TOWARD TRANSLATIONAL IMPACT OF NORMOGLYCEMIC RED BLOOD CELL STORAGE

By

Logan Dallas Soule

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ABSTRACT

Red blood cell (RBC) transfusions are life-saving procedures for a wide variety of patient populations, resulting in nearly 30,000 transfusions each day within the United Sates. However, transfusions can also result in complications for patients, including inflammation, edema, infection, and organ dysfunction. These poor transfusion outcomes may be related to irreversible chemical and physical damages that occur to RBCs during storage, called the “storage lesion”. These damages, including diminished ATP production/release, decreased deformability, increased oxidative stress, and increased membrane damage, may result in poor functionality when transfused. The damage that occurs during storage may be due to the hyperglycemic nature of current anticoagulants and additive solutions used for RBC storage. All FDA approved storage solutions contain glucose at concentrations that are over 8x higher than the blood stream of a healthy individual. Previous work has already shown that storing RBCs at physiological concentrations of glucose (4-6 mM), or normoglycemic conditions, resulted in the alleviation of many storage-induced damages, including an increase in ATP release, increased deformability, reduced osmotic fragility, and decreased oxidative stress. However, this storage technique was also accompanied by many limitations in its translation to clinical practice. The manual feeding of glucose to normoglycemic stored RBCs to maintain physiological levels of glucose introduced both a breach in sterility and unreasonable labor requirements that could not be translated to clinical practice. Additionally, the low-volume storage (< 2 mL) method with custom PVC bags used in previous work may not illicit similar benefits when scaled up to larger volumes with commercially available blood collection bags.

This work overcame these limitations through the design and implementation of an autonomous glucose delivery system that maintained normoglycemia of stored RBCs

autonomously for 39 days in storage, while also maintaining sterility. This system was then used to store RBCs under normoglycemic conditions and monitor key storage lesion indicators, resulting in reduced osmotic fragility, decreased oxidative stress, and reduced morphological changes. There was also no impact on glycolytic activity or hemolysis levels, improving upon previous work which reported significant hemolysis that surpassed the FDA threshold of 1%. These data solidify and improve upon previous results, indicating that normoglycemic RBC storage results in reduced damages in storage that may translate to better *in vivo* function. The autonomous glucose delivery system also significantly advances the applicability of the normoglycemic storage technique to clinical practice, making large scale studies now possible.

An alternative strategy to combat RBC storage-induced damage was investigated through the use of albumin as a novel rejuvenating agent. Albumin, a 66 kDa protein, was able to reverse the echinocytic shape transformations seen during RBC storage, resulting in RBCs closer in shape and size to that of fresh RBCs. These data indicate that this phenomenon is likely due to a change in the Donnan equilibrium that reverses echinocytosis, resulting in a recovery of RBC shape and size. Rejuvenation of stored RBCs with albumin had no impact on phosphatidylserine externalization, reactive oxygen species generation, or osmotic fragility in comparison to a buffer without albumin, supporting the hypothesis that rejuvenation does not occur via lipid interaction, osmotic changes, or antioxidant abilities of albumin, but rather by alteration of the Donnan equilibrium in favor of stomatocytosis. These data highlight a possible mechanism responsible for RBC rejuvenation via albumin that may result in improved *in vivo* function.

I dedicate this work to Mom, Dad, Dakota, Gavin, Lexi, and Butter.
Thank you for your unconditional love and support.
I love you all!

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LIST OF SYMBOLS

E – enzyme

S - substrate

k_{on} – enzyme-substrate association rate constant (M^{-1} s^{-1})

k_{off} – enzyme-substrate dissociation rate constant (s^{-1})

ES – enzyme-substrate complex

k_{cat} – enzyme catalysis rate constant (s^{-1})

P - product

$v_{reaction}$ – enzyme reaction velocity (M s^{-1})

V_{max} – maximum enzyme reaction velocity (M s^{-1})

$[S]$ – substrate concentration (M)

k_m – Michaelis constant (M)

$[E]_{total}$ – total enzyme concentration (M)

k – rate constant

A – pre-exponential factor, related to frequency of molecular collisions

E_a – activation energy (J mol^{-1})

R – gas constant, 8.314 J mol^{-1} K^{-1}

T – temperature (K)

K_2 - rate constant at second temperature

K_1 – rate constant at first temperature

T_2 – second temperature (K)

T_1 – first temperature (K)

r – radius of rotation (m)

F_{cp} – centripetal force (N)

F_{cf} – centrifugal force (N)

m – mass of sample (kg)

u_t – tangential velocity (m s^{-1})

u_s – settling velocity (m s^{-1})

d – diameter of particle (m)

g – acceleration due to gravity, 9.81 m s^{-2}

ρ_p – density of the particle (kg m^{-3})

ρ_f – density of the fluid (kg m^{-3})

η – fluid viscosity (Pa s)

J - mass transfer rate (mol s^{-1})

D – diffusivity coefficient (m^2 s^{-1})

A – surface area of diffusion (m^2)

C – concentration (M)

x – distance (m)

t – time (s)

P – pressure (mm Hg or Pa)

h – height (cm)

ρ – density (kg m^{-3})

v – velocity (m/s)

f – friction factor

L – length (m)

D_t – diameter of tubing (m)

Q – volumetric flow rate ($m^3\ s^{-1}$)

V_d – dispensing volume (mL)

V_0 – initial volume (mL)

$Gluc_{actual}$ – actual glucose concentration of stored red blood cells (mM)

$Glu_{measured}$ – glucose concentration measured from glucometer (mM)

HbSN – hemoglobin in the supernatant (mg/mL)

HbRBC – hemoglobin in the RBC pellet (mg/mL)

Hct% - hematocrit (%)

OF – osmotic fragility (%)

$Abs_{0.0\%}$ - absorbance of RBC supernatant in pure water

$Abs_{0.45\%}$ - absorbance of RBC supernatant in 0.45% NaCl

$Abs_{0.9\%}$ - absorbance of RBC supernatant in 0.9% NaCl

Chapter 1 – Introduction

1.1 Blood Banking and Transfusion: Current Standard of Care

According to the American Red Cross, approximately 29,000 units of red blood cells (RBCs) are needed every day within the U.S.[1] A unit of RBCs is approximately 350 mL in total volume with 50-60% of the volume occupied by RBCs. Though the average RBC transfusion is approximately 3 units, acute hemorrhagic patients such as extreme trauma victims can require as many as 100 units.[1] The supply and demand of RBC units are highly dependent on a variety of factors including natural disasters,[2–4] pandemics/epidemics,[2,5,6] population characteristics,[7,8] geographic regions,[9,10] processing methods,[11] and many other variables.[12] When the supply of blood products is high and demand is low, there is limited burden on patient care. However, during blood shortages, such as the most recent shortage due low donation rates during the COVID-19 pandemic, healthcare providers are forced to make difficult decisions on which patients receive transfusions, generating much greater risk to patient care.[13,14] Unlike many of the modern mass-manufactured drug products such as biologics, vaccines, and chemically synthesized active pharmaceutical ingredients, blood components cannot be manufactured. Therefore, blood banks rely solely on donations to maintain adequate supply. Advancements in the collection, processing, and storage of blood products are therefore crucial to improving the standard of care for transfusion patients.

The current standard of care for blood collection, processing, and storage is outlined in figure 1.1. Approximately 450 mL of whole blood is first collected from an informed and consented donor into an anticoagulant solution. After sufficient mixing, this primary bag is centrifuged to separate the donated blood into its components: platelet rich plasma, leukocytes, and packed RBCs. The platelet rich plasma is removed by aspiration, and the packed RBCs are

then passed through a leukocyte filter to remove unwanted leukocytes from the final storage productand subsequently mixed with an appropriate additive solution for long term storage at 2-6 ºC for up to 42 days, or until the unit is requested for transfusion.[15–17]

RBC transfusions are important to patients for multiple reasons. Not only does the introduction of exogenous RBCs into the circulation raise the hematocrit, or the volume percentage of whole blood that is RBCs, but also the level of hemoglobin in the patient.[18,19] The loading and offloading of oxygen by hemoglobin is the primary source of oxygen for organs and tissues. The RBC is the primary transporter of oxygenated hemoglobin to tissues in need, with hemoglobin making up more than 98% of the total RBC protein mass.[20,21] Though typical hemoglobin concentrations are 14-18 g/dL for men and 12-16 g/dL for women, the Association for the Advancement of Blood & Biotherapies (AABB) recommends that RBC units be transfused only when a patient's hemoglobin level falls below 7 or 8 g/dL for most cases.[22–24] This is due to clinical trial data suggesting that a more restrictive hemoglobin threshold (7-8 g/dL) does not adversely affect patient-important outcomes in comparison to the previously recommended, more liberal

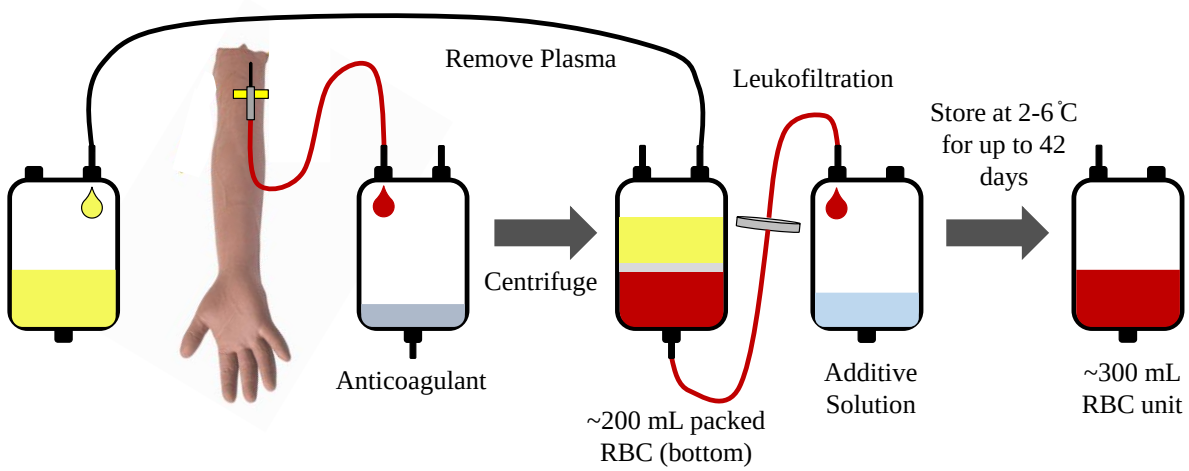

Figure 1.1: **Current standard of care for blood collection and processing for RBC units.** After initial collection and separation into the blood components, the ~200 mL of packed RBCs are leukoreduced and transferred to a separate bag containing an additive solution, resulting in an RBC unit of ~300 mL and 55-60% hematocrit. The unit is stored at 2-6 ºC for up to 42 days.

transfusion threshold (9-10 g/dL).[23]

When patients require a transfusion, especially patients with traumatic injury, there is often a short window of time between admission and requirement of transfusion before suffering severe consequences, such as death. A retrospective study on the turnaround time from ordering RBC units to transfusion initiation found the average time to be 12 minutes for life-saving cases.[25] Without modern blood storage procedures and, consequently, a reliable supply of RBC units on-demand, the relatively short turnaround time for transfusion could not be realized, and patient morbidity and mortality would likely increase. This short timespan for patient care can mostly be attributed to the infrastructure that has been cultivated from decades of research, innovation, and advancements as it pertains to blood collection, processing, storage, and transfusion.

Though undoubtedly beneficial to patients, transfusions are not without their risks, and can sometimes be life-threatening. Many adverse clinical outcomes related to blood transfusion, such as inflammation, iron overload, transfusion related acute lung injury (TRALI), and insufficient nitric oxide bioavailability (INOBA), may be attributed to damages incurred to RBCs from prolonged storage outside of the body.[26] These damages, collectively termed the "storage lesion", negatively impact vital RBC functions, including ATP release, RBC deformability, membrane integrity, oxygen delivery, and many more.[26] The goal of much of the blood storage research and advancements in recent years has focused on reducing the impact of the storage lesion on RBC health and, subsequently, patient outcomes.[26–28] However, in light of published transfusion-related adverse clinical outcomes, it is clear that current blood storage methods fall short of providing the highest quality transfusion product to patients. To advance the current practices in the field of blood storage, however, an appreciation of the history surrounding blood collection and storage is first necessary.

1.2 Advancements in Blood Banking: Principles of Engineering

The potential benefit of transfusing blood into anemic or hemorrhagic patients was recognized long before blood storage was made possible. The first successful blood transfusion was conducted by Richard Lower in 1665 by using a direct whole blood transfer from one dog to another, alleviating the hemorrhagic shock suffered by the recipient canine through adequate resupply of lost RBCs.[29,30] Following his success, much progress was made in the understanding of blood and transfusion medicine over the next several hundred years. This included the first successful human-to-human transfusion and the discovery of blood types.[31–34] However, since blood could not be stored and preserved, all transfusions relied on the direct donor-recipient method, requiring close proximity, multiple personnel, and a readily-available donor.[35] The main barrier to early blood storage, and even some transfusion methods, was the tendency of blood to coagulate once removed from the body.[31,36] This was overcome by the use of sodium citrate as an anticoagulant, leading to the first successful indirect human transfusion with citrated blood by Albert Hustin in 1914.[37] That same year, Richard Weil reported studies on refrigeration of citrated blood and successful transfusions of guinea pigs, dogs, and even humans with up to 5-day old citrated blood stored in hypothermic conditions.[38] The combination of Hustin and Weil's discoveries led to advancements in blood storage that are key to modern day blood banking success.

1.2.1 Metabolism Kinetics of Cold Storage

Although unknown to Weil at the time of his publication on cold-stored blood,[38] the success of his transfusions after 5-day storage was due to a significant decrease in the RBC metabolism during storage. Like all living cells, RBCs require the high-energy molecule adenosine triphosphate (ATP) to perform cellular functions. Unlike many other cell types, however, RBCs

rely solely on anaerobic glycolysis for ATP production.[39] The glycolytic pathway (figure 1.2) within the RBC cytoplasm converts glucose to lactate through a series of enzymatic reactions, generating a net gain of 2 ATP molecules.[40] Within the circulation, RBC glycolytic activity consumes glucose at a rate of 12 mg dL^{-1} hr^{-1} or 1.85 µM s^{-1}, with the conversion of fructose-6-phosphate to fructose-1,6-phosphate by phosphofructokinase being a major rate-limiting reaction

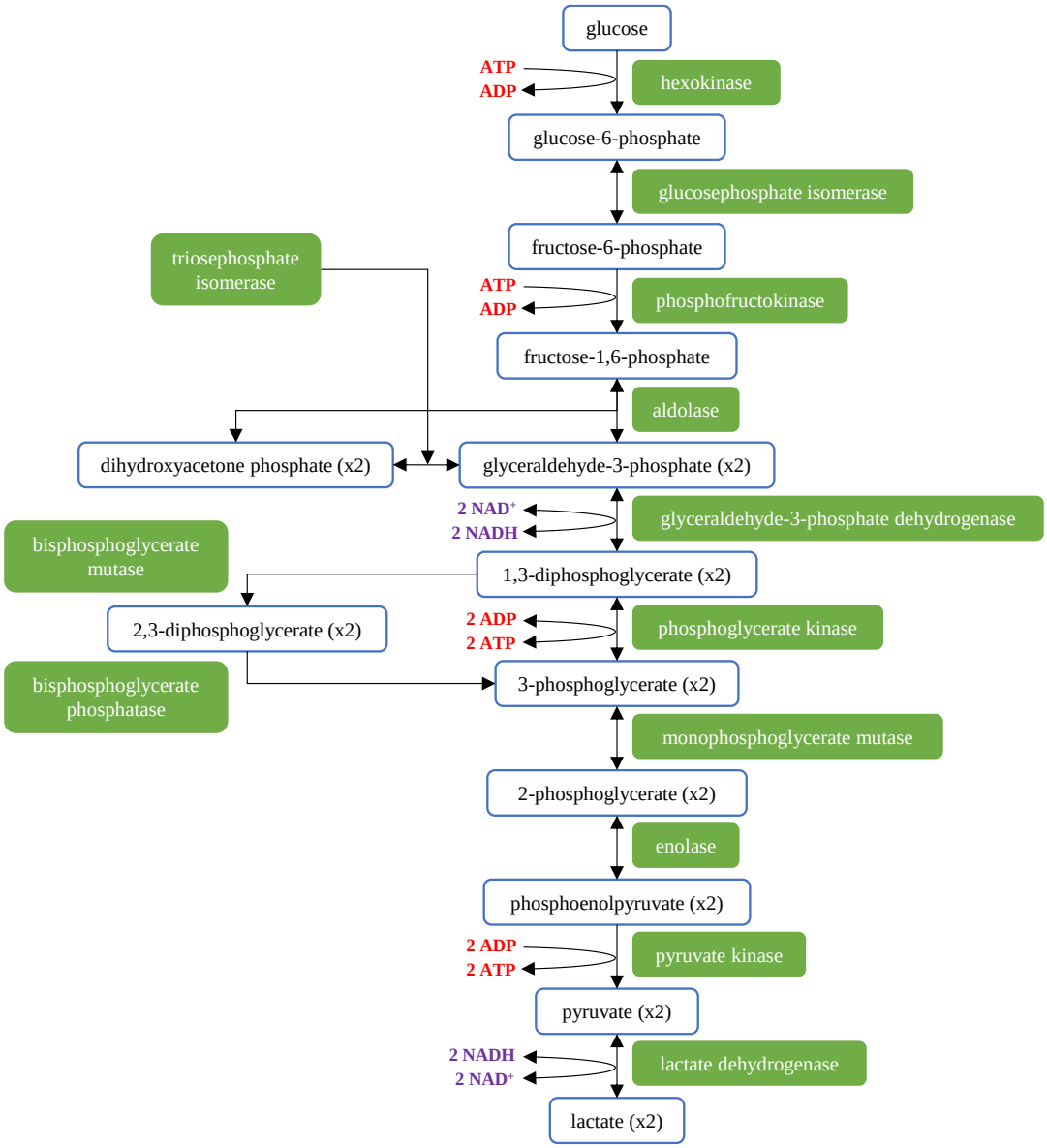

Figure 1.2: **Anerobic glycolysis pathway occurring within RBCs to generate a net of 2 ATP**. This process still proceeds during cold-storage, albeit at a much slower rate. With no waste clearance mechanism, this slower glucose metabolism and decreased waste generation allows the cells to survive for several days.

step.[40,41] However, during cold storage of RBCs, the glycolysis rate can drop by 10-15% per degree Celsius[42] due to the reduction in temperature and its resultant effect on enzyme kinetics. The enzymatic activity of phosphofructokinase and all other enzymes in the glycolytic pathway can be modeled using Michaelis-Menten kinetics. In 1913, through a series of experiments, Michaelis and Menten were able to show that steady state reaction velocities of single-substrate enzymatic reactions were proportional to the free ligand concentration as shown in equations 1.1 and 1.2, which introduced important kinetic parameters including the maximum reaction velocity (V_{max}) and the Michaelis constant (K_m) shown in equations 1.3-1.4.[43,44] Although the Michaelis-Menten equation describes a one-substrate enzyme model, the principles of Michaelis-Menten kinetics still apply to two-substrate systems.[45]

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \quad (1.1)$$

$$v_{reaction} = \frac{V_{max}[S]}{K_m + [S]} \quad (1.2)$$

$$V_{max} = k_{cat}[E]_{total} \quad (1.3)$$

$$K_m = \frac{k_{off} + k_{cat}}{k_{on}} \quad (1.4)$$

Important to note from these discoveries, K_m, V_{max}, and therefore, enzymatic reaction velocities are all dependent on the individual rate constants, k_{on}, k_{off}, and k_{cat}. These reaction rate constants are directly proportional to the temperature of the reaction via the Arrhenius equation shown in equation 1.5.[46]

$$k = Ae^{\frac{-E_a}{RT}} \quad (1.5)$$

A manipulation of equation 1.5 can be used to compare the reaction rates at two different temperatures, shown in equation 1.6.

$$\ln\left(\frac{k_2}{k_1}\right) = \frac{E_a}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \quad (1.6)$$

Utilizing the Arrhenius principle, the reaction kinetics of glycolytic enzymes within RBCs during cold storage can be modeled, specifically the rate-limiting enzyme, phosphofructokinase. Substituting the appropriate activation energy for phosphofructokinase of 29.1 kJ mol^{-1}, the ratio of the rate constant at 37 ºC (k_2) to that at 4 ºC (k_1) can be determined to be approximately 3.8.[47] This means that k_{cat}, and therefore V_{max}, for phosphofructokinase in RBCs is expected to be 3.8 times higher at the physiological temperature of 37 ºC in comparison to the cold storage temperature of 4 ºC. This is indeed similar to values found in the literature, experimentally.[42,48] This significant drop in enzyme activity due to cold-storage enabled Weil to store citrated blood for up to 5 days, as opposed to only a few hours at room temperature. In the circulation at 37 ºC, this relatively higher glycolytic reaction velocity can be sustained through equally efficient waste clearance mechanisms, including the clearance of lactate. The accumulation of lactate is usually metabolized by the liver and kidney, while the associated acidosis is managed by bicarbonate buffering and the exhalation of CO_2.[49,50] However, once blood is removed from the body, these clearance mechanisms are no longer available to compensate for the high glycolytic needs of the RBC, resulting in rapid acidosis, increased lactate production, and decreased glucose availability, which negatively impacts the rate of RBC glycolysis and ATP production, eventually leading to significant hemolysis.[26,51] The storage of RBCs at hypothermic conditions reduces the rate of glycolysis by a factor of 3.8, accompanied by an equivalent decrease in waste production and substrate consumption.[48] The combination of lower energy requirements and decreased waste production during cold storage resulted in RBCs that could survive for up to 5 days *ex vivo* and allowed Weil to report successful transfusions after storage.

1.2.2 Blood Component Separation

In 1916, Francis Peyton Rous and Joseph R. Turner began studies on anticoagulants

containing sucrose and dextrose, discovering that blood could be preserved for up to 4 weeks with the addition of glucose to sodium citrate.[52] However, since the glucose would caramelize and decompose when heated, the Rous-Turner solution could not be autoclaved.[53] This led to citrated blood becoming the standard for blood collection and storage until the addition of glucose was revisited by Loutit and Mollison in 1943. Loutit and Mollison discovered that the acidification of the anticoagulant to a pH below 5.86 prevented glucose caramelization during autoclaving, while still imparting the preservative properties of glucose.[54] The new anticoagulant, acid-citrate-dextrose, or ACD, allowed for the sterile, safe, and effective storage of blood for up to 21 days.[53,54]

It was around this same time, in the 1930s and 1940s, that the advantages of blood component therapy (BCT) were discovered.[55] It was the work of John Elliot, John Scudder, and the "father of the blood bank", Dr. Charles Drew, that highlighted not only the possibility of blood component separation, but that separated plasma could be stored for up to two months and remain suitable as an effective treatment of hemorrhagic shock resuscitation.[55–59] Blood component separation was accomplished through centrifugation and sedimentation. Sedimentation is the process by which particles in suspension will settle to the bottom of a vessel due to a force such as gravity, while centrifugation is the process of separating solution components according to their density by the application of a centrifugal force.[60] Both techniques utilize differences in density of the cell types to separate the blood components. Whole blood consists of many different cell types with varying cell sizes, cell number, and densities, outlined in figure 1.3a. These cell types include RBCs, platelets, and white blood cells (WBCs), all suspended in plasma.[61] The circulating WBCs can then be further categorized into neutrophils, basophils, eosinophils, monocytes, B cells and T cells (lymphocytes).[61,62] Each of these components has a specific and slightly different size and density, which allows these components to be separated by centrifugation and sedimentation.

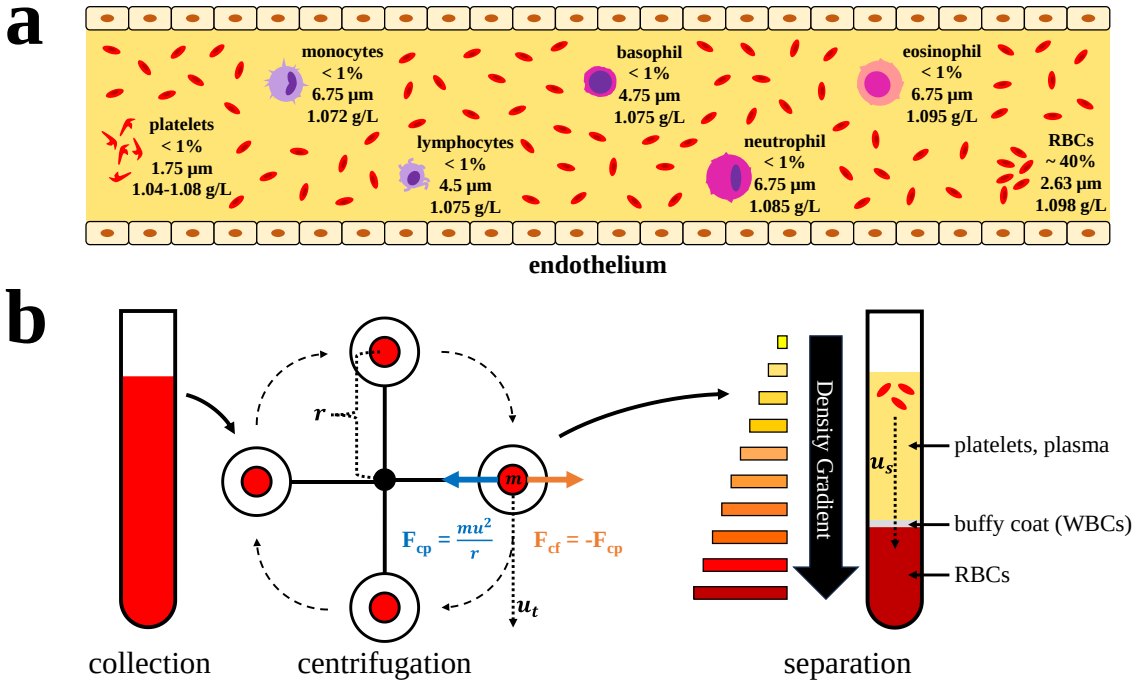

Figure 1.3: **Principles of blood component separation by centrifugation.** (a) A breakdown of all cell types within circulation, identifying their relative volumetric percentage of whole blood, their respective effective radius and cellular density. (b) Process of centrifugation and separation of whole blood into its components, highlighting the engineering principles governing this phenomenon. Centrifugation generates a centripetal force (F_{cp}) and an apparent centrifugal force (F_{cf}) in equal and opposite directions. This force is equal to the mass of the object (m) multiplied by the square of the tangential velocity (u) divided by the radius of rotation about the axis (r).

The velocity (u_s) at which these particles fall is dependent on the particle size, particle density, fluid density, and fluid viscosity. This velocity can be calculated via Stoke's Law in equation 1.7, which is derived from the steady-state equilibrium of buoyant, drag, and gravitational forces.[60]

$$u = \frac{d^2 g(\rho_p - \rho_f)}{18\eta} \qquad (1.7)$$

The settling velocities for each of the cell types within whole blood can be calculated using Stoke's Law and are shown in Table 1.1.[63,64]

As shown in table 1.1, cells with greater size and/or density also exhibit greater settling velocity, however, given sufficient time, it is the cell density that dictates separation order. This is

Table 1.1: **Breakdown of whole blood components, their respective densities and effective radii taken from the literature**.[62,64–66] These densities and radii were utilized in equation 1.7 to calculate the settling velocities of each component. This dictates the length of time required for these components to settle.

Cell Type	**Density ($g\ mL^{-1}$)**	**Effective Radius (μm)**	**Settling Velocity ($\mu m\ s^{-1}$)**
Plasma	1.025	-	-
Platelets (low density)	1.04	1.75	0.022
Platelets (high density)	1.08	1.75	0.082
Monocyte	1.072	6.75	1.0
Lymphocytes (B and T cells)	1.075	4.50	0.49
Basophil	1.075	4.75	0.55
Neutrophil	1.085	6.75	1.3
Eosinophil	1.095	6.75	1.5
RBC (singular)	1.098	2.63	0.24
RBC (aggregate)	1.098	50.0	88

because the surrounding fluid density will increase as denser particles begin to fall, creating a density gradient within the vessel. The cells will continue to fall until their density matches the surrounding fluid density, reaching a settling velocity of 0. It is important to note that although singular RBCs have a lesser settling velocity than the WBCs, it has been reported that RBCs will aggregate when settling, resulting in a much larger pseudo-particle and greater settling velocity.[61,66] The process of RBC sedimentation and these settling velocities is precisely what Oswald H. Robertson relied on when transfusing his patients during WWI.[67] The anticoagulants used by Robertson contained citrate at concentrations that were unsafe for patient administration, requiring the settling of RBCs and removal of the citrate-rich supernatant before transfusion.[42] However, he often waited days for the RBCs to settle by gravity due to the significantly slow

settling velocities shown in table 1.1.[67,68]

Fortunately, centrifugation was able to exploit the principles of sedimentation, while expediting the process through the application of an acceleration force far exceeding that of gravity. Centrifugation is the process of separating solution components by density through the extreme rotation of the solution about an axis. This extreme rotation generates a centripetal force inward toward the axis of rotation and an apparent centrifugal force felt by the sample that is equal and opposite to the centripetal force (Figure 1.3b).[69] In many biological applications, including the separation of blood components, this centrifugal force can be 100-2000 times that of gravity. Applying this acceleration to Stoke's Law in equation 1.7 by replacing the acceleration of gravity (g) with the centrifugal acceleration, settling velocities would be 100-2000 times greater than their respective velocities generated by gravity. This is why the complete separation of blood components can be accomplished in minutes through centrifugation as opposed to hours or days via gravity-driven sedimentation. Again, given sufficient separation time, these cell types will separate via a density-dependent manner, with denser cells migrating to the bottom. Centrifugation of whole blood results in the separation of blood into 3 distinct layers: platelet rich plasma, WBCs or the buffy coat, and the packed RBCs (figure 3.3b).

The application of centrifugation by Dr. Charles Drew in the separation of blood components allowed for the shipment of over 5,000 plasma products to England and the success of the Blood for Britain program in 1940 to 1941.[57] Though initially practiced for plasma collection, a fortunate consequence of blood component separation led to the ability to collect and store blood components independently, though this would not become standard practice for RBC storage until much later in the 1960s.[53]

1.2.3 Molecular Diffusion in Plastic Bag Storage

Before blood component separation was utilized for the isolation and storage of packed RBCs, several advancements were made to whole blood collection and storage. The first was the development of citrate-phosphate-dextrose (CPD) in 1957 by Gibson, which saw the addition of phosphate to the ACD anticoagulant to compensate for the loss of inorganic phosphates from RBCs during storage.[53,70] This improvement led to post-transfusion recoveries at or above 70% for up to 28 days in storage.[70] Post-transfusion recovery percentage, a gold standard for the evaluation of a blood storage technique, represents the percentage of stored RBCs that remain in circulation 24 hours after transfusion. The minimum threshold for recovery percentage set by the Food and Drug Administration (FDA) is 75%, while the maximum threshold for *in vitro* hemolysis is 1%.[71]

Another advancement in blood storage was the development and usage of plasticized polyvinylchloride (PVC) bags for blood storage. Prior to the 1950s, blood collection and storage was performed using glass bottles. These bottles were cumbersome due to their weight, their necessity for decontamination if re-used, and their susceptibility for contamination and breakage.[72,73] It was primarily due to the work of Dr. Carl Walter from 1947 through the 1950s that led to the development of the first plastic blood collection bag.[74] His PVC blood bag plasticized with di-2-ethylhexyl-phthalate (DEHP) revolutionized whole blood collection and storage, resulting in a four-fold decrease in hemolysis, a drop in bacterial contamination rates, decreased air embolism occurrences, optical clarity, and easier transport due to a decrease in weight and fragility.[53,72] His blood collection and storage technique also paved the way for blood component separation through his two- and three-bag collection systems.[74,75]

The PVC polymer is normally a rigid plastic, but the introduction of the DEHP plasticizer at 40% w/w provided flexibility, elasticity, and could withstand high heat during sterilization,

making it suitable for blood collection.[76] An unexpected consequence of DEHP plasticization was its leaching and diffusion into the blood storage product. Fortunately, by the time the presence of DEHP was detected in blood components, many reports had indicated that DEHP was responsible for decreased hemolysis, decreased membrane osmotic fragility, decreased microvesiculation, and increased post-transfusion recoveries. This is due to the incorporation of DEHP into the RBC membrane, compensating for membrane loss.[75–78] The DEHP also made the PVC much more permeable to the diffusion of gases both into and out of the blood storage bag.[75] This enabled the removal of CO_2 generated from the RBC bicarbonate buffering system out of the bag through passive diffusion. Unfortunately, this permeability also allowed the diffusion of oxygen into the stored RBCs, which has been reported to lead to the autooxidation of hemoglobin and resultant generation of reactive oxygen species (ROS).[26] The passive diffusion of DEHP, CO_2, and O_2, across the blood storage container is driven by mass transport and described by Fick's first and second laws of diffusion as shown in equations 1.8 and 1.9 respectively.

$$J = -DA\frac{dC}{dx} \tag{1.8}$$

$$\frac{dC}{dt} = D\frac{d^2C}{dx^2} \tag{1.9}$$

Fick's First Law states that the mass transfer rate (J) through a substance is proportional to the concentration gradient $\left(\frac{dC}{dx}\right)$ within and/or across that substance. This rate of mass transfer is also dependent on the area of transfer (A) and the diffusivity coefficient (D). In the case of the diffusion of gases, the concentration gradient is replaced with a partial pressure gradient $\left(\frac{dP}{dx}\right)$, and the diffusivity coefficient is replaced with the Krogh coefficient, the product of the diffusivity coefficient and solubility. The diffusivity coefficient, or the Krogh coefficient, is specific to both the diffusing molecule and the environment it is diffusing through. For example, diffusivity

coefficients through a gas are generally 10,000 times greater than diffusivity coefficients through a liquid.[79,80] In the context of blood storage, mass transport of O_2, CO_2, and DEHP is limited by their rates of diffusion through the thickness of the PVC bag, since the diffusion rates are at least 10,000 times greater both within the whole blood and the outside atmosphere. A diagram of the relative concentrations of these molecules during blood storage as a function of position is shown in figure 1.4. Note that the blood storage system never reaches steady state regarding the concentrations of these molecules at any position, meaning that concentration is a function of both position and time, requiring the incorporation of Fick's second law (equation 1.9). However, this would complicate the model beyond the necessary scope, so the concentration gradients at time 0 and 42 days is shown in figure 1.4, using reported concentrations from the literature.[74,81–85]

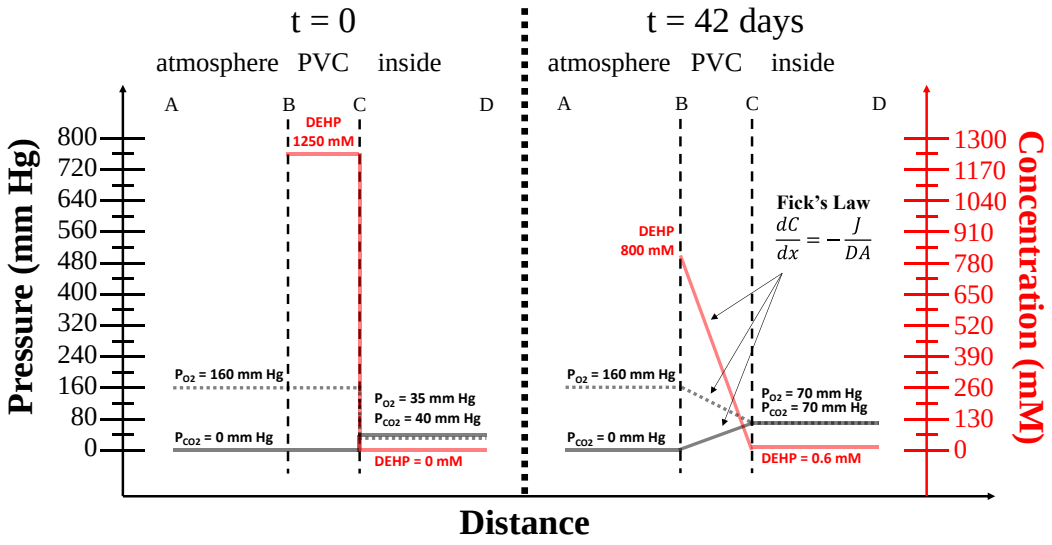

Figure 1.4: **Modeling molecular diffusion within blood storage using Fick's Law**. At time 0, when blood enters the bag, the concentration/pressure gradients are established, driving mass transport into or out of the bag. At the end of storage (42 days), DEHP and O_2 have diffused through the plasticized PVC and into the stored blood, raising the concentrations inside of the bag, while CO_2 has diffused out of the bag. The slopes of the concentration/pressure gradients within the PVC bag represent mass transport via Fick's first law. Although concentration gradients for these molecules would also exist inside the bag, they would be 10,000-fold smaller than the gradients within the plasticized PVC due to the significant increase in diffusivity coefficients through liquids. This 10,000-fold increase in diffusivity creates a near-zero slope in concentration relative to the diffusion within the PVC.

At time zero, the concentration of DEHP within the walls of the PVC bag is assumed to be evenly distributed at 40% w/w. Similarly, the partial pressures of O_2 and CO_2 are assumed to remain at atmospheric levels, both outside and within the PVC walls. The concentrations/pressures of these molecules within the stored blood at time 0 are assumed to be characteristic of whole venous blood (0 mM DEHP, 35 mm Hg O_2, and 40 mm Hg CO_2).[81,82] After 42 days in storage, however, these molecules have had sufficient time to diffuse across the bag via Fick's law. As atmospheric partial pressures do not change, this drives O_2 into the bag and CO_2 out of the bag, reaching a partial pressure of 70 mm Hg for each. Though an increase in CO_2 partial pressure within the stored blood seems counterintuitive due to its diffusion out of the bag, it is important to note that the metabolically active RBCs are continuously generating more CO_2 through their bicarbonate buffering system. The phenomenon of Fick's first law is illustrated in the concentration/pressure gradient slopes within the PVC walls after 42 days of storage. It is important to note that concentration gradients for these molecules would certainly also exist within the liquid stored blood; however, these gradients would be 10,000-fold smaller due to the significant increase in diffusion rates through liquids. This is why these gradients appear as zero-slope lines relative to the gradients within the PVC walls.

The incorporation of DEHP into the PVC, though now extensively studied for its potential toxicological effects within the body, allowed for its passive diffusion into the stored blood and its incorporation into the RBC membrane.[74,78] This led to a four-fold decrease in the hemolysis levels of stored blood, resulting in the FDA approval of this storage method by 1963.[76]

1.2.4 Storage Solution Improvements and Leukoreduction

In the 1960s Simon and colleagues reported the addition of adenine to ACD slowed the onset of ATP loss from RBCs and could extend the shelf-life of stored RBCs.[86] This was attributed

to the loss of adenine during storage and the relationship between decreased ATP levels and lower red cell viability established by Nakao.[87] This led to the development of citrate-phosphate-dextrose-adenine-1 (CPDA-1) in 1968 and its approval for use in 1979.[53] During this 11 year gap, blood component separation was revisited as a method to store RBCs independently from plasma. However, studies using the anticoagulant CPDA-1 showed that RBC post-transfusion recovery was significantly lower when RBCs were stored as packed concentrates in comparison to storage as whole blood.[88] This was later attributed to the insufficient glucose content available for the packed RBCs during storage due to their significantly higher hematocrit (80%) in comparison to whole blood (40%).[89]

To combat glucose depletion when storing RBC concentrates, the development of additive solutions was investigated in the 1970s.[53] Additive solutions provide additional volume and nutrients after plasma separation to allow longer storage of packed RBCs.[53] The first additive solution developed was sodium-adenine-glucose (SAG) by Högman, which lowered the hematocrit to below 60% and allowed RBC concentrates to be stored for 35 days with post-transfusion recoveries greater than 80%.[90] Högman also discovered that the addition of 30 mM mannitol to SAG reduced variability in hemolysis percentage at 35 days of storage due to the free radical scavenging and membrane stabilization abilities of mannitol.[53,91] The new additive solution (SAGM) has since been the standard for blood storage in Europe and has served as a precursor to modern additive solution modifications.[53,92]

An additional improvement to blood storage derived from the leukoreduction of stored RBCs in the 1990s.[93] Leukoreduction is the process of removing leukocytes from a blood donation via a size exclusion filter and cellular adhesion filter.[94] Leukocytes break down during cold storage, releasing harmful proteases and lipases that damage the RBC membrane.[53] The removal

of leukocytes has been reported to increase RBC post-transfusion recovery percentage, decrease hemolysis, and decrease the rate of infection of patients by more than 50%.[53,95] Though leukoreduction has been reported as beneficial, there have also been studies that report no significant reduction in mortality with leukoreduced cells in comparison to non-leukoreduced RBCs.[96,97] These conflicting reports, along with costly implementation, is why it is only universally adopted in a few countries, including Canada, France, and the UK.[93]

Regardless of leukoreduction, the technique of blood component separation and subsequent additive solution supplementation is the current standard of care for RBC storage both in the US and in Europe.[15] However, since the development of SAGM, there have been a variety of different additive solutions that have been investigated, all differing slightly in their components and concentrations. For example, though SAGM is widely used in Europe, the US prominently utilizes AS-1 (Adsol), AS-3 (Nutricell), and AS-5 (Optisol), which mostly contain the same constituents as SAGM with slight differences in concentrations.[98] Tables 1.2 and 1.3 illustrate an up-to-date history of the major anticoagulants and additive solutions developed for blood storage, focusing on their constituents, concentrations, maximum storage duration, and 24-hour post-transfusion recovery percentages.[38,52,53,98–111]

Most modern improvements to blood storage practices now focus primarily on these additive solutions and how they impact the biochemistry of RBC aging during storage. For example, the additive solution knows as PAGGS-M contains guanosine, which increased the activity of phosphofructokinase, thus leading to higher levels of ATP and 2,3-diphosphoglycerate (2,3-DPG) during storage.[112–114] AS-7 and E-Sol 5 are both alkaline and do not contain any chloride ions, leading to a chloride shift mechanism by which intracellular chloride ions diffuse out of the RBCs and hydroxyl ions are transported into the cell to maintain the electrostatic

balance.[108] This mechanism, coupled with the alkaline extracellular pH and increased phosphate/bicarbonate concentrations, inherently raises the intracellular pH of the RBCs, resulting in a greater buffering capacity, increased glycolytic flux, improved membrane integrity, and greater than 75% post-transfusion recovery for up to 56 days of storage.[108]

Whether blood storage improvements originated from engineering principles, such as component separation and passive molecular diffusion, or from the utilization of biochemistry as reported with additive solutions, these advancements have focused on improving the health of the stored RBC *ex vivo* with the hope of improved function *in vivo*. Throughout the history of blood storage, the removal of RBCs from the circulation results in immediate damage to the RBCs that is exacerbated during storage. These can include metabolic, physical, and oxidative damages that disrupt RBC physiology and consequently, their function *in vivo*, as shown by post-transfusion recoveries less than 100%. These storage-induced damages are collectively termed the "storage lesion". Though the many advancements discussed here have proven effective at reducing the impact of the storage lesion, the continuous improvement of RBC storage will inevitably lead to better patient outcomes.

