray, Ireland) was used to determine the total amount of trehalose within the sample following the manufacturer's instructions. RBC samples that did not undergo freezing and thawing were used as a control to correct for residual trehalose after washing of RBCs. The corrected total trehalose quantity was then divided by the mean corpuscular volume (MCV) or the intracellular osmotically active water volume to calculate intracellular sugar concentrations [32]. Osmotically active water volumes were determined by multiplying the total number of cells in the assay by the average MCV of the RBCs determined with an ADVIA 2120i Hematology System (Siemens Medical Solutions USA Inc., Malvern, PA) (59.3 ± 2.7 fL, $n = 13$) and the osmotically active water fraction (73% of the MCV). The MCVs measured for the porcine RBC match previously reported values in the literature [33; 34].

2.11. Scanning Electron Microscopy

RBC samples were imaged with an Apreo C LoVac Field Emission Scanning Electron Microscope (Thermo Fisher, Waltham, MA) before and after freezing and thawing. Samples were resuspended in DPBS or 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1, and kept at room temperature for 30 min before fixation. Cells were fixated by adding 1/5th of the sample volume of a 4 % w/v glutaraldehyde solution every 5 min until a final concentration of 2 % w/v glutaraldehyde was reached. The cells were then dehydrated with consecutive ethanol resuspensions from 50 % – 100 % ethanol. Cells were plated onto aluminum stages and allowed to settle before excess ethanol was removed with a micropipette. The samples were then dehydrated over anhydrous calcium sulfate for 15 min. Stages were sputter-coated with palladium and gold to prevent charging artifacts.

2.12. Statistical Analyses

Data were analyzed with one-way and two-way ANOVA tests using GraphPad Prism 9 (Graphstats Technologies, Bengaluru, India) using a Tukey-Kramer post hoc analysis. Bars on bar graphs represent averages, stars represent individual data points, and error bars represent standard deviations. Standard box plots with stars representing individual data points were also used for data visualization. Different letters in the graphs represent statistically significant differences between sample groups. When describing the number of replicates performed in an experiment, *n* represents the number of times the experiment was performed independently, and nested replicates are multiple measurements obtained within an experiment.

3. Results

3.1. Cryoprotective Solution

RBC recovery was analyzed after freezing and thawing RBCs in different cryoprotective solutions. Initially, the cryoprotective abilities of trehalose were studied at concentrations ranging from 200 mM – 600 mM at a cell concentration of 50 million RBCs/mL (Fig. 1A). The outcomes showed a goldilocks effect, and a precise trehalose concentration was needed for optimal RBC protection during freezing and thawing. The trehalose concentration that conferred the highest percentage of intact RBCs at 50 million RBCs/mL was 300 mM of the sugar. The samples processed at 300 mM trehalose were the only ones showing significantly higher RBC recovery than those processed with 200 mM trehalose ($p < 0.05$, $n = 3$, 3 nested replicates). A similar study at 2.5 billion RBCs/mL showed that the optimal trehalose concertation for RBC recovery during freezing and thawing correlates positively with the RBC concentration (Fig. S2).

Inorganic and organic salts were added to the cryoprotective solution. There was no significant difference in cell recovery when comparing the two salts, sodium chloride (NaCl) and sodium lactobionate (NaLB) ($p > 0.05$). However, with the addition of 60 mM NaCl or NaLB, a significantly improved RBC recovery was observed after freezing and thawing compared to samples without any added salt (Fig. 1B) ($p < 0.05$, $n = 3$, 3 nested replicates). Additionally, the osmotic pressure of the solution was modified by varying the concentration of trehalose (200 mM – 300 mM) and NaCl (75 mM – 150 mM) (Table S1). No difference in RBC recovery was seen at different NaCl concentrations ($p > 0.05$, $n = 3$, 3 nested replicates).

3.2. RBC Thawing Temperature

The percentage of intact RBCs after freezing was determined at different thawing temperatures ranging from 30 °C – 70 °C (Fig. 2). Temperature profiles during thawing were recorded for each condition (Fig. S3). A positive correlation between RBC recovery and thawing temperature was observed until temperatures exceeded 55 °C. Thawing temperatures above 55 °C yielded highly variable results, with no general trend observed. The thawing rate of cryopreserved RBCs significantly impacted the recovery outcomes, with an optimal recovery at 55 °C.

3.3. RBC Concentration

RBC recovery was analyzed after freezing and thawing at different RBC concentrations ranging from 250 million – 2.50 billion RBCs/mL in a cryoprotective solution composed of 300 mM trehalose, 100 mM NaCl, and 20 mM HEPES-NaOH, pH 7.1 (Fig. 3). The percentage of intact cells was negatively correlated with the RBC concentration. Additional studies showed that the negative effect on RBC recovery with increased cell concentration could be mitigated by increasing trehalose concentration (Fig. S2). With a 10-fold increase in cellular volume, the cryoprotective properties of trehalose were still observable.

