TRANSLATIONAL IMPROVEMENTS IN BLOOD BANKING AND DEVELOPMENT OF AN AUXILIARY THERAPEUTIC FOR TYPE 1 DIABETES

By

Stephen A. Branch

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ABSTRACT

With over 10.5 million units of red blood cells (RBCs) transfused in 2021 in the United States alone, blood transfusions are one of the most common hospital procedures. These life-saving interventions are necessary to treat a variety of conditions that result in decreased hemoglobin levels. Common causes include anemia, hemoglobinopathy, cancer, chemotherapy, radiotherapy, and blood loss from trauma or major surgeries.

Despite centuries of research into the storage of RBCs for transfusion, current methods cannot prevent degradation of these cells. Detrimental biochemical and physical changes occur after even short periods of storage. This collection of harmful storage-induced changes is known as the *storage lesion*. The storage lesion can be broadly categorized into oxidative damages and metabolic impairments. Oxidative damages include generation of reactive oxygen species, lipid and protein oxidation, and degradation of cellular structure leading to severe morphological changes. Metabolic impairments lead to accumulation of lactate, acidifying the cellular milieu, and decreases in adenosine triphosphate (ATP) and 2,3-diphosphoglycerate levels.

Transfusion of RBCs significantly impacted by the storage lesion raises questions of patient safety. Though contemporary transfusion medicine has mitigated clinical complications from these procedures, they are not without risk. Complications range from transfusion-transmitted infections to fatal acute reactions, such as transfusion-associated circulatory overload. Minimizing the number of transfusions required is a key objective in blood banking research.

This may be achieved by improving the efficacy of transfusions through reduction of the storage lesion. Here, this is addressed through the use of modified additive solutions, which are used to prolong viability of RBCs in storage, and investigation of post-storage cellular rejuvenation. The additive solutions used today contain extreme amounts of glucose, ranging from $45\,\mathrm{mmol\,L^{-1}}$ to $111\,\mathrm{mmol\,L^{-1}}$. Such hyperglycemic conditions have been implicated in the development of various aspects of the storage lesion.

Previous reports have demonstrated that a normoglycemic additive solution containing just $5.5\,\mathrm{mmol\,L^{-1}}$ glucose is effective in reducing oxidative stress and osmotic fragility in stored RBCs as well as increasing ATP release and cellular deformability. As glucose is metabolized throughout storage, an RBC feeding system was previously developed to automate maintenance of normoglycemic conditions. However, aspects of the design of this system limited experimental control and regulatory

compliance, and therefore translational potential.

Here, a second-generation RBC feeding system is developed and employed in additional normoglycemic RBC storage studies. Beyond validating the performance of this system, benefits of normoglycemic storage such as reduced cellular glycation and hemolysis are confirmed. Expanding on previous reports, rejuvenation of RBCs stored under these conditions via post-storage washing is investigated. This rejuvenation results in significant improvements to the health of stored RBCs. Both cellular deformability and morphology were consistently restored to near-normal.

Next, the rejuvenation potential of C-peptide, a pancreatic hormone, is reviewed. There is a significant overlap between certain aspects of the storage lesion and the dysfunction of RBCs from people with type 1 diabetes (T1D). This includes reduced cellular deformability and ATP release, increased glycation and oxidative stress, and morphological changes. Consistent exposure to hyperglycemic environments may be responsible for these mutual impairments.

Extensive study has shown this proinsulin-derived peptide to interact with RBCs, eliciting beneficial effects. Reports include improvements to cellular deformability, glucose metabolism, and ATP release, including enhanced RBC function *in vivo*. Thus, C-peptide may be an excellent candidate not just as an auxiliary therapeutic for T1D, but as a rejuvenating agent for RBC storage as well.

A comprehensive review of the applications of a functional C-peptide formulation is provided. Preclinical development of this product is initiated, including studies into the efficacy of the proposed formulation. Inconsistencies in C-peptide function then lead to a thorough investigation to identify key developmental roadblocks. A resolution is offered, and a path toward successful *in vivo* clinical studies is laid out.