Table 1.2**: Comprehensive list of all major anticoagulants used for blood collection and storage throughout history**. This table highlights the components and their respective concentrations for each anticoagulant, along with the pH, maximum storage duration and 24-hr post-transfusion recovery for storage as whole blood or as an RBC concentrate. Due to inadequate technology, the post-transfusion recovery percentages for citrate and Rous-Turner solutions were not measured. Additionally, since CD2D is only used in combination with an additive solution, maximum storage duration and post-transfusion recovery % for this anticoagulant have no meaning.

Component (mM)	**Citrate**	**Rous-Turner**	**ACD**	**CPD**	**CPDA-1**	**CPDA-2**	**CP2D**
Trisodium citrate	147	42	75	89	89	89	89
Dextrose	-	214	124	129	161	281	258
Citric Acid	-	-	38	16	16	16	16
Monobasic sodium phosphate	-	-	-	16	16	16	16
Adenine	-	-	-	-	2	4	-
pH	7.8	7.2-7.4	4.5-5.5	5.2-6.2	5.6	5.6	5.6
Maximum storage duration (days)	5	26	21	21	35	42	NA
RBC 24 hr. post-transfusion recovery %	NA	NA	75% (whole)	79% (whole)	79% (whole) 71% (concentrate)	80% (concentrate)	NA

Table 1.3: **Comprehensive list of all major additive solutions used for blood storage throughout history, along with their respective anticoagulants used for collection**. This table highlights the components and their respective concentrations of each additive solution, along with the pH, maximum storage duration, 24-hour post-transfusion recovery %, FDA licensure, and the countries it is widely utilized within. The pH of SAG was not reported. EU = European Union; UK = United Kingdom; USA = United States of America.

Constituent (mM)	**SAG**	**SAGM**	**AS-1**	**AS-3**	**AS-5**	**PAGGS-M**	**MAP**	**E-Sol 5**	**AS-7**
NaCl	150	150	154	70	150	72	85	-	-
Dextrose	45	45	111	55	45	47	40	111	80
Adenine	1.25	1.25	2	2	2.2	1.4	1.5	2	2
Mannitol	-	30	41	-	45.5	55	80	41	55
Citric Acid	-	-	-	2	-	-	1	-	-
Sodium Citrate	-	-	-	23	-	-	5	25	-
Guanosine	-	-	-	-	-	1.4	-	-	-
Na_2HPO_4	-	-	-	-	-	16	-	20	-
NaH_2PO_4	-	-	-	23	-	8	6	-	12
$NaHCO_3$	-	-	-	-	-			-	26
pH	NA	5.7	5.8	5.8	5.5	5.7	5.7	8.4	8.5
Anticoagulant	CPD	CPD	CPD	CP2D	CPD	CPD	ACD	½ CPD or CPD or ACD	CPD
Maximum storage duration (days)	35	42	42	42	42	49	42	49	56
RBC 24 hr. post-transfusion recovery %	83%	78-84%	83%	78-84%	> 75%	74%	83%	78%	82%
FDA Licensure	No	No	Yes	Yes	Yes	No	No	No	Yes
Countries utilized	-	EU, UK, Australia, Canada, New Zealand	USA	USA, Canada	USA	EU	Japan	EU	USA

1.3 The Storage Lesion

The storage lesion is a result of the prolonged *ex vivo* storage of RBCs that leads to irreversible metabolic and physiological damage. This decline in RBC health due to storage has been well-documented throughout the history of blood banking, with these damages being directly linked to proper RBC function and having been reported to be related to poor clinical outcomes.[26] An appropriate understanding of these physical and biochemical changes to RBCs during storage is vital to effectively reducing their impact on transfusion medicine and patient care. The storage lesion can thus be classified into 3 interconnected, yet distinct categories: metabolic impairments, physical changes, and oxidative damages.

1.3.1 Metabolic Impairments

The RBC relies solely on anaerobic glycolysis for ATP production.[40] This necessary metabolic pathway can contribute to the development of the storage lesion through an increase in metabolic waste products, such as lactic acid.[40] The resultant acidosis significantly decreases the rate of glycolysis through a negative feedback loop, leading to decreased ATP production for the cell, falling by more than 60% by the end of storage.[39,115] As ATP is utilized to fuel cellular functions, such as the maintenance of proper membrane integrity through Na^+/K^+ ion pumps, this significant drop in ATP is reported to lead to drastic RBC shape changes in the form of echinocytosis and increased mean cellular volume (MCV).[115,116] ATP levels are not only important for cellular function and membrane integrity, but were also established as a valuable indicator of RBC viability *in vivo* by Nakao.[87]

An additional metabolic pathway equally important as glycolysis is shown in figure 1.2, the Rapaport-Luebering Shunt, which leads to the generation of 2,3-diphosphoglycerate (2,3-DPG). 2,3-DPG allosterically binds to hemoglobin and decreases its affinity to bind oxygen,

thereby increasing the ability of the RBC to offload oxygen to necessary tissues.[117] During storage, as intracellular inorganic phosphates leak out of the RBC, the degradation of 2,3-DPG is accelerated to compensate for phosphate loss and maintain phosphate levels for ATP production.[53,118] These near-zero levels of 2,3-DPG after two weeks of storage lead to a decreased oxygen delivering capacity of the RBCs.[115] Though it is reported that levels of 2,3-DPG and ATP can be recovered to physiological levels within 7-72 hours of transfusion, this rate of recovery may not be sufficient to meet the immediate metabolic needs of trauma patients.[26]

Another consequence of RBC storage on metabolism is the disruption of the redox potential and availability of reducing equivalents. The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) is utilized by the RBC to recycle key antioxidants, such as glutathione, that neutralize reactive oxygen species (ROS).[119] Under elevated glucose conditions and acidic environments, such as those experienced by stored RBCs, the polyol metabolism pathway is upregulated.[120,121] The first step, which converts glucose to sorbitol via aldose reductase, consumes an NADPH molecule, while the next step, converting sorbitol to fructose via sorbitol dehydrogenase, generates an NADH molecule.[120] The upregulation of this pathway during RBC storage causes several problems. The first is the disruption of the $NADPH/NADP^+$ redox balance. The regeneration of a key antioxidant, reduced glutathione (GSH), is highly dependent on the adequate supply of NADPH.[120] As NADPH is consumed in the polyol pathway, this leads to decreased levels of GSH, and increased oxidative stress on the cell.[120,122] The second is the accumulation of cell impermeable sorbitol and fructose within the cell, generating increased osmotic stress.[120] Lastly, the accumulation of NADH has a negative impact on the rate of glycolysis due to the inhibition of glycolytic enzymes that utilize NAD^+ to produce NADH, such as glyceraldehyde-3 phosphate dehydrogenase.[40,120]

To compensate for the loss of NADPH in the polyol pathway, the metabolism of glucose through the pentose phosphate pathway (PPP) can produce NADPH, however this pathway also has decreased activity as storage progresses, and cannot recover baseline NADPH levels.[122]

1.3.2 Physical Changes

It is well established that the storage of RBCs leads to the progression of echinocytosis, and spherocytosis.[26] These processes are characterized by the generation of spicules in the outer membrane due to a relative increase in surface area of the outer leaflet membrane to inner leaflet membrane.[123] As damaged proteins and lipids are exocytosed via microvesicle shedding, the echinocytes transform into spherical cells called spherocytes.[124] These echinocytes and spherocytes are more rigid and prone to further damage, often externalizing phosphatidylserine (PS), a key apoptosis marker, that results in their clearance from circulation via the spleen.[125,126]

The membrane damage and morphological changes consistent with RBC storage are partially exacerbated by metabolic impairments and the decrease in ATP production, as seen previously. An additional culprit is the disruption of the Na^+/K^+ ion pump. Within circulation, these ion pumps maintain intracellular Na^+ and K^+ concentrations by transporting these ions against their concentration gradients at the expense of ATP.[39,115] During storage, at hypothermic temperatures, these ion pumps exhibit decreased activity and fail to maintain appropriate concentrations, resulting in increased sodium content and decreased potassium content within the cell.[39] This shift puts osmotic strain on the cell, resulting in the accelerated depletion of ATP to maintain the appropriate osmotic gradients, and subsequently leading to the loss of membrane integrity.[125]

It has also been proposed that the diffusion of inorganic phosphates into the cell contributes to the progression of echinocytosis.[127] Wong et al. proposed that the complete depletion of 2,3-

DPG causes the slow transport of phosphates into the RBC via band 3 protein, resulting in a greater concentration of inward facing band 3 protein than outward facing. Band 3 protein is directly responsible for the folding and unfolding of the spectrin cytoskeleton via the inward and outward facing configurations respectively. The greater phosphate transport into the cell during storage via band 3 protein consequently leads to contraction of the cytoskeleton and resultant echinocytosis.[127] The extensive membrane damage during storage leads to the externalization of PS and exocytosis of damaged membrane lipids and proteins via microvesicles.[128] The release of microvesicles not only advances the progression of RBCs to more fragile spherocytes, but they often contain free hemoglobin which is reported to scavenge nitric oxide, a known vasodilator, within circulation.[129]

The glycation of intracellular and membranous proteins also contributes to the progression of the storage lesion. The non-enzymatic addition of aldose sugars to amino groups of proteins, or glycation, is responsible for the generation of glycated hemoglobin (HbA1c), which is often utilized as an indicator of the 3-month average of glycemic control for people with diabetes.[130] HbA1c is reported to slightly increase throughout RBC storage, with potential consequences of increased oxygen binding affinity and reduced oxygen delivery to tissues.[130,131] Along with HbA1c, prolonged storage leads to the glycation of other proteins, generating advanced glycation end-products (AGEs), known to induce oxidative stress, and lead to endothelial adhesion *in vivo* through the binding to the receptor for advanced glycation end-products (RAGE).[132]

1.3.3 Oxidative Damages

Many of the storage lesion complications involve oxidative damage. The generation of ROS during storage can lead to both protein and lipid oxidation, resulting in decreased glycolytic enzyme activity, decreased ATP production, membrane damage, and reduced deformability.[26] As mentioned previously, an unfortunate consequence of plasticized PVC bag storage for RBCs was

the permeability of oxygen into the bag. The elevated oxygen levels within the stored RBCs lead to the autooxidation of hemoglobin to methemoglobin, producing superoxide anion (O_2^-) as a byproduct.[26] Superoxide anion is converted to H_2O_2 by superoxide dismutase, a significant ROS and a substrate for free hydroxyl radical (OH•).[26] Significant increases in levels of ROS after two weeks of storage result in the reversible and irreversible oxidation of structural, functional, and metabolic enzymes/proteins, imparting deleterious effects on metabolic activity and RBC shape.[26] Oxidized proteins can bind to the ankryn and spectrin cytoskeleton of the RBC, disrupting its structure, leading to echinocytosis and spherocytosis accompanied by the externalization of PS.[26] The oxidation of membrane lipids via hydroxyl radicals produces oxidized polyunsaturated fatty acids, causing membrane damage and loss through microvesicle formation.[26] The oxidation of hemoglobin can also eliminate its ability to bind to oxygen properly.[26]

Fortunately, RBCs have antioxidant mechanisms, briefly mentioned earlier. The primary defense against ROS generation is through the glutathione antioxidant cycle, depicted in figure 1.5. The reduced form, GSH, is able to reduce ROS such as H_2O_2 by glutathione peroxidase. As mentioned previously, the oxidized form (GSSG) must be recycled to GSH using available NADPH and glutathione reductase. This NADPH is primarily produced through the PPP. However, during RBC storage, the generation of ROS increases, production of NADPH slows,

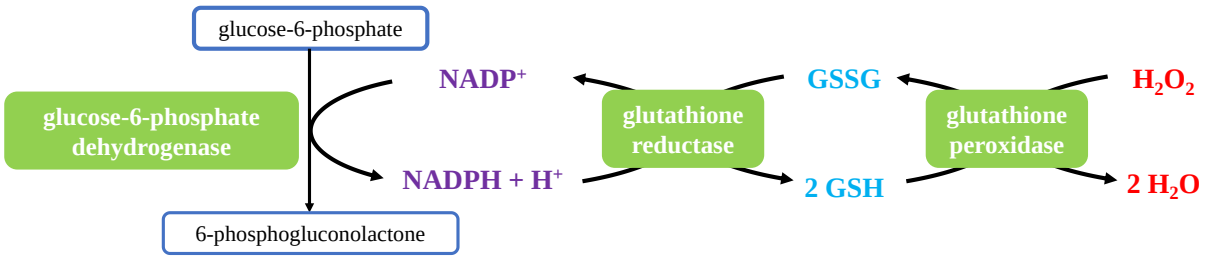

Figure 1.5: **The antioxidant mechanism and recycling of glutathione**. Two GSH molecules are utilized to neutralize ROS such as H_2O_2 into 2 H_2O, producing glutathione disulfide (GSSG) in the process. The NADPH produced via the pentose phosphate pathway provides reducing equivalents to recycle GSSG back to GSH for further ROS neutralization.

and consumption of NADPH accelerates via the polyol pathway, resulting in a significant decrease in the GSH/GSSG ratio and subsequent inability to defend against ROS.[26,120,122]

Whether metabolic, physical, or oxidative in nature, it is clear that the storage lesion consists of many complex interconnected relationships. The clinical impact of the storage lesion, however, remains a debate within the literature and requires further investigation.

1.4 Clinical Significance

Of all critically ill patients in the intensive care unit (ICU), up to 45% require at least one blood transfusion during their stay.[133] For patients with severe impairments, there is clear evidence that transfusions improve survivability, however, there are many inconsistent reports on the effectiveness of transfusions in regard to morbidity and mortality.[133] Some studies, such as a randomized controlled clinical trial on transfusion requirements conducted by Hebert *et al.,* have found that of the 838 enrolled patients, the 30-day mortality was not statistically different between patients who received a liberal versus conservative transfusion threshold (10-12 g/dL Hb versus 7-9 g/dL Hb).[134] In fact, in certain groups, the 30-day mortality was worse for patients who received the more liberal transfusions.[134] Several investigators have linked the relative ineffectiveness and even worse outcomes of transfusions to the consequences of the storage lesion.[26] Though a clear connection can be drawn between RBC impairments in storage to their function *in vivo*, it is naïve to claim that the storage lesion is the sole culprit in poor transfusion outcomes, since patients receiving transfusions are often more susceptible to further complications.[134] Regardless, an evaluation of the clinical sequelae resulting from blood transfusions and their potential link to the storage lesion is necessary to understand the motivation of blood storage research.

Some clinical outcomes can be claimed as being directly caused by transfusions, such as

pathogen transmission, intravascular hemolytic reactions, alloimmunization, transfusion associated circulatory overload (TACO), and some transfusion related acute lung injuries (TRALI).[26] TRALI is characterized by the activation of neutrophils within the lungs from incompatible donor antibodies resulting in edema, while TACO is the result of an overload of volume within the circulatory system, also causing edema.[135] These two complications were the top causes of transfusion mediated death reported to the FDA from 2012 to 2017.[135] Alloimmunization is a result of transfusing mismatched RBC units, causing intravascular hemolytic reactions as the recipients' immune system targets the donors' RBCs. Since the influence of HIV in the 1980s, the testing of donated blood for diseases has caused a significant drop in the pathogen transmission rate; however, disease transmission via blood transfusion still occurs at an incidence of 3.38 per 10,000 transfusions.[136] Though these complications may not be related to the storage lesion, it is still important to understand their clinical impact.

Alternatively, some complications have direct linkages to the development of the storage lesion. An example being the insufficient nitric oxide bioavailability (INOBA) commonly reported after transfusions, causing decreased tissue perfusion and organ dysfunction, potentially leading to delirium.[26] The decrease in NO bioavailability after transfusion can be linked to decreased RBC deformability and the increase in free hemoglobin and microvesicles in RBC storage, known NO scavengers.[26] Additionally, iron overload, a common post-transfusion complication especially in β-thalassemia patients, can cause organ damage to the liver/heart along with increased bacterial infection rates.[26,137] This increase in non-transferrin bound iron (NTBI) may be a result of the increased *in vitro* hemolysis, and subsequent release of NTBI within blood storage.[26] Increased inflammation, hypercoagulability, and RBC adhesion to the endothelium are also significant *in vivo* consequences of transfusion.[26] These may be explained by the increase in oxidative damage

and increased glycation of proteins during RBC storage, causing an immune response. Along with hyperactive immune responses, blood transfusions can also cause immunosuppressive effects, termed transfusion related immunomodulation (TRIM), in which monocyte activity is suppressed resulting in an increased risk for infection.[26] This immunosuppression may be a result of allogenic donor leukocytes and their cytokines, RBC membrane changes, increased free hemoglobin and microvesicles, all potentially inhibiting monocyte activity.[138,139]

Though it may seem impossible that the RBC storage lesion could not have an impact on clinical results, there is much debate in the literature on whether the age of stored RBCs, and therefore the progression of the storage lesion, has an impact on patient care. A cohort study of 9439 transfusion patients from 2005 to 2009 found that there was a near 2-fold increase in mortality rates when patients were transfused with "new" (less than 17 days storage) vs. "old" (greater than 17 days of storage) stored RBC units.[140] An additional study found no impact of the storage duration of RBCs on the 30-day mortality of patients.[141] However, other studies, such as the one performed by Koch *et al*. on over 6,000 patients and nearly 20,000 transfusions found that 1-year mortality was significantly decreased (7.4%) when patients were given newer (14 days or less) blood in comparison to older blood (11.0%).[142] Additionally, this same study found that a composite of post-transfusion complications was more common in patients given older blood as opposed to newer blood (25.9% vs. 22.4%). A meta-analysis of 21 studies conducted by Wang *et al*. concluded that the transfusion of older blood was associated with increased risk of death (odds ratio of 1.16).[143] Due to the inconsistencies in the literature, it is difficult to determine the impact of the storage lesion on patient outcomes.

However, an argument can be made against the studies that utilize mortality as the primary indicator of storage age impact, often seen in investigations that report no impact of the storage

lesion on patient outcomes. Though mortality is important, there is much more to patient care than the binary life or death outcome, which then requires an evaluation of morbidity due to transfusion of old vs. new blood. The issue in evaluating morbidity due to transfusions, however, lies in its ambiguity and presence of biases. This is not only due to the difficulty in diagnosing many of these adverse events, but the exacerbated difficulty of directly linking them to singular storage lesion indicators, and not to other non-transfusion related causes.[26] This problematic relationship also inevitably leads to underrepresentation of these adverse events. Many clinical trials studying the adverse events of blood transfusions also suffer from inherent biases, in that critically ill patient populations receiving blood transfusions are more likely to present with further complications regardless of transfusion status.[133] Lastly, many studies evaluating the transfusion of fresh vs. old RBC units set the threshold for the two at 14-17 days. However, by day 14, the storage lesion has already progressed significantly enough to cause permanent damage to RBCs, potentially leading to conclusions of insignificant differences in clinical outcomes between fresh vs. old RBCs. These relationships complicate the potential causal link between the storage lesion and clinical significance.

Though the clinical implications may remain ambiguous to some, the relationships between storage lesion damages and proper RBC functionality are very clear, resulting in a strong argument that the storage-induced damages imparted to RBCs must be playing some role in clinical outcomes. Due to this, it is of the utmost importance to reverse or prevent the progression of the RBC storage lesion, in the hope that it leads to improved patient care.

1.5 Combatant Strategies

As the history of blood banking has shown, there have been a plethora of significant improvements to blood storage in the pursuit of combating the irreversible damages incurred by

RBCs. Most of these have been discussed previously, including refrigeration, blood component therapy, plasticized PVC storage, and improvements to anticoagulants and additive solutions. These advancements have progressively led to improved patient outcomes as evidenced by the increased storage duration and 24-hour post-transfusion recovery percentages of stored RBCs throughout history. However, there are a few other combatant strategies and modern technologies that merit a discussion of their own.

In the 1950s, Smith *et al*. were able to successfully store RBCs at -79 ºC for up to 8 weeks in a 15% glycerol solution, resulting in minimal hemolysis when thawed.[144] The cryopreservation of RBCs has since been extensively investigated, with promising potential, especially for rare RBC units and special transfusion circumstances.[145] The underlying principles of cryopreservation and its benefit to the storage lesion are similar to that of hyperthermic storage in that it slows metabolism. In the case of cryopreservation, however, the extremely low temperatures are able to halt metabolism and all physiological activity nearly completely.[146] Since most of the storage lesion arises from oxidative damage and metabolic waste accumulation, cryopreservation allows the storage of RBCs for up to at least a decade, with *in vivo* recoveries of greater than 80%.[147,148] Though effective at preventing storage lesion development, cryopreservation is not without its limitations. The cryoprotectant, glycerol, is required for successful preservation as it prevents the formation of damaging ice crystals while freezing that often result in significant cell death.[145] However, glycerol must be removed from the RBCs before transfusion through a series of washing steps, due to its high intracellular concentration and negative impact on osmotic fragility.[145] The washing steps can be time-consuming, require specialized equipment, and therefore be cost-ineffective, while also introducing the potential for contamination.[145,147] Though useful for preserving rare RBC units and special transfusion circumstances such as for the military,

cryopreservation is unfortunately not a universal solution for standard blood storage practices.[149]

As opposed to preventing the development of the storage lesion, another strategy rigorously investigated aims to simply reverse the negative consequences associated with the storage lesion through washing the stored RBCs in rejuvenation solutions prior to transfusion. Many studies have reported that specific physical and biochemical properties of stored RBCs, such as membrane damage and low levels of ATP and 2,3-DPG, can be recovered to near physiological levels if they are washed or rejuvenated in various buffers.[150] These rejuvenation solutions allow an alternative path toward better patient outcomes. Currently, the only FDA approved rejuvenation solution is Rejuvesol, composed of sodium pyruvate, inosine, adenine, and sodium phosphate in water.[151] Studies have reported that incubating stored RBCs in Rejuvesol resulted in increased ATP and 2,3-DPB levels, normalized cell membrane function and hemoglobin oxygen affinity, along with decreased endothelial adhesion, all contributing to improved *in vivo* post-transfusion recoveries.[150,152–154] Rejuvesol, however, is only approved for use with stored RBC units greater than 14 days in storage and requires a 1 hour incubation time and further washing steps to remove the toxic inosine before transfusion.[151] These additional time-consuming steps makes its application costly and limited, considering trauma patients are often in immediate need of transfusion. Though rejuvenation solutions offer a promising avenue to pursue regarding transfusion medicine, their limitations may be difficult to overcome.

A much more recent development in blood banking involves the storage of RBCs under hypoxic conditions. As discussed previously, the diffusion of oxygen into the stored RBC bag is detrimental to the RBCs, leading to increased ROS generation. The innovative technology, only recently FDA approved in late 2023, developed at Hemanext Inc. relies on a simple principle: eliminate oxygen and therefore eliminate ROS generation.[155] As the autooxidation of hemoglobin

is the initial step in ROS generation and can be considered the primary culprit in the development of subsequent storage lesion complications, hypoxic storage of RBCs has the potential to eliminate a significant portion of the transfusion related burden on patient care. Through proprietary information, the Hemanext ONE technology operates by first deoxygenating donated RBC units stored in CP2D/AS-3 and then maintaining this hypoxia (< 3% oxygen saturation) for the remainder of storage.[155,156] A metabolomics report from D'Alessandro *et al*. extensively investigated the metabolic effect of hypoxic RBC storage using this technology and found that RBCs stored under hypoxia displayed increased glycolytic activity, higher levels of 2,3-DPG, improved glutathione homeostasis, decreased purine oxidation and membrane remodeling, along with improved post-transfusion recovery percentages of 90.2% at the end of 42 days in storage.[155] These results are mostly attributed to the decreased oxidative stress on the stored RBCs; however this technology also reduces carbon dioxide levels, resulting in alkalinization of the unit, favoring glycolysis and the PPP.[155] In comparison to cryopreservation and rejuvenation, hypoxic storage has a distinct advantage in that the pre- and post-storage processing remain relatively unchanged. This technology can be accompanied with the current collection solution combination, CP2D/AS-3, and does not require any washing steps before transfusion. Though still early in the clinical utilization of this technology, there are strong indications that hypoxic storage will change the standard of care for transfusion medicine.

These novel and unique strategies to target the prevention/reversal of the storage lesion have clearly been effective, yet still leave room for improvement, as evidenced by post-transfusion recoveries below 100%. This begs further investigation into previously unexplored areas of blood storage research.

1.6 Normoglycemic Storage

Of all blood storage advancements, both modern and historic, it is surprising that none have raised concerns at the relatively high levels of glucose in the stored RBC environment. As seen in Tables 1.2 and 1.3, all anticoagulants and additive solutions contain glucose in concentrations at least 8 times that of physiological levels (5 mM). Though glucose is indeed necessary to maintain energy and redox status of the RBCs through various metabolic pathways, the hyperglycemic environment in RBC storage may be detrimental to the RBCs and contribute to the development of the storage lesion. It is well established in the literature that chronic hyperglycemia seen in type 1 and type 2 diabetes directly contributes to the development of several diabetic complications, including retinopathy, neuropathy, nephropathy, and damages to the cardiovascular system.[157] The development of these complications are all exacerbated by poor microvasculature and blood flow, a common characteristic of patients with diabetes.[158] Interestingly, many of the functional impairments measured in stored RBCs share shocking similarities with diabetic RBCs. Some of these include reduced deformability, membrane shape changes (echinocytosis and spheocytosis), increased HbA1c and AGE formation, greater oxidative stress, reduced ATP release, and

Table 1.4: **Components and concentrations of the traditional anticoagulant and additive solution, CPD and AS-1 respectively, and glucose-modified solutions, CPD-N and AS-1N.**

Component [mM]	**CPD**	**CPD-N**	**AS-1**	**AS-1N**
Sodium citrate	89.4	89.4		
Monobasic sodium phosphate	16.1	16.1		
Citric acid	15.6	15.6		
Dextrose	129	5.5	111	5.5
Sodium chloride			154	154
Adenine			2	2
Mannitol			41	41
pH	5.6	5.6	5.8	5.8

decreased ability to stimulate NO.[26,159–161] This led to the Spence group to investigate the effect of hyperglycemia on stored RBC functionality through the development of novel RBC storage solutions. These storage solutions were modified forms of CPD and AS-1, containing physiologically relevant levels of glucose (5.5 mM) termed CPD-N and AS-1N respectively. The components of each are shown in Table 1.4.

In the subsequent studies, RBCs were collected and processed using either the CPD/AS-1 combination or CPD-N/AS-N combination according to traditional storage methods discussed previously and stored at 2-6 ºC for *in vitro* testing. The resultant publications revealed that normoglycemic storage of RBCs led to increased ATP release (a NO stimulus), reduced oxidative stress, improved deformability, and reduced osmotic fragility in comparison to traditional hyperglycemic storage.[162–165] This was not only seen with the normoglycemic variations of AS-1, but similar results were reported with other common additive solutions, such as AS-3 and AS-5.[164] The beneficial impact of normoglycemic storage on RBC functionality may be explained by glucose metabolism and its resultant effect on oxidative stress. As seen previously, glucose can be metabolized by RBCs by several pathways, with the polyol pathway causing stress on the RBC redox balance. It has been proposed that the excess glucose available in traditional, hyperglycemic storage, is made available to be metabolized through the polyol pathway, producing significantly more sorbitol and disrupting the $NADPH/NADP^+$ ratio. In comparison, normoglycemic storage resulted in decreased levels of intracellular sorbitol and therefore decreased activity of the polyol pathway. The decrease in oxidative stress may lead to less oxidation of proteins/lipids and therefore less membrane damage. This may be able to explain the reduction in osmotic fragility seen in normoglycemic storage. Additionally, since sorbitol is cell impermeable, the lower sorbitol concentrations in normoglycemic storage are likely to also contribute to the decreased osmotic

fragility. The increased ATP release and improved deformability are also likely linked to the improved membrane health of normoglycemic RBCs. Reduced membrane damage and decreased oxidation of membrane proteins/lipids could explain the improved RBC deformability. As deformability and maintenance of the RBC membrane are directly associated with ATP, it is not surprising that normoglycemic stored RBCs also display improved ATP release, especially under flow conditions.

Though the benefit of normoglycemic RBC storage has been established *in vitro*, there are several limitations to the previously published work that require addressing. Firstly, normoglycemic storage of RBCs at a clinical scale would require impossible labor efforts and result in increased risk of contamination. As RBCs utilize glucose for metabolism within storage, they would quickly become hypoglycemic (less than 4 mM) within a few days if initially stored at normoglycemic conditions. This is why the previously published works from the Spence lab have relied on a manual "feeding" of concentrated glucose to the stored RBCs every few days to maintain physiological glucose levels. However, to manually feed the over 11 million RBC units that were collected in 2021 with glucose every day would not only be financially illogical, but logistically impossible.[166] Additionally, the manual feeding of glucose in previous work inherently breaches the sterility of the stored blood, increasing the risk of contamination with each feeding period, resulting in a decrease in patient safety if implemented.

Secondly, the data on normoglycemic RBC storage published thus far by our group utilized in-house prepared custom PVC bags (without DEHP) that held a maximum of 2 mL of stored RBCs. As described previously, the presence of DEHP and Fick's laws of diffusion led to drastic impacts on blood storage that remains absent in previous work. This limitation requires the scale-up to commercially available blood storage bags to understand the impact of these factors on

normoglycemic storage.

Lastly, promising *in vitro* results do not always translate well to *in vivo* outcomes. Though there is strong evidence that normoglycemic storage will lead to better RBC functionality *in vivo*, there have yet to be any animal studies to reinforce its impact clinically. Specifically, the gold standard of 24-hour post-transfusion recovery must show improvements to traditional storage if normoglycemic RBC storage is to be clinically meaningful.

To this end, the work presented here aims to address these limitations and provide improved potential for the applicability of normoglycemic blood storage through the design, development, and implementation of an autonomous glycemic control system that maintains the sterility of RBC storage. This was accomplished first through the design and validation of the autonomous glucose control system, evaluating expected operational outcomes, glycemic control accuracy, and consistency. Subsequently, the system was implemented in the maintenance of normoglycemic conditions of stored RBCs, while key storage lesion indicators were measured to ensure the implementation of the device using commercial storage techniques led to similar benefits as previously reported. Additionally, alternative methods of reversing the RBC storage lesion were investigated, including post-storage washing of RBCs in various buffers reported to improve RBC health.

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Chapter 2 – Autonomous Control of Glucose Levels in Red Blood Cell Storage

2.1 Introduction

Red blood cell (RBC) transfusions are often life-saving procedures. RBCs contribute more than 80% of the body's total cell count, therefore significant decreases in RBC count due to a variety of pathologies, such as aplastic anemia, sickle cell anemia, or hemolytic anemia, can have detrimental consequences, including death.[1] In fact, hemorrhage accounts for 30-40% of trauma mortalities, and of these deaths, 33% to 56% occur before reaching care.[2] Though the maintenance of RBC count is vital for survival, physicians are often hesitant to prescribe a blood transfusion, even when patients exhibit below normal hemoglobin levels (< 12 g/dL).[3] Indeed, the current standard transfusion threshold set by the Association for the Advancement of Blood and Biotherapies (AABB) is a hemoglobin level less than 7-8 g/dL, a 42% decrease from physiological levels.[4] This arguably low and conservative threshold is due to several studies indicating that more liberal transfusion strategies do not lead to reduced mortality rates. The largest and most well-known study, Transfusion Requirements in Critical Care (TRICC), which enrolled over 800 patients in the ICU , showed no difference in 30-day mortality between patients receiving a liberal transfusion strategy (less than 10-12 g/dL hemoglobin) and patients receiving the restrictive strategy (less than 7-9 g/dL hemoglobin).[5] However, the in-hospital mortality rate was significantly lower in the restrictive group compared to the liberal group (22.2% vs. 28.1%).[5] In a similar study of over 600 critically ill pediatric patients, there was also no significant differences in mortality of patients receiving a restrictive transfusion threshold (< 7 g/dL Hb) versus a more liberal threshold (< 9.5 g/dL Hb).[6] However, the percentage of patients not receiving any transfusions was significantly lower in the restrictive threshold (54%) in comparison to the liberal threshold (2%).[6] Clearly, the more restrictive transfusion threshold not only results in a decreased

risk to the patient, but also can alleviate some of the burden on blood storage supply.

As described in chapter 1, the negative outcomes of receiving an RBC unit, or multiple units, can be exacerbated by transfusion-related complications. This is, in part, why physicians are reluctant to transfuse patients who are not actively bleeding and are not below the 7 g/dL hemoglobin threshold. This is especially true for patients suffering from comorbidities or pre-existing inflammation, priming their bodies for potentially worse outcomes, as is the case with transfusion related acute lung injury (TRALI).[7] Due to the potential link between transfusion related complications and the RBC storage lesion, improvements to RBC health *ex vivo* may have a beneficial impact on clinical outcomes. This is precisely the motivation behind normoglycemic RBC storage. Our previous work has shown improvements to RBC function *in vitro*, including increased ATP release, improved RBC deformability, decreased oxidative stress, and decreased osmotic fragility.[8–11] Though not yet shown to be clinically beneficial, the *in vitro* results suggest that normoglycemic stored RBCs may function better *in vivo* than traditionally stored RBCs.

Normoglycemic RBC storage, however, has many limitations and barriers impeding its application to clinical practice. The most significant is its dependency on periodic glucose feeding to maintain normoglycemia. In a previous report, RBC storage without glucose feeding resulted in a hypoglycemic environment and subsequent increase in hemolysis to over 8%.[11] Conversely, periodic glucose feeding maintained hemolysis levels similar to that of traditional storage.[11] Maintenance of the normoglycemic RBC storage environment in our previous work was accomplished via manual opening of stored RBC samples, addition of glucose, and subsequent closure of the sample every 3-4 days.[8–11] Unfortunately, this technique for maintaining normoglycemia of stored RBCs would not be allowed in clinical practice due to an increase in patient risk due to sterility breaches. This obstacle needs to be addressed by development of a

method to feed the cells while maintaining a closed system. However, any attempt at overcoming these barriers to adoption may unintentionally generate additional challenges during blood collection and/or processing. To properly address the limitations of normoglycemic RBC storage, it is first necessary to understand the logistics surrounding the lifecycle of an RBC unit, including collection, processing, storage, and administration.

The World Health Organization estimates blood donation by a minimum of 1% of a country's population is necessary to sustain basic blood transfusion demands.[12] Fortunately, the United States surpasses this threshold with 100% dependence on voluntary donations through blood donor programs, government support, financial resources, and high public trust.[12] In fact, it is approximated that over 11 million RBC units were collected in the United States in 2021, estimated to be 3.3% of the US population assuming 1 donor per RBC unit.[13] A summary of the typical lifecycle of a donor RBC unit is shown in figure 2.1. Whole blood collection is typically performed at blood donation centers or mobile collection venues utilizing one of many FDA approved blood collection systems. These systems can be single, double, triple, or quadruple bag units interconnected with tubing, designed for the complete separation of various cell components without exposure to the atmosphere.[14] After collection, the tubing is closed off, creating a closed and sterile system that is maintained throughout the RBC unit lifecycle.[14] Maintaining a closed system reduces contamination risk and ensures patient safety. In fact, the FDA Code of Federal Regulations regarding standards for human blood and blood products (CFR 21.640) states that "the blood container shall not be entered prior to issue for any purpose except for blood collection or when the method of processing requires use of a different container."[15]

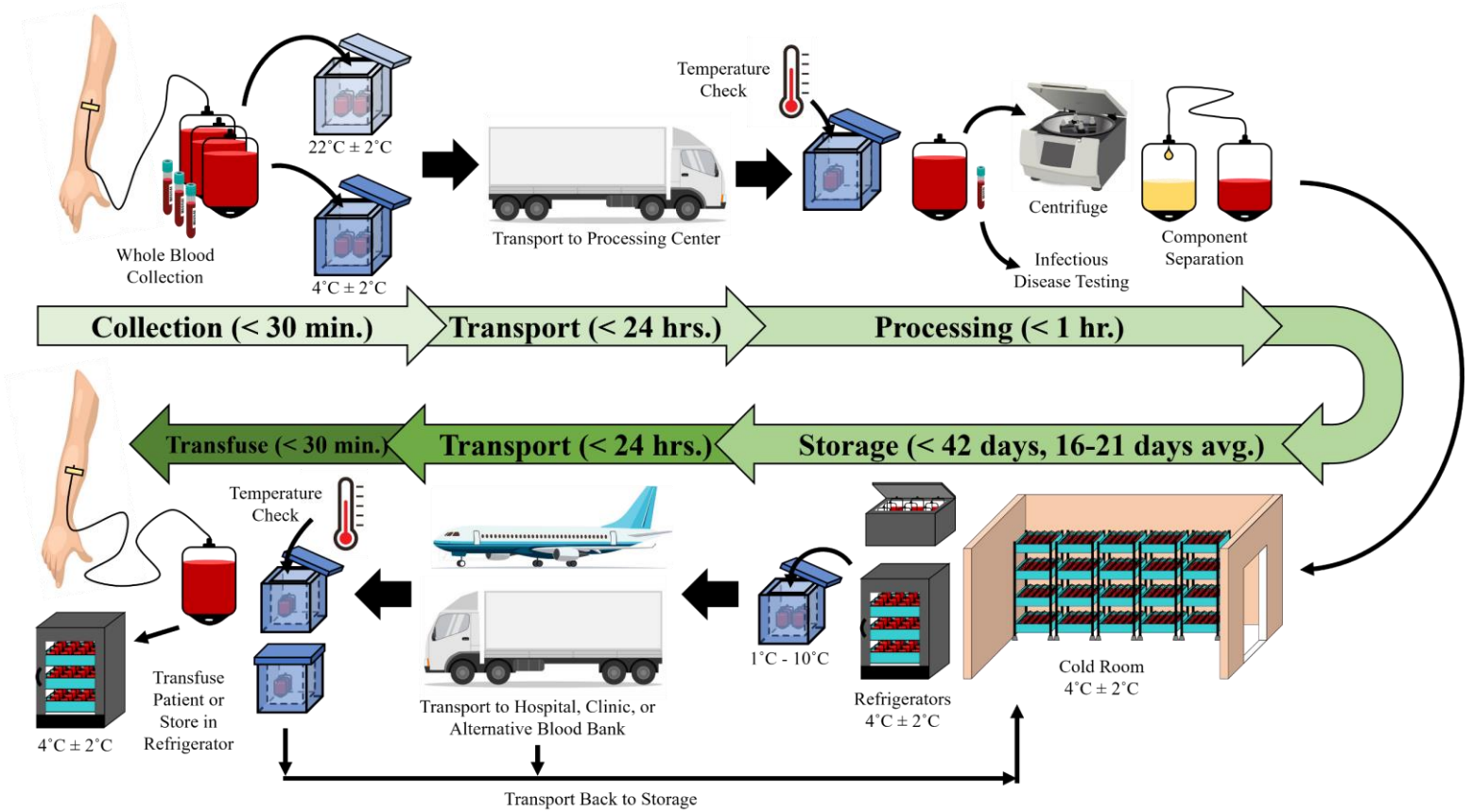

Figure 2.1**: Lifecycle of a RBC unit from collection to transfusion**. The collection of whole blood into FDA approved blood collection apparatus and additional tubes for disease testing must be placed in a temperature-controlled environment within 30 minutes of collection (either 22 ˚C or 4˚C). The blood must be transported to a blood bank processing center within 24 hrs of collection, where the temperature is checked, and the components are separated within a maximum of 1 hr. Following separation, the RBC units, are kept at 4˚C ± 2˚C. The RBC units are stored for up to 42 days, with the average duration being 16-21 days. When requested, up to 30 RBC units are placed into a blood product transport container specifically designed to maintain the temperature between 1˚C - 10˚C for up to 24 hours. Transport to the appropriate hospital, clinic, or alternative venue is conducted via ground or air transportation within 24 hours of release from the blood bank. Upon arrival, the product information is confirmed, and temperature is recorded. The RBC unit must then be administered to the patient or placed at 4˚C ± 2˚C within 30 minutes of arrival. If the RBC unit(s) was/were no longer needed and the container's seal remains intact, it can be sent back to the blood storage facility.