3.4. Cooling Rate

RBC cryostorage outcomes were analyzed when cooling the samples at two different rates. (1) rapid cooling, which utilized plunging them into liquid N_2, and (2) slow cooling by placing samples into a Corning CoolCell container (Corning, NY), which maintains a rate of cooling at -1 °C min^{-1} after transferring the device into an -80 °C freezer. RBCs were placed in a cryopreservation solution of 300 mM trehalose, 100 mM NaCl, and 20 mM HEPES-NaOH, pH 7.1, at 250 million RBCs/mL. Remarkably, few RBCs were recovered after slow cooling (1 ± 1%) compared to fast cooling, which yielded recovery outcomes of 88 ± 8% intact cells (Table S2)."

3.5. Hemolysis Analysis

The percentage of RBC hemolysis after freezing and thawing was determined for samples of 250 million and 1.25 billion RBCs/mL in a cryopreservation solution composed of 300 mM trehalose, 100 mM NaCl, and 20 mM HEPES-NaOH, pH 7.1 (Fig. 4). Hemoglobin lost to the supernatant during cryopreservation was observed in all samples. There was no significant difference between measured hemolysis (Fig. 4) and the loss of intact cells (Fig. 3) calculated based on cell recovery at 0.25 billion RBCs/mL ($p > 0.05$, $n = 3$, 3 nested replicates). This result indicated that RBCs that remain intact retain the hemoglobin within them after cryopreservation.

3.6. Hemoglobin Oxygen Binding

Hemoglobin's oxygen-binding properties were monitored before and after freezing. Ice crystallization did not affect oxygen transport capabilities, and the p50 values and the Hill coefficients were not significantly different before and after freezing (Table 1) ($p > 0.05$, $n = 3 – 5$). The cooperatively regulated binding sites within the hemoglobin multimer were not significantly affected during storage, and the protein maintained its allosteric regulation in the presence of 2,3-BPG after cryopreservation.

3.7. Trehalose Loading

Intracellular trehalose concentrations of RBCs were measured after cryopreservation. Cells were suspended in a solution composed of 300 mM trehalose, 100 mM NaCl, and 20 mM HEPES-NaOH, pH 7.1, and then frozen at -300 °C min^{-1} and thawed at 55 °C. The intracellular trehalose concentration of the frozen and thawed RBCs was 5.2 ± 2.3 mM, or when just considering the water that is osmotically active in RBCs, trehalose was loaded to concentrations of 7.2 ± 3.1 mM ($n = 6$).

3.8. Scanning Electron Microscopy

Scanning electron microscopy was performed on RBCs to evaluate morphological changes in the cell after cryopreservation. Images of cells were taken before or after freezing and thawing. The cells were washed three times in DPBS or a 300 mM trehalose, 100 mM NaCl, and 20 mM HEPES-NaOH, pH 7.1 solution and were allowed to osmotically equilibrate before fixation (Fig. 5). Echinocytes were observed when the RBCs were resuspended in the cryopreservation solution (Fig. 5 A1 and B1), but the cells were able to return to a discocyte morphology when washed and equilibrated in DPBS (Fig. 6 A1 and B1). There were changes to RBC morphology after freezing and thawing, and the cells appeared more osmotically stressed after cryostorage. When RBCs were washed and equilibrated in the hypertonic cryopreservation solution after freezing and thawing, they expressed many different reversible morphologies such as discocytes, echinocytes, stomatocytes, and irreversible spherocytes (Fig. 5 A2 and B2). Cells that were washed and equilibrated in DPBS after freezing and thawing had morphologies of stomatocytes, spherocytes, and irreversible spheroechinocytes, indicating damage accumulated while equilibrating to isotonic conditions (Fig. 6 A2 and B2).