This dissertation is dedicated to my wife, Emily, and my parents, Brent and Leslie; without their unrelenting support, it surely would not exist.

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LIST OF ABBREVIATIONS

2,3-DPG 2,3-diphosphoglycerate
A450 ..... Absorbance at 450 nm
ACD ..... Acid citrate dextrose solution
ADA ..... American Diabetes Association
ADP ..... Adenosine diphosphate
AGE ..... Advanced glycation end-product
AS-1 ..... Additive Solution 1
AS-1N ..... Additive Solution 1 (normoglycemic)
AS-3 ..... Additive Solution 3
AS-5 ..... Additive Solution 5
AS-7 ..... Additive Solution 7
ATP ..... Adenosine triphosphate
BJT ..... Bipolar junction transistor
CAD ..... Computer-aided design
CI ..... Confidence interval
CO_2 ..... Carbon dioxide
CP2D ..... Citrate phosphate double dextrose solution
CPD ..... Citrate phosphate dextrose solution
CPDA-1 ..... Citrate phosphate dextrose adenine solution
CPD-N ..... Citrate phosphate dextrose (normoglycemic) solution
DC ..... Direct current
DCCT ..... Diabetes Control and Complications Trial
DEHP ..... Diethylhexyl phthalate
DHCP ..... Dynamic Host Configuration Protocol
DKA ..... Diabetic ketoacidosis
DLS ..... Dynamic light scattering
DNS ..... Domain Name System
EDIC ..... Epidemiology of Diabetes Interventions and Complications study
ELISA ..... Enzyme-linked immunosorbent assay
ELS ..... Electrophoretic light scattering
eNOS ..... Endothelial nitric oxide synthase
EV ..... Extracellular vesicle

FDA ..... Food and Drug Administration

g ..... Gravitational force equivalent

GPIO ..... General-purpose input/output

GSH ..... Glutathione

GSSG ..... Glutathione disulfide

H_2CO_3 ..... Carbonic acid

H_2O ..... Dihydrogen monoxide

H_2O_2 ..... Hydrogen peroxide

HbA1c ..... Glycated hemoglobin

HCO_3^- ..... Bicarbonate

HCT ..... Hematocrit

HHS ..... Hyperosmolor hyperglycemic state

HPLC ..... High-performance liquid chromatography

ICH ..... International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

IL-6 ..... Interleukin 6

INOBA ..... Insufficient nitric oxide bioavailability

IP ..... Internet Protocol

KDE ..... Kernel density estimate

LLOQ ..... Lower limit of quantification

MI ..... Morphological index

NAD^+ ..... Oxidized nicotinamide adenine dinucleotide

NADH ..... Reduced nicotinamide adenine dinucleotide

$NADP^+$ ..... Oxidized nicotinamide adenine dinucleotide phosphate

NADPH ..... Reduced nicotinamide adenine dinucleotide phosphate

NO ..... Nitric oxide

NTBI ..... Non-transferrin bound iron

O_2 ..... Dioxygen

PBS ..... Phosphate buffered saline

PBS-A ..... Phosphate buffered saline with albumin

PCB ..... Printed circuit board

PDI ..... Polydispersity index

PSS ..... Physiological salt solution

PVC ..... Polyvinyl chloride

PWM ..... Pulse-width modulation
RBC ..... Red blood cell
RMSE ..... Root mean squared error
ROS ..... Reactive oxygen species
SAG ..... Saline adenine glucose solution
SAGM ..... Saline adenine glucose mannitol solution
SBC ..... Single-board computer
SBM ..... Single-board microcontroller
SD ..... Standard deviation
SEM ..... Scanning electron microscopy
SPR ..... Surface plasmon resonance
T1D ..... Type 1 diabetes
TACO ..... Transfusion-associated circulatory overload
TNF ..... Tumor necrosis factor
TRALI ..... Transfusion-related acute lung injury
TRIM ..... Transfusion-related immunomodulation
USB ..... Universal Serial Bus
V_{in} ..... Input voltage

CHAPTER 1

INTRODUCTION TO BLOOD BANKING AND TRANSFUSION MEDICINE

1.1 Background

The first successful human-to-human blood transfusion was administered by James Blundell in 1825, marking an important milestone in the beginning of a critical field of modern medicine.[1] In 2021, the National Blood Collection and Utilization Survey estimated that over 10.5 million units of red blood cells (RBCs) are transfused every year in the United States alone.[2] From the management of anemias to trauma stabilization, the availability of high quality blood products is key to proper patient care. In order to have these products readily available, effective protocols for the storage of RBCs are of incredible importance.