After collection, the whole blood must be placed in a temperature controlled container or refrigerator within 30 minutes.[16] This can be at either 22 ºC ± 2 ºC or 4 ºC ± 2 ºC for up to 24 hours. These blood transportation containers are specifically designed to maintain tight control of temperature, have a fixed geometry, and be capable of holding 10-30 RBC units.[16] The transportation of the RBC units to a blood bank processing facility must be accomplished within 24 hours of collection to ensure adequate temperature control.[16] Upon arrival to the processing facility, key quality control checks are executed, including the measurement of temperature, and recording of the collection time.[16] If either are outside of the acceptable limits, the donation is discarded.[16] Processing of the whole blood donation proceeds as described previously (see chapter 1), including the separation of blood components, blood typing, and testing for infectious diseases (HIV, hepatitis, etc.)[16] The processing steps must be completed within 1 hr of receipt, with RBC units being stored upright or at a slight angle at 4 ºC ± 2 ºC for up to 42 days.[16] The cold storage of RBCs can be accomplished via upright refrigerators, smaller chest refrigerators, or walk-in cold rooms specifically designed for blood product storage.[16] These all vary in their storage capacity ranging from 25 to over 1000 RBC units for refrigerators, and significantly more for cold rooms.[16] However, it is important to note that in all cases, the RBCs are stored on tightly stacked shelves, categorized by blood type.[16] There are also several redundant quality control steps that ensure the sustained maintenance of the temperature between 2-6 ºC.[16] If requested by a hospital, clinic, or alternative blood storage facility, the appropriate units (maximum of 30) will be packaged into blood transportation containers that are ensured to maintain a temperature of 1-10 ºC for up to 24 hours.[16] Depending on the location of the destination, this can be accomplished via ground or air transportation.[16] Due to the perishability of blood products, hospitals often do not maintain a large inventory of RBC units, and thus rely on the efficient supply chain management at blood

distribution centers, or blood banks.[17] The speedy turnaround time from request to issue and transfusion is a necessity for emergency orders, often requiring less than minutes in total turnaround time.[17] Upon arrival, the units are again checked for quality via appearance and temperature.[16] Within 30 minutes, the unit(s) should be either transfused into the patient, or placed into small satellite refrigerators to maintain a short supply of units when needed, most commonly practiced with the universal donor blood type, O negative.[16] If any requested units are no longer needed, the sealed, temperature controlled container can be sent back to the blood bank for re-distribution.[16]

The 9-minute turnaround time from patient arrival to initial transfusion for transfusion recipients suggests the supply chain management of blood products in the United States (US) is efficient, cultivated by a well-established infrastructure of blood collection through to distribution.[18] This is why future improvement to blood storage methods that threatens to disrupt this infrastructure and its focus on patient safety will not likely be implemented. For normoglycemic RBC storage to exhibit potential for clinical applicability, the method of glycemic control for each individual RBC unit has specific design constraints that must be acknowledged.

Firstly, as stated previously, the method of periodic glucose addition to individual RBC units must be completely autonomous and maintain sufficient confidence in sterility as to not impact patient safety. These design necessities can now be better appreciated through the analysis of figure 2.1. Any attempt to manually feed the over 11 million RBC units received per year, as they are distributed into/out of blood banks and hospitals, may create a financial burden and logistical problems in execution. Additionally, the nearly zero contamination risk exhibited by RBC units throughout their entire lifecycle makes it difficult for any new blood storage method to gain interest if it threatens this sterility. Because of these non-negotiable design constraints,

normoglycemic RBC storage inherently demands a physical device/product able to maintain normoglycemia completely autonomously for a 42-day lifecycle, while ensuring sterility standards comparable to current methods.

Beyond autonomy and sterility, there are several other important design considerations. For example, the device cannot create a significant disruption to the current collection and processing procedures. Considering the relative consistency in collection procedures, blood collection bags, necessary processing equipment, and personnel training across the US, any design that impacts any of these areas would have trouble with adoption. Additionally, the blood collection bag must also remain relatively unchanged. Transportation containers, refrigerators, and processing equipment are all specifically designed for the geometry of the current blood collection bags. Deviations from the standard collection bag could result in unforeseen challenges in handling. As shown in figure 2.1, the maintenance of temperature throughout the RBC unit lifecycle is vital to ensure the quality of the blood product. The quality control checks involved in this maintenance are called “cold chain management”. The importance of temperature control and its implications in RBC health indicate that any glucose feeding design cannot significantly delay established collection, processing, or transportation time allocations. Lastly, the ability to distribute an RBC unit and readminister it back into the storage supply is a necessity to maintain. A crucial aspect of the supply chain management of stored RBCs is the transport of RBC units to hospitals or other blood banks and the redistribution back into storage. The ability to easily remove a unit from storage and place it back into the supply must be maintained in normoglycemic storage.

There are also several design constraints for a glucose feeding device that may not be necessary for the applicability of normoglycemic storage but would be helpful in the persuasion for its adoption. These include its adherence to current FDA regulations, simplicity in the design,

relative expense, and precise and accurate glycemic control. As noted previously, FDA regulations state that "blood containers shall not be entered prior to issue for any reason except for collection or when the method of processing requires use of a different container".[15] This, of course, generates a problem for autonomous glucose feeding due to its inherent necessity of glucose addition to the RBC unit after collection. However, chapter 1 covered rejuvenation solutions that are incubated with stored RBCs to "rejuvenate" key RBC metabolites.[19] The FDA approved rejuvenation solution, Rejuvesol, requires the invasion of the stored RBC bag and subsequent washing of the RBCs before transfusing the patient.[19] Clearly, the FDA authorizes, at least in certain circumstances, invading stored RBC units prior to transfusion, which is why adherence to FDA guidelines is not a necessity for this design. Simplicity of the design is preferred for easier installation and operational comprehension by technicians and staff that would be utilizing the device/system. Also, while glycemic control is necessary, precise control may not be necessary. Physiological glucose levels can be considered to lie somewhere between 3.3 and 7.8 mM.[20] However, because there is some ambiguity in an exact cutoff for these boundaries and that traditional blood storage glucose is significantly greater than these levels (> 40 mM), it is unclear whether or not precise glucose control is necessary to result in a beneficial impact.[20,21] The necessary and preferred design characteristics are summarized in table 2.1.

Table 2.1**: Summary of both necessary and preferred design characteristics for glucose feeding device in the application of normoglycemic RBC storage.**

Necessary	**Preferred**
Autonomous glycemic control (42 days)	FDA guideline adherence
Comparable confidence in sterility	Simplistic
Limited disruption of blood processing	Inexpensive
No change to blood collection bags	Precise glycemic control
No impact to cold chain management	
Ability to distribute and readminister unit	

Considering the design constraints, the success of the design requires well-defined validation methods and expected deliverables. Though certain design characteristics cannot be quantitatively measured in the lab, such as the design's disruption to blood processing procedures or its impact on the cold chain management, there are several deliverables that can and must be quantitatively validated. These include consistency in glucose delivery, duration and precision of glycemic control, sterility, *in vitro* benefit, *in vivo* benefit, and patient safety. The work described here addresses several of these deliverables while adhering to the design constraints listed in table 2.1. The design, implementation, and validation of a novel, autonomous and sterile glucose feeding device for application in normoglycemic RBC storage is described. The successful operation of this device will allow for further *in vitro* data collection on normoglycemic RBC storage and will open the opportunity for *in vivo* studies.

2.2 Methods

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2.2.1 Device Design

A computer aided design (CAD) of the autonomous glucose feeding device is shown in figure 2.2. The glucose delivery system described here controlled stored RBC glucose levels through periodic addition of a concentrated glucose solution into the storage bag. Similar to a traditional intravenous piggyback (IVPB) delivery system, the glucose feeding device utilized gravity to drive fluid flow. Approximately 150 mL of feeding solution (100 mM glucose in 0.9%

saline) was placed into a blood collection bag to serve as the feeding reservoir. This was connected to a two-way, 12V DC solenoid valve using plasticized PVC tubing. The bottom end of the solenoid valve fed into additional PVC tubing that, when in operation, was connected to the stored RBC unit after collection and processing. The stored RBC bag sat upright on the benchtop, while the solenoid valve was attached to a shelf 52 cm above the top of the RBC bag. The feeding solution was then also hung approximately 90 cm above the top of the RBC bag. The periodic delivery of glucose boluses was controlled by opening and closing of the solenoid valve.

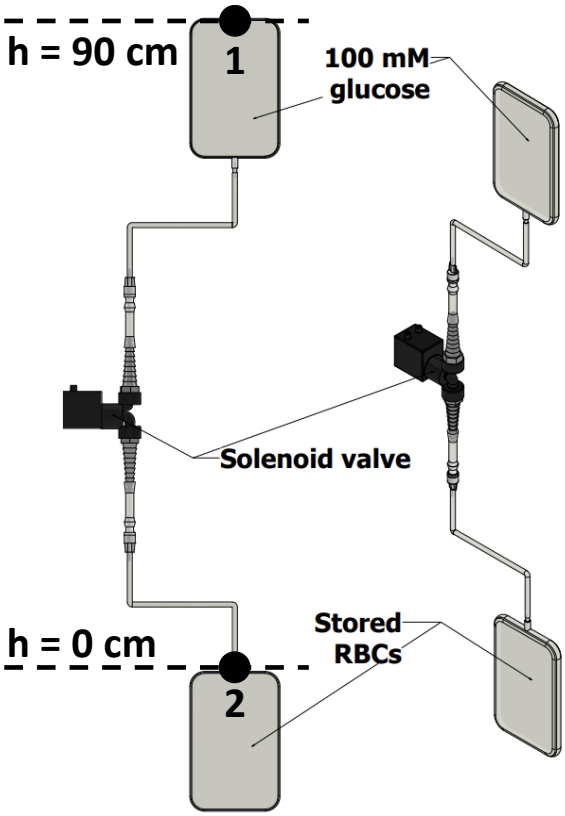

Figure 2.2**: CAD drawing of automated glucose feeding device for normoglycemic stored RBCs.** The pressure difference created between the 100 mM glucose bag and the stored RBC bag is driven by gravity, since there is a 90 cm difference in height between the two. When the valve is closed, there is no fluid flow since the valve's inability to expand exerts an equal pressure on the fluid. However, when it is opened, the pressure differential is realized due to the flexibility of the plasticized PVC bag. This drives fluid flow into the bag, therefore allowing feeding volume and frequency to be controlled by the opening and closing of the solenoid valve.

2.2.2 Device Operation

As stated previously, the fluid flow in this system was driven by gravity and pressure differentials. When closed, the solenoid valve eliminates the pressure differential created between the feeding solution bag and the stored RBC bag, thus preventing fluid flow. However, when it is open, this pressure differential due to gravity drives the feeding solution into the stored RBCs. Thus, fluid flow is controlled by the opening and closing of the solenoid valve. The frequency and duration of the opening and closing of the valve was controlled by an Arduino Uno microcontroller connected to a solderless breadboard. The circuit utilized to control the valve is shown in figure 2.3.

The design, following an approach outlined elsewhere, utilizes low side switching and a TIP120 Darlington NPN transistor to control the valve.[23] As shown in figure 2.3, the voltage is delivered to the solenoid valve constantly, however, the NPN transistor ensures that the circuit is not complete, acting as the switch in this circuit. The solenoid negative terminal connects directly

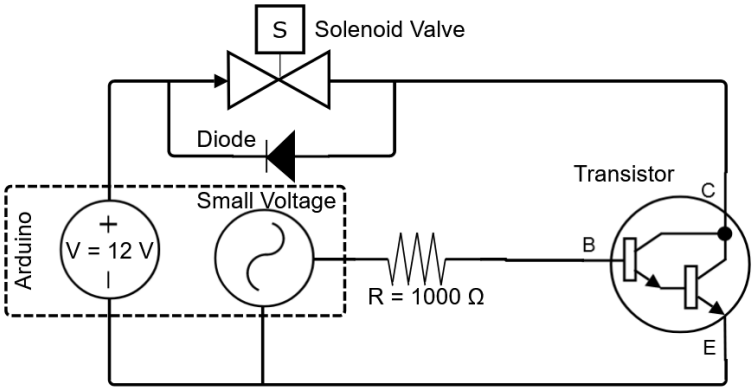

Figure 2.3: **Circuitry diagram for controlling the solenoid valve.** This circuitry diagram follows the flow of current in controlling the valve. The transistor acts as a switch in the circuit between the collector pin (C) and the emitter pin (E). The 12 V circuit is only completed when a small oscillating voltage is sent from the Arduino to the base of the transistor (B) after passing through a resistor. This allows current flow between C and E, thus opening the solenoid valve. Controlling the frequency and duration of the small voltage applied to B allows the complete control of the opening and closing of the valve. The diode serves to dissipate any transient voltages generated from the solenoid valve after turning off.

to the collector portion of the NPN transistor (C). The transistor will then complete the circuit to the emitter portion of the transistor (E) only when a small voltage is applied to the base end of the transistor (B). Once applied, the circuit completes, and the solenoid valve will open. In this low side switching approach, controlling the small voltage applied to the base end of the transistor enables the solenoid valve to be opened and closed within milliseconds. The snubber diode (1N4001) connecting the two terminals of the solenoid valve acts to dissipate any transient voltage created by the solenoid valve when it is turned off.

The program to operate the Arduino Uno was written using the Arduino coding language, similar to C++. Implementing a time delay between the opening and closing of the valve controls the amount of feeding solution delivered to the RBCs. Additionally, the script was set to loop, allowing for feeding of glucose over the 42-day storage period with a delay period of 3 days after glucose delivery.

2.2.3 Device Modeling

The valve system's operation was modeled utilizing Bernoulli's principle with a pressure drop term. Briefly, Bernoulli's principle states that the pressure of a fluid flowing through a system is equivalent at any two points as shown in equation (2.1) where P is initial pressure (Pa), ρ is the density of the fluid (kg/m^3), g is the acceleration due to gravity (m/s^2), h is the height relative to the reference point (m), v is the velocity of the fluid (m/s), and ΔP is the magnitude of any additional pressure drop term (Pa).

$$P_1 + \rho g h_1 + \frac{1}{2}\rho v_1^2 = P_2 + \rho g h_2 + \frac{1}{2}\rho v_2^2 + \Delta P \qquad (2.1)$$

In the context of the feeding system, the volume flow rate was determined through Bernoulli's principle with careful selection of reference points. As shown in figure 2.2, point 1

marked the top of the feeding solution bag while point 2 was the outlet of the feeding solution into the stored RBC bag, which also represented the reference height of 0 cm. Determining the pressure at each of these points allowed for the volumetric flow rate to be calculated. P_1 and P_2 were removed from the equation because both points are subjected to atmospheric pressure. Additionally, due to the large diameter and volume of the feeding solution reservoir relative to the tubing, the velocity at point 1 relative to the velocity at point 2 is significantly smaller and was assumed to be 0 m/s, eliminating the velocity term on the left side of equation 2.1. Point 2 is located at the reference point and its height is 0 cm, therefore the pressure due to gravity term was eliminated on the right side of equation 2.1. The equation was then simplified to equation (2.2) below.

$$\rho g h_1 = \frac{1}{2}\rho v_2^2 + \Delta P \qquad (2.2)$$

The magnitude of the pressure drop due to wall shear stress was determined using equation (2.3) where *f* is the friction factor, *L* is the length of tubing (m), and *D* is the diameter of the tubing (m).

$$\Delta P = f\frac{L\rho v^2}{2D_t} \qquad (2.3)$$

A Moody diagram was used to determine the value of the friction factor (0.015) because the friction factor is dependent on the Reynold's number and the roughness of the tubing. Substituting equation 2.3 into equation 2.2 and solving for v_2 yielded equation (2.4) below.

$$v_2 = \sqrt{\frac{2gh_1}{1+\frac{fL}{D_t}}} \qquad (2.4)$$

After solving for velocity, the volumetric flow rate out of the tubing at point 2 was determined using equation (2.5) below where Q is the flow rate (m^3/s), A is the cross-sectional area (m^2), and v is the calculated velocity (m/s) from equation 2.4.

$$Q = Av \tag{2.5}$$

This volumetric flow rate was then multiplied by various valve delay periods, resulting in theoretical dispensing volumes. These volumes outputs were plotted against the valve delay periods alongside experimental volumes measured from calibration of the device.

2.2.4 Experimental Calibration of the Device

After modeling the device operation, it was necessary to experimentally confirm the expected dispensing volumes through the calibration of the device. A mass subtraction method was implemented using water with a density of 1 g/mL. This method determines the volume of dispensed solution by massing a sample collection tube before and after dispensing for various time delays. The device was set up as described in figure 2.1, with the substitution of water for the 100 mM glucose, and the terminal end of the tubing left open to dispense into sample tubes, as opposed to connected to a stored RBC unit. Various valve delay periods (25 – 2000 msec) were tested by adjustment of the Arduino script. Before testing each delay period, 10 microcentrifuge tubes (1.7 mL each) were massed and recorded. The feeding device then dispensed a single bolus of water into each sample tube. This process was repeated for all 10 microcentrifuge tubes. The 10 microcentrifuge tubes were then massed and recorded afterward. The difference in mass between the two time points (measured in grams) for each sample tube was representative of the volume dispensed in mL since the density of pure water is 1 g/mL.[24] This mass subtraction method was repeated for each delay period tested. The averages and standard deviations of volumes dispensed were plotted against delay periods to develop a calibration curve for the valve. The expected

volumes calculated from Bernoulli's principle were also plotted.

2.2.5 Device Validation

The operation of the device was validated by measuring glucose levels after delivering expected volumes of 400 mM glucose to samples with known glucose concentrations. First, 3 sample tubes were filled with 50 mL of a normoglycemic version of a traditional RBC storage solution, AS-1N (see table 2.2). The automated glucose feeding device was set up as described in figure 2.1, with the substitution of 400 mM glucose for the 100 mM glucose, and the terminal end of the tubing left open to dispense into sample tubes, as opposed to connected to a stored RBC unit. The valve was programmed to open for a specific delay period, delivering a single bolus into each of the 3 sample tubes. The glucose concentration of each sample tube was then measured using a commercially available blood glucometer (AimStrip Plus). Utilizing the calibration curve developed in 2.2.5, the expected increase in glucose concentration for each sample tube was calculated based on the expected volume dispensed. This was repeated for a total of 6 valve delay periods, with experimental glucose increases plotted against theoretical glucose increases.

2.2.6 Blood Collection, Processing, and Storage

After device validation, it was then tested for glycemic control of stored RBCs. All blood collection procedures from informed and consented donors were approved by the Biomedical and Health Institutional Review Board at Michigan State University. Anticoagulants and additive solutions were prepared in-house. Citrate-phosphate-dextrose (CPD) was prepared from its components at the specified concentrations[25]. The additive solution, AS-1, was prepared in the same manner.[26] Components of normoglycemic test solutions, CPD-N and AS-1N, are shown in Table 2.2. The solutions were autoclaved at 121 ºC at 21 bar for 30 minutes to sterilize.

Prior to collection, 1 mL of the CPD or CPD-N anticoagulants was injected into non-

Table 2.2**: Anticoagulant and additive solution components and concentrations**. The FDA approved anticoagulant and storage solutions (CPD and AS-1 respectively) contain glucose concentrations 20-fold greater than physiological levels. The proposed counterparts, CPD-N and AS-1N, are similar in composition except for lower glucose content.

Component [mM]	**CPD**	**CPD-N**	**AS-1**	**AS-1N**
Sodium citrate	89.4	89.4		
Monobasic sodium phosphate	16.1	16.1		
Citric acid	15.6	15.6		
Dextrose	129	5.5	111	5.5
Sodium chloride			154	154
Adenine			2	2
Mannitol			41	41
pH	5.6	5.6	5.8	5.8

siliconized and untreated (no anticoagulant) 10 mL glass Vacutainer tubes (BD, Franklin Lakes, NJ). Next, approximately 7 mL of whole venous blood were collected into each tube from a consented donor. A total of 10 tubes were collected from each donor and split evenly into traditional processing and normoglycemic processing (5 hyperglycemic tubes, 5 normoglycemic tubes). The collected blood was allowed to sit for 30 minutes at room temperature, then centrifuged at 2000*g* for 10 minutes. The plasma and buffy coat layer were removed by aspiration along with the top 2 mm layer of packed RBCs to minimize retention of white blood cells in the stored product. No leukoreduction was performed due to the small blood volumes used in this work and it is noted that this limitation may have an impact on the stored RBCs. The packed RBCs were then combined with AS-1 or AS-1N using a 2:1 volume ratio. The RBCs were injected into commercially available, sterile, 100 mL veterinary blood collection bags (ABRI Cat# BG-DD 100 BAG) for a total stored RBC volume greater than 20 mL. The bags were subsequently connected to the automated feeding system at 2-6 ˚C for up to 44 days. All non-sterile equipment/reagents

were autoclaved or UV sterilized, while the valve was sterilized using 10% bleach followed by a sterile water flush and priming of the feeding solution. Hyperglycemic stored RBCs (AS-1) were fed with 0.9% saline, while normoglycemic stored RBCs (AS-1N) were fed with 100 mM glucose in 0.9% saline. Although saline addition is not the standard practice for RBC storage, this was necessary to ensure that any differences in RBC quality were due to normoglycemia, and not the addition of saline or volume. All RBC processing was conducted inside of a biosafety cabinet. The stored RBC units were kept upright inside of beakers without mixing or agitation. However, the RBC units were inverted and mixed thoroughly before all sampling, including for glucose measurements.

2.2.7 Feeding Regimen

The glucose feeding frequency was set to every 3 days, which was supported by previous publications and preliminary data indicating that glucose utilization was approximately 0.4 mM/day.[10] The amount of glucose to deliver every 3 days was initially determined through this average glucose utilization rate. The volume of 100 mM glucose in 0.9% saline to be dispensed every 3 days was determined using equation 2.6 below, where V_d is the dispensing volume (mL), V_0 is the stored RBC bag volume, and ΔC is the change in glucose concentration over 3 days. The 94.5 denominator is related to the glucose concentration in the feeding solution (100 mM) minus the target concentration (5.5 mM). Using a higher or lower glucose concentration in the feeding reservoir would change the denominator, accordingly.

$$V_d = \frac{V_0 * (\Delta C)}{94.5} \qquad (2.6)$$

Considering the 3-day average glucose utilization (ΔC) of 1.2 mM (0.4 mM/day x 3 days), the initial dispensing volume was calculated based on the initial volume of stored RBCs (V_0). However, as concentrated glucose is added to the stored RBCs, the volume increases, thus

requiring a larger dispensing volume to maintain a target of 5.5 mM. To compensate for this, individual theoretical dispensing volumes were calculated for each 3-day feeding period over a 44-day storage period. The average of these dispensing volumes was used as the glucose dispensing volume for each 3-day feeding period during device operation. This volume was then implemented in the interpolation of the feeding device calibration curve to determine the appropriate valve delay period to implement into the Arduino script.

This initial feeding regimen was utilized with the glucose feeding device in a semi-autonomous manner, meaning that the Arduino script, and subsequently the glucose delivery, was manually implemented every 3 days by turning the Arduino on and then off. By doing so, the glucose levels both before and after delivery could be measured to determine a more accurate average daily glucose utilization by the RBCs. The improved average was then implemented into equation 2.6 to determine a more accurate average dispensing volume that was used for subsequent autonomous glucose delivery of stored RBCs for 44 days. This feeding regimen will be referred to as the constant volume delivery method, as it delivered the same volume of concentrated glucose every 3 days. This method was implemented completely autonomously, dispensing glucose every 3 days without intervention. However, it is important to note that if glucose ever measured outside of the 4-6 mM range, action was taken to correct these deviations including forcing an additional glucose delivery or turning the device off, therefore breaking complete autonomy.

2.2.8 Glucose Monitoring and Hematocrit Measurement

Glucose measurements of stored RBCs were taken every 2-4 days to monitor glycemic control of the automated feeding device. Glucose levels were measured using a blood glucometer (AimStrip Plus) and blood glucose electrode strips (AimStrip). For the determination of glucose utilization rates using the semi-autonomous feeding approach (see 2.2.7), glucose levels were

measured before and after glucose delivery. Glucose utilization was determined by subtracting the glucose level at one time point from the previous time point and dividing by the number of days elapsed.

A sample of the stored RBCs was taken every 7 days for the measurement of hematocrit, or the volume percentage occupied by RBCs within a given volume. The hematocrit of removed aliquots of RBCs was determined with a hematocrit centrifuge (CritSpin M960-22, StatSpin, Westwood, MA) and a microcapillary reader (StatSpin).

2.2.9 Sterility of Stored RBCs

The sterility of the stored RBC units was determined by using a simple agar plate streaking method. At the end of the 44-day storage period, approximately 25 µL of the stored RBCs were removed and placed onto blood agar plates using aseptic technique. The sample was streaked across the plate using a sterile, disposable inoculating loop, and incubated at 37 ºC for 7 days. Evaluation of sterility was determined on the basis of any microbial growth seen on the agar at the end of the 7-day incubation period.

2.2.10 Device Improvements

Several improvements to the device and its operation were made following initial data collection. Firstly, a modified feeding regimen was implemented after initial data collection utilizing the constant volume delivery approach with a 3-day feeding frequency. Although the 3-day feeding frequency was maintained, the variation in glucose utilization by the stored RBCs was integrated into the Arduino script to result in storage-dependent volume delivery. The data generated from the glucose utilization measurements enabled variable valve delay periods that were dependent on the number of days that the RBC unit had been in storage. Glucose concentrations were also monitored to determine how this feeding regimen impacted glycemic

control. This feeding regimen will be referred to as the storage-dependent delivery method, as the volume of concentrated glucose delivered was dependent on the number of days in storage. Lastly, an additional solenoid valve was tested with a smaller orifice diameter. The valve utilized for the storage of RBCs was a generic valve with an orifice diameter of 5.16 mm, not designed for medical applications (Grainger Cat# 20HL65). To achieve lower volume dispensing limits and improve the applicability of the design, a medical-grade solenoid valve with a significantly smaller orifice diameter (2.72 mm) was also calibrated similar to 2.2.5.

2.3 Results

2.3.1 Device Operation

The device as described in 2.1.1-2.1.2 was modeled using Bernoulli's principle and calibrated to validate its successful operation. The predicted and experimental dispensing volumes were each plotted against their corresponding valve delay periods. The volume dispensed from the valve was directly proportional to the valve delay period (figure 2.4). The lower limit of dispensing volume for the system was 176 ± 21 µL at a 50 msec delay. Any valve delay period below this threshold resulted in comparable volumes dispensed. This may be due to the transient pressure differential created from the opening and closing of the valve, regardless of the amount of time it remained open.

The theoretical dispensing volume calculated using pressure differentials in Bernoulli's principle is also shown in figure 2.4, with a relative error in the slope of 0.67% and a coefficient of variation of the root mean square error of 4%. These low error percentages indicated that the valve was well characterized utilizing Bernoulli's principle and operated as expected with precise control of volume delivery down to 176 uL.

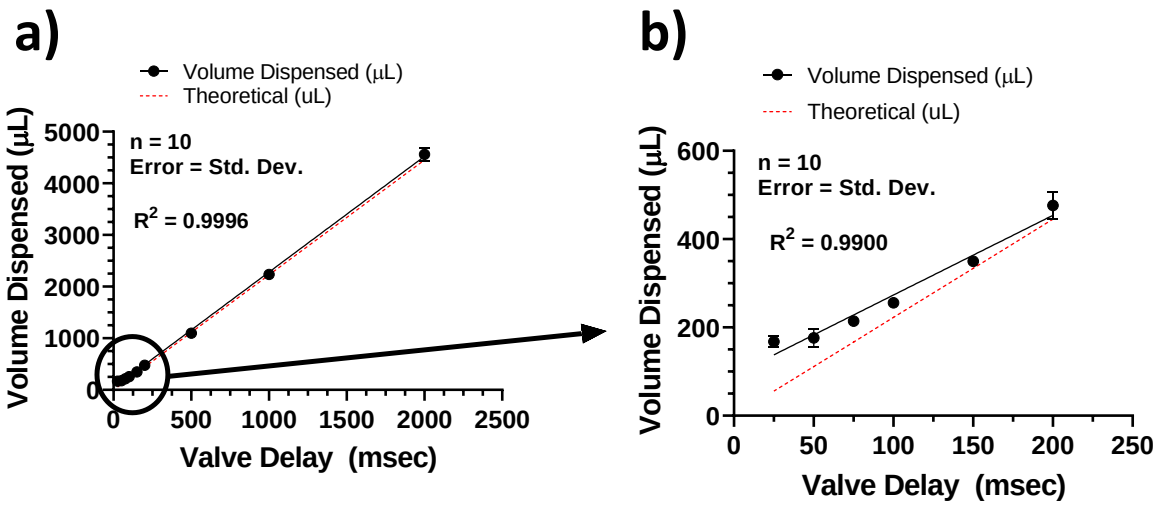

Figure 2.4**: Calibration curve depicting volume dispensed as a function of solenoid valve delay period**. (a) The volume dispensed from the automated feeding system using various valve delay periods (25 – 2000 msec) was determined by utilizing water (density = 1 g/mL) and a mass subtraction technique. The calibration curve is linear and can be used to determine the appropriate valve delay period that corresponds to the desired volume to dispense. The valve is also well characterized utilizing Bernoulli's principle (red dotted line) (n = 10, error = standard deviation). (b) Blown up view of the valve calibration at lower valve delay periods (25-200 msec), also depicting strong linearity and a close match to expected dispensing volumes (n = 10, error = standard deviation).

2.3.2 Device Validation

To further confirm the feeding device's operation, the calibration curve generated in figure 2.4 was used to open the valve for specified delay periods, delivering expected volumes of a 400 mM glucose solution into samples of known glucose concentration. The experimental glucose increases were plotted against theoretical glucose increases, shown in figure 2.5. The red dotted line with a slope of 1 is shown as an example of an ideal system where measured glucose increases exactly match theoretical glucose increases. This line can be compared to the experimental values (shown in black) to determine their relative closeness to an ideal system. The experimental values were also fit to a linear regression equation (black line) resulting in a slope of 0.98 ± 0.19 with a relative error of 2%. Due to the slope closely matching that of an ideal system (slope of 1), it can

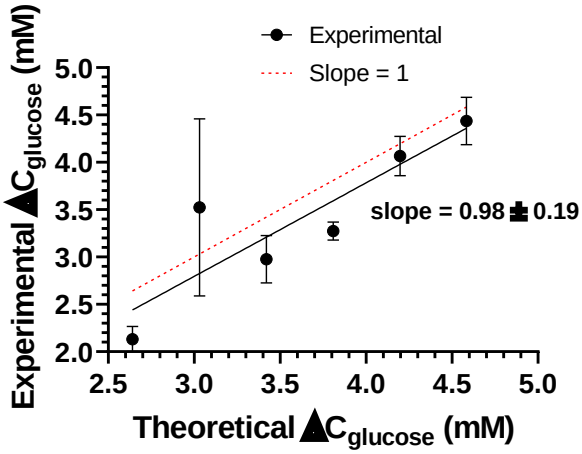

Figure 2.5**: Validation of automated glucose delivery device through the plotting of experimental vs. expected glucose concentration ($\Delta C_{glucose}$) increases of samples.** The automated feeding device was programmed to deliver 400 mM glucose to samples of known glucose concentration at various valve delay periods (60-250 msec). The glucose levels were then measured after dispensing. Utilizing the calibration curve in figure 2.5, experimental glucose increases were plotted against theoretical increases. The red line indicates an ideal system where measured glucose increases perfectly match theoretical increases. The experimental data (black), fit with a linear regression line, closely match an ideal system, exhibiting a slope of 0.98 ± 0.19. (n = 3, error = standard deviation).

be concluded that the glucose delivery system was able to deliver accurate and precise volumes of concentrated glucose solution that correlated well to expected increases in glucose concentration. This data is even more compelling considering the valve delay periods tested in figure 2.5 (60-250 msec), and subsequent volumes delivered (175 – 600 µL), were within the range of expected values to be used in device operation for the maintenance of glucose in stored RBCs.

2.3.3 Glycemic Control and Glucose Utilization

Glucose levels of stored RBCs were monitored for up to 44 days to determine average glucose utilization rates . The glucose utilization of AS-1N stored RBCs is shown in figure 2.6, starting at 1.3 ± 0.3 mM/day on day 2 and steadily dropping throughout storage, reaching 0.25 ± 0.07 mM/day by the end of storage, more than an 80% decrease in glucose usage. The average glucose utilization across the entirety of storage time points was determined to be 0.33 ± 0.42

mM/day (red dotted line) and was implemented in the determination of the dispensing volume in the constant volume delivery method for autonomous glucose control (see 2.2.7).

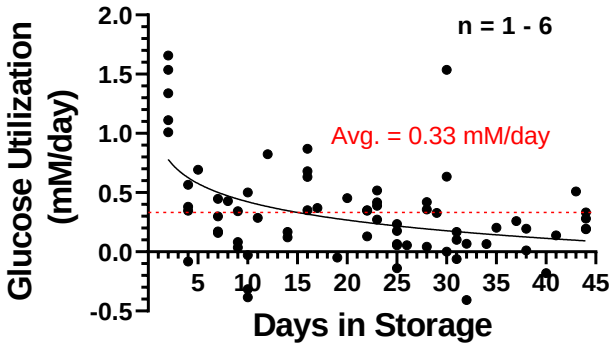

Figure 2.6**: Glucose utilization rates of AS-1N stored RBCs as a function of storage time**. The average glucose utilization across all storage time points was 0.33 ± 0.42 mM. The data was subsequently fit with a logarithmic trend line for prediction of storage-dependent glucose utilization (n = 1- 6).

Implementing the average glucose utilization rate (0.33 mM/day) from figure 2.6 in the determination of glucose volume delivery (equation 2.6), autonomous glucose control of stored RBCs was attempted using the constant volume delivery method (see 2.2.7) and glucose was monitored for up to 44 days, shown in figure 2.7a. It is important to note that the blood glucometer used to measure glucose levels saturated at 33 mM, reporting "high" when above this level. The glucose concentration dropped steadily throughout storage in AS-1 (AS-1 stored RBCs were fed with only 0.9% saline), reaching 19.5 ± 2.5 mM at the end of storage, still over 3x greater than physiological conditions. Though AS-1 stored RBCs read above saturation at early time points, a trend line fit to the values below saturation were extrapolated back to time 0, estimating the initial glucose concentration to be 35 mM, similar to the expected concentration of 40 mM. As noted by the green circles in figure 2.7a, AS-1N stored RBCs supplemented with glucose every 3 days were maintained within a physiologically relevant glucose concentration (4-6 mM) for 44 days of storage. However, this was not consistent across all samples through the entirety of storage, as

shown by the green circles with red outline in figure 2.7a. These deviations from normoglycemia (4-6 mM) for particular RBC units can be explained by donor variation in glucose metabolism and time-dependent glucose utilization changes, as shown in figure 2.6. Manual intervention was implemented when deviating from the 4-6 mM range due to the importance of normoglycemic maintenance for biochemical measurements. These interventions included additional glucose deliveries when glucose was below 4 mM and turning the device off when glucose was above 6 mM. This constant volume delivery method resulted in approximately 4 deviations from normoglycemia per sample throughout the 44-day storage period, but maintained glucose levels of all samples between 2.8 and 8.0 mM for the entirety of storage.

Though not consistent for all samples, glucose control within a 4-6 mM range is possible with this device. Using the constant volume delivery method, figure 2.7c showcases two normoglycemic stored RBC samples that were maintained between 4-6 mM autonomously and without intervention for up to 37 days. The operation of the device with the constant volume delivery method was also modeled and is shown in figure 2.7c. This model assumed a 3-day feeding frequency, a constant feeding volume based on average glucose utilization (figure 2.6), glucose depletion interpolated from the trend line in figure 2.6, and an average volume removal of approximately 0.7 mL per week (for running experiments). Though the experimental data did not match the model perfectly, the data exhibits the same parabolic, upward trend of glucose concentration throughout storage predicted by the model. This trend can be explained through several factors. Firstly, the RBCs consume more glucose earlier on in storage in comparison to the end (figure 2.6a). The model exhibited a parabolic trend because the constant volume delivery method did not account for this variation in glucose consumption rates. Secondly, the volume dispensed using the constant volume delivery method did not account for volume removal due to

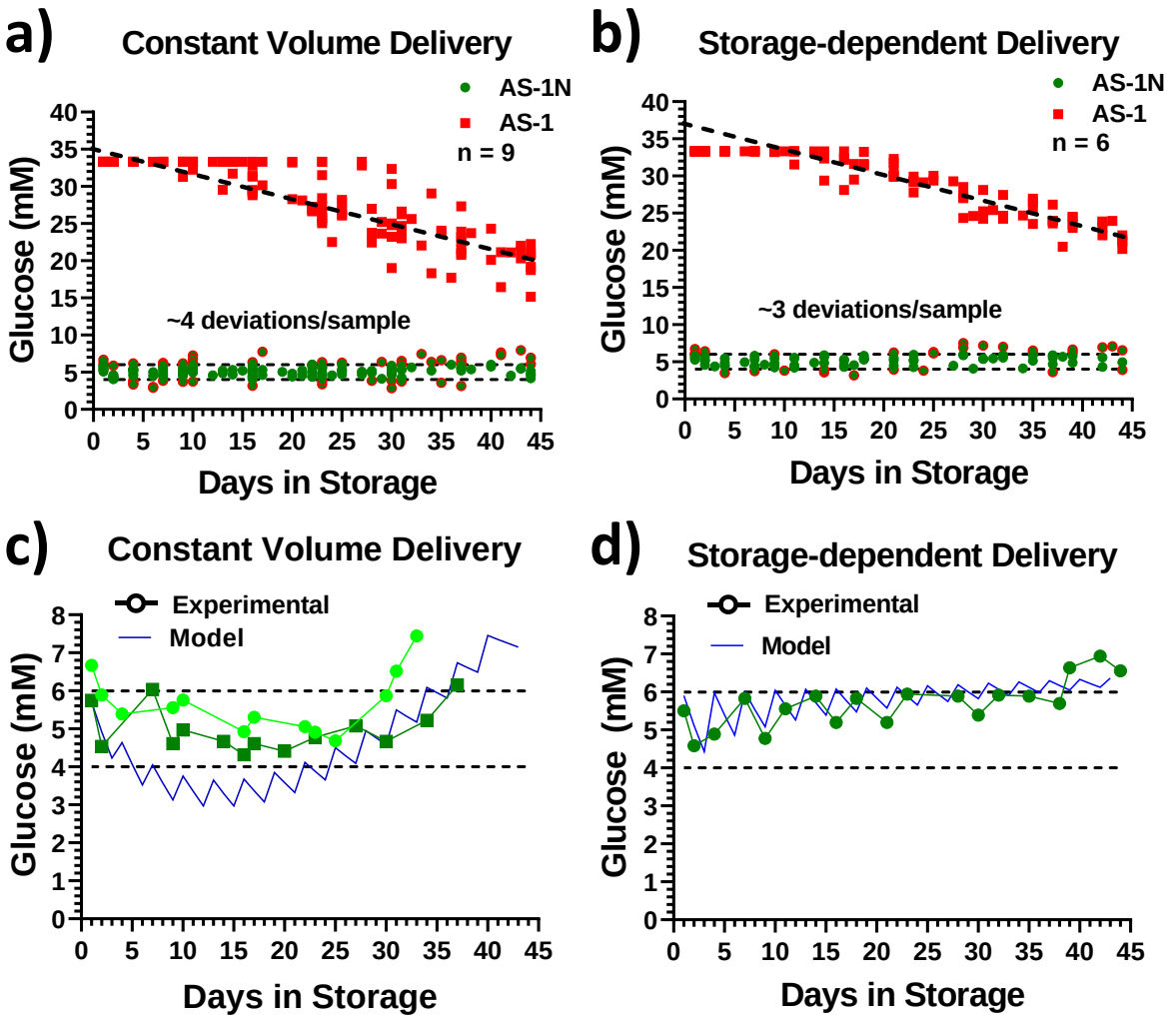

Figure 2.7: **Glycemic control of stored RBCs**. (a) Glucose levels of AS-1 and AS-1N stored RBCs fed using the constant volume delivery method for up to 44 days of storage. Glucose levels of AS-1 stored RBCs steadily dropped throughout storage, reaching 19.5 ± 2.49 mM by day 44. Though glucose read at saturation above 33 mM, a trend line extrapolated back to time 0 predicted a day 0 glucose concentration of 35 mM for AS-1 stored RBCs. The AS-1N stored RBCs were mostly maintained between 4-6 mM for 44 days of storage, resulting in approximately 4 deviations outside of this range per sample (n = 9). (b) Glucose levels of AS-1 and AS-1N stored RBCs fed using the storage-dependent delivery method. Glucose levels of AS-1 stored RBCs steadily dropped to 21.2 ± 0.77 mM by day 44. The AS-1N stored RBCs were well maintained between 4-6 mM, resulting in approximately 3 deviations per sample (n = 6). (c) Glucose levels of 2 normoglycemic RBC samples fed completely autonomously using the constant volume delivery method, successfully maintaining a 4-6 mM range for up to 37 days and exhibiting a similar parabolic upward trend predicted by the model. (d) Glucose levels of a single normoglycemic stored RBC unit fed completely autonomously using the storage-dependent delivery regimen, successfully maintaining a 4-6 mM range for up to 39 days, closely matching the model.

sampling. This caused the constant feeding volume to have a larger impact on glucose concentration at later feeding periods in comparison to initial feeding periods.