4. Discussion

This study demonstrates the effectiveness and feasibility of using trehalose as a CPA for RBC cryopreservation. The cryopreservation solution containing 300 mM trehalose, 100 mM NaCl, and 20 mM HEPES had a theoretical osmolarity of 520 mOsm/L, significantly higher than the osmolarity of the porcine plasma. Slow cooling led to significant hemolysis, which is in agreement with previous findings [23]. However, after rapid cooling with LN_2 and thawing, RBC loss was low, and hemoglobin was well retained within the cells. Considering RBC recovery in the 250 million RBCs/ mL trials were measured after a one-step 50-fold dilution with DPBS (270 – 300 mOsm/kg) after thawing, it is unlikely RBCs will lyse due to osmotic injury during transfusion (Fig. 2)[35; 36; 37]. The higher osmolarity of the cryoprotective solution also caused water loss from the RBC cytosol minimizing the probability of intracellular ice formation (Fig. 5 A1 and A2)[38]. Intracellular dehydration due to the hyperosmotic solution combined with the preferential exclusion of trehalose from the surface of biomolecules aid in the protection of phospholipid bilayers during freezing and thawing [39; 40]. Dou et al. (2019) showed a similar effect using hypertonic cryopreservation solutions comprised of trehalose and L-proline[24]. Additionally, the cryopreserved RBCs were able to be stored for up to 84 days after being transferred from LN_2 to -80 °C with no difference in RBC recovery or hemoglobin oxidation during storage (Fig. S4 & S5) ($p > 0.05$, $n = 3$, 3 nested replicates).

Furthermore, RBCs were shown to become permeable to trehalose during rapid cooling or thawing, accumulating ~7 mM trehalose intracellularly. The data from SEM imaging may suggest that RBCs from the same batch have varying amounts of intracellular water contents after freezing and thawing due to their array of morphologies (Fig. 5 A2). This result could be due to disproportional compound loading among the cell population resulting in different intracellular osmotic pressures with varying morphologies despite being exposed to the same tonicity of the extracellular solution. Zhang et al. (2016) showed that less than 50 % of fibroblast cells become loaded with an impermeant compound when frozen in LN_2. The cells that had significant compound uptake after rapid cooling in LN_2 accumulated higher intracellular compound concentrations than observed with incubation and slow cooling loading techniques [41]. A range of intracellular trehalose concentrations in the cell population is hypothesized to be due to the local environments experienced by RBCs when being forced randomly into the freeze concentrated solution during ice formation and thawing, causing a wide range of phospholipid properties.

The oxygen-binding parameters of porcine hemoglobin were unaltered after cryopreservation utilizing trehalose as a cryoprotectant. When analyzing the p50 value and Hill coefficient before and after preservation, no change was observed ($p > 0.05$) (Table 1), and the maintenance of allosteric regulation extends to 2,3 BPG. These encouraging results indicate that cryopreserved RBCs, which employ trehalose as a cryoprotectant, will effectively bind oxygen at the lungs and discharge the gas at other tissues after transfusion.

The current study reinforces the potential of utilizing trehalose as a CPA in the cryopreservation of RBCs, but some additional considerations should be addressed in future studies. Limitations of the current study include porcine blood collection and the age distribution of the RBCs before cryopreservation. Ideally, whole blood would be collected in an anticoagulant citrate phosphate dextrose (CPD) solution, washed to remove plasma, leukoreduced to remove white blood cells, and immediately cryopreserved. Unfortunately, this was not performed for the discussed study and could affect the results obtained by reducing RBC recoveries and providing more variability. However, like blood obtained from the local abattoir, commercially available blood is, on average, also only available after testing and processing, which may take several days on average, and cryopreservation immediately after collection is challenging. Moreover, the morphology of the RBCs observed in Fig. 6 A2 & B2 could be skewed due to hemolysis of RBCs with fragile morphologies during the change in osmotic pressure when being transferred from the cryoprotective solution to PBS.

We anticipate that with further optimization, RBC concentrations approaching transfusion unit levels (~60% hematocrit) can be successfully frozen and thawed using trehalose as a CPA. As shown in early works, the packing effect may play a critical role in phospholipid damage that accumulates at high hematocrit concentrations during rapid cooling, suggesting that intermediate cooling rates may be required to reduce hemolysis [42]. Additionally, the study presented here utilized 1 mL sample volumes processed in 2 mL cryotubes. When the container volume is increased, typically, the diameter increases as well. The reduced volume-to-surface area ratio will have a negative effect on RBC recovery due to the increased time required for thawing. A small diameter bag could be developed to reduce the thawing durations to solve this problem. Studies are currently underway to determine the influence of the packing effect when using trehalose as a CPA, the upper limit in molecular mass of molecules that can be loaded during freezing and thawing, and to develop a theoretical framework of the basic mechanisms that form the foundation of this compound loading technique. Furthermore, long-term stability tests are ongoing as the current study only analyzed cryopreserved RBCs stored for 84 days. This storage time is incomparable to the traditional method that uses glycerol as a CPA and allows RBCs to be stored for up to 30 years, but our initial results obtained over 84 days of storage are highly encouraging.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Figure Captions

Figure 1: Percentage of RBC recovery after freezing in a cryoprotective solution containing: **A)** various concentrations of trehalose (200 mM – 600 mM) and 50 million RBCs/mL in 20 mM HEPES-NaOH, pH 7.1 ($p < 0.05$, $n = 3$, 3 nested replicates), and **B)** inorganic (NaCl) and organic (NaLB) salts at 50 million RBCs/mL, 200 mM trehalose, 20 mM HEPES-NaOH, pH 7.1 ($p < 0.05$, $n = 3$, 3 nested replicates). Thawing was performed at 40 °C.