Notably, virtually all contemporary RBC transfusions continue to use donor-derived cells.[3] Donations of these products are necessary due to the complexities of RBC function *in vivo*; all synthetic attempts have either had inferior oxygen transport and delivery capabilities or resulted in too many detrimental side effects.[3–5] While there continues to be ongoing development of RBC replacements to negate the need for donation, no approved commercially available solutions exist.[3–5]

Additionally, RBCs cannot simply be cultured or grown *in vitro*. They follow a complex differentiation from hematopoetic stem cells, going through several steps before maturing into a full erythrocyte, or RBC.[6] Thus, donation, and the subsequent processing and storage, of RBCs remains the only viable option to maintain the supply of these life-saving products.[3–5]

While Blundell's 1825 transfusion utilized whole blood taken directly from the donor onsite, modern practices have undergone significant evolution.[1,7] In the United States, the Food and Drug Administration (FDA) now regulates every step of contemporary blood drawing, processing, storage, and transport.[7–9] A diagram showing a standard blood collection process is shown in Figure 1.1. Note that collection and processing procedures may vary depending on donation purpose, method, and regulatory requirements.

Briefly, venipuncture is used to extract approximately 450 mL to 500 mL of whole blood from a donor.[10,11] This whole blood is drawn into a bag prefilled with an anticoagulant to prevent clotting and subsequent spoilage.[10,11] The entire bag is centrifuged to separate the blood components by density; RBCs settle to bottom, with a thin buffy coat separating them from the relatively less dense

plasma. The plasma and buffy coat are removed, typically by transferring them into a separate storage bag for use in other procedures.[10,11] The packed RBCs are then also transferred into a storage bag, passing through a leukofiltration apparatus on the way.[10] This leukofiltration helps reduce the number of unwanted white blood cells in the RBC product. Finally, the RBCs are mixed into the prefilled additive solution that helps keep them healthy throughout the storage period.[10,11] Each unit of RBCs has a final volume around 330 mL to 350 mL at a hematocrit (HCT) of 60 % to 80 %, and can be kept in cold storage at 1 °C to 6 °C for up to 42 days.[10]

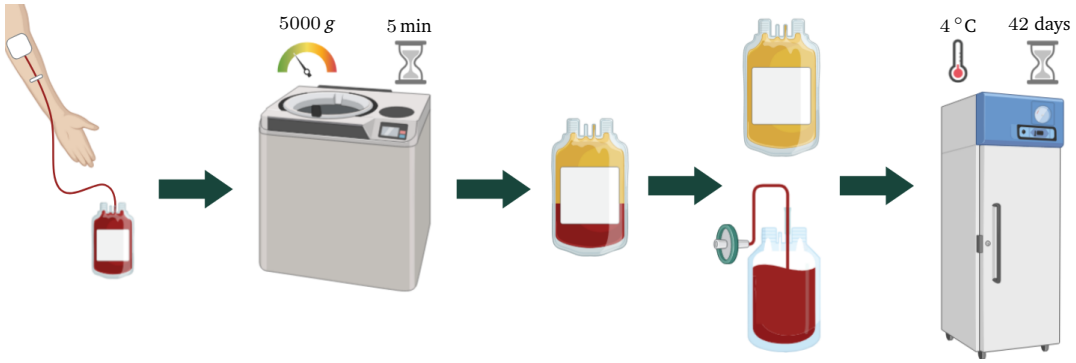

Figure 1.1: Diagram of a standard blood draw for donation.[10,12,13] Whole blood is drawn into an anticoagulant solution before being separated into its components via centrifugation. The plasma is then removed, followed by leukofiltration to purify the packed RBCs. These packed RBCs are then transferred into an additive solution before entering cold storage.