Due to the changes in glucose utilization during storage, a modified storage-dependent delivery regimen was implemented (see 2.2.10). This modified feeding program interpolated figure 2.6 to determine expected reductions in glucose concentrations at specific storage days and subsequently calculated appropriate dispensing volumes to make up for these reductions, leading to customized dispensing volumes for each feeding period. This feeding regimen was implemented into the autonomous glucose control of 6 normoglycemic stored RBC units with glucose monitored for up to 44 days, as shown in figure 2.7b. Similar to the constant volume delivery method, this feeding regimen also led to deviations that required manual intervention, shown as the green circles with red outline in figure 2.7b. However, this method led to a tighter control of glucose levels for all samples (3.2 – 7.6 mM) and less deviations from normoglycemia (3 per sample), indicating its superior ability to control glucose. This is further supported by figure 2.7d, showing the predicted model for this feeding regimen and autonomous glycemic control of a single AS-1N stored RBC unit, successfully maintaining the 4-6 mM range for up to 39 days in storage. The model does not exhibit the same parabolic trend in glucose levels that is characteristic of the constant volume delivery method due to its ability to match glucose delivery to time-dependent glucose consumption, resulting in tighter glucose control and less deviations from normoglycemia.

Though neither method was able to maintain precise glycemic control between 4-6 mM completely autonomously for a full 42-day storage period, it is clear that autonomous glycemic control of stored RBCs is possible with this device and storage-dependent glucose delivery was superior in glucose maintenance, evidenced by a tighter range of glucose concentrations, less deviations, and a less variable expected model.

2.3.4 Hematocrit and Sterility of Stored RBCs

The hematocrits of the stored RBCs from figure 2.7a using the constant volume delivery feeding regimen were measured throughout storage and are shown in figure 2.8. As expected, the hematocrit dropped throughout storage for both AS-1 and AS-1N stored RBCs. This is due to the periodic addition of 0.9% saline (AS-1) or 100 mM glucose in saline (AS-1N) throughout storage. Like other characteristics of the device operation, the hematocrit drop was also modeled, shown as the solid black line in figure 2.7. The AS-1 stored RBCs exhibited hematocrit decreases well characterized by the model, starting at 58.7% ± 1.2% on day 2 and dropping to 50.4% ± 3.8% by day X. The hematocrit of the AS-1N stored RBCs exhibited similar behavior, beginning at 58.5% ± 0.8% on day 2 and dropping to 52.8% ± 1.3%. Though AS-1 and AS-1N stored RBC hematocrits were statistically equivalent at all time points, both were well characterized by the model, resulting

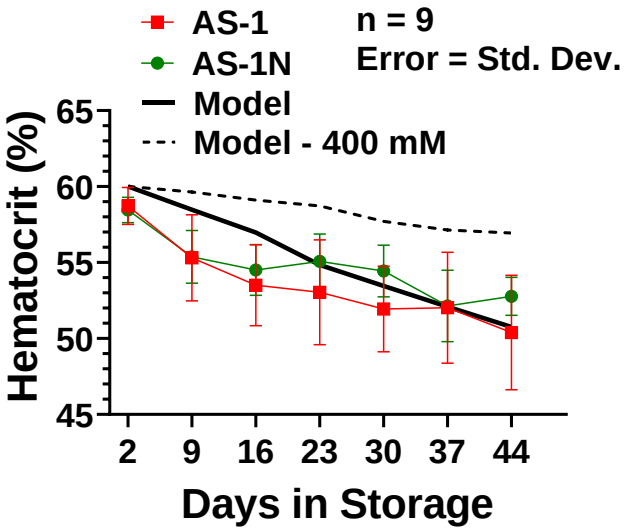

Figure 2.8: **Hematocrit changes of stored RBCs using the constant volume delivery feeding regimen**. The AS-1 stored RBCs start at a hematocrit of 58.7% ± 1.23% on day 2 and drop to 50.4% ± 3.78%., closely matching the values predicted from the model. The AS-1N stored RBCs also exhibited a decrease in hematocrit, from 58.5% ± 0.84% on day 2 to 52.8% ± 1.26% on day 44, and were statistically equivalent to AS-1 stored RBC hematocrits. Utilizing more concentrated glucose (400 mM) would result in a significantly smaller decrease in hematocrit of 3% (dotted line) (n = 9, error = standard deviation).

in a drop in hematocrit of almost 10%.

Additionally, figure 2.8 shows the expected hematocrit decrease using 400 mM glucose as the feeding solution (dotted black), as opposed to 100 mM glucose (solid black). The more concentrated glucose solution would inherently lead to smaller dispensing volumes and lower drops in hematocrit, resulting in a 3% total decrease in hematocrit.

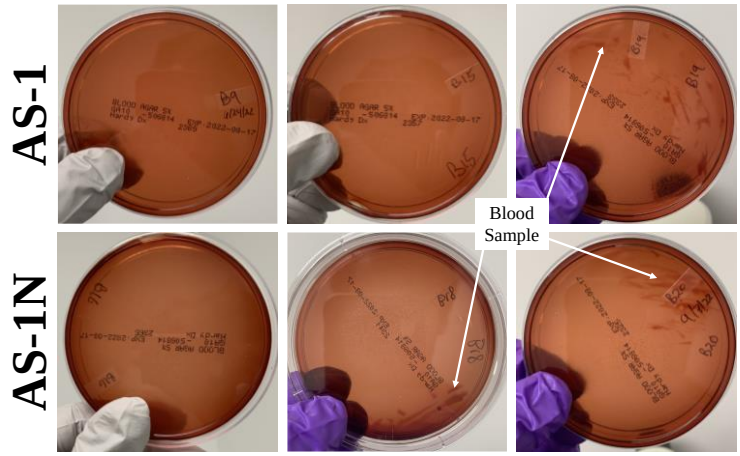

Figure 2.9**: Blood agar plates streaked with stored RBC samples after 44 days in storage.** The agar plates were incubated at 37 ˚C for 7 days and monitored for growth. No growth was seen in any of the samples tested.

The stored RBCs were also tested for sterility at the end of storage to determine if the glucose feeding device and/or regimen introduced any contamination during storage. Three donor RBC units for each storage condition (AS-1 and AS-1N) were fed utilizing the autonomous glucose feeding device and sampled at the end of storage for streaking on 5% sheep blood agar. Blood agar was used to determine the presence of any hemolytic microorganisms in the samples. The plates, after 7 days of incubation, are shown in figure 2.9. As seen in all conditions, there was no growth of any kind, providing strong evidence that the device did not introduce any contamination into the storage bags. With certain plates, a larger sample of blood was streaked onto the plate and should not be mistaken with microbial growth. It is noted, however, that some microorganisms may not be able to be detected using this method, specifically viruses and mold.

More rigorous testing would be required to ensure complete confidence in the sterility of the feeding device.

2.3.5 Device Improvements

Beyond the change in feeding regimen discussed in 2.3.4, an additional modification to the device involved the calibration of a new solenoid valve. The new valve (Humphrey Cat# 35031500) exhibited a smaller orifice diameter (2.72 mm) and better resistance to corrosion, an

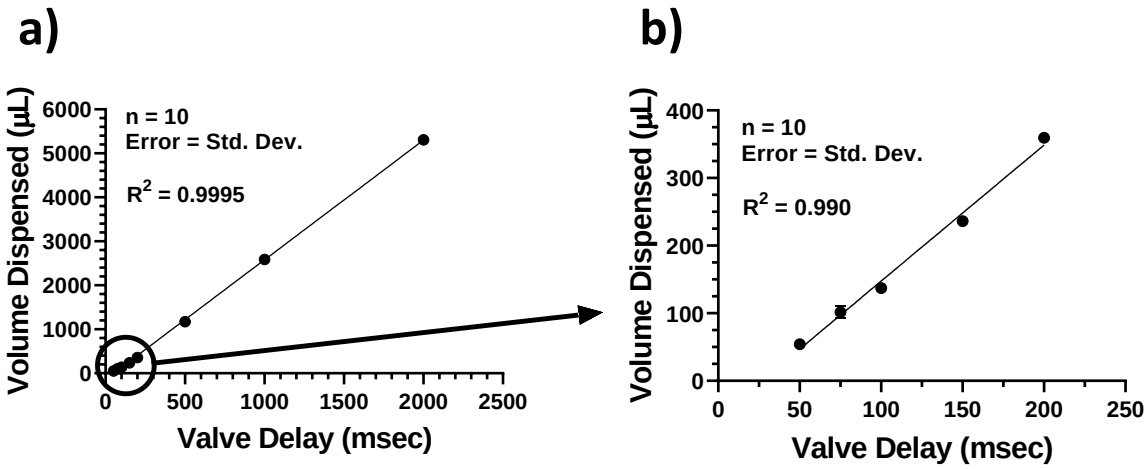

Figure 2.10: **Calibration curve depicting volume dispensed as a function of solenoid valve delay period utilizing a clinically approved valve with a smaller orifice diameter**. (a) The volume dispensed from the automated feeding system using a smaller solenoid valve at various valve delay periods (50 – 2000 msec) was determined. The calibration curve is linear and achieved a significantly lower minimum dispensing volume of 54.3 µL ± 1.96 µL (n = 10, error = standard deviation). (b) Blown up view of the valve calibration at lower valve delay periods (50-200 msec), also depicting strong linearity (n = 10, error = standard deviation).

observed issue using the generic solenoid valve. This valve was calibrated similar to the methods described in 2.2.5. The data is shown in figure 2.10. This data indicates that this valve, with a significantly smaller orifice diameter, was able to achieve a much lower minimum dispensing volume of 54.3 µL ± 2.0 µL, while maintaining linearity in its dispensed volumes throughout all valve delay periods tested. Though this valve was not used in the maintenance of glucose levels of stored RBCs, this data highlights the ability of the design to achieve lower dispensing volumes.

Additionally, lower dispensing volumes could also be achieved by decreasing the distance in height between the feeding reservoir and the stored RBC unit.

2.4 Discussion

Previous investigations reported that normoglycemic stored RBCs exhibit superior functionality in comparison to RBCs processed with traditional storage methods.[8–11] However, the implementation of normoglycemic RBC storage to clinical practice has been severely limited by its necessity for periodic glucose feeding. This manual feeding strategy required the injection of concentrated glucose into stored RBCs every 2-4 days, resulting in unrealistic labor requirements and sterility breaches that would not lead to clinical adoption. This necessitated the design of an autonomous and sterile glucose maintenance strategy for stored RBCs that could also be integrated into the current blood collection, processing, and storage infrastructure. The device described here satisfies these requirements along with adhering to most of the design constraints outlined in table 2.1.

Arguably, the most important deliverable for this device was the autonomous glycemic control of stored RBCs for up to 42 days. This was attempted utilizing both the constant volume delivery method as well as the storage-dependent delivery method shown in figure 2.7c and 2.7d, respectively. Neither approach was consistent in its glycemic control between 4-6 mM for a full 42-day storage period. Although certain units maintained normoglycemia for 37-39 days (figure 2.7c and 2.7d), the inconsistencies in glycemic control remain a limitation in the application of this device. The inconsistencies and deviations from normoglycemia were most likely due to donor variation in glucose utilization along with gas pockets that developed within the RBC unit, often traveling upward into the feeding tube. It is well known that gaseous exchange occurs within the semi-permeable PVC storage bags, leading to increased O_2 partial pressure and an increase in CO_2

partial pressure due to the bicarbonate pH buffering system.[27,28] These gasses and increase in pressure may have caused gas pockets to travel into the feeding solution, resulting in subsequent feeding periods delivering less than intended volumes of glucose. Though the constant volume delivery approach inherently exhibited deviations from normoglycemia even when operating as intended (figure 2.7c), the device's inability to consistently match stored RBC glucose levels to those predicted by the model were most likely due to these gas pockets and donor variation in glucose usage. It is also possible that the deviations from normoglycemia were due to variable volume delivery from inconsistent valve opening and closing, however, this is not likely. Figures 2.4 and 2.5 illustrate the ability of the device to consistently deliver precise volumes of feeding solution with minimal error that is characterized well by its appropriate model and led to expected increases in glucose concentration experimentally. Regardless of cause, however, it is noted that the consistency of this glucose delivery approach must be improved for clinical application.

Though not consistent, the data indicate that autonomous glycemic control of stored RBCs is possible (figures 2.6c and 2.6d), overcoming a significant obstacle in the application of normoglycemic RBC storage. When operating as intended, both feeding regimens exhibited similar trends to their respective models, maintaining the normoglycemic range for up to 37-39 days. Though incapable of maintaining normoglycemia for 42 days, the average age of RBC units transfused to ICU patients is 16-21 days, with this average being lower during blood shortages.[29] Therefore, it may not be necessary to consistently maintain normoglycemia for a full 42-day storage period. Regarding the two feeding regimens, the data illustrate that the storage-dependent delivery method was superior in glycemic control, resulting in less deviations from the 4-6 mM range and a tighter range of glucose values. This can be attributed to the change in glucose utilization exhibited by the RBCs throughout storage, and the storage-dependent delivery method

compensating for these changes. The RBCs metabolized a larger amount of glucose earlier on in storage than later in storage. This is likely due to their removal from the body and metabolic adjustment to hypothermic storage. Within the body, RBCs are subject to metabolic waste clearance mechanisms from the liver and also exhibit metabolic rates that reflect physiological temperature (37 ˚C).[30,31] Within storage, however, these waste mechanisms are unavailable, and temperature drops significantly. As discussed in chapter 1, the buildup of metabolic waste products and drop in temperature negatively impact the rate of glycolysis and other glucose metabolism pathways, leading to a significant drop in glucose utilization following the first few days in storage. This is why the storage-dependent delivery method was superior in glycemic control, adjusting glucose delivery to the needs of the RBCs.

While our data suggest it is possible to achieve glycemic control of stored RBCs using the device described here, it is unclear whether the precise 4-6 mM glucose range is necessary to illicit the beneficial effects of normoglycemic storage. Even in comparison to 8 mM glucose (the highest recorded glucose level of AS-1N stored RBCs), AS-1 stored RBCs exhibited glucose concentrations over 2 times that of AS-1N stored RBCs at the end of 44 days in storage. As noted previously, there is some ambiguity in the exact cut-offs for a normoglycemic range of blood glucose.[20,21] A healthy person can experience blood sugar levels down to 3.3 mM and upwards of 7.8 mM.[20] However, other reports indicate that fasting blood glucose above 7 mM is considered hyperglycemic and fasting blood glucose between 5.6 mM and 7 mM is pre-diabetic.[32] Due to this variation, attempting to maintain a 4-6 mM glucose range would be preferable and was established as the normoglycemic target. But due to the variability in thresholds for normoglycemia, the precise control of glucose within this 4-6 mM range may not be necessary, especially if they are transient deviations such as many of the deviations shown in figures 2.7a and 2.7b. In fact, our

previous publications on normoglycemic storage of RBCs occasionally depicted glucose levels of normoglycemic stored RBCs deviating outside of the 4-6 mM range, yet still led to improved RBC functionality over RBCs in hyperglycemic storage.[10] So although this device did not achieve precise glucose control, it is most likely unnecessary, and the fully autonomous glycemic control of stored RBCs in figure 2.7d can be considered successful for a full 44 days, despite the rise in glucose above 6 mM after day 39.

The hematocrit of stored RBCs in additive solutions is typically between 55-60%.[33] Physicians and patients rely on this presumed RBC concentration to raise hemoglobin levels of transfused patients by an average of 1 g/dL per unit of stored RBCs. It is important to maintain these hematocrit percentages in stored RBCs since lower hematocrits inherently would require more stored RBC units to achieve the same increase in patient hemoglobin levels. Apart from further burdening the stored RBC supply, transfusing additional units to patients may put them at increased risk for transfusion associated circulatory overload (TACO) due to increased transfusion volumes.[34] Periodic addition of 100 mM glucose via the autonomous feeding device led to a drop in hematocrit to 50.4% ± 3.8% in the most extreme case. Though this is outside of the typical 55-60% range characteristic of traditional RBC storage, it is unclear whether this lower hematocrit of stored RBCs would significantly impact patient outcomes. A study from 2021 investigated over 1900 RBC units transfused into sickle cell patients, finding that the average hematocrit was 63.3% with a range of 54.6% - 72.9%.[35] Considering this study, it is not unreasonable to assume that RBC units that drop to 50% could still be used for transfusion. However, as noted in figure 2.8, using a 400 mM glucose feeding solution would lead to a theoretical decrease in hematocrit of less than 3%, a much less significant drop that could reasonably be administered to a patient. The 100 mM glucose solutions were implemented as the feeding reservoir primarily due to the minimum

dispensing volume of the valve at 176 µL ± 21 µL indicated by figure 2.4b. Using a more concentrated glucose feeding solution would require 3-day dispensing volumes lower than this limit, which was not achievable. Further design improvements that could lower this dispensing limit could utilize a more concentrated glucose solution and result in smaller drops in hematocrit.

The maintenance of complete sterility of the stored RBCs is vital to limit the infection risk to transfused patients. Due to the completely closed nature of multi-bag blood processing systems and the rigorous testing for infectious diseases after donation, the rate of septic transfusion reaction is 1 in 500,000 and that of transfusion-transmitted infections (HIV, syphilis, hepatitis, etc.) is less than 1 in 1,000,000.[36,37] Any new blood storage/transfusion technology that would threaten to increase these rates is likely to experience difficulty with adoption. The autonomous glucose feeding device described here is indeed able to maintain confidence in the sterility of the stored RBCs and is not expected to increase patient risk in relation to microorganism contamination. Since the valve, tubing, and feeding solutions were all sterilized prior to attachment to the RBC units, the completely closed system was expected to maintain complete sterility for the entire duration of storage. This was confirmed through plate streaking in figure 2.9, resulting in no growth of any microorganisms. However, it should be noted that this device currently does not comply with FDA regulations regarding blood collection, processing, and storage. As previously mentioned, the FDA Code of Federal Regulations (CFR 21.640) states that "the blood container shall not be entered prior to issue for any purpose except for blood collection or when the method of processing requires use of a different container."[15] Obviously, the device implemented here fails to comply with this regulation as the stored RBC bag is "entered" periodically with a concentrated glucose solution. However, due to the FDA approval of rejuvenation solutions for stored RBCs, which also invade the blood bag, the failure to abide by this FDA regulation is not a concern,

especially if future *in vivo* clinical trial evidence of normoglycemic RBC storage results in improved post-transfusion recovery percentages and no impact on patient infection rate.[19]

Beyond glycemic control and sterility, there were many other necessary design characteristics for the glucose feeding system. These included the limited disruption of blood processing procedures, no change to blood collection bags, no impact on the cold chain management of RBCs, and the ability to distribute and readminister an RBC unit. Firstly, since this automated glucose feeding system was designed to be attached to stored RBCs units only after blood collection and processing, this device has no impact on blood processing. Although novel anticoagulant and additive solutions would be necessary to implement (CPD-N and AS-1N), the procedures for collection and processing would remain unchanged. Thus, no significant additional training would be required using this normoglycemic storage system, nor would additional equipment be necessary beyond the valve and feeding reservoir. This includes blood collection bags. The autonomous feeding device described here was designed to be attached to any pre-existing blood collection bag. Not only would a custom blood collection bag require further FDA approval in the future, but it would be an additional challenge to compete with the over 15 blood bag manufacturers for a market share. The device designed and described here also does not impact any aspect of the cold chain management. As described previously, cold chain management is a crucial aspect to blood processing and storage that directly impacts RBC health. One study found that stored RBCs kept at 6 ˚C exhibited greater levels of non-transferrin bound iron (NTBI) and increased cell-free hemoglobin in comparison to RBCs stored at 2 ˚C, illustrating just how important cold chain management is and how sensitive RBCs are to fluctuations.[38] The autonomous glucose feeding device was designed to be residential to blood banks or RBC storage facilities within the already tightly temperature controlled cold rooms and refrigerators, thus

imparting no impact to cold chain management. Stored RBCs are also often distributed to hospitals, but then sent back to storage if no longer needed. A study conducted at the University of Pittsburgh Medical Center found that 49% of its surgery patients were issued at least one RBC unit with 72% of the units sent back to the blood bank for reissue.[39] This may seem high, but often, these are issued so they are available for the patient without delay if necessary, which is only made possible by the ability to send the units back to the blood bank. Due to this, it was critical that any glycemic control system maintain this ability to issue and put back into storage as necessary. With the feeding device as described, it would be simple to remove a stored RBC unit from the feeding tubing for issue, and just as easy to readminister a stored RBC unit back into the feeding system at any storage day. This assumes that the valve and feeding solution reservoir are residential to blood storage facilities. Regarding the storage-dependent delivery feeding regimen, modification would be required to indicate the age of the RBC units sent back to the blood bank, which would be a simple adjustment to the device's program and could be accomplished via user input in future design iterations.

Other preferred, but not necessary, design constraints included the simplicity of the design and its relative inexpensiveness. The method of gravity-driven fluid flow, similar to IVPB systems, was implemented to maintain simplicity in the design. Since IVPB is already such a well-established technique for solution delivery in clinical practice, this same technique was adopted for autonomous glucose control and was thought to be better received by medical professionals due to this simplicity and familiarity.

Though meeting most of the design constraints outlined in table 2.1, the design for the autonomous glycemic control of stored RBCs illustrated here is not without its limitations. Firstly, and most importantly, the device currently fails to accommodate to current blood storage physical

infrastructure. The device relies on gravity-driven fluid flow and a precise operational distance between the feeding reservoir and the RBC unit. Though this simplicity inherently presents some benefit as described previously, it also requires a fixed geometry for implementation that is not compatible with current blood storage infrastructure. Many of the blood storage refrigerators maximize space by implementing several shelves, allowing for as many as several thousand RBC units to be stacked on top of each other.[16] Not only would the autonomous glucose feeding device require additional physical space in these refrigerators, but would also require the maintenance of exact operational distances which would be near impossible to accomplish. Although a singular feeding reservoir could be used for multiple RBC units and many valve calibrations could be conducted at various operational heights, each RBC unit would still require its own valve, which is likely unrealistic to implement for several thousand RBC units. Additionally, the device's inconsistency in glycemic control is another barrier that is necessary to overcome. Since glucose levels cannot be monitored for each of the over 13 million annual whole blood donations, implementing normoglycemic RBC storage not only requires that autonomous glycemic control be possible, but that the control is consistent enough to ensure confidence in the normoglycemia of stored RBCs at any point during storage. This is indeed critical regarding deviations away from normoglycemia toward hypoglycemia. Under hypoglycemic conditions, the RBCs cannot metabolize enough glucose to sustain their energy needs, leading to hemolysis.[40] This was illustrated through a previous study on normoglycemic RBC storage without periodic glucose feeding that led to significant hemolysis, over 20 times that of traditional storage.[11] This is why consistent glycemic control is necessary for the implementation of normoglycemic RBC storage at a wide scale. Though the storage-dependent delivery feeding regimen made improvements to this consistency, it still was not adequate to be confident in the device's infallibility. Lastly, it is

important to note that normoglycemic RBC collection, processing, and storage may consequently have a negative impact on other blood component products. As mentioned previously, whole blood is often collected in multi-bag systems for the complete separation of the components (RBCs, plasma, platelets, etc.). Implementing normoglycemic collection/storage of RBCs would then inherently lead to normoglycemic conditions experienced by the other blood components, specifically platelets. Considering platelets are currently stored at room temperature for up to 5-7 days, it is likely that they would metabolize glucose significantly faster than hypothermic stored RBCs and may deplete glucose to hypoglycemic conditions relatively quickly.[41] Though hyperglycemia actually leads to overactivation of platelets, hypoglycemia is also detrimental, resulting in platelet hyperaggregability and increased coagulation cascade factors.[42,43] Even if normoglycemic storage is beneficial to platelets, the use of normoglycemic storage solutions, as described here, for blood collection would most likely lead to platelet hypoglycemia during storage and potentially introduce unintentional adverse effects to stored platelets.

2.5 Conclusion

Previous work has already established the *in vitro* benefit of normoglycemic RBC storage. However, its application was significantly impeded by its dependence on periodic glucose addition to the stored RBCs to maintain normoglycemia. This periodic glucose "feeding", previously accomplished by manual addition of concentrated glucose, introduced repeated risk for contamination and would not be possible to translate to current blood storage practices due to unreasonable labor requirements. The autonomous glucose feeding device designed, validated, and implemented within this chapter was able to successfully overcome these limitations while also adhering to many other design constraints that would lead to limited impact to the current blood collection, processing, and storage standards. Utilizing a gravity-driven fluid flow approach,

similar to a simple IVPB system, coupled with a controllable solenoid valve, precise volumes of concentrated glucose were delivered to stored RBCs periodically over a period of 44 days. The device's operation was well characterized by engineering principles that led to expected increases in glucose concentrations experimentally. The precise control of volume delivery, down to the microliter range, allowed for its implementation in the normoglycemic maintenance of stored RBCs for 44 days via periodic dispensing of 100 mM glucose. After observing time-dependent changes to RBC glucose utilization during storage, a modified feeding regimen that reflected the changing glucose needs of the RBCs was applied to the device, resulting in glycemic control with less deviation from normoglycemia and a tighter glucose concentration range in comparison to the constant volume delivery approach. Though neither approach consistently maintained glucose concentrations between 4-6 mM for the full storage period, a few RBC units were successfully maintained within normoglycemia completely autonomously for up to 37 and 39 days, highlighting the possibility of sterile and autonomous glucose control of stored RBCs. Furthermore, the stored RBC units showed no signs of contamination and led to expected drops in hematocrit that would likely have little to no impact on expected patient hemoglobin increases. This work makes significant progress toward the application of normoglycemic RBC storage. Though consistency of glycemic control must be improved, the device characterized within this chapter illustrates that autonomous glycemic control of stored RBCs is possible, while still maintaining sterility, overcoming two of the most challenging limitations of this technology. Not only did the autonomous glucose feeding device overcome these barriers, but it also would result in little to no impact on the current standards of blood collection and processing. Since the procedures for blood collection and subsequent processing for storage are so well-established and universal, creating a design that could maintain normoglycemia without impacting these standards was crucial. Though

the device exhibited several limitations, including physical compatibility with blood storage facilities and consistency in glycemic control, much work is currently being done to address these. Even if physical compatibility with blood banks cannot be overcome, this technology may still have potential for implementation with rare blood types or special transfusion circumstances where superior RBC functionality is necessary, assuming normoglycemic RBC storage also yields improved *in vivo* outcomes. Overall, the work described here offers a novel strategy for the autonomous control of glucose concentrations in stored RBCs, greatly improving the potential for normoglycemic stored RBCs to be translated to clinical practice.

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Chapter 3 – Red Blood Cell Storage Lesion Progression in Normoglycemic Storage

3.1 Introduction

Transfusions are necessary procedures due to the functions performed by red blood cells (RBCs) throughout the circulation. As a key transporter of molecules throughout the body, adequate RBC supply is critical to the proper function of other tissues.[1] The most important role of the RBC is its ability to deliver oxygen to cells, tissues, and organs that rely on oxidative phosphorylation for energy metabolism.[1–3] The total oxygen consumption in the body is usually between 110 and 160 mL min^{-1} m^{-2}, or nearly 350 L per day on average.[4] Hypoxia, or the inadequate oxygen content in tissues, can lead to very serious issues including organ deterioration, organ failure, and even death.[5] In fact, the kidney and liver can tolerate hypoxia for only 15-20 minutes before severe irreversible damage, while the brain exhibits more stringent tolerance at less than 3 minutes.[6] Hypoxia in the tissues is often due to low RBC supply or low oxygen content in the blood (hypoxemia).[5] This is why maintaining sufficient RBC levels in the circulation, and subsequently ensuring adequate oxygen delivery, is vital to the health and survival of transfusion patients.

Oxygen delivery by RBCs is dependent on several related characteristics of RBC physiology, such as their deformability, or ability to change shape when circulating through small vessels. Within their average 120-day lifespan in the circulation, RBCs traverse through arteries, veins, arterioles, venules, and small capillaries. These blood vessels range from 3 cm down to 5 µm in diameter, requiring the 8 µm diameter RBCs to deform their shape to squeeze through these vessels and deliver oxygen.[7–9] The characteristic discocytic shape of RBCs is what allows them to deform and travel through small capillaries. Their high surface area to volume ratio enables them to adapt their shape to the relatively small diameter of capillaries.[9] Due to their lack of organelles

and greater than 96% hemoglobin content (dry weight percentage), these shape changes do not disrupt their cellular functions and allows the RBC to withstand a maximum shear stress of 1,500 dynes cm^{-2} without permanent cell damage.[10–12] These phenomena are directly related to the RBC membrane integrity and composition.[11] Without proper cell deformability, RBCs can often block small capillaries and other blood vessels, inhibiting oxygen delivery and perfusion to tissues.[11]

An additional RBC characteristic related to oxygen delivery and deformability is adenosine triphosphate (ATP) release. ATP is a high energy molecule used for RBC functions, including the maintenance of a tightly controlled ion balance across the cell membrane.[13] ATP is also important in facilitating interactions between the cytoskeleton and the cell membrane, which helps retain its shape.[14,15] When released from the RBC to the extracellular matrix, ATP acts as a stimulus of nitric oxide (NO) production in multiple cell types, a known vasodilator.[16] Under response from such stimuli as shear stress or hypoxia, RBCs release ATP into the extracellular space where it binds to endothelial cell P2Y receptors.[17] This results in an influx of Ca^{2+} into the cell where it binds to calmodulin. This Ca^{2+}-calmodulin complex stimulates endothelial nitric oxide synthase (eNOS) activity, resulting in an increase in NO concentration as a byproduct of L-citrulline production from L-arginine.[17] The increased NO diffuses into vascular smooth muscle, resulting in cyclic guanosine monophosphate production (cGMP) and subsequent vasodilation,[17] which enables increased blood flow and increased delivery of RBCs to target tissues. This ATP release, and subsequent NO production and vasodilation, allows for greater blood flow and oxygen delivery to tissues.[18,19] When blood oxygen content falls (hypoxemia), RBCs will release ATP in response, stimulating NO production and vasodilation to compensate for the limited oxygen delivery to the tissues.[17,20] Similarly, RBCs traversing small capillaries and other small vessels will exhibit increased shear stress, resulting in ATP release that leads to vasodilation and improved blood

flow.[20] The mechanism of RBC derived ATP release is well established as a contributor to improved blood flow and has also been indicated as playing a role in many pathologies including type 1 diabetes and multiple sclerosis.[14,17,21–24]

Maintaining proper RBC membrane shape and integrity, accompanied by sufficient ATP release is vital for proper blood flow and oxygen delivery *in vivo*. Stored RBCs, however, exhibit decreased deformability and reduced ATP production and release, often leading to clearance from the circulation when transfused.[25] This may be attributed to the development of the storage lesion. The storage lesion is the collective accumulation of several metabolic, membrane, and oxidative damages imparted to RBCs during hypothermic storage. These include decreased ATP production, lipid/protein oxidation, membrane loss, morphological changes, protein glycation, and many other impairments.[13] More specifically, a combination of dysfunctional ion pumps, acidosis, metabolic waste, protein/lipid oxidation, and glucose metabolism impairment, are all reported to negatively impact ATP production and RBC membrane shape/integrity during storage.[13] Considering the importance of these properties to RBC function, it is not surprising that many RBCs are cleared from circulation after transfusion due to senescence. In fact, the average 24-hour post transfusion recovery percentage, or the percentage of transfused RBCs still circulating 24 hours after transfusion, was 73.5% ± 13.7% in 10 patients who received 25-35 day old stored RBCs, a clearance of over 25% of the transfused RBCs.[26] The potential link between the RBC storage lesion and clinical outcomes is precisely the motivation driving blood storage research, aiming to limit or reverse the progression of the storage lesion. Though many strategies have been implemented and have been successful at accomplishing this, including novel rejuvenation solutions and hypoxic RBC storage, none have addressed the hyperglycemic conditions exhibited by all current FDA approved RBC storage solutions.[27–31]

The Spence lab believes that the hyperglycemic conditions present in RBC storage is, in part, responsible for the development of the storage lesion. This is supported by evidence suggesting that RBCs from people with diabetes exhibit similar RBC impairments as stored RBCs, specifically decreased ATP release, reduced deformability, increased protein glycation, and increased oxidative stress.[17,32] A common characteristic between diabetes and blood storage is hyperglycemic environment, although glucose levels are ~ 5 times more concentrated in blood storage than in the blood stream of people with diabetes.[33] In fact, chronic hyperglycemia in diabetes patients is directly associated with increased protein glycation and increased oxidative stress, leading to further microvascular and cardiovascular complications.[34–36] Though progressing at a much slower rate at a lower temperature, many of these same hyperglycemia-related impairments may contribute to the development of the RBC storage lesion. Similarities between the properties of stored RBCs and RBCs from patients with diabetes motivated the Spence lab to investigate RBC storage lesion progression under normoglycemic conditions. Interestingly, it was found that storing RBCs at physiological glucose concentrations alleviated many aspects of the storage lesion, specifically in function-defining attributes, such as ATP release and deformability.[37–40] As discussed previously, these two RBC characteristics are pivotal to RBC function *in vivo*. As shown in figure 3.1 borrowed from previous work in the lab, both ATP release and RBC deformability remained statistically unchanged throughout storage under normoglycemic conditions, whereas hyperglycemic storage resulted in significant decreases.[38,39] ATP release and relative deformability recovered for hyperglycemic-stored RBCs when incubated in normoglycemic buffers, although after 1 week of storage the effect of the hyperglycemic storage

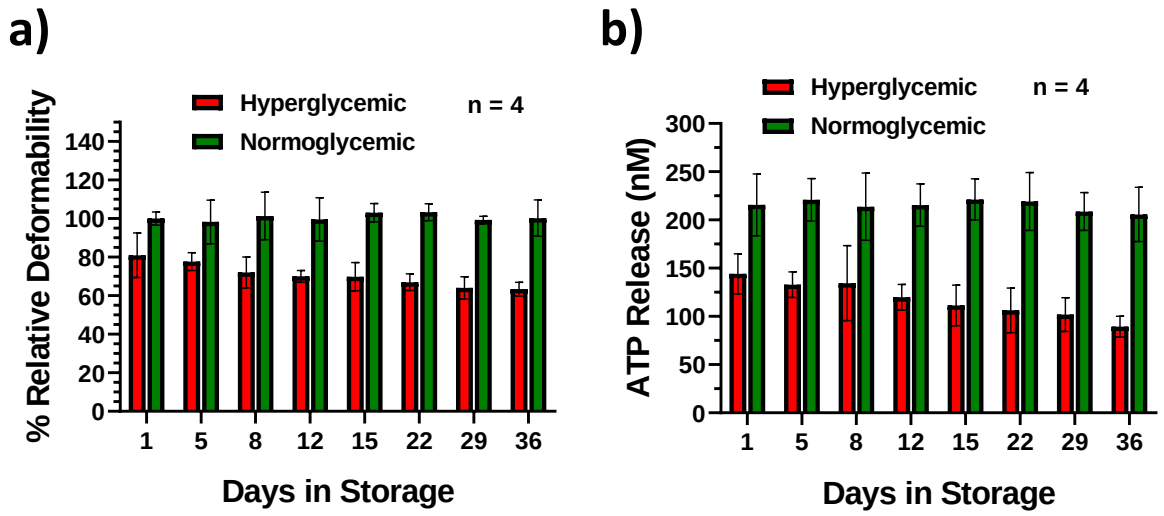

Figure 3.1**: ATP release and deformability of stored RBCs under hyperglycemic and normoglycemic storage conditions.** (a) ATP release of normoglycemic stored RBCs (AS-1N) exhibited sustained levels at approximately 200 nM throughout 36 days of storage. Hyperglycemic stored RBCs (AS-1), however, resulted in significantly reduced ATP release that continued to decrease throughout storage. (b) Relative deformability of normoglycemic stored RBCs was maintained throughout 36 days of storage, while hyperglycemic stored RBCs resulted in significantly reduced deformability relative to normoglycemic storage (n = 4, error = SEM).

conditions were mostly irreversible.. Additionally, normoglycemic storage of RBCs resulted in decreased osmotic fragility and decreased sorbitol production.[38] The rates of hemolysis, an additional known consequence of RBC storage, were comparable between hyperglycemic and normoglycemic storage, though above Food and Drug Administration (FDA) allowable levels.[37] Though *in vivo* benefit is yet to be demonstrated, clearly normoglycemic stored RBCs exhibited improved function *in vitro.*

The benefits of normoglycemic storage may be explained by decreased membrane damage and reduced oxidative stress. Under physiological glucose concentrations, RBCs mainly metabolize glucose through the Embden-Meyerhof-Parnas pathway and the pentose phosphate pathway, producing ATP, reducing equivalents, and 2,3-disphosphoglycerate (2,3-DPG).[41] However, glucose can also be metabolized via the polyol pathway, which disrupts the redox

balance of RBCs, depleting NADPH and producing cell impermeable sorbitol.[42,43] All of these pathways are summarized in figures 1.2 and 1.5 in chapter 1. The polyol pathway is often upregulated in patients with diabetes and results in increased levels of reactive oxygen species (ROS) and advanced glycation end products (AGEs).[42,43] This phenomenon, which exhibits increased activity under hyperglycemic conditions such as those found in RBC storage solutions, may exacerbate storage lesion progression in two parts. Firstly, the depletion of NADPH and subsequent increase in ROS can lead to oxidation of membrane proteins/lipids and cytoskeletal proteins, leading to membrane loss and cell rigidity.[42,44,45] Secondly, increased metabolic flux through the polyol pathway generates increased concentrations of sorbitol and fructose, which are nearly cell impermeable.[42] This draws fluid into the RBC, imparting greater osmotic stress to the cell. These processes may be able to explain the results in previous publications on normoglycemic blood storage, such as the data in figure 3.1. Under normoglycemic conditions, reduced sorbitol levels may be due to decreased polyol activity, resulting in decreased osmotic fragility and decreased membrane damage that allows the RBCs to maintain sufficient deformability, and therefore ATP release.

Normoglycemic RBC storage yields beneficial outcomes on stored RBC functionality *in vitro*. However, as discussed in previous chapters, previous work utilized a manual glucose feeding regimen (requiring bag invasion) to maintain normoglycemia of stored RBCs that would not be permissible in clinical practice. Additionally, previous experiments were performed at small-scale storage (< 2 mL) with custom storage bags made in-house. Therefore, small volume storage may lead to mixing profiles that would not be present at larger-volume, commercial scale storage thereby leading to a disconnect between in-house and commercial storage systems employed in the clinical setting. In addition, commercial blood storage bags are semi-permeable to gases such

as oxygen and carbon dioxide. Semi-permeability of the storage bags fabricated in-house was low due to absence of plasticizers in the bag material. DEHP also renders the storage bag permeable to gases, including oxygen and carbon dioxide.[46] Commercial blood storage bags are often plasticized with di(2-ethylhexyl)phthalate (DEHP) that is thought to leach into the stored RBCs and incorporates into the membrane. To improve the rigor of our data, it was anticipated that scaling to larger storage with commercially available bags and subsequent autonomous glucose control was requisite to fully understand the translation of normoglycemic storage to clinical practice. Additional measurements regarding RBC metabolism under such conditions are also necessary to confirm previous findings.

Previous data on normoglycemic RBC storage indicate that reduced oxidative and membrane damage are responsible for improved RBC health in normoglycemic storage. A further investigation into these mechanisms, especially through more direct measurements, will provide a clearer understanding of the benefits of normoglycemic RBC storage. For example, it is well established that sorbitol levels can serve as a surrogate for oxidative stress in cells.[42,47] However, the increase in oxidative stress generated through the polyol pathway is due to a depletion of the reducing equivalent NADPH, not direct ROS generation.[42] Depletion of NADPH leads to less antioxidant activity due to its role in recycling the key antioxidant glutathione (GSH). Reduced GSH levels then leads to reduced ROS clearance.[42] A more direct measurement of ROS levels under hyperglycemic and normoglycemic RBC storage would confirm that normoglycemic storage results in reduced ROS generation.