Figure 2: Percentage of RBC recovery after freezing in 200 mM trehalose, 20 mM HEPES-NaOH, pH 7.1, and 50 million RBCs/mL and thawing at different temperatures ($p<0.05$, $n = 3$, 3 nested replicates). Letter (**A**) represents a significant difference from 40 °C.

Figure 3: Percentage of RBC recovery after freezing in a cryoprotective solution containing 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1, and different concentrations of RBCs (0.25 – 2.5 billion RBCs/mL). Thawing was performed at 55 °C ($p < 0.05$, $n = 3$, 3 nested replicates).

Figure 4: **Grey**) Percent hemolysis in RBC samples that did not undergo freezing and thawing ($n = 3$, 2 nested replicates). **White**) Percent hemolysis of cells after freezing and thawing ($n = 3$, 3 nested replicates). All samples were placed in a cryoprotective solution composed of 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1, and different RBC concentrations. Thawing was performed at 55 °C.

Figure 5: Scanning electron microscopy images of 2.5 billion RBCs/mL exposed to 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1 after: **1)** resuspension in the cryopreservation solution, or **2)** resuspension in the cryopreservation solution, frozen with LN_2, and thawed at 55 °C. RBCs were imaged at magnifications of **A)** 3500X and **B)** 15000X.

Figure 6: Scanning electron microscopy images of 2.5 billion RBCs/mL that were centrifuged, decanted, and resuspended three times in DPBS after **1)** resuspension in a cryopreservation solution composed of 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1, or **2)** resuspension in the cryopreservation solution, frozen with LN_2, and thawed at 55 °C. RBCs were imaged at magnifications of **A)** 3500X and **B)** 15000X.

Figure S1: Temperature recordings with a 3 mm hermetically sealed wire RTD probe. The probe was inserted into a plastic cryotube with a 1 mL sample volume and submerged into LN_2. The composition of the liquid sample was 300 mM trehalose, 100 mM NaCl, and 20 mM HEPES (pH 7.1) (n = 5).

Figure S2: Percent of RBC hemolysis after freezing in a cryoprotective solution containing different concentrations of trehalose (0 mM – 600 mM) and 2.5 billion RBCs/mL, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1. Thawing was performed at 55 °C ($p<0.05$, n = 3, 3 nested replicates).

Figure S3: Temperature profiles of RBCs when thawing in a water bath at temperatures of 30 °C (black), 40 °C (pink), 50 °C (teal), 55 °C (dark purple), 60 °C (light purple), and 70 °C (light blue) (n = 4 – 5). The insert shows the thawing profiles (-25 °C – 70 °C). Arrows indicate when the phase transition from solid to liquid was complete, and the RBCs were removed from the water bath for analysis. The RBCs were thawed for 145 sec at 30 °C, 103 sec at 40 °C, 93 sec at 50 °C, 87 sec at 55 °C, 73 sec at 60 °C, and 58 sec at 70 °C.

Figure S4: Spectrums of porcine hemoglobin from λ = 500 – 650 nm. Hemoglobin was collected from control RBCs **(A)** and frozen and then thawed RBCs after 0 days **(B)**, 28 days **(C)**, 56 days **(D)**, and 84 days **(E)** of storage at -80 °C (n = 3). The spectrums indicate no oxidation of oxyhemoglobin to methemoglobin, indicated by the absence of absorbance at λ = 630 nm [43].