Of key importance are the anticoagulant and additive solutions used for RBC storage. Prior to the development of these solutions, first started in 1915 by Francis Rous and J.R. Turner, blood could not be stored or transported before transfusion; rather, donated blood had to be used immediately.[11] In addition to enabling blood banking, these solutions contribute to the mitigation of storage-induced degradations in RBC health that impair transfusion efficacy.[11] These degradations, collectively referred to as the storage lesion, are discussed in further detail in Section 1.2.3.

Anticoagulation serves to prevent the formation of clots in whole blood and is often achieved through the use of citrate.[10,11,14] Citrate acts as a calcium chelator, preventing calcium-dependent steps of the coagulation cascade from proceeding, thus inhibiting clot formation.[10,14] Anticoagulant solutions generally also contain dextrose to support RBC metabolism.[10,11]

Indeed, the original Rous-Turner solution consisted solely of citrate and dextrose.[11] Unfortunately, heat sterilization via autoclave destroys Rous-Turner solution by caramelizing the dextrose, rendering it of little use in storage due to concerns of bacterial contamination.[11] Luckily, this

caramelization can be inhibited by lowering the pH of the solution below 5.8 via the addition of citric acid.[11] This acid citrate dextrose solution (ACD) was developed and used to great effect during World War II by the British and American militaries.[11]

Contemporary anticoagulants include the addition of phosphate to support adenosine triphosphate (ATP) synthesis.[11] The addition of monobasic sodium phosphate to ACD led to the development of citrate phosphate dextrose solution (CPD).[11] In some cases, adenine is also used to slow the loss of ATP during storage.[11] This is referred to as citrate phosphate dextrose adenine solution (CPDA-1).[11]

Iterations to improve RBC longevity in storage has led to the development of additional anticoagulant solutions. Besides CPD and CPDA-1, citrate phosphate double dextrose solution (CP2D) has also been approved for whole blood storage by the FDA.[15] This solution is nearly identical to CPD, but it contains twice as much dextrose to provide additional fuel for cellular metabolism.[10] Additional experimental anticoagulants have been developed for storage and are undergoing investigation, but have not yet received regulatory approval for use. Table 1.1 provides a summary of the anticoagulants described thus far.

Table 1.1: Concentrations of components and descriptive information of various anticoagulant-preservation solutions.[10,11,16–19] Currently, only CPD, CP2D, and CPDA-1 are approved for storage use by the FDA.[10] Values are approximate and may vary slightly from manufactured pharmaceutical products.

	Component ($\mathrm{mmol\,L^{-1}}$)				
	Rous-Turner	ACD	CPD	CP2D	CPDA-1
Trisodium citrate	42	75	89	89	89
Dextrose	214	124	129	258	161
Citric acid	–	38	16	16	16
Monosodium phosphate	–	–	16	16	16
Adenine	–	–	–	–	2
Year developed	1915	1943	1957	1983	1968
pH	7.2–7.4	4.5–5.5	5.2–6.2	5.6	5.6
Osmolarity ($\mathrm{mOsm\,L^{-1}}$)	382	462	533	662	567
Approved storage days	–	–	21	21	35
Approved whole blood ratio	–	–	0.14	0.14	0.14

While anticoagulants themselves can be effective preservatives, additive solutions are required to attain a full 42-day storage period. The term *additive solution* in the context of blood banking was

purportedly coined by Gerald L. Moore in 1987, and was, in part, defined as “an isotonic solution containing some or all of the nutrients needed for extended red cell storage”.[18] The benefits of additive solutions are twofold: in addition to providing additional nutrients and preservatives, they reduce the HCT of packed RBCs, and therefore viscosity, for easier transfusion.[11]

The first additive solution, developed in 1978, was a saline adenine glucose solution (SAG).[20] As the name suggests, this solution contained sodium chloride for tonicity, along with adenine and glucose for cellular metabolism. RBCs stored in SAG were shown to stay healthy for up to 35 days of storage, with successful *in vivo* transfusions at higher flow rates.[20]