The improved deformability and decreased osmotic stress reported in normoglycemic RBC storage indicates that other, more direct, membrane-related measurements may also highlight differences between hyperglycemic and normoglycemic RBC storage. As discussed previously,

the shape of RBCs directly impacts the relative deformability of the cells. Considering that normoglycemic stored RBCs exhibited increased relative deformability in comparison to hyperglycemic stored RBCs, it is possible that there may also be differences in the RBC shape.[39] It is well known that RBCs lose membrane via microvescicle shedding throughout storage, due to protein and lipid oxidation.[13,48] As a method to maintain membrane health and an initial step in programmed cell death, RBCs release microparticles composed of damaged membrane proteins/lipids.[48] Additionally, the hypothermic temperatures of cold storage disrupt the activity of membrane bound Na^+/K^+ ATP-ase pumps, causing leakage of sodium and potassium ions that results in osmotic stress and increased ATP consumption to maintain proper ion gradients.[13,49] As ATP becomes depleted, these cation leaks become exacerbated, driving fluid flow into the cell and increasing the mean corpuscular volume (MCV).[49] The combination of cation leaks, membrane loss, and cytoskeletal protein oxidation result in echinocytosis of the RBCs, characterized by a relative increase in outer leaflet membrane surface area relative to the inner leaflet.[11,13,48–51] As membrane loss is progressed, the RBCs transition to a more sphere-like shape, eventually resulting in spherocytes.[11,13,49] Reports have indicated that ATP depletion is directly correlated with the progression of these shape changes, likely due to ATP dependence on maintaining appropriate intracellular ion gradients and transient interactions between the spectrin cytoskeleton and the cell membrane.[15,49] Additionally, the conformation of the membrane-bound ion transporter, band 3 protein, may also determine RBC shape, with clustering and inward facing conformations favoring echinocytosis.[52,53] It was discovered that band 3 protein has several cytoplasmic phosphorylation cites and exhibits slow ion transport when ATP levels are low, implicating a relationship between ATP and band 3 protein, causing shifts toward echinocytosis when ATP is low.[54] These echinocytes and spherocytes are known to be more rigid and less deformable than healthy

discocytes, resulting in decreased perfusion *in vitro* and increased cell clearance *in vivo*.[50,55] The shape transformation from discocyte to echinocyte through to spherocyte is shown in figure 3.2a. In the context of normoglycemic RBC storage, there may be less severe echinocytic shape changes due to the increased relative deformability of the RBCs in comparison to hyperglycemic storage

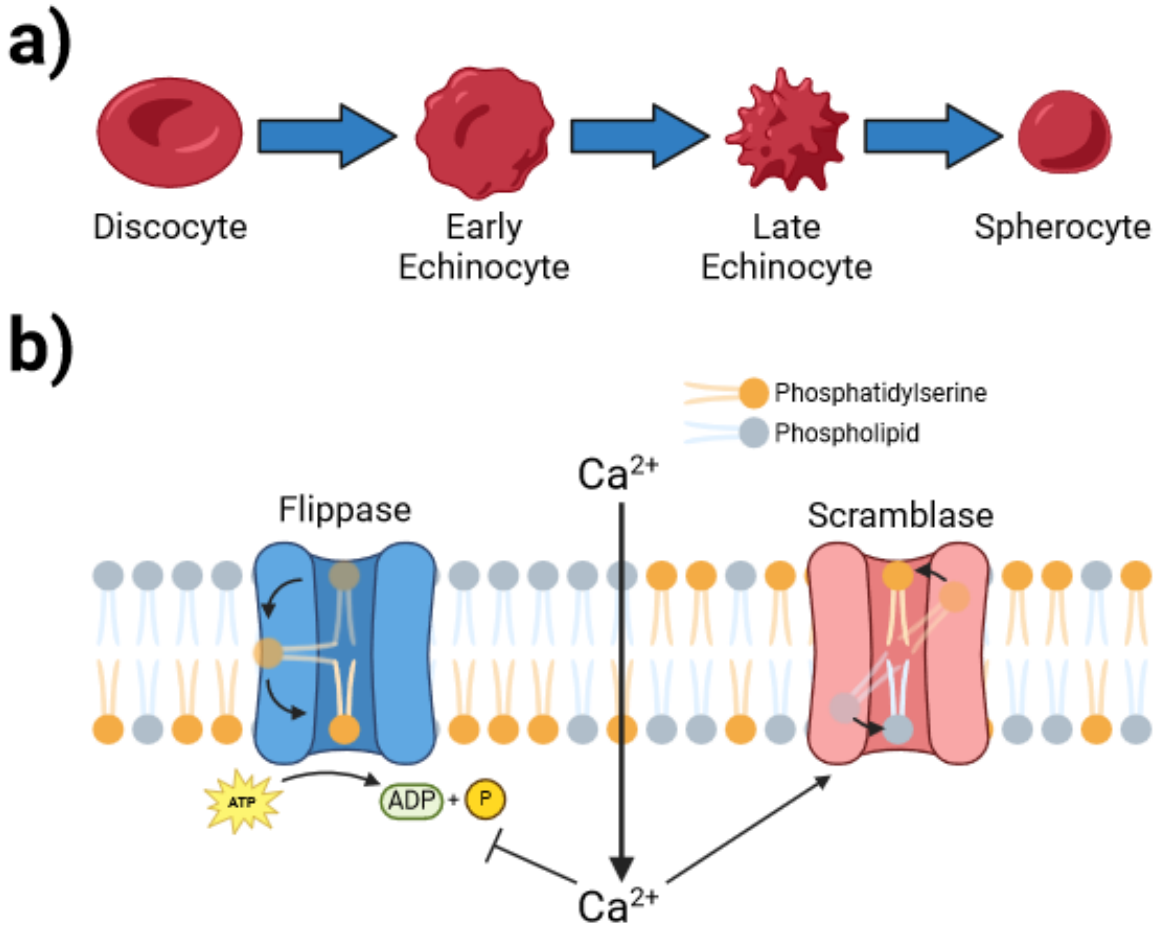

Figure 3.2: **Progression of RBC shape changes and membrane damage during storage**. (a) As RBCs lose portions of their membrane during hypothermic storage along with the ability to maintain appropriate ion concentration gradients and cytoskeletal interactions, their shape progresses through echinocytosis and spherocytosis, generating spicules and becoming more sphere-like. These shape changes lead to increased membrane fragility and rigidity. (b) The process of PS externalization within the RBC membrane is a result of a balance between flippase and scramblase activity. Flippase transmembrane proteins maintain the asymmetrical distribution of PS to the inner leaflet of the membrane, consuming ATP in the process. Scramblase, an ATP-independent transmembrane protein, non-specifically translocates phospholipids from the inner leaflet to the outer leaflet as well as the opposite. Increased levels of Ca^{2+} lead to increased PS exposure due to its inhibitory effect on flippase and activation effect of scramblase.

Accompanying storage-induced membrane damage of RBCs is the externalization of phosphatidylserine (PS). PS is an aminophospholipid that is contained almost exclusively to the inner leaflet of the membrane in many cell types, including RBCs.[56] The asymmetry of PS distribution into the inner leaflet is well maintained by an ATP-dependent transmembrane flippase protein, such as ATP11C.[57,58] This flippase protein translocates PS from the outer leaflet to the inner leaflet, exhausting ATP in the process.[58] During programmed cell death, however, PS is externalized to the outer leaflet in an ATP-independent manner via Ca^{2+} activated scramblase, which non-specifically flips inner leaflet lipids to the outer leaflet.[56–58] Increases in intracellular Ca^{2+} is often reported as an inducer of this process as it inhibits flippase activity and activates scramblase activity.[57] The externalization of PS *in vivo* serves as a signaling molecule for phagocytic cells to engulf and remove these cells from circulation (in the case of RBCs) due to damage or senescence.[56] PS externalization in stored RBCs increases throughout storage, as membrane damage progresses and PS rich membrane vesicles are shed.[49,56,59] It is likely that this increase in PS closely follows the progression of RBCs toward the spherocytic shape, due to ATP-dependence on flippase activity and the decrease in ATP during storage.[25,60] The process of PS externalization is shown in figure 3.2b. Though membrane changes and increases in PS externalization during storage may recover to some extent after transfusion, increased PS exposure leads to increased phagocytic removal of RBCs from circulation.[59,61] Therefore, measurements of PS externalization during RBC storage may provide a more direct measurement of RBC membrane damage and senescence.

In review of the probable link between normoglycemic RBC storage and membrane integrity and oxidative status, it is important to remember the impact of these mechanisms on deformability and ATP release. Improvements to stored RBC deformability and ATP release may

have significant impact on RBC function *in vivo* due to their relation to proper blood flow and oxygen delivery. The work presented in this chapter aims to confirm *in vitro* benefit of normoglycemic RBC storage utilizing commercial blood storage bags with autonomous glycemic control. Considering scaling up to larger blood storage with autonomous glucose feeding may impact the response of RBCs to normoglycemic storage, hemolysis, osmotic fragility, and intracellular sorbitol levels were measured to confirm data from previous reports. Additionally, further *in vitro* measurements related to metabolic activity, membrane integrity and oxidative stress of stored RBCs were performed to illustrate a clearer understanding of the mechanism driving the benefits of normoglycemic RBC storage. These included extracellular lactate levels, pH, cell size/shape, externalization of PS, and ROS generation.

3.2. Methods

The remaining content of this chapter expands upon previously published work and is re-printed with permission from ACS Journal of Pharmacology and Translational Science.[62]

3.2.1 RBC Collection, Processing, and Storage

All blood collection procedures from informed and consented donors were approved by the Biomedical and Health Institutional Review Board at Michigan State University. Blood collection procedures were performed according to 2.2.7 using traditional and modified storage solutions. These included citrate-phosphate-dextrose (CPD) and AS-1 for hyperglycemic RBC storage, and normoglycemic modified counterparts, CPD-N and AS-1N, for normoglycemic RBC storage. The constituents and concentrations of these solutions is shown in table 2.2. The collected blood was also attached to the automated glucose feeding system. Stored RBC units were stored at 4 ºC ± 2 ºC for up to 44 days. RBCs collected and stored using the traditional, hyperglycemic storage solutions will be referred to as AS-1 storage, whereas the normoglycemic alternative will

be referred to as AS-1N storage.

Glucose feeding was performed by utilizing both the constant volume delivery method as well as the storage-dependent delivery method. RBCs stored using AS-1 were periodically fed a 0.9% NaCl solution, while normoglycemic AS-1N stored RBCs were fed with 100 mM glucose in 0.9% NaCl. It should be noted that data collection regarding morphological changes, PS externalization, and ROS generation did not implement a 0.9% saline feeding regimen for the AS-1, hyperglycemic storage condition. This was in response to a reviewer suggesting to remove the feeding regimen from AS-1 storage so storage conditions were a closer match of traditional storage practices. Glucose was measured every 2-4 days using an AimStrip Plus blood glucometer to ensure normoglycemic maintenance. It should be noted that the data collected from morphology, PS externalization, and ROS generation experiments implemented a correction factor for glucose measurements of the stored blood since it was determined that the blood glucometer was not measuring accurate results for stored blood due to the increased hematocrit (60% in storage vs. 40-45% in whole blood). Due to this, the glucose reading from the glucometer was corrected using equation 3.1 below.

$$Gluc_{actual} = \frac{Gluc_{measured} * 0.575}{(1 - Hct\%)} \quad (3.1)$$

As discussed in chapter 2, neither of these methods were consistent in their glucose maintenance between 4-6 mM. Whenever the glucose deviated from this range, manual intervention was implemented by either forcing additional dispenses of glucose or shutting off the device. This resulted in a range of glucose values from 2 – 8 mM and should be noted that these deviations may have impacted the results of the biochemical measurements.

3.2.2 Measuring Stored RBC Hemolysis

Hemolysis was determined through the measurement of visible light absorbance of stored

RBC supernatants. Due to the direct relationship between cell-free hemoglobin levels and RBC hemolysis, absorbance of RBC sample supernatants at 540 nm wavelength light (hemoglobin absorbance wavelength) was utilized to determine the hemolysis percentage. Every 7 days, a 300 µL aliquot of stored RBC sample was removed from the respective storage bag and centrifuged at 2000*g* for 15 minutes. Approximately 70-90 µL of the supernatant and 20 µL of the cell pellet were collected and separated. Six hemoglobin (Sigma Aldrich) standards were then prepared in the range of 0 – 1 mg/mL hemoglobin by diluting the stock in Drabkin's reagent (Sigma Aldrich) to create 1 mL standards. The hemoglobin absorbance bands were measured at 540 nm using a spectrophotometer (Molecular Devices, SpectraMax M4). Using equation 3.2 below, the lysis percentage was determined for each sample collected at each time point where *[HbSN]* is the supernatant hemoglobin concentration (mg/mL), *Hct%* is the hematocrit percentage of the original sample, and *[HbRBC]* is the hemoglobin concentration of the packed RBC samples (mg/mL).

$$\%\, Lysis = \frac{[HbSN](100-Hct\%)}{[HbSN]+[HbRBC]} * 100 \qquad (3.2)$$

3.2.3 Lactate Accumulation and pH Measurement

Metabolic flux through the glycolytic pathway in both hyperglycemic and normoglycemic stored RBCs was monitored via extracellular lactate accumulation and pH measurements. Every 7 days, a 300 µL sample of stored RBCs was drawn and centrifuged at 500*g* for 5 minutes. The supernatant was collected and pH measured using a micro pH electrode (OHAUS, STMICR05). Extracellular lactate was measured using a fluorescent enzymatic assay with lactic acid dehydrogenase. The separated supernatant samples were first cleared of residential LDH via centrifugation in 10 kDa molecular weight cut-off spin filters. L-(+)-lactic acid (Sigma Aldrich) standards were prepared in the range of 0-80 mM in water. All sample filtrates and standards were diluted 1:1000 in water. A 100 µL aliquot of the diluted sample or standard was then added to 100

µL of either the enzyme assay mixture or blank assay mixture in a 96-well plate. The blank assay mixture contained 0.1 M Tris and 5 mM NAD^+ in water. The enzyme assay mixture saw the addition of 20 U/mL lactic acid dehydrogenase (LDH) to the blank assay mixture. The samples were allowed to incubate in the dark at 37 ºC for 15 minutes. LDH converts extracellular lactate to pyruvate and NADH, with NADH exhibiting fluorescence proportional to the concentration of lactate. The fluorescence of the samples was measured on a spectrophotometer (Molecular Devices, SpectraMax M4) (340 nm excitation and 460 nm emission) and compared to standards to determine lactate concentration. Any signal resulting from pre-existing NADH in the sample was removed by subtraction of the blank assay buffer signal. This allowed for detection of signal resulting only from the enzymatic conversion of lactate.

3.2.4 Osmotic Fragility and Intracellular Sorbitol Measurement

The propensity of RBCs to lyse under hypotonic stress is a well-established method for the determination of the osmotic fragility of RBCs.[63] Though this is normally performed using a series of saline dilutions from 0% - 0.9% to determine the salt concentration at which 50% of the RBCs lyse, a modified approach was implemented here for simplicity. First, a 300 µL aliquot of stored RBCs was drawn from each RBC storage bag. Exactly 25 µL of each sample was added to 500 µL of each of 3 solutions: 0.9% NaCl, 0.45% NaCl, and 0.0% NaCl (pure water). The samples were inverted 3 times and allowed to incubate at room temperature for 30 minutes. All samples were centrifuged at 1500*g* for 5 minutes. Afterward, the supernatants were collected and diluted in 18.2 MΩ-cm purified water at a ratio of 1:10 for analysis. Next, 200 µL of each sample was added to a clear 96-well plate in triplicate with absorbance read at 540 nm. The osmotic fragility of each sample was determined through measurement of total RBC cell lysis in a hypotonic 0.45% NaCl solution. The lysis percentage was determined using equation 3.3 below. This method assumes

total RBC lysis in pure water and zero lysis in isotonic 0.9% NaCl.

$$OF\ \% = \frac{Abs_{0.45\%} - Abs_{0.9\%}}{Abs_{0.0\%} - Abs_{0.9\%}} * 100 \qquad (3.2)$$

As mentioned previously, intracellular sorbitol levels can be utilized as a surrogate for relative oxidative stress determination. RBC intracellular sorbitol was measured enzymatically every 7 days of storage beginning on day 2. First, sorbitol (Sigma Aldrich) standards were prepared in the range of 5 – 320 µM in pure water. Each week, 700 µL of stored RBCs was collected and centrifuged at 500g for 5 minutes. The supernatant was removed and the RBCs washed 4 times with a physiological salt solution (PSS, 8.21 g/L NaCl, 0.35 g/L KCl, 0.295 g/L $CaCl_2$•$2H_2O$, 0.142 g/L $MgSO_4$, 2.55 g/L tris(hydroxymethyl)aminomethane, 1 g/L dextrose, 5 g/L bovine serum albumin, pH = 7.4), centrifuging at 500g for 5 minutes between washes. After the final wash, exactly 100 µL of each sample or standard was added to 500 µL of 18.2 MΩ-cm purified water to lyse the RBCs. Then 100 µL of 0.3 M $ZnSO_4$ and 100 µL of 0.475 M NaOH (Sigma Aldrich) were added to the samples to separate proteins, specifically hemoglobin. After centrifugation at 2,000 g for 10 minutes, 100 µL of the supernatant was added to 50 µL of either the blank assay mixture, or the enzyme assay mixture. The blank assay mixture consisted of 0.15 M glycine, 3 mM NAD^+, and 10 mM EDTA (Sigma Aldrich) in 18.2 MΩ-cm purified water (pH = 9). The enzyme assay mixture was identical to the blank assay mixture with the addition of 2 kU/L of sorbitol dehydrogenase (SDH) (Sigma Aldrich).

After adding the samples and standards to the blank or enzyme assay mixtures in a black 96-well plate, the plate was allowed to incubate at 37 ºC for 30 minutes. Afterward, the fluorescence was measured on a spectrophotometer (Molecular Devices, SpectraMax M4) (340 excitation and 460 nm emission). The relative fluorescence units of each samples‘ blank (blank assay mixture) was subtracted from the enzyme assay mixture signal to determine the amount of

fluorescence originating from the conversion of sorbitol to fructose, which creates the fluorescent byproduct NADH. The readings were then compared to the standards to determine sorbitol concentrations.

The concentration of hemoglobin was determined for each sample, following a similar procedure to 3.2.2. The amount of hemoglobin in each sample was used to normalize the data, dividing the intracellular sorbitol concentration by the hemoglobin concentration. These values were plotted against storage duration to determine the effect of both duration and storage condition on sorbitol accumulation.

3.2.5 Flow Cytometry Measurements

Flow cytometry was utilized for more direct measurements of membrane damage, ROS generation, and relative cell size of stored RBCs during hyperglycemic or normoglycemic storage. Membrane damage was evaluated through PS externalization and the binding of fluorescently conjugated Annexin V, while ROS generation was determined using CellROX™ green dye (Thermo Fisher Scientific), an ROS indicator that becomes fluorescent upon reaction with ROS. Every 7 days, a 300 µL aliquot was sampled from the stored RBC bags. The hematocrit of each stored RBC sample was determined using a hematocrit centrifuge (CritSpin M960-22, StatSpin, Westwood, MA) and a microcapillary reader (StatSpin). The samples were then diluted to 0.5% hematocrit in their respective additive solutions supplemented with 2 mM $CaCl_2$. Annexin V binding is a calcium dependent process. Approximately 2.5 µL of phycoerythrin-conjugated (PE) Annexin V indicator (Annexin V Apoptosis Detection Kit, eBioscience™, Thermo Fisher Scientific CAT# 88-8102-74) or 1 µL of 50 µM CellROX™ green dye were added to 100 µL of the 0.5% hematocrit RBC sample. These were allowed to incubate in the dark at room temperature for 30 minutes. Each sample was then added to a 96-well plate for fluorescence measurement on

the flow cytometer.

Additionally, a fresh blood sample was drawn each week from an informed and consented donor to serve as the control. The whole blood sample was centrifuged at 1500*g* for 5 minutes with the plasma and buffy coat aspirated off. The remaining packed RBCs were resuspended in an albumin-free version of PSS (AF-PSS) and the hematocrit was measured. Three fresh RBC samples at 1% hematocrit were prepared in AF-PSS up to 1 mL to serve as positive and negative controls. Exactly 1.54 µL of 2.54 mM ionomycin or 50 µL of 40 mM H_2O_2 were added to two of the 1% hematocrit fresh RBC samples and incubated at 37 ºC for 1 hour under agitation at 300 RPM. Ionomycin is a Ca^{2+} ionophore that induces the externalization of PS on the RBC surface. Hydrogen peroxide is a key ROS often generated in the RBC cytosol during storage. After incubation, the samples were centrifuged at 500*g* for 5 minutes and the supernatant was removed and replaced with fresh AF-PSS. The samples were then diluted in 100 µL AF-PSS to a 0.5% hematocrit. The PS induced sample was incubated with 2.5 µL of PE-Annexin V, while the ROS positive sample was incubated with 1 µL of 50 µM CellROX™ green dye for 30 minutes at room temperature in the dark. After incubation, the samples were added to a 96-well plate for fluorescence measurement on the flow cytometer. All concentrations of fluorescent indicators and RBCs were optimized in preliminary titration experiments.

The flow cytometer (BD Accuri C6 Plus) was gated using forward scatter and side scatter to only collect singlet RBCs, collecting 100,000 events at a flow rate of 14 µL/min. The RBCs were excited using a 488 nm wavelength laser. After collection, relative cell size was determined through median forward scatter signal, while relative cell shape was determined through median side scatter signal. The percentage increase or decrease in signal relative to each donor's fresh blood diluted down to 0.25% hematocrit in PBS was determined for each measurement.

Fluorescence gating for positive and negative events was performed based on the population distributions of the negative and positive fresh blood controls. The percentage of PS positive and ROS positive cells was determined through these gates using the FCS Express 6 flow cytometry analysis software.

3.2.6 RBC Shape and Size through Scanning Electron Microscopy

To accompany the measurement of PS externalization, stored RBC samples were evaluated for their cell shape and size using scanning electron microscopy (SEM). For this study, a pooled sampling method was implemented. A total of 4 donors had donated blood according to 3.2.1, resulting in 4 hyperglycemic stored RBC bags and 4 normoglycemic stored RBC bags. Every 7 days, approximately 100 µL of stored RBC sample was drawn for processing. The hematocrit of each stored RBC sample was determined and each sample was diluted down to a 12.5% hematocrit in their respective additive solution (AS-1 or AS-1N) up to 100 µL. Next, the samples were fixed by the drop-wise addition of 100 µL of 4% glutaraldehyde in 0.1 M sodium phosphate buffer. The samples were allowed to incubate for 60 minutes and then pooled according to their storage condition (e.g. 125 µL of each of the 4 fixed hyperglycemic RBC samples were combined to create a 500 µL sample of fixed RBCs from 4 different donors).

Two drops of 1% Poly-L-Lysine (Sigma Aldrich, Cat# P1399) were placed on a plastic Petri dish and a 12 mm round glass coverslip was placed on top of the drop and allowed to stand for 10 min. The coverslip was removed and gently washed with several drops of water. The coverslip was drained but was not allowed to dry. Two drops of the cells fixed in suspension were placed on the side of the coverslip treated with Poly-L-Lysine. The suspension was allowed to settle for 10 min. The coverslip was then gently washed with several drops of water and placed in a graded ethanol series (25%, 50%, 75%, 95%) for 10 min at each step and with three 10 min

changes in 100% ethanol. Samples were critical point dried in a Leica Microsystems model EM CPD300 critical point dryer (Leica Microsystems) using carbon dioxide as the transitional fluid. Samples were mounted on aluminum stubs using epoxy glue and System Three Quick Cure 5 (System Three Resins, Inc). Samples were coated with osmium (≈20 nm thickness) in an NEOC-AT osmium CVD (chemical vapor deposition) coater (Meiwafosis Co., Ltd.). Samples were examined in a JEOL 7500F (field emission emitter) scanning electron microscope (JEOL Ltd.).

Each sample was imaged using 5.0 kV beam voltage at a working distance of 12 mm. Images of each sample were taken at a magnification of 1200x. From each image, all RBCs inside of the field of view (140-180 RBCs) were classified according to the classification system established by Bessis.[64] Each of the RBCs were assigned a number from -1 to 5 according to their shape (stomatocyte = -1, discocyte = 0, echinocyte I = 1, echinocyte II = 2, echinocyte II = 3, sphero-echinocyte I = 4, sphero-echinocyte II = 5). The data was subsequently bootstrapped, a technique described in depth elsewhere.[65] The 130-180 RBCs classified in each image were randomly sampled 1000 times, selecting 100 RBCs for each sample. The means of each 1000 sample sets was determined, with the average of these means representing the mean morphological index of the sample. The calculated means of the 1000 sample sets was also used to determine a 95% confidence interval about the mean. A higher morphological index indicated a progression toward echinoctyosis and spherocytosis.

3.2.7 Statistical Analysis

All statistical testing was performed using two- or three-way ANOVA followed by Tukey's Honest Significant Difference testing at a 95% significance level. GraphPad Prism Software was used for statistical analysis. Statistical analysis data is only shown for data that displayed statistical significance. Anywhere from 2-10 donors were used for assay measurements depending on

availability and measurement difficulty.

3.3 Results

3.3.1 Hemolysis, Lactate Accumulation, and pH

The extent of cell lysis in both hyperglycemic and normoglycemic RBC storage was measured and is shown in figure 3.3. The hemolysis levels at each time point were statistically equivalent between the two storage conditions throughout the storage period of 44 days. Though both storage conditions exhibited increased RBC hemolysis during storage, both were statistically equivalent to 1% hemolysis by the end of 44 days storage. These data differ from our previous work, where hemolysis percentage in storage exceeded 1.5% by day 36.[37] This may be due to the incorporation of the plasticizer, DEHP, into the RBC membrane which has been reported to leach into stored RBCs, leading to a 4-fold reduction in hemolysis.[31,66,67] Previous work utilized custom blood storage bags without this plasticizer, which may have caused the increased hemolysis. Regardless of cause, this data provides strong evidence that normoglycemic storage does not significantly increase RBC hemolysis over the FDA threshold of 1%.

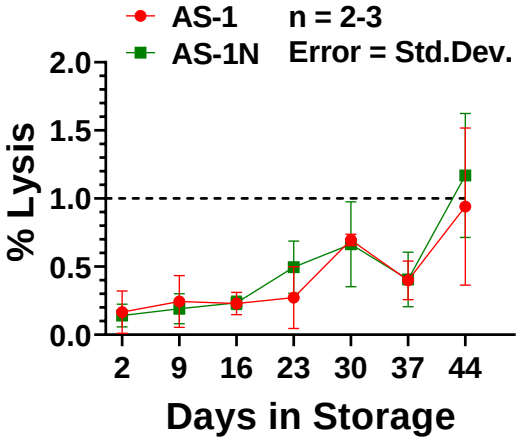

Figure 3.3**: Hemolysis was statistically equal in both AS-1 and AS-1N stored RBCs at each time point.** Additionally, hemolysis at the end of storage was statistically equivalent to the 1% threshold (dashed line) required for transfusions set by the FDA. (n = 2-3, error = standard deviation).

Extracellular lactate accumulation and pH were monitored in both normoglycemic and hyperglycemic storage to determine whether lower glucose conditions had any impact, positively or negatively, on glycolysis rates. As shown in figure 3.4a, both AS-1 and AS-1N stored RBCs exhibited initial extracellular lactate concentrations of 4.2 ± 0.7 mM, which is slightly greater than the physiologically relevant 2 mM but similar to previous findings.[37,68] The lactate levels in both storage environments steadily increased throughout the storage period, reaching 39 ± 9.4 mM in AS-1 and 36 ± 2.6 mM in AS-1N. This trend of lactate accumulation is consistent with the literature.[13] The lactate levels were statistically equivalent between storage conditions at each time point. The change in pH during storage is shown in Figure 3.4b. The pH in both conditions began at 7.7 ± 0.2 on day 2 and steadily dropped to 6.6 ± 0.1 on day 44. This drop in pH is consistent with the literature as well as the lactate accumulation data, indicating that an increase in metabolic waste products, such as lactic acid, is associated with a steady drop

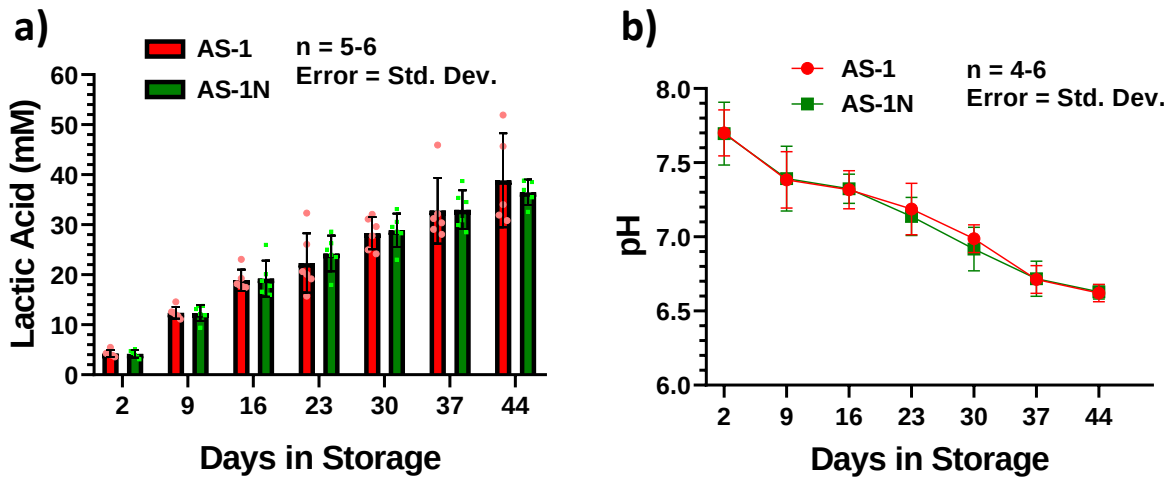

Figure 3.4: **Extracellular lactic acid (lactate) and pH show no differences between storage conditions**. (a) In both AS-1 and AS-1N stored RBCs, the lactate in the extracellular space increased throughout storage. There was no difference in lactate accumulation between the two storage conditions (n = 5-6, error = standard deviation). (b) pH for RBCs stored in both AS-1 and AS-1N began at a physiologically relevant 7.7 and steadily dropped throughout storage as waste products from metabolism including lactate accumulated. There was no statistical difference in pH measurements between RBCs stored in AS-1 or AS-1N at any time point. (n = 4-6, error = standard deviation).

in the pH.[13] There was no statistically significant difference in the pH of the stored RBCs between storage conditions at any time point measured. These data offer the indirect conclusion that normoglycemic RBC storage has no positive or negative impact on glycolysis rates. This is an important finding considering AS-1 stored RBCs have more than 8 times the glucose content of AS-1N stored RBCs. As alluded by figure 3.4, the rate of glycolysis appears to remain unchanged when decreasing the glucose content to a normoglycemic range, which means the excess glucose in hyperglycemic RBC storage remains available for other potentially harmful glucose-dependent pathways including protein glycation and the polyol pathway.

3.3.2 Osmotic Fragility and Intracellular Sorbitol Levels of Stored RBCs

The propensity of RBCs to lyse under hypotonic stress is closely related to their shape, membrane integrity/composition, hydration, and within certain limitations, their susceptibility to *in vivo* destruction.[63,69] The percentage of stored RBCs that lyse when subject to hypotonic conditions (0.45% NaCl) was measured in both AS-1 and AS-1N stored RBCs, shown in figure figure 3.5a. RBCs stored in AS-1N exhibited decreased osmotic fragility up to 23 days of storage in comparison to AS-1. The hypotonic hemolysis percentage of RBCs stored in AS-1N began at 12.2% ± 6.9% on day 2 and rose throughout storage until reaching 74.7% ± 20.7% on day 30, statistically equivalent to the level of lysis in AS-1 stored RBCs. The AS-1 stored RBCs began at 77.9% ± 9.3% lysis on day 2 and rose to 90.8% ± 0.3% lysis on day 44 but was statistically equivalent throughout storage. These results confirm and improve upon previous data that RBCs stored in normoglycemic conditions are more resistant to osmotic stress. This data, however, shows that this resistance persists for up to three weeks of storage, unlike our previous reports which indicated only a few days of benefit.[38]

Additionally, sorbitol levels were monitored throughout storage as an indicator of the

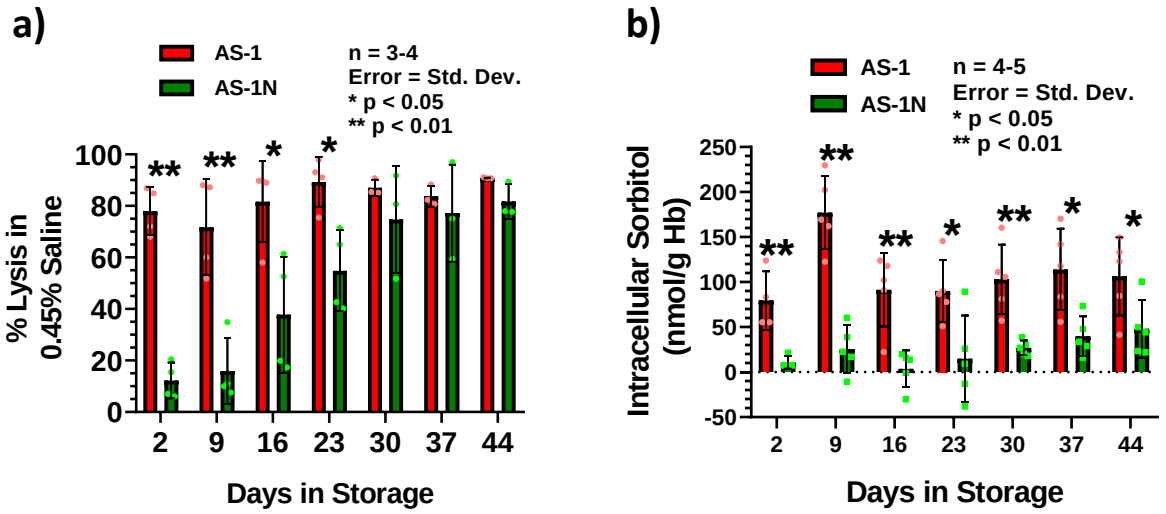

Figure 3.5**: Osmotic fragility and intracellular sorbitol indicate that normoglycemic storage results in RBCs that are less prone to lysis and produce less markers of oxidative stress**. (a) AS-1 stored RBCs exhibited significant hemolysis above 70% at each time point and were statistically equivalent throughout storage. AS-1N stored RBCs were more resistant to osmotic stress initially, with cell lysis of 12.2% ± 6.9% on day 2. AS-1N stored RBC lysis was statistically lower than AS-1 stored RBC lysis at days 1, 9, 16, and 23 (n = 3-4, error = standard deviation, * $p < 0.05$, ** $p < 0.01$). (b) Intracellular sorbitol was measured enzymatically, showing that sorbitol levels were significantly lower in normoglycemic storage at each time point. Though rising throughout storage for both AS-1 and AS-1N stored RBCs, day 44 sorbitol levels using AS-1N remained below that of day 2 sorbitol levels using AS-1. (n = 4-5, error = standard deviation, * $p < 0.05$, ** $p < 0.01$).

polyol pathway and indirectly, oxidative stress. Intracellular RBC sorbitol levels for both AS-1 and AS-1N storage are shown in figure 3.5b. Sorbitol levels for AS-1N storage initially measured 10.9 ± 7.1 nmol gHb^{-1} and rose to 48.0 ± 31.9 nmol gHb^{-1} by day 44. The sorbitol levels for RBCs stored in AS-1 were higher than RBCs stored in AS-1N at each time point, beginning at 79.5 ± 32.4 nmol gHb^{-1} on day 2 of storage and rising to 106 ± 43.4 nmol gHb^{-1}. Though not statistically significant, figure 3.5b also shows that the sorbitol levels of AS-1N on day 44 were lower than the day 2 measurement of AS-1 stored RBCs. These results show there is more sorbitol production, accumulation, and therefore, polyol activity in traditional storage relative to normoglycemic storage.

The results taken together in figure 3.5 confirm and improve upon previous work,

highlighting the benefit of normoglycemic RBC storage and illustrating its application using commercially available blood storage materials with autonomous glycemic control.

3.3.3 PS Externalization, ROS Generation, and Cell Size Using Flow Cytometry

Flow cytometry was implemented for the evaluation of relative cell size, PS externalization, and ROS generation. Flow cytometry detects singular cellular fluorescence and light scattering signal by passing individual cells through a laser of specific wavelength. This causes portions of the laser light to scatter, with light scattering parallel to the line of the laser termed the forward scatter signal (FSC) and light scattering perpendicular to the laser line called side scatter signal (SSC). The forward scatter signal is directly proportional to the cell size, whereas side scatter is directly proportional to cell complexity, or density of organelles/granules. Additionally, any fluorochromes present within the cell can be excited by the laser and emit fluorescence that can be detected through various channels and series of filters, allowing for single cellular fluorescence detection. This technique was implemented in the determination of relative RBC size, PS externalization, and ROS generation in hyperglycemic and normoglycemic stored RBCs. The relative percentage change in FSC and SSC signal is shown in figure 3.6, normalized to each donor's fresh RBCs washed and diluted in PBS. As shown in figure 3.6a, the FSC signal relative to that of fresh blood immediately began at 137.8% ± 10.2% and 133.7% ± 11.3% on day 2 of storage for AS-1 and AS-1N RBC storage respectively. This signal slowly decreased throughout storage, reaching 121.8% ± 5.54% and 114.8% ± 4.10% by 23 days in storage for hyperglycemic and normoglycemic storage respectively. The signal then increased thereafter for both storage conditions, reaching 132.9% ± 3.53% and 125.0% ± 3.02% by the end of storage for AS-1 and AS-1N respectively. These results indicate that stored RBCs slowly lose membrane and increase in volume during storage, which is indeed in agreement with the decrease in surface area

to volume ratio in the literature.[70,71] The shape transformation of stored RBCs to echinocytes is represented by the decrease in cell size in the first 3 weeks of storage. However, as shape transformation progresses toward irreversible spherocytosis in the remaining 3 weeks of storage, an increase in volume is observed, resulting in an increase in cell size. The FSC signal in AS-1N stored RBCs was lower than RBCs stored in AS-1 at all time-points, exhibiting statistical significance at day 37, indicating that normoglycemic stored RBCs may be smaller than hyperglycemic stored RBCs, either by reduced volume increases or increased membrane loss due to spherocytosis.

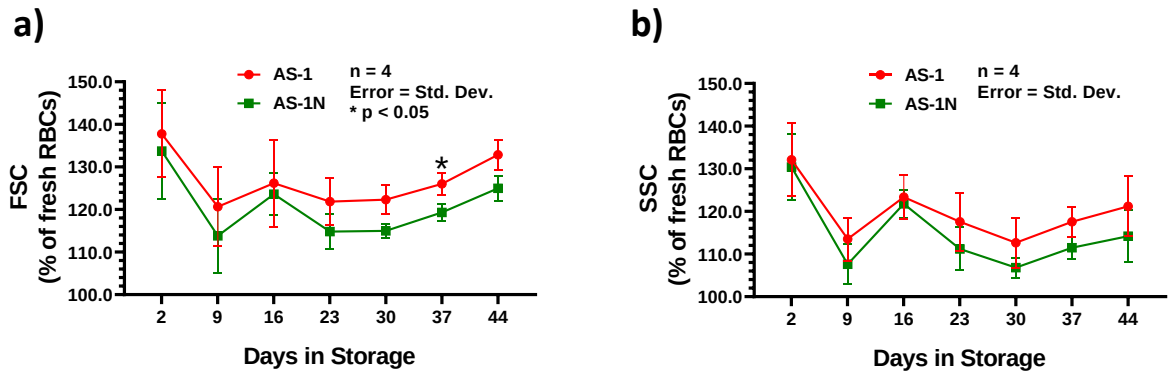

Figure 3.6: **FSC and SSC signal for stored RBCs in both hyperglycemic and normoglycemic storage**. (a) The FSC signal percentage was higher than fresh blood at 136.8% ± 8.42% and 132.2% ± 8.7% on day 2 of storage for AS-1 and AS-1N RBC storage respectively. The signal steadily fell throughout storage, and then rose after day 23 for each storage condition. AS-1N stored RBCs displayed a lower FSC signal in comparison to AS-1 stored RBCs, exhibiting statistical significance at day 37 (n = 4, error = standard deviation, * $p < 0.05$). (b) The SSC signal relative to fresh blood was also higher than fresh blood at 132.8% ± 9.78% and 130.5% ± 7.987% on day 2 of storage for AS-1 and AS-1N respectively. This fell throughout storage, reaching 121.2% ± 6.98% and 114.2% ± 6.02% by the end of storage for AS-1 and AS-1N respectively. (n = 4, error = standard deviation).

Similar to the FSC data, the SSC data shown in figure 3.6b follows a similar trend. The SSC signal, relative to each donor's fresh blood, initially started at 132.1% ± 8.52% and 130.4% ± 7.84% on day 2 of storage for AS-1 and AS-1N respectively, steadily decreasing throughout storage to 121.2% ± 6.98% and 114.2% ± 6.02% by day 44. Often, SSC signal is associated with

the relative complexity of the cell contents such as organelles/granules. However, since RBCs do not have organelles or granules, the SSC signal may be redundant. It should be noted that the SSC signal in figure 3.6b shows that AS-1N stored RBCs exhibit reduced signal in comparison to AS-1 stored RBCs, though this is not significant at any time point.