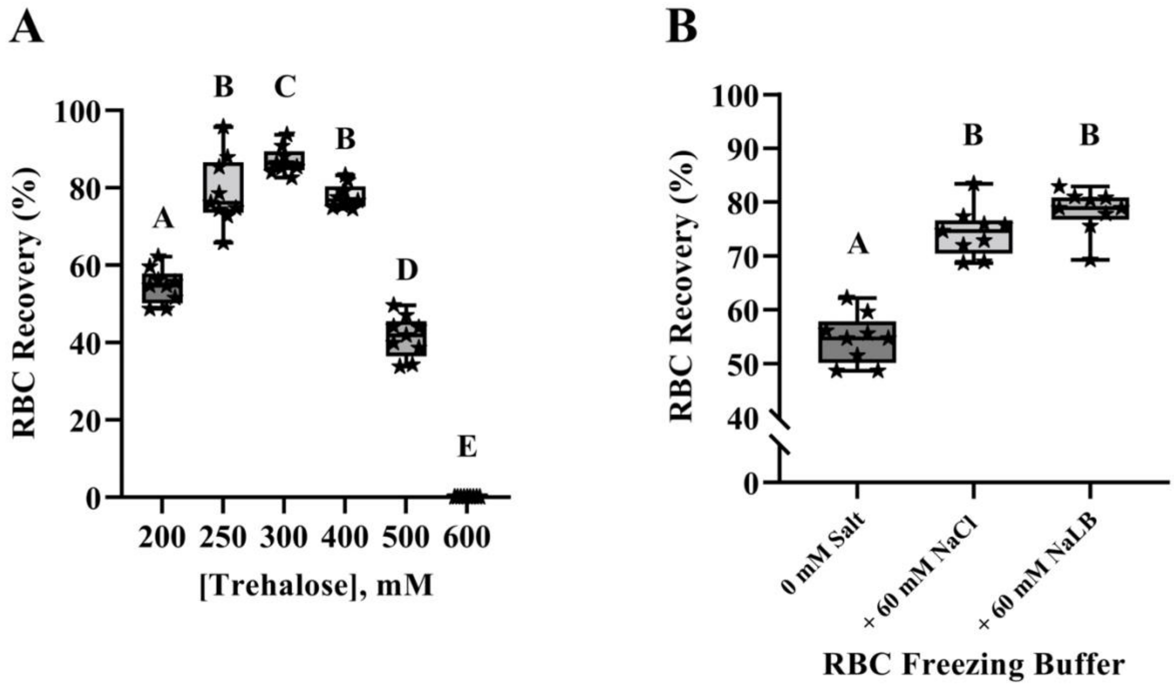
Figure S5: Percentage of RBC recovery after freezing in a cryoprotective solution containing 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1, and 250 million RBCs/mL. After freezing, samples were stored at -80 °C. Thawing was performed after various times (0 – 84 days) at 55 °C ($p > 0.05$, $n = 3$, 3 nested replicates).

Table 1: p50 values and Hill coefficients of hemoglobin before and after freezing and thawing. Trial were conducted with and without 10 mM 2,3 BPG to determine hemoglobin's capability to be allosterically regulated after freezing and thawing.

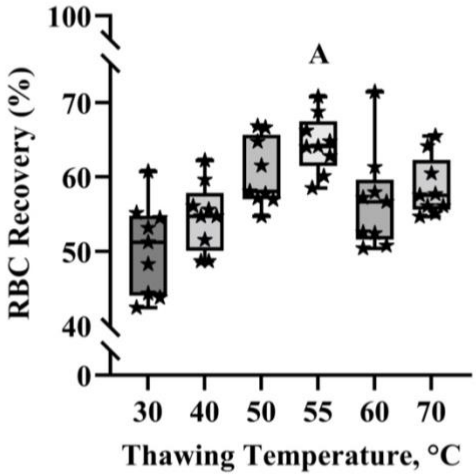
Table S1: Percent of RBCs recovered after freezing in a cryoprotective solution containing different concentrations of trehalose (200 mM – 300 mM) and NaCl (75 mM – 150 mM) using 250 million RBCs/mL in 20 mM HEPES-NaOH, pH 7.1. Thawing was performed at 40 °C ($n = 3$, 3 nested replicates). There was no statistical significance between different NaCl concentrations.

Table S2: Percent of RBCs recovered after slow (-1 °C min^{-1}) and rapid cooling (-300 °C min^{-1}) in a cryoprotective solution containing 250 million RBCs/mL, 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1. Thawing was performed at 55 °C ($n = 6 – 9$).

Figure 1

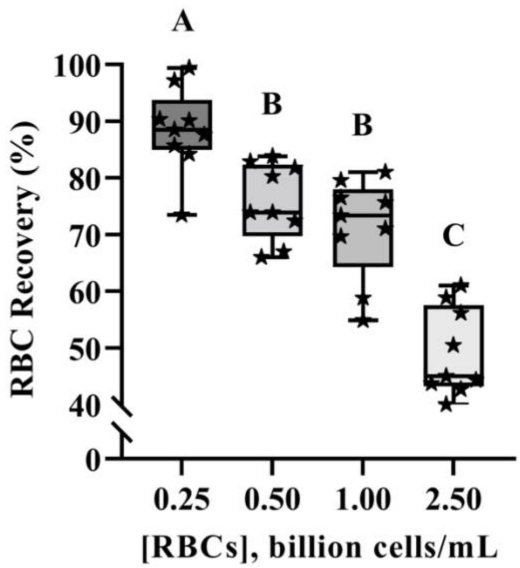

A
B
RBC Recovery (%)
100
80
60
40
20
0
A
B
C
B
D
E
200 250 300 400 500 600
[Trehalose], mM
RBC Recovery (%)
100
90
80
70
60
50
40
0
A
B
B
0 mM Salt
+ 60 mM NaCl
+ 60 mM NaLB
RBC Freezing Buffer