Shortly after the development of SAG, the addition of mannitol in 1981 led to the creation of a saline adenine glucose mannitol solution (SAGM).[21] Mannitol, a sugar alcohol and sorbitol isomer, acts to reduce hemolysis during storage.[22] The exact mechanism of this is unknown, but may be due to its effects on RBC osmotic pressure and ability to act as a free-radical scavenger.[23] SAGM resulted in an approximately $50\,\%$ reduction in hemolysis, and also enabled storage up to 42 days with acceptable post-transfusion RBC recovery rates.[21,24]

While SAGM is commonly used outside the United States, Additive Solution 1 (AS-1), Additive Solution 3 (AS-3), and Additive Solution 5 (AS-5) are the only solutions approved by the FDA and used today.[10,25] Additive Solution 7 (AS-7) was recently approved by the FDA in 2013, but has not yet been introduced to the market.[26,27] Notably, AS-7-stored RBCs have an approved storage span of 56 days in Europe, higher than any other additive solution to date.[27]

These solutions are based on SAGM and vary mainly in their concentrations of salts and sugars.[11,25] Other additive solutions do exist and are used in other countries, though they differ only slightly from SAGM and do not have any significant advantages.[11] Table 1.2 provides a summary of SAGM and the FDA-approved additive solutions discussed here. Note that due to its low sugar content, AS-3 can only be used with CP2D as an anticoagulant.[28]

The final component of RBC storage systems is the plastic storage bags themselves. Replacing glass storage bottles in the 1960s, they offer distinct advantages including ease of manufacturing, increased sterility during blood component separation, and increased durability.[11,29] With the transition to plastic-based storage containers came the unintentional benefits seen in reduced hemolysis during storage. Specifically, storage in polyvinyl chloride (PVC) bags plasticized with diethylhexyl phthalate (DEHP) results in an approximate fourfold decrease in hemolysis throughout storage compared to

glass or other plastics.[30] Morphological alterations and microvesiculation in RBCs is also reduced in the presence of DEHP.[30]

Table 1.2: Concentrations of components and descriptive information of various additive solutions.[10,11,18,19,25,27] Values are approximate and may vary slightly from manufactured pharmaceutical products.

	Component (mmol L^{-1})				
	SAGM	AS-1	AS-3	AS-5	AS-7
Sodium chloride	150	154	70	150	–
Adenine	1.25	2	2	2.2	2
Dextrose	45	111	55	45	80
Mannitol	30	41	–	45.5	55
Monosodium phosphate	–	–	23	–	–
Trisodium citrate	–	–	23	–	–
Citric acid	–	–	2	–	–
Sodium bicarbonate	–	–	–	–	26
Disodium phosphate	–	–	–	–	12
Year developed	1981	1983	1984	1988	2013
pH	5.7	5.8	5.8	5.5	8.5
Osmolarity	376	462	337	393	237
Approved storage days	42	42	42	42	42(56)
Approved whole blood ratio	0.22	0.22	0.22	0.22	0.22
Day 42 characteristics (%)					
Post-transfusion RBC recovery	77	76	84	80	88
ATP (of initial)	70	60	59	68.5	90
2,3-DPG (of initial)	<10	<5	<10	<5	–
Hemolysis	0.3	0.5	0.9	0.6	0.29

This plasticizer, required to give PVC bags their flexibility, makes up approximately 40 % of the weight of the bag material.[31] DEHP leaches out of the PVC throughout storage, resulting in concentrations up to 180 µmol L^{-1} in the stored product.[29,31,32] Although DEHP has been shown to be carcinogenic in murine models, a causal link between exposure to it and cancer development in humans has not been established.[33,34] One recently proposed mechanism of action for DEHP stabilizing RBC membranes suggests that it may be acting as a synthetic phospholipid scramblase.[35]