PS externalization and ROS generation were monitored using common indicators, phycoerythrin conjugated Annexin V and CellROX™ green for PS and ROS detection respectively. The percentage of stored RBCs exposing PS in their outer membrane (figure 3.7a) showed no statistical difference between the storage conditions at any time point. The percentage of PS externalization for AS-1 stored RBCs started at 2.53% ± 1.14% on day 2 of storage and did not significantly increase or decrease, reaching 1.86% ± 0.61% by the end of 44 days in storage. Similarly, AS-1N stored RBCs exhibited 2.79% ± 1.94% PS externalization on day 2 that maintained relatively constant, reaching 2.09% ± 0.55% by day 44. Figure 3.7b highlights the percentage of RBCs generating ROS in both hyperglycemic and normoglycemic stored RBCs

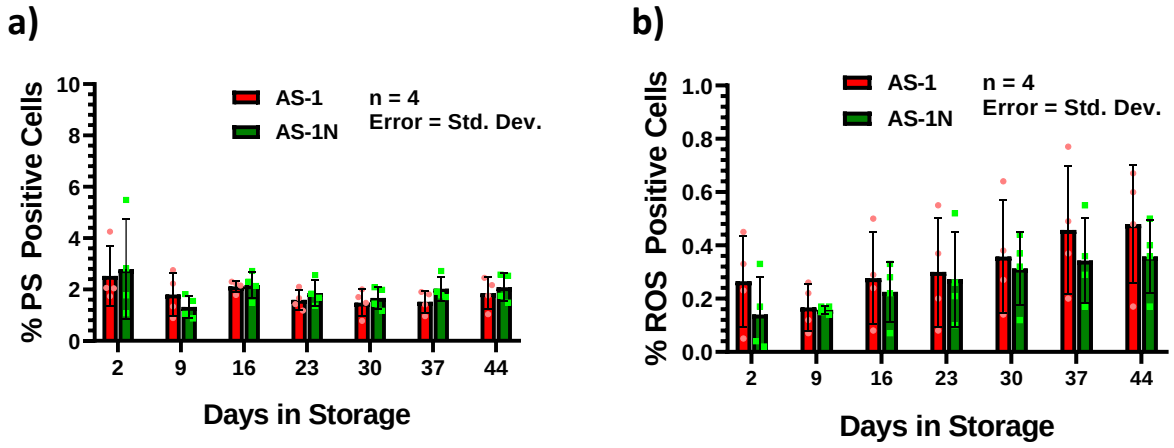

Figure 3.7**: Percentage of RBCs externalizing PS and exhibiting ROS generation within storage**. (a) Percentage of stored RBCs expressing phosphatidylserine on the outer membrane within hyperglycemic and normoglycemic storage (n = 4, error = standard deviation, * $p < 0.05$). (b) Percentage of stored RBCs generating ROS within hyperglycemic or normoglycemic storage (n = 4, error = standard deviation, * $p < 0.05$).

throughout storage. Though the exact amount/quantity of ROS cannot be quantified with this technique, the relative difference in signal originating from ROS generation within the cell can be compared between the two storage conditions. RBCs stored in AS-1 exhibited 0.27% ± 0.17% ROS positive cells on day 2 that steadily rose to 0.48% ± 0.22% by the end of storage, while RBCs stored under normoglycemic conditions began at 0.14% ± 0.14% on day 2 and rose to 0.36 ± 0.14% by day 44. Though also not statistically significant, these data suggest that AS-1N stored RBCs may exhibit decreased generation of ROS, which is supported by the indirect oxidative stress measurement via sorbitol accumulation.

3.3.4 Morphological Indices via Scanning Electron Microscopy

Scanning electron microscopy was implemented to image stored RBCs under hyperglycemic and normoglycemic storage conditions. The classification system established by

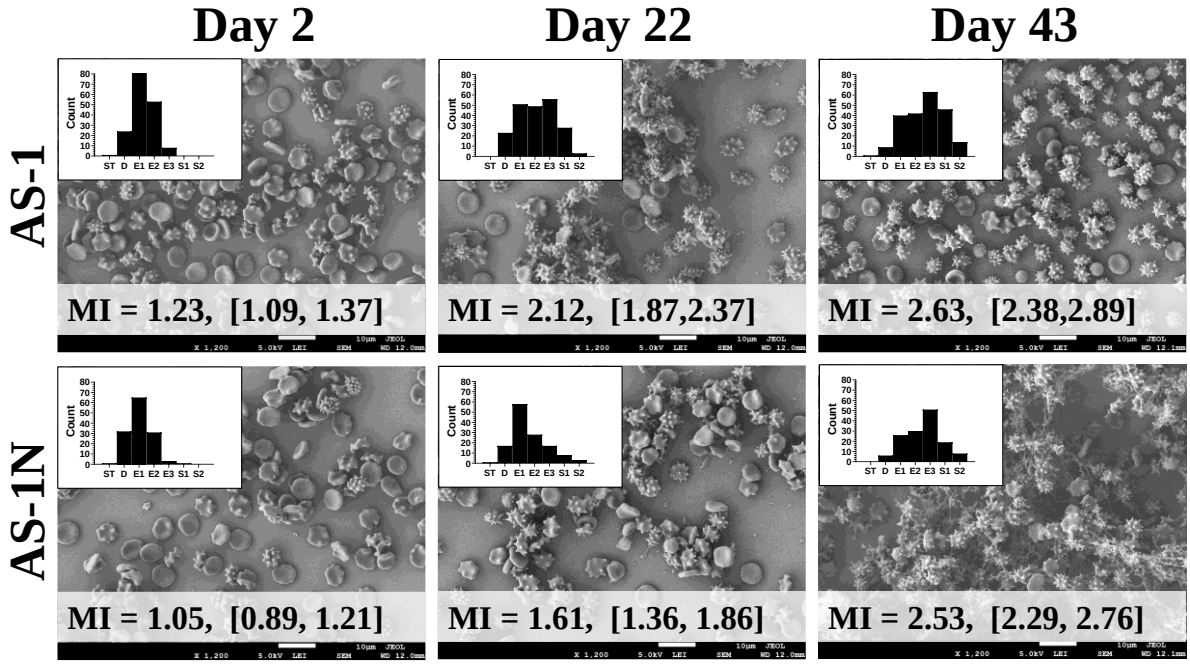

Figure 3.8: **Morphological changes of stored RBCs in both normoglycemic and hyperglycemic storage**. SEM images of AS-1 and AS-1N stored RBCs at day 2, 22, and 43 of storage highlighting the progression of discocytic RBCs transforming to echinocytes and spherocytes (n = 4 donors pooled, error = 95% confidence interval).

Bessis was used to assign a morphological index to each storage condition and time point.[64] As seen through the SEM images (figure 3.8), both AS-1 and AS-1N stored RBCs progressively transform into echinocytes and spherocytes, losing membrane and increasing in volume.[51,53] However, AS-1N stored RBCs resulted in lower morphological indices at all time points (figure 3.9a), indicating that AS-1 stored RBCs exhibit more echinocytic and spherocytic shape changes during storage. The 95% confidence intervals do not overlap on day 22 of storage, highlighting these differences. Additionally, AS-1N stored RBCs exhibited a greater percentage of discocytes at all time points of storage (figure 3.9b). Though no statistical testing was completed, sampling from more donors would confirm that normoglycemic storage leads to less echinocytosis and spherocytosis. This data is also in agreement with the flow cytometry FSC signal collected in figure 3.6a, indicating that stored RBCs decrease in size and increase in volume throughout storage, which is supported by the literature.[50]

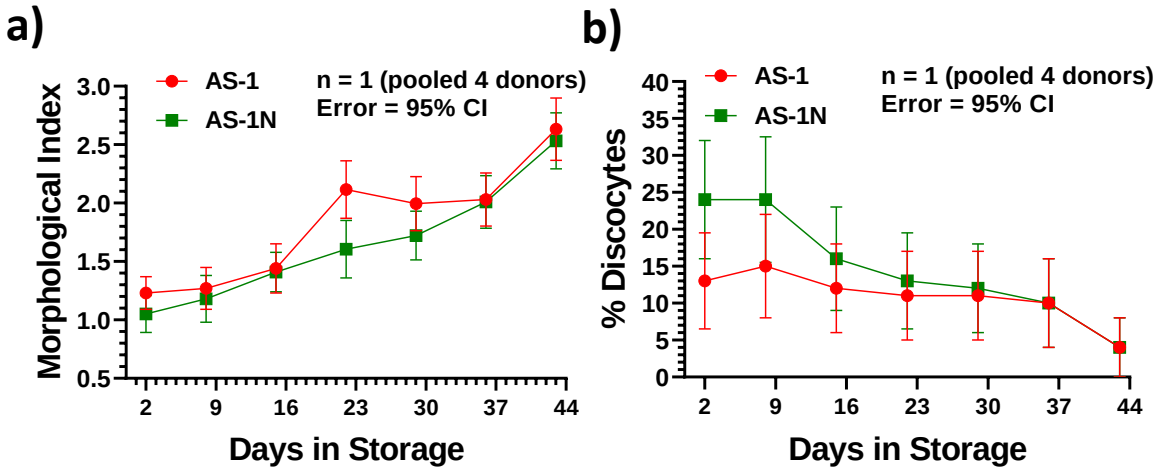

Figure 3.9**: Morphological indices and discocyte percentage of stored RBCs**. (a) Morphological indices of AS-1 and AS-1N stored RBCs throughout storage, classified and numbered according to Bessis (stomatocyte = -1, discocyte = 0, echinocyte I = 1, echinocyte II = 2, echinocyte III = 3, sphero-echinocyte I = 4, sphero-echinocyte II = 5, n = 4 donors pooled, error = 95% confidence interval). (b) Percentage of discocytes in normoglycemic and hyperglycemic storage (n = 4 donors pooled, error = 95% confidence interval).

3.4 Discussion

RBCs must be intact to function. Therefore, hemolysis is a frequently used metric of stored RBC health.[48] As RBCs age throughout storage, an increase in metabolic waste and oxidative stress, along with a decrease in pH contribute to increased hemolysis.[13] Hemolysis releases free hemoglobin into the extracellular space which is known to scavenge nitric oxide (NO), a vasodilator that facilitates blood flow.[13,72] Donadee *et al*. showed that transfusion of the supernatant from stored RBCs with increased hemolysis and free hemoglobin led to increased vasoconstriction in comparison to stored RBCs with reduced hemolysis and free hemoglobin.[73] This phenomenon was attributed to reduced bioavailability of NO after transfusion, a well-known issue in transfusion medicine. This is, in part, why the hemolysis threshold defining an acceptable RBC product for RBC transfusion in the United States is set to 1%.[74] In this study, AS-1N and AS-1 stored RBCs were below or statistically equivalent to 1% for the entirety of storage. Hypoglycemia is associated with increased hemolysis, therefore it was crucial to ensure that normoglycemic storage, and occasional, transient hypoglycemia, did not cause increased cell lysis. Clearly, even during slight deviations from normoglycemia as discussed in chapter 2, there was no impact on hemolysis level. The hemolysis data discussed here also improves upon our previous hemolysis studies of RBC storage, which resulted in significant lysis after 36 days in storage.[37] The reduced lysis reported here may have been due to the incorporation of the plasticizer, DEHP, which is known to integrate into the RBC membrane and lead to decreased hemolysis levels.[31,67]

It is well established that membrane damages occur during RBC storage, leading to the generation of echinocytes and spherocytes, increased cell fragility, and decreased deformability.[13] These membrane damages, along with increased osmotic pressure from metabolic waste accumulation, are associated with increased osmotic fragility, or increased propensity for RBCs to

lyse under hypotonic stress.[75,76] Additionally, it is also well known that increased oxidative stress during RBC storage can oxidize membrane proteins and lipids, leading to further increased RBC membrane damage.[13] The osmotic fragility and sorbitol accumulation data in figure 3.5 show that not only does normoglycemic RBC storage lead to reductions in both measurements, but highlight that these two storage lesion indicators may be tightly correlated.

The polyol pathway first converts glucose to sorbitol, consuming a NADPH molecule, followed by the conversion of sorbitol to fructose, producing a NADH molecule.[42] This pathway is markedly increased in diabetes patients and is reported as a significant contributor of oxidative stress.[42] This is due to the increased consumption of reducing equivalents in the form of NADPH. NADPH is a vital molecule in the recycling of a key antioxidant, glutathione (GSH). With increased sorbitol accumulation, there will be an accompanied equivalent consumption and depletion of NADPH, leaving less available to recycle GSH and therefore, more oxidative damage. This proposed decrease in NADPH and GSH during RBC storage are, indeed, supported by the literature.[13,77]

The excess sorbitol accumulation in AS-1 RBC storage and consequently increased glucose metabolism through the polyol pathway may be explained by the elevated glucose levels in hyperglycemic storage relative to normoglycemic storage. Indeed, RBCs from patients with diabetes have been reported to exhibit increased sorbitol levels that was directly proportional to their degree of hyperglycemia, suggesting that hyperglycemia renders excess glucose available for the polyol pathway, resulting in increased ROS levels.[42,78] The relative reduction of sorbitol production in normoglycemic storage, therefore, indicates that normoglycemic RBCs may exhibit less oxidative stress than RBCs stored under hyperglycemic conditions. This may also explain the reduced osmotic fragility of AS-1N stored RBCs due to potentially increased oxidative damage in

RBC storage causing accelerated membrane damage.

Additionally, the differences in osmotic fragility could also be explained by a buildup of metabolic waste products. The accumulation of waste, such as lactate, imparts osmotic stress onto the RBC, causing greater influx of water when subject to hypotonic 0.45% saline.[76] This is most likely why the normoglycemic RBCs deteriorate and increase in their osmotic fragility between days 9-30, resulting from a build-up of intracellular lactate that generates greater osmotic stress for the cell. Along with lactate, sorbitol and fructose (both polyol pathway by-products) can contribute to osmotic stress as they are both membrane impermeable.[42] The significant reduction in sorbitol accumulation, and therefore reduced osmotic stress within the RBC during normoglycemic storage, could also explain the reduced osmotic fragility exhibited by normoglycemic stored RBCs.

It is important to note that osmotic fragility differences could also be due to inherent differences in osmolarity of the additive solutions (AS-1 = 464 mOsmolar, AS-1N = 359 mOsmolar), or the osmolarity changes due to the addition of glucose/saline from periodic feeding. However, this is not likely, as previous reports evaluating osmotic fragility utilizing other additive solutions characterized by lower osmolarity, such as AS-3 (291 mOsmolar), and without periodic saline addition showed similar results to the data shown here with AS-1.[38]

Regardless of the mechanism, these data highlight a key relationship between sorbitol accumulation and osmotic fragility, indicating that normoglycemic storage can improve both, potentially leading to improved RBC health in storage. This relationship, and its link to hyperglycemia, is indeed supported by the literature. Jain et al. revealed that incubating RBCs in elevated glucose environments led to increased membrane lipid peroxidation and osmotic fragility, which was eliminated when the RBCs were treated with glucose metabolism inhibitors,

antioxidants, ROS scavengers, or other oxidative pathway inhibitors.[79] In traditional, hyperglycemic storage, more glucose is available to be metabolized in the polyol pathway, leading to the increased levels of sorbitol and subsequently increased osmotic stress.

Though excess available glucose in this study led to a detrimental oxidative effect (figure 3.5), previous studies showed that traditional, hyperglycemic storage resulted in increased intracellular ATP and increased lactate production in comparison to normoglycemic storage without feeding.[37] The increase in intracellular ATP and lactate production was slightly higher than AS-1N storage with feeding, suggesting increased glycolytic activity. A reappraisal of these results using automated and controlled feeding of the AS-1N solutions was highlighted in figure 3.4. Though the lactate accumulation is consistent with the drop in pH, this data contradicts our previous reports, but may be explained by better glycemic control. The tight control of glucose at physiologically relevant levels discussed in chapter 2 likely supplied enough glucose to saturate the glycolytic pathway. In previous reports, the manual glucose feeding regimen maintained a low-glucose environment, however, it was not monitored as frequently and may have led to longer hypoglycemic periods.[37] Additionally, the statistically equivalent levels of lactate between the two storage conditions also eliminates the possibility of lactate accumulation as the culprit for the difference in osmotic fragility between AS-1 and AS-1N stored RBCs.

RBC shape and size are highly correlated with their ability to deform and traverse small capillaries. The process of echinocytosis during RBC storage disrupts the RBC shape and can result in clearance *in vivo*. Indeed, Rousell et al. showed that perfusion of echinocytes and spherocytes through human spleen *ex vivo* resulted in 61% clearance, whereas perfusion of stored RBCs (a population of discocytes, echinocytes, and spherocytes) resulted in 15% clearance.[80] This highlights the importance of maintaining RBC shape and size during storage. Considering the SEM

images, morphological indices, discocyte percentage, and FSC data presented here, it is possible that normoglycemic stored RBCs would exhibit less clearance *in vivo* due to their maintained RBC shape and size relative to hyperglycemic storage. This is especially compelling considering the average age of transfused RBCs does not exceed 23 days, where normoglycemic storage exhibits significantly less echinocytic shape transformation than hyperglycemic storage.[81] Echinocytosis is reported as a result of a variety of factors, including osmotic stress, ATP depletion, oxidative damage, and band 3 protein clustering.[82] Wong has proposed, however, that echinocytosis, specifically in RBC storage, is caused by ATP depletion, and its impact on band 3 protein conformation. Band 3 protein is an important anion transporter in the RBC membrane that is anchored to the RBC cytoskeleton.[53,83] This protein is vital to the RBC buffering capacity, operating via a ping-pong mechanism to transport bicarbonate anions ($HCO3^-$) and chloride ions (Cl^-) into and out of the cell.[83] This ping-pong mechanism results in inward and outward conformations of this protein, which is reported to contract and relax the RBC cytoskeleton respectively.[84] The contraction and relaxation favor echinocytosis and stomatocytosis respectively. Therefore, the ratio of inward to outward conformations of band 3 protein is proportional to the degree of echinocytosis of stored RBCs. Wong has proposed that ATP depletion during storage forces degradation of 2,3-DPG to make up for the ATP loss, eventually also depleting the cell of inorganic phosphates.[53] The slow outward transport of inorganic phosphates via band 3 protein initially favors stomatocytosis, however, once phosphates are depleted within the RBC, transport of inorganic phosphates into the RBC favors the inward facing conformation of band 3 protein, which results in echinocytosis.[53] Other investigations also report ATP depletion as a driving force of echinocytosis during storage, resulting in reduced lipid asymmetry and reduced cytoskeletal interaction with the membrane.[25,85] It is possible these mechanisms may be responsible for the

reduced echinocytic progression seen in hyperglycemic storage in comparison to normoglycemic. Previous reports have shown that intracellular ATP levels are higher in hyperglycemic storage in comparison to normoglycemic storage, which may help to maintain band 3 conformations that favor stomatocytosis.[37] Alternatively, increased osmotic stress and oxidative damage can also encourage echinocytosis This may be why AS-1N stored RBCs have lower morphological indices and greater discocyte percentages during storage. Increased protein/lipid oxidation can lead to increased microvesicle shedding and a greater decrease in surface area/volume ratio, forcing the RBC to a spherocytic shape.[50,51,85] Additionally, the increase in intracellular osmolarity due to metabolic waste buildup, such as lactate and sorbitol, causes greater flux of fluid into the cell, increasing its volume and driving the cell toward spherocytosis, which also may be why spherocytes and echinocytes exhibit increased osmotic fragility.[86] The reduced morphological indices seen in AS-1N stored RBCs is likely due to reduced osmotic stress and oxidative damage. The reduced morphological index in normoglycemic RBC storage also agrees with previous data regarding deformability, which showed that normoglycemic stored RBCs exhibited increased deformability relative to hyperglycemic storage.[39] These findings suggest that normoglycemic stored RBCs may result in less clearance *in vivo*.

These findings are more compelling considering that echinocytosis seen in normoglycemic RBC storage may be further prevented by modifying the feeding solution to omit NaCl which may be affecting band 3 protein conformations. Band 3 protein primarily transports bicarbonate and chlorine anions into and out of the cell. This transport is dictated by the Donnan equilibrium, or the unequal distribution of charged diffusible ions across a cell membrane that balances opposing forces from concentration gradients and electrical charge.[53,87] The ratio of outward facing conformation (band 3_o) to inward facing conformation (band 3_i) is equivalent to the ratios of

intracellular to extracellular concentrations of chlorine and bicarbonate ions: (band 3_o)/(band 3_i) = $[Cl^-_i]/[Cl^-_o]$ = $[HCO_3^-{}_i]/[HCO_3^-{}_o]$.[53] An increase in intracellular chlorine or bicarbonate favors stomatocytosis, while increases in extracellular chlorine or bicarbonate favors echinocytosis. As discussed in the methods, the morphology studies did not implement a 0.9% NaCl feeding regimen for the hyperglycemic condition, whereas the normoglycemic stored RBCs were fed with 100 mM glucose in 0.9% NaCl. Removing the feeding regimen from the hyperglycemic condition was in response to a reviewer suggesting the removal of the feeding regimen would be a closer match to current storage practices. By feeding the normoglycemic stored RBCs with 100 mM glucose in 0.9% NaCl every 3 days, the extracellular concentration of free chloride ions was increasing throughout storage, thereby decreasing the Donnan ratio and favoring echinocytosis. Though normoglycemic storage exhibited decreased echinocytosis, in comparison to hyperglycemic storage, the addition of NaCl during glucose delivery may have contributed to increased shape transformation. By implementing a feeding solution that is isotonic and without NaCl, such as 300 mM glucose, echinocytic transformations of normoglycemic stored RBCs may be alleviated further.

RBC stress and membrane damage are strongly associated with PS externalization. Increased PS exposure can contribute to recognition and removal of old and damaged cells from circulation.[59] Additionally, RBC PS externalization has been reported to activate coagulation and induce thrombosis *in vivo*.[88] The significant echinocytosis in RBC storage was expected to be accompanied by increased PS externalization. However, the percentage of PS expressing cells was equivalent between hyperglycemic and normoglycemic storage at each time point and did not rise significantly throughout storage. Though this may seem contradictory to the SEM and morphological index data, Verhoeven et al. showed that PS externalization in storage is primarily

driven through reduced flippase activity, and not increased scramblase activity.[89] In fact, scramblase activity remained virtually absent during storage, which may explain the low levels of PS externalization seen in this work.[89] This further supports the hypothesis that reduced echinocytosis in normoglycemic storage was likely due to decreased osmotic fragility and oxidative stress in comparison to hyperglycemic storage. As PS externalization is a marker for apoptosis, or programmed cell death, PS externalization on the RBC results in increased clearance from circulation when transfused and may also be linked to other negative clinical outcomes, including delayed hemolytic transfusion reaction.[59,90,91] Although PS externalization may be equivalent between normoglycemic and hyperglycemic storage, RBC clearance within circulation may be reduced when transfusing normoglycemic stored RBCs considering the difference in morphology between hyperglycemic and normoglycemic conditions.

Oxidative damage during RBC storage is a well-known contributor of the storage lesion and may be the underlying cause of other storage-induced damages.[29] In fact, the D'Alessandro lab showed that hypoxic storage of RBCs resulted in decreased oxidation of enzymes and hemoglobin, preserved RBC morphology, and resulted in increased post-transfusion recovery percentage *in vivo*.[92–95] Previous investigations, as well as the sorbitol accumulation data discussed here, support the hypothesis that hyperglycemic storage of RBCs results in increased oxidative stress in stored RBCs via a disruption of the redox balance.[38] A more direct measurement using flow cytometric methods, however, revealed that increased polyol activity may not yield similar increases in ROS levels during hyperglycemic RBC storage. Though the average percentage of ROS positive RBCs was lower in AS-1N stored RBCs in comparison to AS-1 stored RBCs, the data did not display statistical significance. It is possible that antioxidant defense systems within the RBCs stored under either hyperglycemic or normoglycemic conditions were significantly

impaired, despite differences in polyol activity. The data reported here support the conclusion that hyperglycemic stored RBCs exhibit increased metabolic flux through the polyol pathway, depleting NADPH and disrupting the redox balance. However, other factors can disrupt antioxidant ability, NADPH/GSH recycling, and the overall redox balance. One such factor is a decrease in hexose monophosphate pathway (HMP) flux (the sole pathway responsible for NADPH regeneration).[77] If normoglycemic RBCs fail to regenerate sufficient NADPH through the HMP to continue recycling GSH, then decreased polyol activity relative to hyperglycemic RBC storage would have limited impact on ROS levels regardless of NADPH consumption through this pathway. Further investigation into NADPH levels and reducing equivalent regeneration is required to support this possibility. This data further supports the claim that increased oxidative stress in hyperglycemic RBC storage may be contributing to increased echinocytosis and membrane damage in comparison to normoglycemic storage.

3.5 Conclusion

The data presented here validates the benefit of normoglycemic RBC storage at commercial scale with autonomous glycemic control, providing significant contributions toward the application of normoglycemic RBC storage to *in vivo* studies and clinical practice. Additionally, many other RBC storage lesion indicators related to RBC membrane integrity and oxidative stress were evaluated under hyperglycemic and normoglycemic RBC storage, resulting in continued support for normoglycemic RBC storage and its benefit to RBC health. Though some of the data may not have led to any significant differences between the storage conditions, including hemolysis, pH, lactate accumulation, ROS generation, PS externalization and RBC adhesion, many of the measurements showed promising evidence for further investigation into the membrane and oxidative damage pathways involved in hyperglycemic and normoglycemic RBC

storage. Relatively lower levels of ROS generation, RBC adhesion to endothelium, and decreased cell size/shape exhibited by normoglycemic stored RBCs provide encouraging support for *in vivo* benefit of normoglycemic RBC storage. Other measurements of RBC health showed stark differences between hyperglycemic and normoglycemic storage. Significant differences in cell size/shape on day 37 indicate that normoglycemic storage may result in less echinocytosis and reduced volume increase. These findings support previous data showing improved deformability of RBCs under normoglycemic storage. The significant decrease in osmotic fragility and sorbitol accumulation in normoglycemic RBC storage indicate that normoglycemic storage of RBCs may provide beneficial *in vivo* functionality when transfused, though further investigation is required to confirm these claims. The implications of these studies open many additional investigative opportunities into the clinical impact of normoglycemic RBC storage. With autonomous glycemic control of stored RBCs not only possible, but also validated to illicit similar *in vitro* benefit as previous work, a plethora of *in vivo* studies on normoglycemic RBC storage are now possible through the allowance of many normoglycemic stored RBC bags to be sufficiently maintained at normoglycemia without extensive labor requirements.

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Chapter 4 – Stored RBC Rejuvenation and Storage with Albumin

4.1 Introduction

Normoglycemic RBC storage has shown promising *in vitro* benefit to RBC functionality that has been illustrated both in previous work as well as the data discussed here.[1–3] These data highlighted that normoglycemic RBC storage may be correlated with improved membrane integrity and/or decreased oxidative damage. The membrane damages and shape transformations toward echinocytes and spherocytes seen in RBC storage may result in poor performance when transfused. However, some of our previous work has shown that certain stored RBC properties, including ATP release and deformability, can be somewhat recovered when hyperglycemic stored RBCs are incubated in a physiological salt solution (PSS).[1] This "rejuvenation" ability of PSS may also result in similar improvements to RBC morphology.

Outside of modifications to the way we store RBCs, rejuvenation of stored RBCs is also a promising alternative to addressing the RBC storage lesion. Rejuvenation solutions have been extensively studied as a way to "rejuvenate" RBCs, recovering key molecules to their original levels, such as adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG).[4,5] These rejuvenation solutions, including the FDA approved Rejuvesol, were able to recover stored RBC deformability, 2,3-DPG levels, and ATP levels comparable to fresh blood.[4] Transfusion of rejuvenated RBCs also led to improved oxygen delivery, and myocardial, renal, and pulmonary function in comparison to stored RBCs.[4] An advantage of stored RBC rejuvenation in comparison to modifications to storage solutions is its wide-scale applicability. RBC rejuvenation can be implemented to any RBC unit stored under any conditions with any storage solution. Though normoglycemic RBC storage shows promising potential for RBC benefit, it may be difficult to implement due to its competition with current standards in RBC storage. Rejuvenation solutions

provide an attractive alternative to reversing the progression of the RBC storage lesion.

Most rejuvenation solutions, including Rejuvesol, contain variable concentrations of inosine, pyruvate, phosphate, and adenine.[4] These components restore glycolytic flux via several mechanisms that replenish metabolites associated with glycolysis and the Luebering-Rapoport shunt.[4] However, an alternative RBC rejuvenation method that has relatively little investigation focuses on albumin and its ability to recover RBC shape characteristics. Albumin is a 66 kDa protein that represents half of the total plasma protein content and is the most abundant plasma protein.[6] Albumin is a significant modulator of plasma oncotic pressure an acts as a transporter for a wide variety of endogenous molecules as well as exogenous drugs.[6] Recently, albumin has been investigated as a unique protein responsible for rejuvenating stored RBC morphology to the standard discocytic shape. It is well established that stored RBCs increase in volume and lose membrane during storage due to protein/lipid oxidation and ATP exhaustion, resulting in a shape change from discocytes to echinocytes and spherocytes.[7] These shape changes are associated with decreased deformability, an important and defining characteristic of dysfunctional RBCs.[7,8] However, washing stored RBCs in albumin containing buffers has been reported to reverse these shape changes, recover ATP and glutathione (GSH) levels, and result in improved perfusion through an artificial microvascular network.[9–11] These benefits have been confirmed to be specific to albumin due to no effect seen when RBCs were washed with proteins of similar molecular weight (dextran 70, heat shock protein, and protein C).[12] Reinhart et al. conducted a rigorous investigation into the optimum albumin percentage, hematocrit, and washing duration necessary to illicit these benefits, discovering that shape recovery was inversely associated with hematocrit and directly associated with albumin weight percentage and washing duration.[10] The optimum washing parameters were reported to be 0.2% (w/w) albumin, 2-4% hematocrit, and 10-100s for

wash duration.[10] Though washing RBCs in isotonic buffers (e.g. 0.9% saline, phosphate buffered saline) has also been reported to somewhat recover RBC properties, washing in albumin containing buffers provided superior benefit to stored RBCs.[9,11] Interestingly, one of these studies also found via ektacytometry that stored RBC deformability was not improved after washing in an albumin containing buffer, despite reporting shape recovery.[9] These results seem contradictory, considering the relationship between RBC shape and deformability. Additionally, one may argue that RBC rejuvenation via albumin washing is unnecessary due to the likely shape recovery of stored RBCs once transfused into the albumin-rich bloodstream (5% w/w). However, RBC shape recovery was not observed when stored RBCs were washed in fresh plasma, indicating that morphological recovery may not occur once transfused. Clearly, further work is necessary to understand the complex mechanism that governs albumin's ability to rejuvenate stored RBCs.

The mechanism for these benefits is not well understood. Wong has proposed that albumin acts to decrease the total NaCl and KCl concentrations, resulting in the outward transport of inorganic phosphates, favoring the outward conformation of band 3 protein, which is a stomatocytogenic event (the opposite process of echinocytosis).[13] Alternatively, Reinhart et al. has proposed that the mechanism responsible for these shape reversals may be due to albumin's interaction with and removal of membrane lipids.[9] Echinocytosis is characterized by greater outer leaflet membrane surface area relative to the inner leaflet, with greater expression of lysophosphatidylcholine (lysolecithin) generated during RBC storage, inducing echinocytosis.[9] Removal of outer leaflet membrane lipids would result in shape recovery. Albumin is known to interact with the RBC membrane and able to remove lysolecithin, which may be responsible for its mechanism in reversing storage-induced echinocytosis.[9] Additionally, albumin exhibits antioxidant capabilities within circulation.[14] Considering many of the storage induced damages to

RBCs, including lipid peroxidation, are reported to be a result of extensive oxidation, albumin may also derive its beneficial effect from its antioxidant ability.[7] Indeed, Reinhart et al. reported increased levels of GSH, a key antioxidant, after washing stored RBCs in an albumin containing buffer.[9] Though washing stored RBCs in albumin buffers is established as a novel rejuvenation technique, the mechanism of rejuvenation by albumin is poorly understood and requires further investigation. Considering albumin's many roles in the bloodstream, including the maintenance of oncotic pressure and its antioxidant properties, the mechanism responsible for rejuvenation is likely a combination of many of these characteristics.

Washing stored RBCs in albumin containing buffers may offer a unique alternative to storage solution improvements in preventing the progression of the storage lesion. However, rejuvenation of stored RBCs inherently increases patient risk for contamination due to the necessary processing steps and exposure to the atmosphere. Storing RBCs in additive solutions containing albumin may reap the same benefits of albumin washing, while avoiding the additional contamination risk associated with washing RBCs. Currently, only one study has attempted to store RBCs with albumin, which saw no beneficial effect on morphology in comparison to traditional storage.[10] However, the RBCs were stored in 0.2% (w/w) human serum albumin, which was reported by this same study to be ineffective at reversing RBC echinocytosis at a hematocrit of 40%, 20% lower than typical RBC storage.[10] Increasing the albumin percentage in RBC storage to physiological levels (5% w/w) may be able to maintain RBC shape as opposed to recovering RBC shape as seen when washing RBCs in albumin containing buffers.

The benefits of albumin rejuvenation of stored RBCs may also be exaggerated when applied to normoglycemic RBC storage. This is supported by the work of Lu et al., which showed that extreme morphological changes, characteristic of spherocytes, could not be reversed upon

albumin washing, indicating that there is a threshold of membrane damage that renders these changes irreversible. Indeed, previous reports show that hyperglycemic storage results in irreversible RBC damages after approximately 2 weeks in storage.[1,7] In contrast, normoglycemic stored RBCs are able to maintain ATP release and deformability relative to initial levels throughout the entirety of storage.[1] Albumin rejuvenation may yield greater benefit to normoglycemic stored RBCs due to improved deformability, ATP release, osmotic resistance, and morphology seen in normoglycemic storage in comparison to hyperglycemic storage.[1,2] The literature on normoglycemic storage and albumin rejuvenation of stored RBCs indicate that RBCs function better under normoglycemic conditions and in the presence of albumin. This should not be surprising considering these conditions are a closer mimic of the physiology seen *in vivo*.

The work presented in this chapter aims to uncover further benefits of albumin washing that have not previously been reported. This includes evaluating the impact of albumin washing on both hyperglycemic and normoglycemic storage through morphology, phosphatidylserine (PS) externalization, and osmotic fragility to understand if rejuvenation has a greater effect on normoglycemic stored RBCs. Storing cells in albumin is also investigated to capture the benefits of albumin rejuvenation without the requirement of additional processing steps.

4.2 Methods

4.2.1 Albumin rejuvenation and storage

Both hyperglycemic and normoglycemic stored human RBCs were collected, processed and stored according to previous methods (see chapter 2, 2.2.7). The storage of these human RBCs implemented the FDA approved, hyperglycemic additive solution AS-1 and the normoglycemic-modified counterpart, AS-1N.[15] Additionally, the storage of RBCs with albumin was investigated. The hyperglycemic additive solution, AS-1, was modified to contain 5% (w/w) bovine serum

albumin, referred to as AS-1A, and used in the storage of human RBCs. These AS-1 and AS-1A stored RBCs were not fed with any feeding solution or mixed for up to 14 days in storage. Normoglycemic stored RBCs were attached to the automated glucose feeding system and was maintained between 4-6 mM via periodic delivery of 100 mM glucose in 0.9% saline using the volume-dependent delivery method described previously (see chapter 2, 2.2.7, 2.2.8, 2.2.11). It is important to note that this study did not feed the hyperglycemic condition with 0.9% NaCl, unlike previous work.[16] This was implemented to more closely resemble traditional RBC storage practices, which do not feed the stored RBCs with any solution. The hyperglycemic storage condition will be referred to as AS-1 storage, with normoglycemic storage referred to as AS-1N storage and hyperglycemic storage with albumin referred to as AS-1A. The stored RBCs were kept at 2 – 6 ºC for up to 44 days.

Rejuvenation of both hyperglycemic and normoglycemic stored RBCs via isotonic buffer washing with and without albumin was also investigated. According to Reinhart et al., rejuvenation of stored RBCs via washing with albumin containing buffers is dependent on hematocrit, albumin percentage, and wash duration.[10] This study reported that there was minimal benefit to RBC shape recovery when washing RBCs below 2-4% hematocrit, above 0.2% albumin, and longer than 1-2 minutes.[10] Though it is important to note that surpassing these thresholds did not yield any negative impact on the RBCs.[10] The data presented here implemented two rejuvenation solutions, phosphate buffered saline (PBS), and phosphate buffered saline with 0.4% human serum albumin (PBSA). Hyperglycemic and normoglycemic stored human RBCs were sampled each week and washed at a 1-2% hematocrit with either PBS or PBSA for 10 minutes. The samples were subsequently centrifuged at 2000*g* for 10 minutes and resuspended in their respective buffer up to a 60% hematocrit.

4.2.2 Glucose Monitoring

Glucose measurements of stored RBCs were taken every 1-3 days to monitor glycemic levels of the stored RBCs. Glucose levels were measured using a blood glucometer (AimStrip Plus) and blood glucose electrode strips (AimStrip). See 3.2.1 for glucose correction.

4.2.3 Hemolysis, Osmotic Fragility, and Sorbitol Measurements

Osmotic fragility was measured in AS-1A stored human RBCs along with PBS and PBS-A rejuvenated stored human RBCs using methods described previously (see chapter 3, 3.2.2 and 3.2.4) for up to 44 days in storage. The osmotic fragility measurements of AS-1A stored RBCs also implemented a series of hypotonic saline solutions ranging from 0% - 0.9% to highlight the saline concentration that results in 50% hemolysis of the stored RBCs.

4.2.4 Flow Cytometry and Scanning Electron Microscopy

Flow cytometry was used to measure relative cell size, phosphatidylserine (PS) externalization, and reactive oxygen species (ROS) generation in rejuvenated stored human RBCs. These procedures are outlined previously (see chapter 3, 3.2.5).

Scanning electron microscopy (SEM) of rejuvenated stored human RBCs and AS-1A stored RBCs was performed as described elsewhere (see chapter 3, 3.2.6). This was done for all storage time points, with the exception of the PBS rejuvenation condition on day 2. These images were taken to illuminate the relationship between RBC shape and other storage lesion indicators (e.g. forward scatter, PS externalization).

4.2.5 Statistical Analysis

All statistical testing was performed using two- or three-way ANOVA followed by Tukey's Honest Significant Difference testing at a 95% significance level. GraphPad Prism Software was used for statistical analysis. Statistical analysis data is only shown for data that displayed statistical

significance. Anywhere from 1-4 donors were used for assay measurements depending on availability and measurement difficulty.

4.3 Results

4.3.1 Stored RBC Shape Following Rejuvenation

Both hyperglycemic and normoglycemic stored RBCs were imaged throughout storage via SEM after rejuvenation with PBS and PBSA (figure 4.1). The cells were subsequently classified according to their morphology and each condition was assigned a morphological index (figure 4.2). As discussed previously, both hyperglycemic and normoglycemic stored RBCs without rejuvenation increased in their morphological index during storage, indicating a trend toward echinocytosis. However, AS-1N stored RBCs exhibited decreased shape transformation, evidenced by the lower morphological index of AS-1N stored RBCs in comparison to AS-1 stored

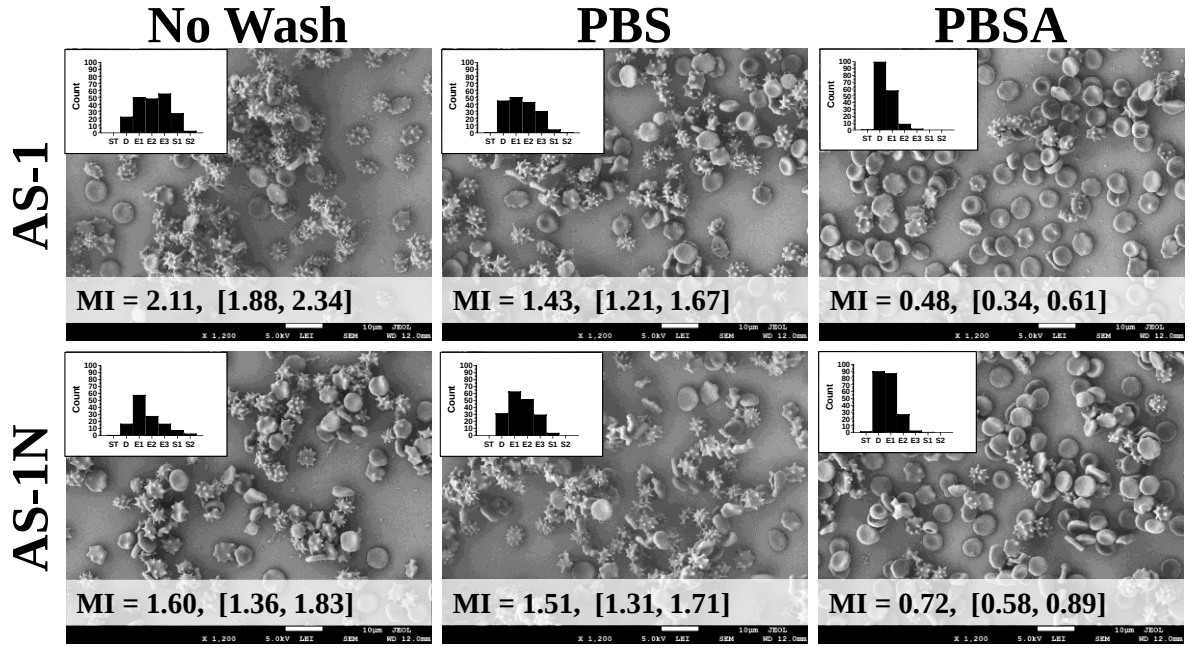

Figure 4.1: **SEM images of hyperglycemic and normoglycemic stored RBCs rejuvenated with PBS or PBSA on day 23 of storage (n = 4, pooled sample, error = 95% confidence interval)**. (top-left) AS-1 non-rejuvenated. (top-center) AS-1 rejuvenated in PBS. (top-right) AS-1 rejuvenated in PBSA. (bottom-left) AS-1N non-rejuvenated. (bottom-center) AS-1N rejuvenated in PBS. (bottom-right) AS-1N rejuvenated in PBSA.