450 **Figure 2**



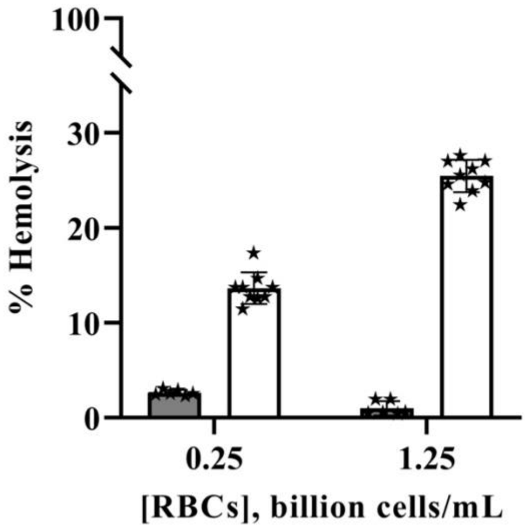
451

Figure 3



A
B
B
C
RBC Recovery (%)
100
90
80
70
60
50
40
0
0.25
0.50
1.00
2.50
[RBCs], billion cells/mL

454 **Figure 4**



455

Figure 5

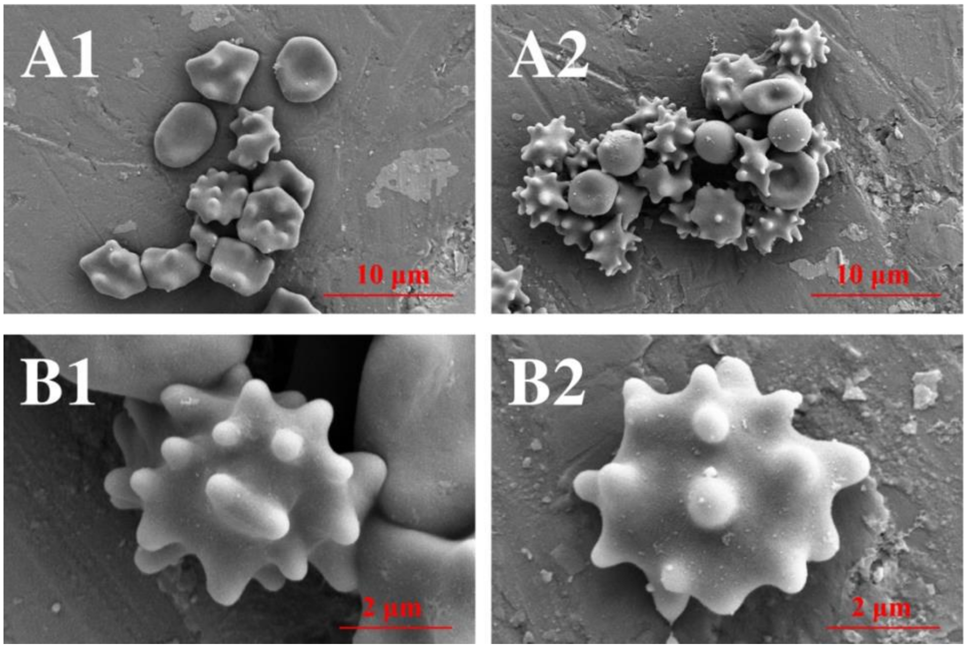


Figure 6

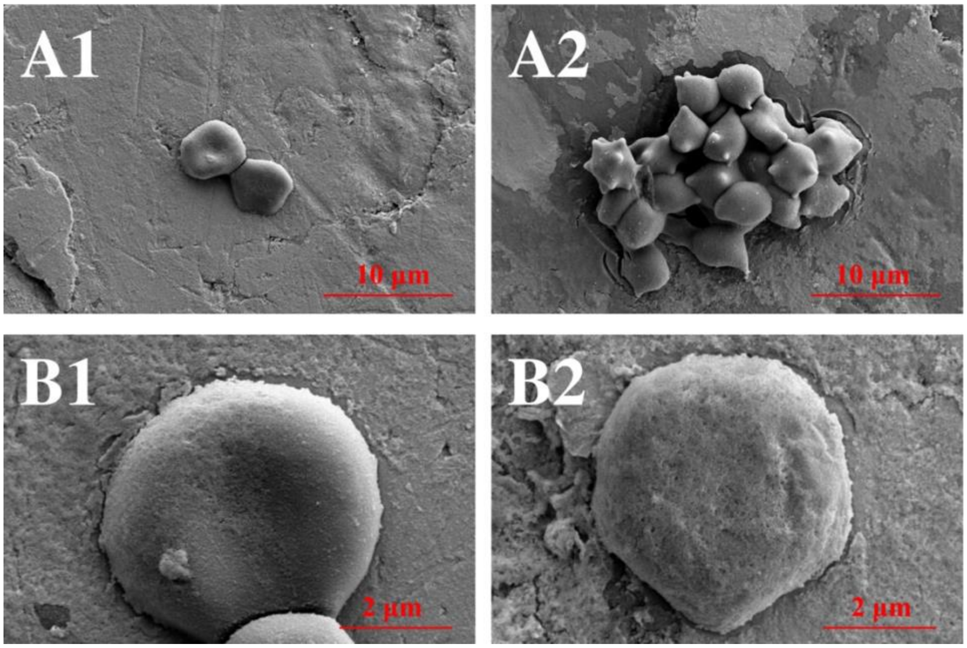
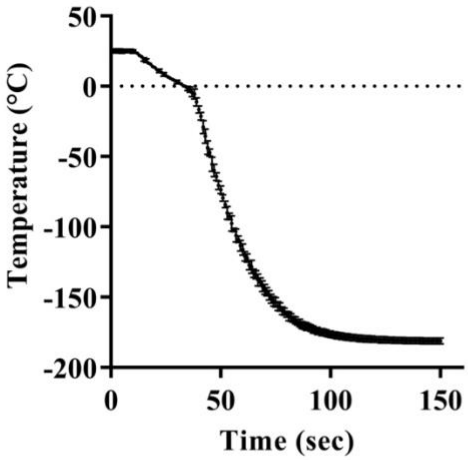
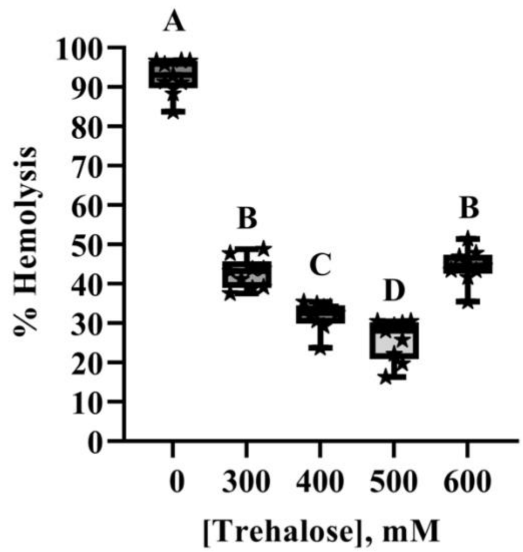


Figure S1

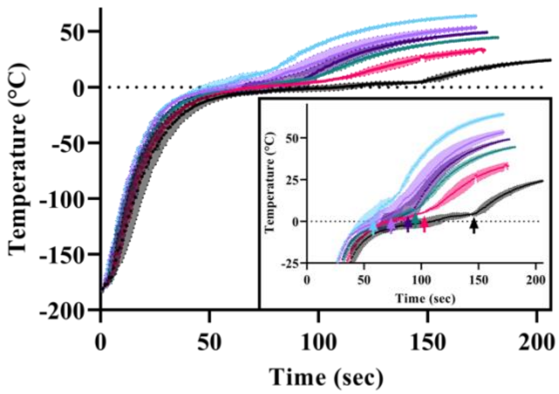


462 **Figure S2**



463

Figure S3



Temperature (°C)
50
0
-50
-100
-150
-200
0
50
100
150
200
Time (sec)
Temperature (°C)
50
25
0
-25
0
50
100
150
200
Time (sec)