RBCs at each time point. Though no statistical testing was completed, the 95% confidence intervals for morphological indices do not overlap at day 23 of storage, indicating that normoglycemic storage may lead to less echinocytosis than normoglycemic storage. Echinocytosis rapidly progressed during storage for both storage conditions reaching morphological indices of 2.53 ± 0.24 and 2.63 ± 0.27 for normoglycemic and hyperglycemic storage respectively. Both PBS and PBSA rejuvenation were able to recover storage induced shape changes, lowering the morphological indices of both hyperglycemic and normoglycemic stored

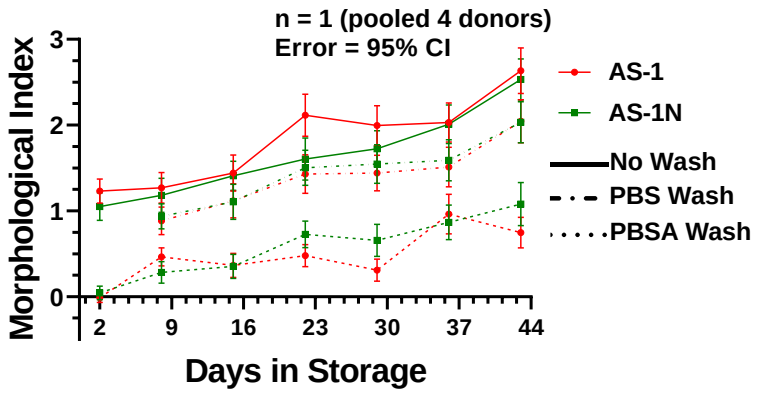

Figure 4.2: **Morphological indices of hyperglycemic and normoglycemic stored RBCs throughout storage rejuvenated with PBS or PBSA** (n = 4, pooled samples, error = 95% confidence interval about the mean).

RBCs at each storage time point. However, the extent of shape recovery for both PBS and PBSA rejuvenation was reduced throughout storage, evidenced by the increasing morphological indices for both PBS and PBSA washed RBCs during storage. Stored RBCs washed in PBSA, however, exhibited greater shape recovery in comparison to PBS rejuvenated RBCs, regardless of storage condition. This is supported by the greater percentage of discocytes in the SEM images as well as the significantly lower morphological indices throughout storage when washed in PBSA as seen in figure 4.3. Rejuvenating stored RBCs in PBSA on day 2 resulted in almost complete recovery of echinocytic shape changes back to normal discocytes, regardless of storage condition. This

decreased the morphological index of AS-1 stored RBCs from 1.23 to -0.01 and decreased the morphological index of AS-1N stored RBCs from 1.05 to 0.05. Considering the 95% CI bars for PBSA rejuvenated RBCs do not overlap with the PBS rejuvenated RBCs or non-rejuvenated RBCs at any storage time point tested, these changes are likely statistically significant, indicating that rejuvenating stored RBCs with albumin provides greater shape recovery than without. It is also worth noting that rejuvenation in either PBS or PBSA resulted in a greater recovery and lower morphological indices in hyperglycemic storage at days 22 and 29.

4.3.2 Impact of Rejuvenation on Stored RBC Size

To accompany the SEM images, stored RBCs rejuvenated with PBS or PBSA were analyzed for relative cell size via forward scatter signal using flow cytometry (figure 4.4). As discussed previously, the RBC size initially increases upon storage and subsequently decreases as the RBCs shed membrane via microvesicles. This is supported by the decreasing forward scatter signal seen in non-rejuvenated stored RBCs in figure 4.4, starting at 137.8% ± 10.2% and 133.7%

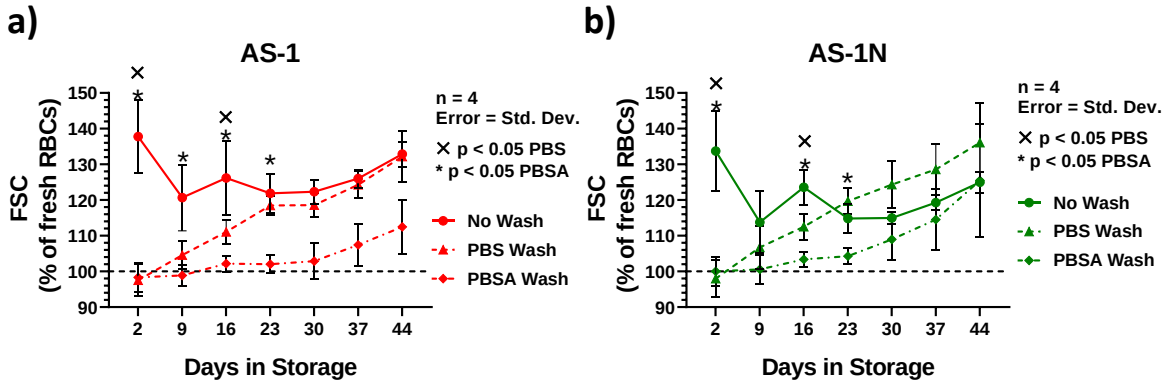

Figure 4.4: **Relative stored RBC size via forward scatter signal after rejuvenation in PBS or PBSA**. (a) Forward scatter signal of AS-1 stored RBCs without rejuvenation (solid line), after PBS rejuvenation (red dashed line), and after PBSA rejuvenation (dot-dashed line) (n = 4, error = standard deviation, × $p < 0.05$ comparing "No Wash" to "PBS Wash", * $p < 0.05$ comparing "No Wash" to "PBSA Wash"). (b) Forward scatter signal of AS-1N stored RBCs without rejuvenation (solid line), after PBS rejuvenation (green dashed line), and after PBSA rejuvenation (dot-dashed line) (n = 4, error = standard deviation, × $p < 0.05$ comparing "No Wash" to "PBS Wash", * $p < 0.05$ comparing "No Wash" to "PBSA Wash").

± 11.3% on day 2 of storage for AS-1 and AS-1N RBC storage respectively, dropping to 132.8% ± 3.53% and 125.0 ± 3.02% by the end of storage. However, rejuvenation of stored RBCs in both PBS and PBSA was able to somewhat recover stored RBC size to that of fresh RBCs. This is evidenced by the relative stored RBC size in AS-1 storage dropping from 136.8% ± 8.42% on day 2 to 96.9% ± 4.04% in PBS and 97.6% ± 3.68% in PBSA. However, as the RBCs age, the extent of size recovery via rejuvenation is reduced, indicated by the decreasing difference in cell size between non-rejuvenated RBCs and PBS/PBSA rejuvenation conditions throughout storage. Additionally, cell size recovery is also reduced when rejuvenating in PBS in comparison to PBSA, illustrated by a greater reduction in cell size when rejuvenating in PBSA in comparison to PBS at each storage time point. Although AS-1N stored RBCs exhibit similar trends when rejuvenating with PBS or PBSA, the degree of size recovery via these rejuvenation solutions is reduced in AS-1N stored RBCs in comparison to AS-1 stored RBCs. For example, on day 30, AS-1 stored RBCs recovered cell size from 121.5% ± 3.63% to 102.2% ± 3.85% after PBSA rejuvenation, a reduction in size of 19.4%. However, AS-1N stored RBCs recovered cell size from 114.2% ± 2.26% to 108.2% ± 4.56% after PBSA rejuvenation, a reduction in size of only 6.00%.

4.3.3 Osmotic Fragility of Stored RBCs Following Rejuvenation

The propensity of stored and rejuvenated RBCs to lyse under hypotonic stress was evaluated throughout storage (figure 4.5). Both PBS and PBSA rejuvenation were able to lower the osmotic fragility of AS-1 stored RBCs (figure 4.5a) to levels comparable to AS-1N stored RBCs at each storage time point. However, the reduction in osmotic fragility when rejuvenated with PBS or PBSA was diminished as the cells aged throughout storage. This is evidenced by over 50% reduction in osmotic fragility when AS-1 stored RBCs were rejuvenated by PBS or PBSA on day 2, but only a 25% reduction when rejuvenated on day 44. This highlights irreversible damage

to the stored RBCs that slowly progresses and cannot be recovered by rejuvenation. Additionally, though not significant, the osmotic fragility of AS-1 stored RBCs remained higher when rejuvenated in PBSA than when rejuvenated with PBS. This same trend is seen when AS-1N stored RBCs were rejuvenated in PBS or PBSA, with PBSA rejuvenated RBCs exhibiting greater osmotic

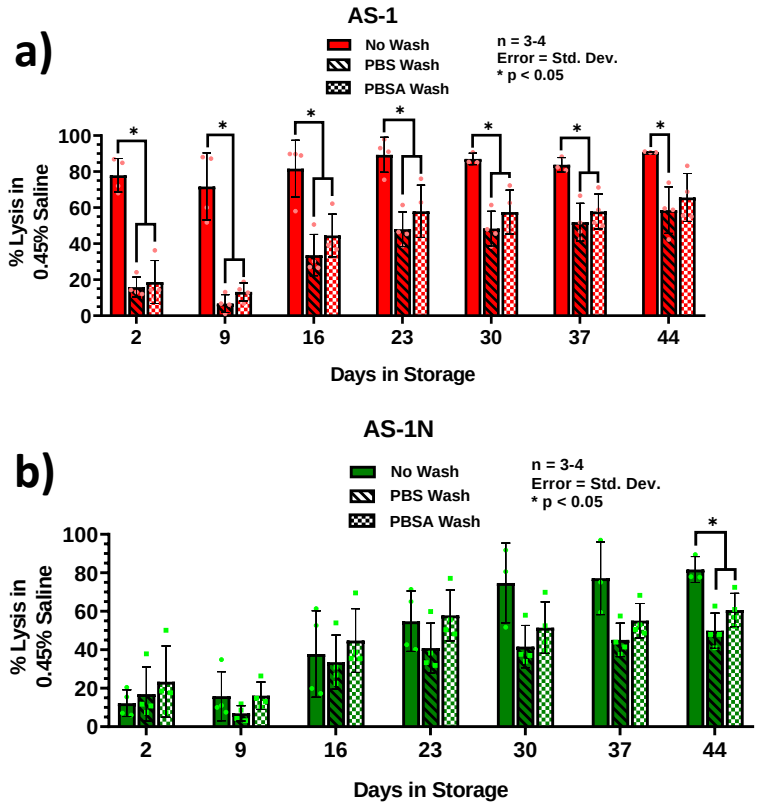

Figure 4.5: **Osmotic fragility of AS-1 stored and AS-1N stored red blood cells after rejuvenation in PBS or PBSA**. (a) PBS and PBSA rejuvenation of AS-1 stored RBCs were able to reduce osmotic fragility during storage with reduced rejuvenation capabilities as the cells aged in storage, indicating the slow progression of irreversible damage (n = 4, error = standard deviation, * $p < 0.05$). (b) Rejuvenation of AS-1N stored RBCs with PBS or PBSA did not reduce osmotic stress of AS-1N stored RBCs, indicating a maximum resistance to hypotonic stress under normoglycemic RBC storage (n = 4, error = standard deviation).

fragility than PBS rejuvenated cells. However, unlike AS-1 stored RBCs, AS-1N stored RBCs did not exhibit significant reductions in osmotic fragility when rejuvenated with PBS or PBSA. These data together indicate three important conclusions. Firstly, membrane shape recovery due to

rejuvenation is not associated with reduced osmotic fragility, considering osmotic fragility was lower when rejuvenating in PBS as opposed to PBSA, yet membrane shape recovery is reduced when rejuvenating with PBS. Secondly, rejuvenation does not improve osmotic fragility when implemented in normoglycemic storage, indicating that normoglycemic storage offers maximum resistance to hypotonic stress. Lastly, the mechanism of rejuvenation by the presence of albumin is not related to alleviating intracellular osmotic pressures.

4.3.4 PS Externalization and ROS Generation Following Rejuvenation

The externalization of PS and generation of ROS was measured in AS-1 and AS-1N stored RBCs after rejuvenation in PBS or PBSA (figure 4.6 and figure 4.7). Interestingly, PS

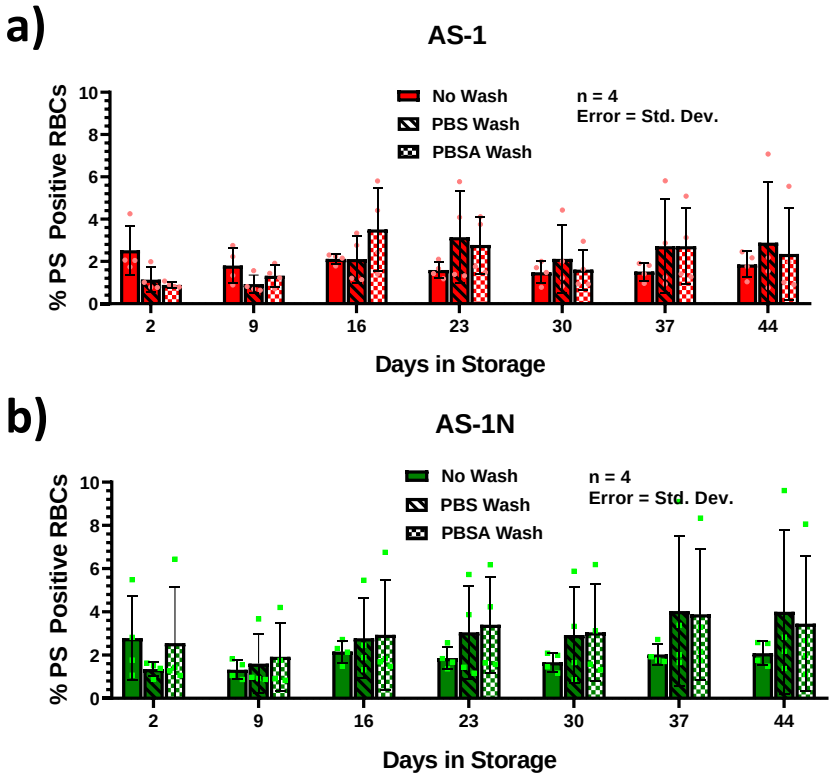

Figure 4.6: **PS externalization of stored RBCs after rejuvenation in PBS and PBSA**. (a) Rejuvenation of AS-1 stored RBCs revealed no change in PS externalization at any storage time point (n = 4, error = standard deviation). (b) Rejuvenation of AS-1N stored RBCs revealed no change in PS externalization at any storage time point (n = 4, error = standard deviation).

externalization in both AS-1 and AS-1N stored RBCs did not significantly decrease after rejuvenation in PBS or PBSA at any time point. In fact, PS externalization increased at certain points, though not statistically significant. ROS generation also did not benefit from rejuvenation, resulting in an increase in ROS generation after rejuvenation, which was more exaggerated for PBSA rejuvenation and under normoglycemic storage. These data indicate that RBC shape recovery via albumin rejuvenation is not related to PS externalization or ROS generation.

4.3.5 Storage of RBCs with Bovine Serum Albumin

Hyperglycemic storage of RBCs supplemented with 5% BSA (AS-1A) was implemented to determine if albumin could prevent RBC membrane shape transformation during storage, as

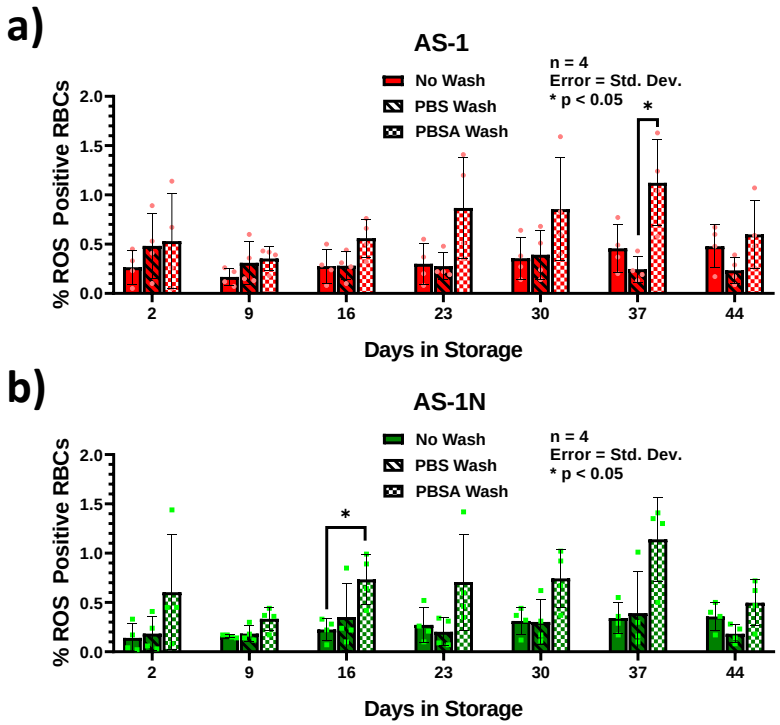

Figure 4.7: **ROS generation of stored RBCs after rejuvenation in PBS and PBSA.** (a) Rejuvenation of AS-1 stored RBCs with PBS or PBSA revealed increased ROS generation at each time point, though there was no significance (n = 4, error = standard deviation, * $p < 0.05$). (b) Rejuvenation of AS-1N stored RBCs with PBS and PBSA also revealed increased ROS generation, specifically larger increases when rejuvenating in PBSA (n = 4, error = standard deviation, * $p < 0.05$).

opposed to rejuvenating the shape changes. The SEM images and morphological indices for AS-1 and AS-1A stored RBCs at day 14 of storage (figure 4.8) show that storage of RBCs with 5% BSA resulted in increased echinocytosis rather than decreased echinocytosis, evidenced by the increased morphological index of 1.25 in AS-1A as opposed to 1.05 in AS-1. Though not significant, these data indicate that RBC shape recovery via albumin is exclusive to rejuvenation and cannot be prevented in storage. Furthermore, figure 4.8 also shows SEM images and morphological indices of AS-1 and AS-1A stored RBCs after rejuvenation in PBSA. Recovery of stored RBC shape via albumin rejuvenation was more exaggerated in AS-1 storage as opposed to AS-1A storage, indicating that storing cells in albumin not only results in greater echinocytosis, but reduced capacity for rejuvenation via albumin washing. The morphological indices between AS-1 and AS-1A stored RBCs after rejuvenation in PBSA also do not overlap in their 95% confidence intervals, likely representing statistical significance, though no testing was performed. A closer evaluation of AS-1 and AS-1A stored RBCs via SEM is shown in figure 4.9. These images show that AS-1A storage potentially resulted in protein accumulation on the membrane of the stored RBCs.

Osmotic fragility was also tested for RBCs stored in AS-1 or AS-1A (figure 4.10), showing that osmotic fragility was not improved when storing RBCs with bovine serum albumin. In fact, osmotic fragility was increased in AS-1A stored RBCs at all time points tested, indicating that

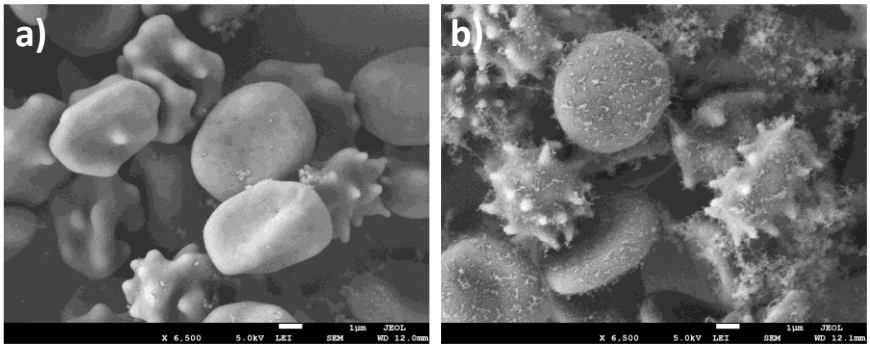

Figure 4.9: **High magnification SEM images of AS-1 (a) and AS-1A (b) stored RBCs at day 14 of storage.**

storing cells with 5% BSA results in greater osmotic stress for the cells. This is further illustrated in figure 4.10b which shows that on day 14 of storage, 50% of the RBCs lyse at a higher NaCl concentration when RBCs were stored with albumin. A higher NaCl concentration that results in 50% hemolysis indicates greater osmotic fragility. These data highlight that storage of RBCs with albumin does not yield the same benefit as albumin rejuvenation.

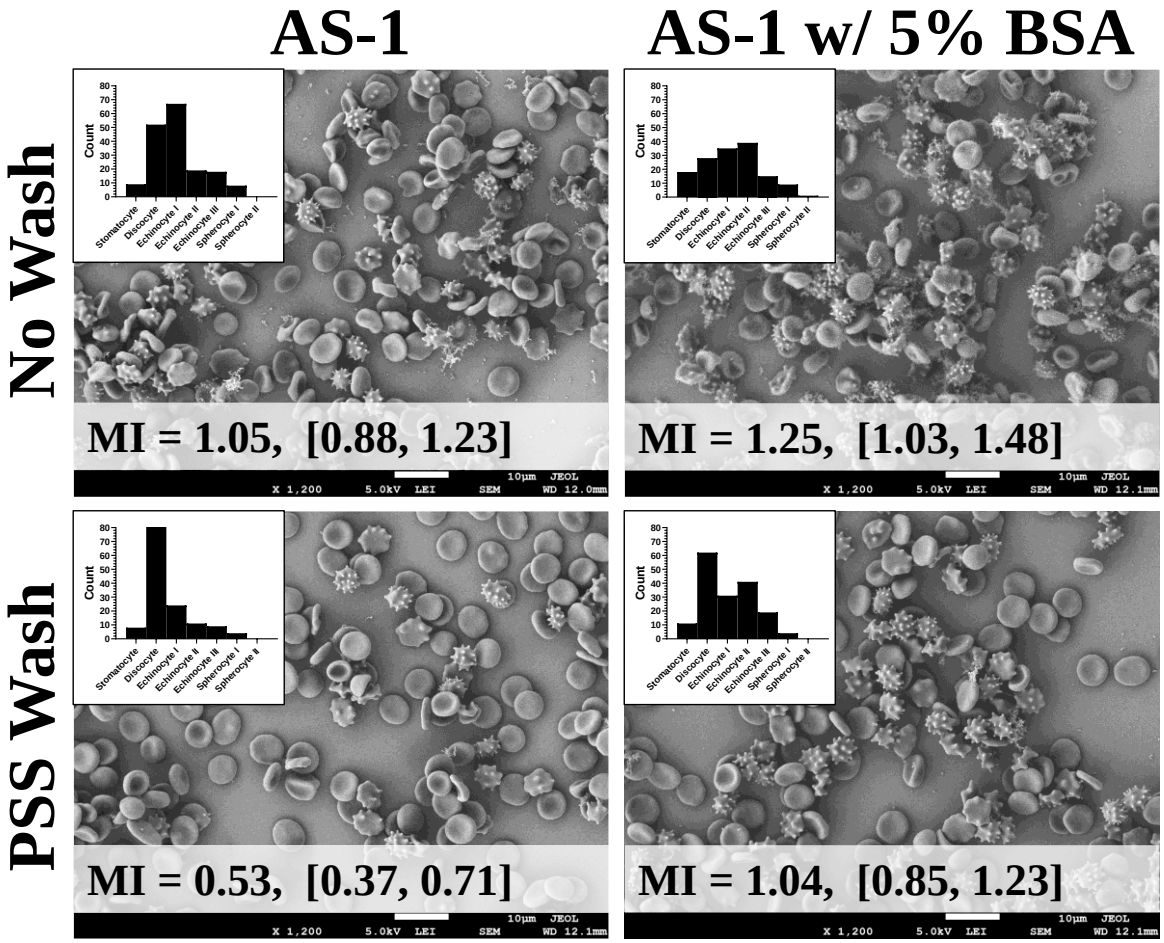

Figure 4.8: **SEM images with shape classification histograms of RBCs stored in AS-1 or AS-1 supplemented with 5% bovine serum albumin (AS-1A) at day 14 of storage.** The AS-1 stored RBCs (top left) exhibited a decreased morphological index than AS-1A stored RBCs (top right), though not significant. AS-1 stored RBCs after rejuvenation in PBSA (bottom left) resulted in a lower morphological index than AS-1A stored RBCs rejuvenated in PBSA (bottom right) that did not have overlapping confidence intervals (n = 1, error = 95% confidence interval).

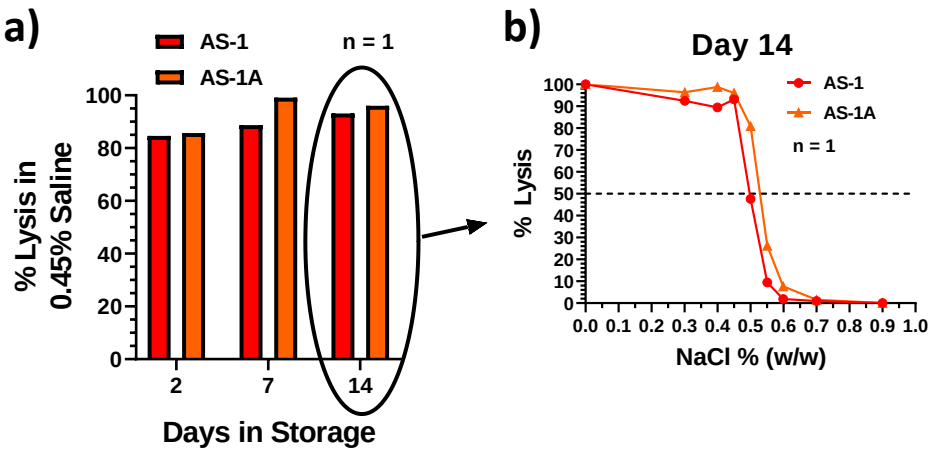

Figure 4.10: **Osmotic fragility of AS-1 and AS-1A stored RBCs**. (a) Storage of RBCs in AS-1 supplemented with 5% bovine serum albumin resulted in increased osmotic fragility. (b) An osmotic fragility test on day 14 of storage using a series of NaCl concentrations revealed a higher NaCl concentration in AS-1A storage that resulted in 50% hemolysis in comparison to AS-1 storage.

4.4 Discussion

Recovery of stored RBC shape via albumin washing has been reported by many studies.[9–12] However, the mechanism by which albumin alleviates these storage induced damages is yet to be reported. Indeed, the data here also support the rejuvenating ability of albumin in recovering storage induced membrane changes of RBCs. Not only was the morphological index lowered upon albumin rejuvenation, but the relative RBC size was also recovered closer to the size of fresh RBCs than when rejuvenated with PBS alone. However, the data presented here also indicate there is a threshold of irreversibility regarding these storage-induced morphological damages, evidenced by the increasing morphological index and reduced recovery of RBC cell size after rejuvenation in PBS or PBSA throughout storage. This is also supported by an investigation by Lu et al. that studied single cellular morphology changes of stored RBCs after rejuvenation in an albumin containing buffer.[11] This study discovered that RBCs with severe morphological damages, specifically spherocytes, could not recover their shape after rejuvenation and often lysed when

rejuvenated with an isotonic buffer, illustrating their severe fragility.[11] This may be due to increased membrane vesiculation that surpasses a threshold that cannot be recovered by rejuvenation. This suggests that rejuvenation by PBS or PBSA is only able to recover echinocytic shape changes, and not spherocytic membrane damages. This finding provides evidence that rejuvenation with albumin does not add or remove lipids from the RBC membrane. It has previously been proposed that albumin may be interacting with the RBC membrane and removing lysophosphatidylcholine (lysolecithin), an inducer of echinocytosis, from the membrane, resulting in alleviation of echinocytosis.[9] However, this is likely not the mechanism of albumin rejuvenation considering there are conflicting reports on the levels of lysolecithin during storage.[9,10,17] Additionally, this mechanism would suggest that increased lysolecithin would result in more severe membrane damages and more spherocytes, allowing albumin rejuvenation to provide benefit to lysolecithin-rich spherocytes. Considering albumin rejuvenation cannot recover spherocytic damages, this proposed mechanism is not likely. This is also supported by the PS externalization data presented here. Rejuvenation via PBS or PBSA had little effect on PS externalization, and in some cases, led to an increase in PS externalization. This provides evidence that PS expression is not associated with membrane shape/integrity and albumin rejuvenation does not remove PS from the membrane, suggesting that albumin does not remove phospholipids from the membrane.

Alternatively, albumin has important antioxidant properties in the blood stream that has also been proposed as a mechanism of stored RBC rejuvenation.[18] Echinocytosis of stored RBCs is reported as a result of many factors, including changes in the actin-spectrin network, Band 3 protein clustering, loss of phospholipid asymmetry, and oxidative damage.[11] Protein and lipid oxidation can trigger aggregation and blebbing of the membrane from the spicules generated

through echinocytosis.[11] It is possible that albumin may act as an antioxidant during rejuvenation, scavenging reactive oxygen species and alleviating oxidative damages to the membrane.[11,18] Indeed, one study reported that washing stored RBCs in an albumin containing buffer lowered oxidized glutathione levels (GSSG), an important antioxidant in its reduced form.[9] Though albumin rejuvenation may offer some antioxidant benefit, this is also not likely the mechanism driving shape recovery. The same study that discovered lowered GSSG levels also reported that reduced glutathione levels (GSH) were not significantly changed. Additionally, scavenging of ROS via albumin may result in reduced progression of echinocytosis due to reduced protein/lipid oxidation, but it is unclear if albumin could also reverse protein/lipid oxidative damage, which would be necessary for the reversal of echinocytosis in this proposed mechanism. Indeed, the ROS data in this study refutes this proposed mechanism, revealing that ROS generation is increased after rejuvenation in PBS or PBSA, not decreased. It is unclear why ROS generation would be increased after rejuvenation, but it is clear that RBC shape recovery via albumin rejuvenation does not occur through a reduction of ROS.

Albumin also plays a significant role in determining oncotic pressure in the blood stream.[6] Oncotic pressure, or the colloid pressure exerted on the RBC due to proteins, is primarily responsible for driving fluid into our out of blood vessels.[19] Due to the large size of albumin at 66 kDa, it cannot passively diffuse through the cell membrane and into the cytosol, generating greater osmotic pressure outside of the RBCs. This may result in fluid driven outside of the stored RBC when incubated in albumin containing buffers in comparison to albumin-free buffers. This is supported by a reduction in stored RBC volume reported after washing in an albumin-containing solution.[9] However, the data presented here indicate that shape recovery due to albumin rejuvenation is not driven by osmotic changes. Though osmotic fragility was decreased after

rejuvenation in PBSA, it was also reduced after washing in PBS, indicating that the osmotic benefit was not a result of albumin rejuvenation. In fact, osmotic fragility for stored RBCs was higher after PBSA rejuvenation in comparison to PBS rejuvenation. These results suggest that rejuvenation of stored RBCs may alleviate intracellular osmotic pressure, but this is not driven by albumin interaction. This is further supported by work from Reinhart et al. which illustrated that incubation of stored RBCs with proteins of similar molecular weight to albumin, such as dextran 70, heat shock protein, or protein C, did not result in the reversal of echinocytosis exhibited by albumin.[12]

The rejuvenation capacity of stored RBCs due to albumin is potentially driven by modification of the Donnan equilibrium, first proposed by Pierre Wong.[13] As discussed previously, the Donnan equilibrium describes the unequal distribution of charged diffusible ions across a cell membrane that balances opposing forces from concentration gradients and electrical charge.[20] This equilibrium is important as it impacts the behavior of band 3 protein, the primary anion transporter in the RBC membrane.[21] Band 3 protein transports chlorine and bicarbonate ions via a ping-pong mechanism that results in both an inward and outward confirmation, favoring echinocytosis and stomatocytosis respectively via cytoskeletal contraction and relaxation.[13,22] The ratio of band 3 protein confirmations is equivalent to the Donnan ratio, dictated by the ratio of intracellular to extracellular chlorine, bicarbonate and H^+ ions: (band 3_o)/(band 3_i) = $[Cl^-_i]/[Cl^-_o]$ = $[HCO_3^-{}_i]/[HCO_3^-{}_o]$.[13] Wong has proposed that echinocytosis during RBC storage progresses through ATP depletion, resulting in inward transport of inorganic phosphates into the RBC and inward facing band 3 confirmations, favoring echinocytosis. Wong has also proposed that the reversal of echinocytosis of stored RBCs by rejuvenation with albumin may be driven by a dehydration effect, drawing NaCl and KCl out of the RBC.[13] Though this dehydration effect may exist and can explain

the significant decrease in RBC size after rejuvenation due to fluid driven out of the cytosol, albumin is more likely to have an impact on the Donnan ratio. Above its isoelectric point of ~5, albumin has a charge of -17.[23] Due to its inability to travel through the membrane, this significantly disrupts the Donnan equilibrium, forcing anions, such as Cl^- into the RBC and cations, such as Na^+, out of the RBC. This has several effects. Firstly, the increased osmotic pressure outside of the cell due to the albumin protein drives fluid outside of the cell and into the extracellular space, causing a decrease in cell volume and size. Additionally, removal of intracellular Na^+ alleviates the stress imparted on the Na^+/K^+ ATP-ase by reducing intracellular Na^+, known to increase from leakage into the RBC during storage.[24] This may result in increased ATP available for further maintenance of RBC membrane integrity. Indeed, Reinhart et al. observed greater intracellular ATP after rejuvenation of stored RBCs with an albumin-containing buffer.[9] Lastly, and most importantly, the extreme negative charge of albumin may drive chlorine into the cell, increasing intracellular chlorine ions, and therefore increasing the Donnan ratio. An increase in the Donnan ratio favors outward transport of anions via band 3, resulting in stomatocytosis. These mechanisms may be why rejuvenation of stored RBCs with albumin containing buffers results in a recovery of RBC size and shape. This also implies that other proteins with similar size and charge may cause a similar recovery of shape. There is a limit to this recovery, however, evidenced by the data presented here. This is potentially a result of decreased membrane loss that surpasses a threshold beyond the ability of albumin to recover shape. Indeed, RBC storage results in a decrease in both activity and quantity of band 3 protein via clustering, protein oxidation, and microvesiculation.[7,24,25] Severe storage-induced membrane transformations toward spherocytosis resulting in significant loss of membrane surface area and band 3 protein may prevent the ability of albumin to recover the discocytic RBC shape.

Interestingly, rejuvenation of stored RBC shape/size via PBS or PBSA was reduced in normoglycemic storage in comparison to hyperglycemic storage. This may be due to increased irreversible damage of AS-1N stored RBCs in comparison to AS-1 stored RBCs, resulting in limited recovery via rejuvenation. However, considering the morphological indices were lower in normoglycemic storage, reduced recovery due to irreversible damage may not be the cause. Alternatively, rejuvenation may have a greater effect on hyperglycemic stored RBCs due to the significant difference in osmotic pressure between hyperglycemic and normoglycemic stored RBCs. Further investigation into intracellular solute concentrations both before and after rejuvenation would confirm this.

Considering the rejuvenation capabilities of albumin, it was hypothesized that storage of RBCs with albumin may result in a similar effect without the necessary washing steps that rejuvenation requires. The washing step inherently increases the potential contamination risk for the patient and may be avoidable through storage of RBCs with albumin. Currently, only one study had attempted to store RBCs with albumin, which did not result in any significant change in the morphology.[10] However, this study stored RBCs at an albumin percentage of 0.2% (w/w) which may have been too low considering the physiological albumin concentration is 5%.[10] In fact, this same study discovered that the stomatocytogenic effect of albumin is inversely proportional to the hematocrit and directly proportional to the albumin percentage, reporting that incubating RBCs with 0.2% albumin at a hematocrit of 64% had no impact on the morphology.[10] Considering that RBCs are stored at approximately 60% hematocrit, increasing the albumin concentration in storage may result in a similar effect as albumin rejuvenation. However, the SEM images and morphological indices shown here indicate that storage of RBCs with a higher albumin concentration did not cause a decrease in morphological index. In fact, the progression of

echinocytosis appeared to be greater when storing RBCs with albumin, with subsequent rejuvenation leading to a diminished recovery in comparison to rejuvenation of RBCs stored without albumin. Additionally, storing RBCs with albumin did not lead to any reduction in osmotic fragility of the RBCs, indicating that intracellular solute concentration and volume were not reduced during storage with albumin. These results highlight a key finding regarding the stomatocytogenic effect of albumin, indicating that it may not be continuous. Though it may result in an initial stomatocytogenic effect, the driving mechanism of echinocytosis during storage may still persist regardless of albumin presence.

4.5 Conclusion

Normoglycemic RBC storage has been shown to exhibit *in vitro* benefit when compared to traditional hyperglycemic storage. However, rejuvenation of stored RBCs is also a promising alternative to modifications to storage solutions. Rejuvenation of stored RBCs via isotonic buffer with or without albumin was shown to result in improved morphology and size, with reduced osmotic fragility. However, these rejuvenation strategies did not result in reductions in PS or ROS, eliminating these mechanisms as an explanation for this rejuvenation. The recovery of RBC shape via albumin rejuvenation may be due to cell dehydration and an increase of the Donnan ratio, favoring stomatocytosis. Investigation into other proteins with similar molecular weight and charge may reinforce the proposed mechanism via manipulation of the Donnan ratio. This rejuvenation mechanism is limited, however, due to irreversible membrane damage occurring to stored RBCs, preventing the membrane recovery of spherocytes. Storage of RBCs with albumin at physiological concentrations did not result in the same stomatocytogenic effect seen with rejuvenation. It instead resulted in increased membrane damage and echinoctyosis with a reduced recovery when rejuvenated in an albumin-containing buffer. This highlights the transient nature of

stomatocytosis induced by albumin rejuvenation, indicating that storage with albumin cannot sustain the morphological benefit seen in rejuvenation. Though normoglycemic stored RBCs exhibited increased echinocytosis and decreased shape recovery via rejuvenation throughout storage, modifying the feeding solution to omit NaCl may alleviate the irreversible membrane damage and result in improved morphology in comparison to hyperglycemic storage. This is further supported by the decreased osmotic fragility, oxidative stress, and volume changes seen in normoglycemic storage. Further investigation into normoglycemic RBC storage using an isotonic glucose feeding solution without NaCl is necessary to illuminate morphological changes. Feeding with isotonic glucose without NaCl may result in decreased echinocytic transformations in normoglycemic storage that is observed in the first 2 weeks of storage.

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Chapter 5 – Conclusions and Future Directions

5.1 Conclusions

5.1.1 Autonomous and Sterile Glycemic Control of Stored Red Blood Cells

Normoglycemic storage of red blood cells (RBCs) has been shown in several studies to provide functional benefit to RBCs *in vitro* by slowing the progression of the storage lesion.[1–6] These studies have highlighted encouraging support for normoglycemic RBC storage to result in improved transfusion response *in vivo*. However, several limitations of the normoglycemic storage strategy have inhibited its application clinical translation. Normoglycemic storage of RBCs may provide functional benefit but requires periodic glucose addition to prevent hypoglycemia.[4] This was accomplished in previous studies via manual injection of concentrated glucose into the stored RBC bags. However, this technique requires unrealistic labor demands and introduces repeated risk of contamination when translating to clinical practice. The work discussed here has overcome these limitations through the design, validation, and implementation of an autonomous glucose delivery system that maintains a completely closed and sterile environment throughout the entirety of storage.

The autonomous glucose feeding device utilized gravity to drive fluid flow and was able to achieve consistent, microliter volume delivery that followed a linear relationship with the valve delay period. Well-modeled by Bernoulli's principle, the autonomous glucose delivery system was validated to result in expected glucose increases. When applied to the glycemic control of stored RBCs, the device was successful in controlling normoglycemia completely autonomously for up to 37 days. However, limitations of the device's operation were discovered, specifically in its consistency regarding glycemic control. Though most samples were maintained within 4-6 mM glucose during the storage duration, the glucose levels sometimes fell outside of this range, which

required manual intervention to correct these deviations. This may have been due to the constant volume delivery approach initially implemented. The device initially assumed an average RBC glucose utilization of 1.2 mM every 3 days, dispensing a constant volume to compensate for this average glucose reduction. However, measurements of glucose utilization revealed that the stored RBCs consume significantly more glucose in the first 3 days of storage than in the remaining weeks, where glucose utilization continues to steadily drop. This reduction in glucose utilization during RBC storage was consistent with reports in the literature, which indicate that rates of glycolysis are significantly reduced in storage due to hypothermic temperatures and metabolic waste buildup.[7] By implementing a constant volume delivery approach in the feeding regimen, the device did not deliver enough glucose early on in storage and delivered too much toward the end of storage, resulting in significant deviations. Modifying the feeding regimen, however, to a storage-dependent delivery approach with glucose delivery that matched glucose utilization resulted in more consistent glycemic control. This modified feeding regimen resulted in an increase in consistency of 25% in comparison to the constant volume delivery approach. Additionally, a single RBC unit was maintained between 4-6 mM completely autonomously for up to 39 days, highlighting the potential for this device to allow for clinical translation of normoglycemic RBC storage. Though design changes must be implemented to further improve consistency and reduce deviations, the glycemic control of this device has overcome a significant limitation to the application of normoglycemic RBC storage, allowing this technique to be applied to clinical practice. Additionally, the glycemic control device makes larger scale studies significantly easier to accomplish considering the reduction of labor requirements in maintaining normoglycemia.