Figure S4

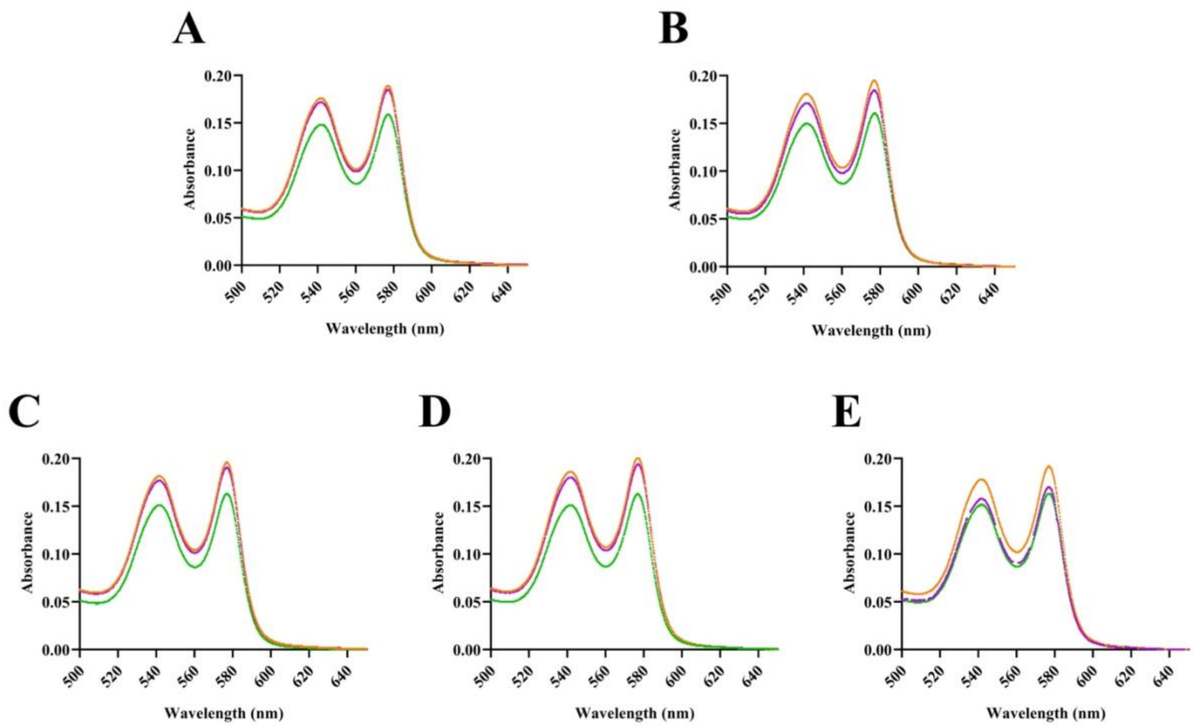

A
B
C
D
E
Absorbance
Wavelength (nm)
0.00
0.05
0.10
0.15
0.20
500
520
540
560
580
600
620
640

Figure S5

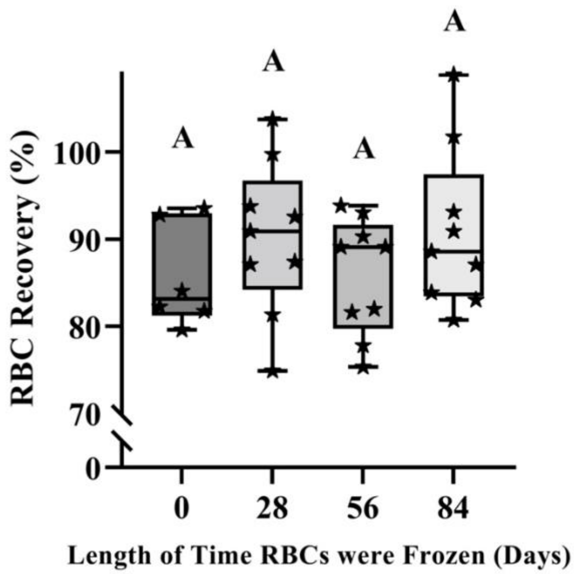

A
A
A
A
RBC Recovery (%)
100
90
80
70
0
0
28
56
84
Length of Time RBCs were Frozen (Days)

Table 1

p50 Values						
	0 mM 2,3 BPG			10 mM 2,3 BPG		
Condition	*M*	*SD*	*N*	*M*	*SD*	*N*
Control	7.45	0.11	5	12.07	1.97	5
Freeze/Thaw	7.20	1.12	3	12.60	0.54	3
Hill Coefficients						
Control	2.82	0.22	5	2.87	0.36	5
Freeze/Thaw	2.61	0.29	3	2.39	0.27	3

Table S1

Cryopreservation Solution Composition

[Trehalose]	% of Cells with Intact Membranes		
200 mM	79 ± 5	73 ± 8	74 ± 8
250 mM	74 ± 9	83 ± 7	75 ± 6
300 mM	85 ± 7	86 ± 12	83 ± 6
[NaCl]	75 mM	100 mM	150 mM

Table S2

Cooling Rate	RBC Recovery (%)
-1 ºC min^{-1}	1 ± 1 %
-300 ºC min^{-1}	88 ± 8 %

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