Beyond autonomous glycemic control, many other specifications were necessary to meet

in the design of a glucose delivery device. Maintaining sterility of the blood collection and storage process is a high priority in minimizing patient risk. Fortunately, many years of advancements in blood storage techniques have resulted in near 0 infection rates from transfusion.[8,9] The autonomous glucose delivery device described here was able to maintain this confidence in sterility, resulting in no contamination of any of the normoglycemic stored RBC units fed using the glucose delivery device. Additionally, the well-established infrastructure of blood collection, processing, and storage has allowed for efficient turnaround times between patient arrival and initial transfusion, as quickly as 9 minutes in emergency situations.[10] This is due to stringent quality assurance steps and engineering controls that ensure adequate supply of stored RBC units.[11] Any novel device/procedure that would threaten to disrupt these procedures would experience difficulty in adoption. Fortunately, the normoglycemic RBC storage approach outlined in this work is applicable to any current blood collection bag system and requires minimal capital investment from the blood storage facilities. This minimal disruption would allow for this blood storage technique to be implemented without difficulty into current practices. Overall, the autonomous glucose feeding device designed and implemented in this work provides encouraging evidence that normoglycemic RBC storage is possible to achieve in a clinical setting, significantly improving its potential for translation to common practice.

5.1.2 Normoglycemic Storage of Red Blood Cells Alleviates Storage Lesion Progression

Previous reports, including the work described here, have provided strong evidence for normoglycemic RBC storage to alleviate these storage-induced damages and prevent the progression of the storage lesion.[1–6] This previous work showed that storing RBCs at physiological glucose concentrations resulted in increased ATP release, improved deformability, reduced osmotic fragility, decreased oxidative stress, and increased capacity to stimulate nitric oxide

production.[1–6] These studies, however, utilized small-scale storage (< 2 mL), with custom PVC bags. Additionally, the manual glucose feeding approach previously implemented was accompanied by mixing, which ensured a homogenous distribution of glucose. These limitations cause concern for normoglycemic RBC storage to illicit similar response at a larger scale with autonomous glucose delivery that cannot provide adequate mixing profiles. Implementing the autonomous glucose feeding device with commercially available blood storage bags and subsequent measurement of storage lesion indicators resulted in confirmation that normoglycemic storage leads to improved RBC function, providing evidence for benefit in a clinical setting.

Transfused RBCs must circulate to be effective. This is why the level of *in vitro* hemolysis during RBC storage is arguably one of the most important consequences of the storage lesion. Considering the goal of transfusions is to raise patient hematocrit and hemoglobin levels, significant hemolysis during storage would directly prevent this objective. Fortunately, normoglycemic RBC storage did not result in significant hemolysis, maintained at or below the 1% threshold throughout storage. These results are a significant improvement to previous work which reported increased hemolysis above 1% after just 28 days in storage.[4]

Beyond *in vitro* hemolysis, intravascular hemolysis after transfusion is just as important in avoiding transfusion-related complications. The propensity of RBCs to lyse due to hypotonic stress may contribute to post-transfusion intravascular hemolysis. The osmotic fragility of stored RBCs was significantly reduced under normoglycemic conditions, resulting in a 65% reduction in osmotic fragility on day 2 of storage when compared to hyperglycemic conditions. Normoglycemic storage continued to illicit benefit in comparison to hyperglycemic storage for up to 23 days. The osmotic benefit of normoglycemic RBC storage seen in this work improves upon previous data which observed no significant benefit of normoglycemic storage after only 7 days.[1]

The reduced osmotic fragility of normoglycemic stored RBCs may be due to reduced production of sorbitol through the polyol pathway. The polyol pathway is a glucose metabolism pathway that is upregulated under hyperglycemic conditions, such as patients with diabetes.[12] Increased metabolic activity through the polyol pathway consumes NADPH, a key reducing equivalent used to recycle glutathione, a major antioxidant utilized by RBCs.[12] Intracellular sorbitol levels, an intermediate of the polyol pathway, serves as an indicator of metabolic activity through this pathway. Normoglycemic RBC storage led to significant reductions in intracellular sorbitol, indicating there may be less oxidative stress in normoglycemic storage. Additionally, sorbitol is cell impermeable, thus generating greater osmotic stress for stored RBCs under hyperglycemic storage.[12] The increased levels of cell impermeable sorbitol in hyperglycemic storage may be responsible for the increased osmotic fragility seen in traditional storage systems.

Excess glucose in hyperglycemic storage of RBCs may also allow for increased glycolysis and ATP production. Indeed, Wang et al. noted increased intracellular ATP and lactate levels in hyperglycemic RBC storage relative to normoglycemic storage, indicating that glycolysis may be increased in traditional RBC storage. However, the lactate accumulation and pH data presented here indicate that glycolysis rates are equivalent between hyperglycemic and normoglycemic RBC storage. Excess glucose in hyperglycemic storage is therefore available for metabolism through harmful pathways, such as the polyol pathway.

Many of the benefits of normoglycemic RBC storage, such as osmotic resistance, improved deformability, increased ATP release, and reduced oxidative stress indicate that normoglycemic storage may derive its benefit from improved membrane integrity and decreased reactive oxygen species (ROS) generation. To confirm this hypothesis, a direct measurement of ROS generation and additional measurements of membrane damage were collected. These data revealed that

normoglycemic RBC storage resulted in reduced morphological transformations toward echinocytosis and spherocytosis. Echinocytosis is the RBC shape transformation process characterized by greater outer membrane surface area relative to inner membrane surface area.[13] This results in echinocytes and spherocytes that are smaller in size and exhibit spiky projections, or spicules, on their outer surface due to RBC crenation. This process is driven by many factors during storage including osmotic stress, protein/lipid oxidation, cation leakage, ATP depletion, and band 3 protein clustering.[14] Both echinocytes and spherocytes are smaller in size, are more osmotically fragile, and exhibit decreased deformability relative to healthy discocytes, resulting in significant clearance *in vivo*.[15,16] Normoglycemic RBC storage resulted in less echinocytic and spherocytic shape transformation in comparison to hyperglycemic storage, resulting in greater discocyte percentages throughout storage. Additionally, normoglycemic stored RBCs were smaller in size in comparison to hyperglycemic stored RBCs. The increase in mean corpuscular volume (MCV) of stored RBCs is a known consequence of storage that further exacerbates echinocytosis and spherocytosis.[7,17] These results taken together provide further evidence that normoglycemic RBC storage results in improved membrane integrity that may explain previous data regarding increased deformability relative to hyperglycemic storage. This data is also in agreement with the reduction in osmotic fragility under normoglycemic storage. Increased intracellular sorbitol and osmotic stress under hyperglycemic storage may also result in increased RBC swelling, leading to greater MCV. Indeed, the larger RBC size in hyperglycemic storage seen in this work is likely due to greater MCV, which imparts greater strain on the RBC cytoskeleton and further progresses RBC shape transformation to spherocytosis.[18]

Interestingly, phosphatidylserine (PS) externalization did not significantly increase or decrease throughout storage regardless of storage condition. PS externalization is a key indicator

of cell senescence and is recognized by phagocytic cells in circulation as a marker for eryptosis, or programmed cell death, resulting in their clearance from circulation. Though the percentage of PS expressing RBCs was greater than fresh RBCs, there was no significant change during storage. Though this may seem contradictory considering the increased progression of echinocytosis and spherocytosis, Verhoeven et al. also observed that PS externalization during RBC storage did not significantly increase, which was attributed to virtually absent activity of scramblase, the protein responsible for externalizing PS.[19] This data highlighted a key relationship between PS externalization and membrane shape changes, indicating that the two are unrelated, suggesting that stored RBC clearance *in vivo* is predominantly dictated by physical membrane damages, and not recognition of PS by phagocytic cells.

Considering the increased activity of the polyol pathway in hyperglycemic RBC storage, a more direct measurement of ROS generation was implemented to identify if increased polyol activity was accompanied by increased ROS. Though there was no statistical difference in ROS generation between the two storage conditions, ROS was lower in normoglycemic storage at each time point. This suggests that there may be less oxidative stress in normoglycemic storage, but significant disruption of the redox balance due to increased polyol activity did not translate to equivalent differences in ROS generation.

These results have confirmed that normoglycemic RBC storage alleviates storage induced damages and may provide benefit to RBC function *in vivo*. Further evidence has been provided to identify the mechanism(s) that drive(s) the benefit of normoglycemic RBC storage. The excess glucose available in hyperglycemic storage generates greater osmotic stress for the RBC due to increased glucose metabolism through harmful pathways. This results in greater strain on membrane integrity due to increased swelling, causing greater membrane shape transformation

toward echinocytosis and spherocytosis. Additionally, increased oxidative stress in hyperglycemic storage may also contribute to these damages. These results provide encouraging support for normoglycemic RBC storage to result in improved *in vivo* response, considering RBC clearance in circulation is reported to be primarily exclusive to irreversibly damaged spherocytes.[18]

5.1.3 Role for Albumin in Red Blood Cell Storage

Washing stored RBCs in an albumin-containing buffer offers a novel alternative approach to preventing post-transfusion complications by reversing the storage lesion as opposed to preventing its progression during storage. Albumin is a 66 kDa globular protein and is the most abundant protein within circulation, responsible for controlling oncotic pressure and transporting endogenous/exogenous molecules throughout the body.[20] Rejuvenation of stored RBCs via albumin has recently been reported to reverse echniocytic shape transformation, and result in greater perfusion *in vitro*.[14,21,22] The shape recovery has the potential to result in less RBC clearance *in vivo* considering echinocytes and spherocytes are less deformable than discocytic RBCs.[15,16] The mechanism of stomatocytosis (reverse process of echinocytosis) due to albumin rejuvenation, however, remains unknown. Several hypotheses have been proposed regarding albumin's role in this process, including lipid removal from the outer membrane via interaction with albumin, an antioxidant effect of albumin, and manipulation of band 3 protein conformations.[21–23] The work presented here has highlighted this same benefit of albumin rejuvenation and has provided further data to uncover the mechanism driving this process. Stored RBC shape recovery was observed after washing in a phosphate buffered saline (PBS) as well as PBS supplemented with 0.4% human serum albumin (PBSA). However, this shape recovery was diminished throughout storage, highlighting the irreversible membrane damage that occurs during storage. Additionally, PBSA exhibited greater ability to recover RBC shape in comparison to PBS,

illustrated by lower morphological indices throughout storage. This trend was also seen in the relative cell size measured via flow cytometry, resulting in improved cell size recovery to that of fresh RBCs when stored RBCs were washed in PBSA relative to PBS. This phenomenon is likely due to a disruption of the Donnan ratio via albumin, in favor of stomatocytosis. The Donnan equilibrium describes the unequal distribution of charged diffusible ions across a cell membrane that balances opposing forces from concentration gradients and electrical charge.[24] The Donnan ratio of intracellular chorine/bicarbonate anions to extracellular chlorine/bicarbonate anions dictates the conformation of band 3 protein (outward facing or inward facing): (band 3_o)/(band 3_i) = $[Cl^-_i]/[Cl^-_o] = [HCO_3^-{}_i]/[HCO_3^-{}_o] = [H^+o]/[H^+i]$.[23] Band 3 protein plays important roles in both anion transport as well as cytoskeletal contraction/relaxation, favoring contraction or echinocytosis with greater inward conformations and favoring relaxation or stomatocytosis with greater outward conformations.[23,25,26] The extreme negative charge of albumin at physiological pH (-17) would force the intracellular anion concentration to increase due to the impermeability of albumin and the resultant equilibrium established by the Donnan effect. The increase in intracellular anion concentration raises the Donnan ratio and thus favors cytoskeletal relaxation or stomatocytosis. This process would also draw cations, such as Na^+, out of the cell, relieving the stress imparted on the Na^+/K^+ ATP-ase by reducing intracellular Na^+, known to increase from leakage into the RBC during storage.[27] The removal of solutes from within the RBC due to washing would also drive fluid out of the RBC, resulting in a reduction in volume, known to increase during RBC storage and cause stress on the cytoskeleton.[7,17] These resultant consequences of albumin rejuvenation may be the reason why washing in PBSA resulted in greater shape and size recovery of stored RBCs relative to PBS washing. Other mechanisms have also been proposed, however, including albumin's interaction with the RBC membrane, removing lipids from the outer

membrane.[21] However, the PS externalization data presented here did not result in any significant decrease after PBS or PBSA rejuvenation, suggesting that shape recovery via rejuvenation is not associated with phospholipid removal. Though there are certainly other phospholipids that albumin may interact with and remove from the membrane, the unchanged expression of PS after rejuvenation provides evidence that phospholipid distribution is not manipulated by albumin. Additionally, albumin rejuvenation has been hypothesized to act as an antioxidant, alleviating oxidative stress of stored RBCs.[21] However, the ROS generation data in this work did not show a decrease after incubation in PBSA. Furthermore, stored RBC shape/size recovery is not due to decreased osmotic stress, considering the osmotic fragility of stored RBCs after rejuvenation was similar in both PBS and PBSA washing conditions. This is further supported through the osmotic fragility remaining constant after rejuvenation of normoglycemic stored RBCs, despite the recovery of RBC shape/size seen in the SEM data. Additionally, rejuvenation with PBSA resulted in increased osmotic fragility relative to PBS at each time point. These data suggest that rejuvenation of stored RBCs via albumin does not result in greater reduction in intracellular solutes in comparison to rejuvenation in isotonic buffers. These data have provided key findings regarding a possible mechanism driving stored RBC rejuvenation via albumin washing, concluding that it is likely not due to osmotic changes, reductions in oxidative stress, or lipid removal. The most probable explanation is a shift in the Donnan ratio that favors stomatocytosis.

Interestingly, storing RBCs in the presence of albumin has had relatively little investigation. One study had attempted RBC storage with human serum albumin, discovering that it did not lead to any advantage over traditional storage regarding RBC morphology.[22] However, this same study discovered that RBC shape recovery via albumin was inversely proportional to the hematocrit, reporting no difference in RBC morphology when washing RBCs with 0.2% albumin

at a 40% hematocrit.[22] Washing RBCs at a higher hematocrit required greater albumin concentrations to illicit a similar morphological recovery effect. Considering RBCs are stored at an average 60% hematocrit, there may not have been enough albumin within the storage solutions to illicit any benefit. It was hypothesized that increasing this albumin percentage to physiological levels within storage would result in a similar benefit to RBC morphology that is seen with albumin rejuvenation. This work, however, observed no difference in morphology between RBCs stored with or without physiological levels of albumin. Interestingly, the ability of RBCs to recover shape after rejuvenation with an albumin containing buffer was significantly reduced in the RBCs that were stored with albumin. These data highlight an important conclusion. Albumin may offer a benefit to stored RBC morphology when rejuvenating at low hematocrit and low albumin concentration. However, albumin may result in a net negative effect on RBC morphology at higher concentrations, potentially due to increased osmotic stress. Rejuvenation of stored RBCs with PBSA reduced the osmotic fragility of the RBCs but remained slightly greater than RBCs rejuvenated in PBS without albumin. This highlights a negative effect of albumin as a rejuvenating agent which may explain its failure to maintain RBC shape in storage. Significantly increasing the albumin concentration to 5% for storage with RBCs may have resulted in a more exaggerated increase in osmotic fragility that led to a negative effect on RBC shape transformation. It is possible, however, that reducing the concentration(s) of other salts within storage solutions may compensate for the increased osmotic stress imparted by albumin within RBC storage.

Though storage of RBCs with albumin may not illicit a morphological benefit, it is clear that rejuvenation of stored RBCs via albumin-containing buffers offers a novel alternative strategy at combatting the storage lesion. Considering RBC shape/size is strongly associated with deformability, albumin rejuvenation of stored RBCs has the potential to result in significantly

reduced clearance *in vivo* which may result in improved outcomes for transfusion patients. The data presented here have provided evidence to help identify the mechanism that drives the benefit of albumin rejuvenation, indicating that it may be related to the equilibrium of anion distribution and its resultant impact on band 3 protein conformation.

5.2 Future Directions

5.2.1 Improvements to Autonomous Glycemic Control

The glycemic control of normoglycemic stored RBCs was accomplished in this work by implementing an electronically actuated valve to deliver concentrated volumes of glucose via gravity-driven fluid flow. This resulted in glucose concentrations well-maintained between 4-6 mM, allowing for complete autonomous control within this range for up to 39 days. However, the device was not consistent, resulting in 3-4 deviations from this range per sample that required manual intervention to correct. Though it is unclear whether a 4-6 mM glucose range is necessary to illicit benefits of normoglycemic RBC storage, greater consistency and reliability in glycemic control would be beneficial for translation to clinical practice. One way to accomplish this would be to exchange the valve for a pump in order to achieve lower and more consistent dispensing volumes. The valve design in this work was limited to a lower dispensing volume of 176 µL due to the lower limit on valve delay period at 25 msec. A method to achieve smaller dispensing volumes would be to decrease the distance in height between the feeding reservoir and stored RBC unit, which would reduce the pressure differential and therefore the volumetric flow rate. However, a superior alternative would be to implement a peristaltic pump instead of a valve. Peristaltic micropumps can achieve volume flow rates of less than 10 µL/min.[28] Additionally, they are much more consistent than gravity-driven fluid flow considering gravity-driven flow is dependent on pressure differentials that are controlled by feeding reservoir volume. Larger feeding reservoir

volumes would result in greater pressure and thus greater fluid flow. As concentrated glucose is dispensed into stored RBC units, the feeding reservoir volume decreases, which can lead to inconsistent volume dispensing. Though the volumes dispensed in this work were low enough to have little to no impact on total feeding reservoir volume, it is possible that a clinical application of the device as described could result in volume delivery inconsistencies due to this phenomenon. A peristaltic pump, however, is not dependent on gravity-driven pressure differentials as it uses rollers to establish a pressure differential and drive fluid flow. Additionally, peristaltic pumps have already been established as infusion devices to deliver drugs, such as insulin, to patients.[28,29] Exchanging the valve with a peristaltic pump may result in lower dispensing volumes and more consistent glycemic control of normoglycemic stored RBCs.

Beyond exchanging the valve for a pump, other design improvements could significantly improve glycemic control, but currently face difficulty in implementing. As seen in this work, glucose utilization by stored RBCs is storage-dependent, significantly falling after just a few days of storage. Additionally, there was significant variation in glucose utilization across donors as well. This is partly why there were deviations from the normoglycemic range even when implementing the storage-dependent delivery feeding regimen. A more personalized feeding regimen that could compensate for glucose needs of individual stored RBC units would also result in improved consistency in glycemic control. This may be accomplished by an *in situ* continuous glucose monitoring device located within each stored RBC unit that would relay real-time glucose concentrations to the dispensing device that could then deliver the appropriate volume of concentrated glucose to compensate for the exact deficit. Fortunately, this technology has already been extensively developed within the area of diabetes research. Continuous glucose monitors (CGMs) are common in clinical practice and often used by diabetes patients to monitor glucose

levels and control HbA1c levels.[30–33] Most of these CGMs use enzymatic technology in the determination of glucose concentration, catalyzing a reaction with glucose molecules that transfers electrons to the electrode surface, generating a current that is proportional to the glucose concentration.[32] Implementing similar technology into the RBC storage bag would allow for real-time glucose sensing and personalized glucose delivery, significantly improving the consistency in glycemic control. However, several barriers exist that prevent this technology from being integrated into the feeding device design. Firstly, CGMs often must be recalibrated multiple times per day to maintain accurate readings.[31,32] Also, CGM electrode sensors only have a lifespan of up to 14 days, requiring them to be changed afterward.[30–33] This is because these electrodes begin to foul from biological material, such as proteins, which inhibits analyte contact with the electrode surface.[34] The buildup of protein and other biological material on the electrode surface slowly decreases the electrode activity and signal generation, requiring them to be exchanged every 14 days in the case of CGMs.[32] Considering the goal of glycemic control of normoglycemic stored RBCs is to maintain complete autonomy, the exchange of an electrode every 14 days would not be feasible in translation to clinical practice. Additionally, a CGM for normoglycemic RBC storage would inherently require the manufacture of custom RBC storage bags that can house the CGM. Though continuous glucose monitoring may be better for glycemic control of stored RBCs, a custom RBC collection/storage bag would require blood collection facilities to collect all blood into these specialty bags. This may be difficult to accomplish, would require additional FDA approvals, and could potentially disrupt the current blood collection practices. Due to these concerns, normoglycemic RBC storage must first be established to illicit improved patient outcomes before personalized glucose feeding is necessary.

5.2.2 Future *in vivo* Measurements in Animal Models

Though this work has provided evidence for *in vitro* benefit of normoglycemic RBC storage, this must translate to improved *in vivo* response for this storage technique to be meaningful. Beyond *in vitro* measurements of storage lesion indicators, the grant that funded this project also proposed measurement of *in vivo* responses in a sheep model. Sheep share many similarities with humans regarding their circulatory system and immune response. Their total blood volume is 60 mL/kg, or 3-5.4 L for a 50-90 kg sheep, which is similar to the 5 L average exhibited by humans.[35] Sheep also have an average RBC lifespan of approximately 120 days, similar to humans.[36] Additionally, sheep exhibit similar average heart rates and respiration rates, at 60-80 beats per minute and 16-30 breaths per minute respectively.[35] Sheep and pigs are also often used as animal models in vascular surgery due similarity in blood vessel size, length, and structure to that of humans.[37] Beside similarities within the circulatory system, sheep and humans also exhibit close similarity in their immune system response. A study comparing the gene expression of sheep and human peripheral blood in response to known inflammatory stimulants, lipopolysaccharides, found that there was a similar increase (1.5-2 fold) in the expression of key inflammatory indicators, such as IL-1, IL-6, and TNF-α.[38] Additionally, many other studies have also implemented a sheep model in the study of transfusion medicine.[39–41] These similarities make a sheep model ideal for the measurement of *in vivo* transfusion outcomes, such as post-transfusion recovery percentage and immune activation.

Before *in vivo* measurements in a sheep model can be measured, the *in vitro* benefit of normoglycemic RBC storage must first be established in this sheep model, considering there may be differences in the RBC physiology that impacts their response to normoglycemic storage. Preliminary data measuring levels of hemolysis, osmotic fragility, and intracellular sorbitol of

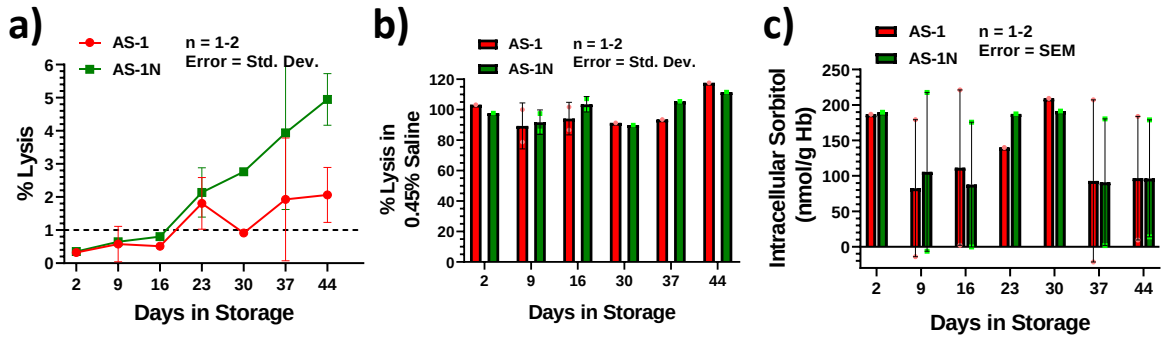

Figure 5.1: **Stored ovine RBC hemolysis, osmotic fragility, and intracellular sorbitol**. (a) Hemolysis steadily increased throughout storage, surpassing the 1% threshold by day 23 for both hyperglycemic and normoglycemic storage. Though AS-1N stored RBCs exhibited greater hemolysis at 4.95% ± 0.79% in comparison to AS-1 stored RBCs at 2.06% ± 0.83% by the end of storage, there was no statistical significance (n = 1-2, error = standard deviation). (b) Osmotic fragility of stored sheep RBCs resulted in greater than 80% hemolysis in a hypotonic solution for both storage conditions at each time point in storage. No statistical difference (n = 1-2, error = standard deviation). (c) Intracellular sorbitol indicated no difference between hyperglycemic and normoglycemic stored RBCs (n = 1-2, error = SEM).

stored sheep RBCs under normoglycemic and hyperglycemic conditions are shown in figure 5.1. These data highlight key differences in RBC behavior between human and sheep RBCs, resulting in significant hemolysis, and no differences in osmotic fragility or sorbitol between hyperglycemic and normoglycemic stored RBCs using a sheep model. These inconsistent results regarding the storage lesion indicators may be related to differences in glucose metabolism between ovine and human RBCs. Indeed, Budtz-Olsen reported that sheep red blood cells metabolize glucose at a rate that is 20-30% that of human RBCs.[42]Additionally, glucose is reported to have difficulty in penetrating into the RBC, while the activity of glycolytic enzymes such as hexokinase and pyruvate kinase is significantly lower than humans and other animal models such as the canine.[43,44] Measurement of glucose utilization of these stored sheep RBCs reveals a similar conclusion, shown in figure 5.2. The glucose utilization of stored ovine RBCs followed a similar trend to

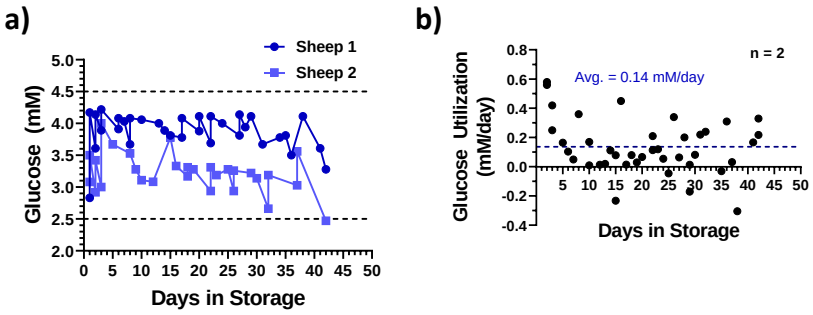

Figure 5.2: **Glycemic control and utilization of AS-1N stored ovine RBCs**. (a) The glucose levels of 2 units of stored ovine RBCs were monitored over 44 days and were well maintained between 2.5 – 4.5 mM (n = 2). (b) Glucose utilization rates of AS-1N stored ovine RBCs initially began at 0.57 ± 0.01 mM/day and dropped to an average of 0.14 mM/day (n = 1-2).

human RBCs, initially beginning high on day 2 at 0.57 ± 0.01 mM/day and falling quickly thereafter reaching an average of 0.14 mM/day. This average glucose utilization is less than half the average rate of human RBCs at 0.33 mM/day. This decreased glucose metabolism may be the reason for minimal differences in osmotic fragility and intracellular sorbitol.

These data demonstrate that normoglycemic RBC storage provides a benefit to stored RBCs by reducing the activity of harmful glucose metabolism pathways. In the case of a sheep model, where the glucose metabolism is significantly reduced, this same benefit is not realized. Although many similarities exist between sheep and human cardiovascular physiology, these data suggest that an animal model for normoglycemic RBC storage may require RBCs that metabolize glucose at a similar rate to that of humans. A potential alternative would be a canine model. On average, a 35 kg dog (~75 lbs) would have a total blood volume of 2.1 L, allowing for 210 mL to be drawn at one time.[45] Though this is lower than the total blood volume of humans, it is comparable in the order of magnitude. Additionally, and equally as important, canine RBCs exhibit glycolytic enzyme activity that is 2-4 times greater than sheep glycolytic enzyme activity, resulting in RBC glucose metabolism that is more similar to human RBCs.[43,46] Canines also exhibit similarities to humans regarding their cardiovascular anatomy/physiology, including heart rate,

vessel size, blood glucose levels, and heart geometry.[47–49] Additionally, many studies have implemented a canine model to evaluate *in vivo* responses to transfusion and have exhibited great success.[50–54] Future studies measuring *in vivo* response to normoglycemic stored RBC transfusions may benefit from a canine model. After *in vitro* behavior is established to be similar to the storage of human RBCs, the measurement of 24-hour post-transfusion recovery percentages and immune responses would indicate if normoglycemic RBC storage results in improved *in vivo* outcomes.

5.2.3 Adhesion of RBCs to Endothelium and Novel Rejuvenation Strategies

Alongside animal studies with measurements of post-transfusion responses, other *in vitro* experiments that mimic *in vivo* mechanisms can be performed. Specifically, the grant that funded this work had proposed measuring stored RBC adhesion to a cultured endothelium. An additional consequence of RBC membrane damage and increased ROS generation during RBC storage is increased endothelial adhesion in circulation.[7] In many hemoglobinopathies and microvascular pathologies, such as sickle cell disease, β-thalassemia, and diabetes, RBCs often adhere to the endothelium, disrupting blood flow and causing further complications such as pain, inflammation, and endothelial activation.[55–59] Though many cellular and plasmatic factors have been reported to be involved in this adhesion mechanism, including vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion factor-4 (ICAM-4), increases in membrane damage, protein glycation, and oxidative stress in RBCs are reported to lead to increased endothelial adhesion.[56–59] Considering normoglycemic RBC storage shows strong evidence for reduced oxidative stress, decreased membrane damage, and inherently lower glucose available for protein glycation, RBC adhesion to endothelium is likely reduced in normoglycemic storage. Evaluation of RBC adhesion to endothelium would also elucidate a cell-cell interaction, which is more applicable to potential *in vivo* response than measurements of RBC properties.

Preliminary experiments have attempted to accomplish this through a novel analytical measurement technique that utilizes the radioisotope, technetium-99m (Tc^{99m}), to label red blood cells and measure their adhesion to a cultured endothelium. This was achieved by first culturing bovine pulmonary artery endothelial cells (BPAECs) on flat, circular polystyrene inserts (10 mm diameter) that were covered with or without an extracellular matrix (ECM) mimic via blow-spun polystyrene fibers (figure 5.3).[60] Stored RBCs labeled with Tc^{99m} were then incubated with these inserts for 2 hours, and subsequently removed, leaving any adhered RBCs on the surface of the endothelium. By measuring the gamma emission from the inserts, the number of stored RBCs adhered to the endothelium was determined.

The data shows that there was greater adhesion of stored RBCs to a BPAEC endothelium than to the polystyrene insert alone (figure 5.4a), solidifying the capability of this technique to measure RBC adhesion. However, there was no statistically significant difference across storage time points or between hyperglycemic and normoglycemic stored RBCs. However, it should be noted that adherence of RBCs to the BPAEC-lined substrates was lower under normoglycemic

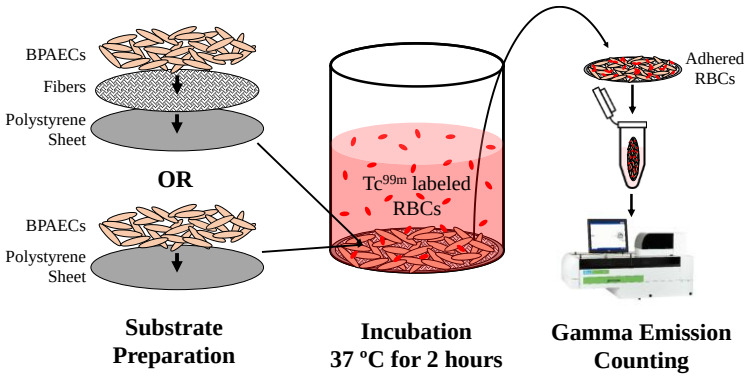

Figure 5.3: **Experimental design implemented in the determination of stored RBC adhesion to a monolayer of endothelial cells.** The substrate preparation resulted in circular polystyrene inserts with or without fibers and BPAECs. These inserts were placed into the wells of a 48-well plate followed by Tc^{99m} labeled stored RBCs which was allowed to incubate with the inserts for 2 hours at 37 ^{O}C. The inserts were then washed 7 times with PSS, then placed into sample tubes for gamma emission counting. Comparison to standards allowed the determination of numbers of RBCs adhered to the surface of the endothelium.

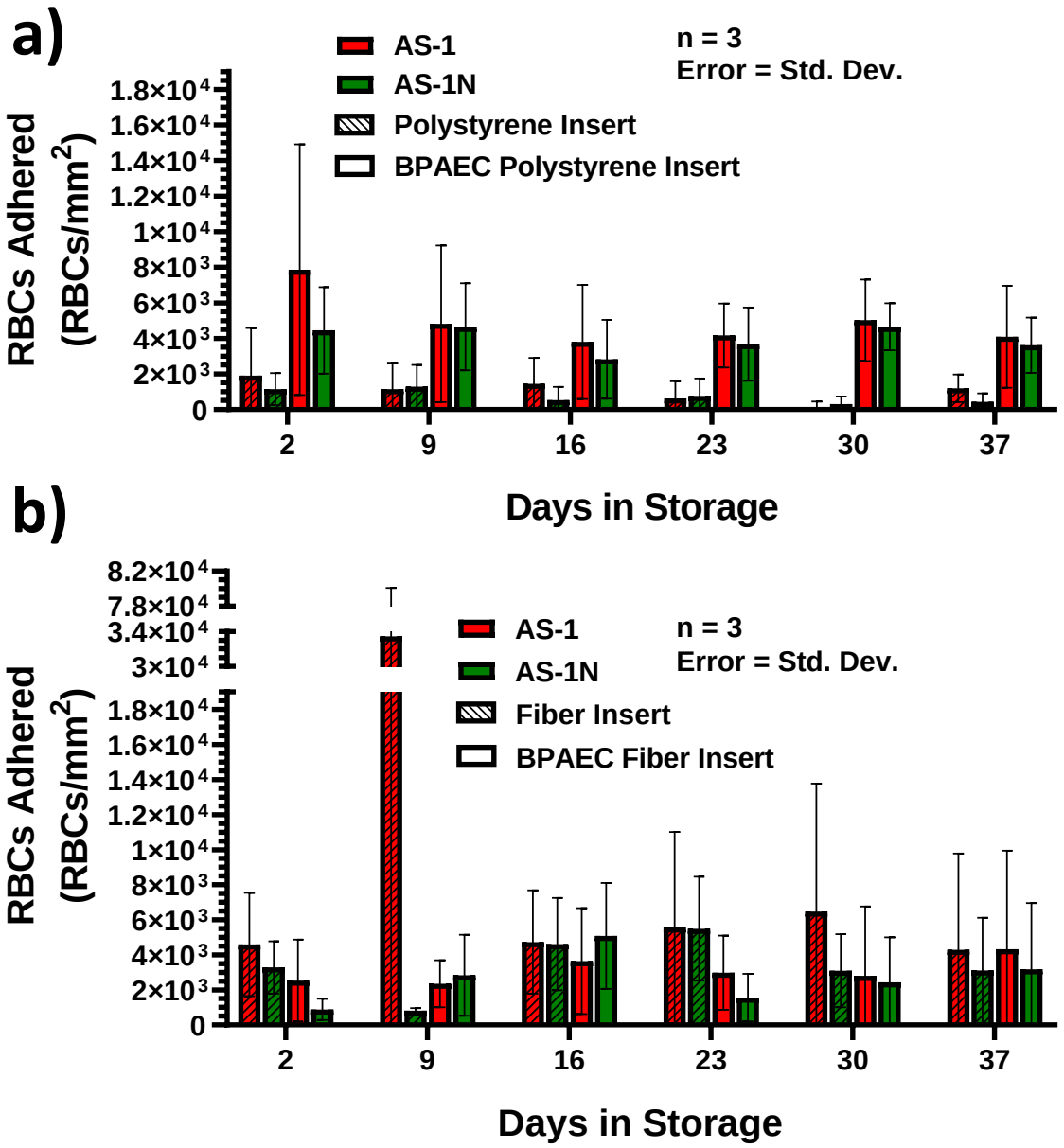

Figure 5.4**: Stored RBC adhesion to polystyrene sheet and polystyrene fiber inserts grown with and without a BPAEC endothelium monolayer**. (a) Stored RBC adhesion to polystyrene sheet inserts over the course of 37 days in storage, resulting in greater RBC adhesion to the inserts in the presence of a BPAEC monolayer. Three-way ANOVA revealed statistical significance in the substrate type (BPAEC Polystyrene vs. Polystyrene), though Tukey's test could not identify which data points were significant. Though there was no statistical significance, RBCs exhibited decreased adhesion to the endothelium under normoglycemic storage (n = 3, error = standard deviation). (b) Stored RBC adhesion to blow-spun fiber inserts with or without BPAEC endothelium over the course of 37 days in storage. No statistical significance was reported in any condition due to the large variability exhibited by each treatment group (n = 3, error = standard deviation).

storage conditions in comparison to hyperglycemic stored RBCs at each time point, though not significant. This data may indicate that normoglycemic stored RBCs exhibit reduced adhesion to endothelium. It is likely that the reported RBC adhesion is derived from non-specific binding of RBCs to the endothelium, and not related to any of the mechanisms that encourage RBC adhesion. This may be due to a variety of reasons. Firstly, this experiment utilized human RBCs with bovine endothelium, which may not interact in a similar manner to what is reported in the literature. In fact, a study which examined bovine RBC adhesion to bovine pulmonary endothelium reported decreased adhesion with increased RBC PS externalization, contradicting reports regarding this mechanism in human physiology.[58] Additionally, even if the BPAECs were expressing RBC adhesion proteins/receptors, they may not be able to interact with human RBCs due to species specificity of the receptors. Many other reports that study the adhesion of RBCs to endothelial cells use human umbilical vein endothelial cells (HUVECs), which may be required in future experimentation.[55,61,62] Lastly, a primary function of endothelial cells is to release NO to stimulate vasodilation and improve blood flow.[64] Nitric oxide released from the endothelium also plays a role in inhibiting cellular adhesion. Though the NO levels were not measured in the RBC adhesion experiment, it is possible that significant levels of NO contributed to the prevention of RBCs to adhere to the surface of the endothelial cells. However, the endothelium most likely provided a morphologically rougher surface than the bare polystyrene insert, leading to increased RBC adhesion through non-specific binding.

The data also revealed inconsistent RBC adhesion, regardless of storage condition or duration, when implementing the ECM mimic onto the inserts (figure 5.4b). This may be due to the nature of the fiber inserts. Due to the difficulty in flattening the polystyrene fibers onto the polystyrene sheets, the fiber inserts often developed folds, holes, and other blemishes within the

fiber network that was inconsistent across the samples. During testing, this led to inconsistent RBC adhesion within the same donor and/or treatment group due to variable RBC adhesion within the folds and holes of the fiber inserts. Improvements to the consistency in the fabrication of the fiber inserts may allow for a more reliable conclusion to be drawn on the relationship between normoglycemic RBC storage and endothelial adhesion.

These adhesion experiments established a promising initial step toward measuring a novel cell-cell interaction that may be affected by the storage condition of the RBCs. However, many improvements must be made to draw meaningful conclusions. Future studies that implement a more consistent ECM mimic with a HUVEC endothelium may provide more reliable and consistent data regarding this mechanism.

This work revealed that albumin plays a role in reversing echinocytic shape transformation of stored RBCs through a mechanism that is not related to osmotic stress, reactive oxygen species, or phosphatidylserine (PS) externalization. As discussed previously, the most probable explanation for the rejuvenation ability of albumin is due to the disruption of the Donnan equilibrium through the significant negative charge of albumin (-17). Considering this hypothesis does not require a specific binding model of albumin to the RBC to illicit these shape changes, it is possible that other proteins of similar size and charge may also cause a similar shape recovery of stored RBCs. Reinhart et al. incubated echinocytic RBCs with other proteins of similar size to albumin, including dextran 70, protein C, and heat shock protein, but found no change in the morphological index of the RBCs, indicating that this phenomenon was specific to albumin. This may have been due to differences in the charge of these proteins relative to albumin. Rejuvenating stored RBCs with a protein of similar size as well as charge may result in a similar shape recovery benefit. One such protein may be vitamin D binding protein (DBP). Both albumin and DPB belong to the albuminoid

family of proteins.[65] DBP shares many similarities with albumin, being a globular protein, exhibiting a similar size (58 kDa), and has a similar charge (-14).[66] Washing stored RBCs with DBP may illicit a similar shape recovery that is seen when rejuvenating stored RBCs with albumin.

In addition to albumin containing buffers, C-peptide has shown promising potential as a novel rejuvenating solution for stored RBCs. C-peptide is a 31-amino acid polypeptide that is co-secreted from the pancreatic β cells with insulin.[67] Long considered bioinert, C-peptide has recently been shown to illicit functional benefits for RBCs *in vitro*, including increasing ATP release and deformability.[68–70] Considering these properties are known to decline during storage, rejuvenating stored RBCs with C-peptide may recover ATP levels and deformability to that of fresh RBCs, potentially resulting in improved *in vivo* behavior. Interestingly, Liu et al. discovered that C-peptide delivery to RBCs requires the presence of albumin, while Castiaux et al. showed that C-peptide specifically binds to albumin.[69,71] Additionally, Geiger et al. provided evidence that C-peptide binds to RBCs via a complex of albumin, C-peptide, and Zn^{2+}, increasing measurable GLUT1 levels in the membrane, the main glucose transporter for RBCs.[72] This may allow for more glucose uptake, more efficient glucose utilization, and potentially more ATP production, a known problem of the RBC storage lesion.[7] These data taken together indicate that a rejuvenating solution containing C-peptide, albumin, and Zn^{2+} may be able to recover key characteristics of stored RBCs. Indeed, Liu et al. observed C-peptide binding to stored RBCs that resulted in greater ATP release and improved deformability, which was exaggerated when RBCs were stored under normoglycemic conditions.[3] Further investigation into other functional benefits of C-peptide rejuvenation of stored RBCs is necessary to determine its potential as a novel rejuvenation solution.

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