Modern PVC storage bags also provide enhanced gas exchange compared to glass.[29] This presents both benefits and detriments to RBC storage. RBCs, due to their large amount of carbonic anhydrase, possess an ability to buffer the pH of their cellular milieu via the bicarbonate buffering system, shown in Equation (1.1).[36] This produces carbon dioxide (CO_2), which is able to diffuse out of storage

bags to enable further buffering and reduce acidification of the stored RBCs.

$$HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \overset{\text{carbonic anhydrase}}{\rightleftharpoons} CO_2 + H_2O \tag{1.1}$$

The permeability of the storage bags poses a problem in the form of oxygen exchange, however. As oxygen is allowed to diffuse into the bags, it may react with hemoglobin, the protein that facilitates for oxygen transport, to produce methemoglobin.[37] A byproduct of this reaction is hydrogen peroxide (H_2O_2), a potent reactive oxygen species (ROS) and generator of hydroxyl radicals, another potent ROS.[37] These contribute to oxidation reactions that are known to cause a great deal of damage in stored RBCs.[37] This will be further addressed in Section 1.2.3.

1.2 Red Blood Cell Cold Storage

1.2.1 Glucose Metabolism

RBCs, also known as erythrocytes, lack any organelles and are therefore only able to metabolize glucose via anaerobic glycolysis.[38] A graphical overview of glycolysis is shown in Figure 1.2.

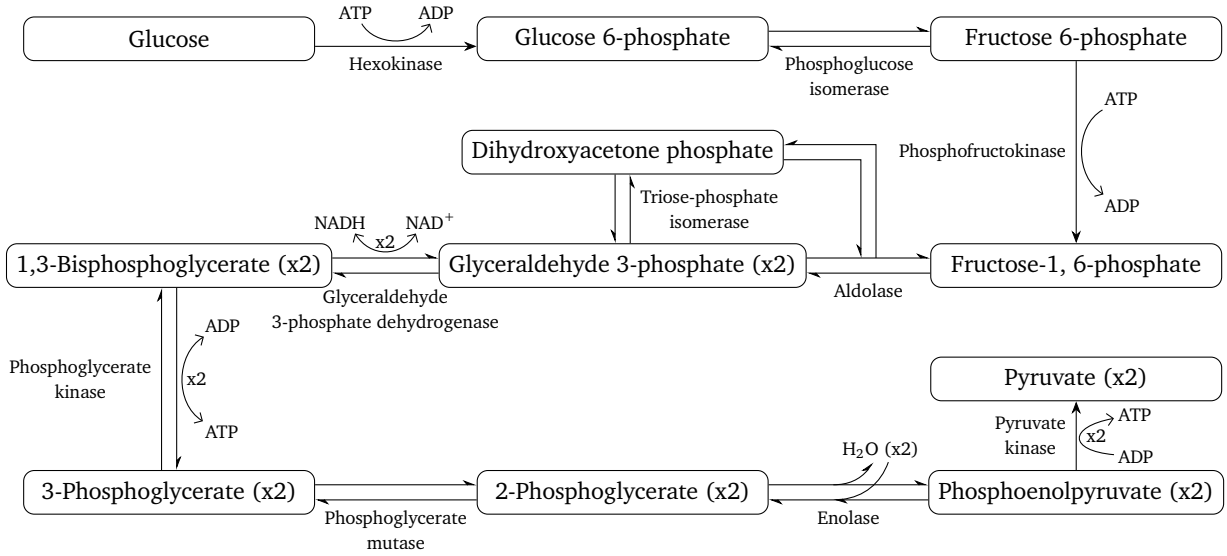

Figure 1.2: Embden-Meyerhof-Parnas pathway of anaerobic glycolysis.[39] The reaction consumes one molecule of glucose to produce two molecules of ATP. It also produces two molecules of NADH, a reducing agent.

Following this, the resulting pyruvate is converted into lactate as shown in Figure 1.3. While glucose is always required to fuel RBCs, the rate at which they metabolize this glucose is not consistent. At physiological temperatures, RBC metabolism is approximately an order of magnitude faster than

that of RBCs stored at $4\,^{\circ}$C.[40] Even throughout storage, RBC metabolism slows; glucose utilization rates decay to less than $20\,\%$ of the starting rate by day 42.[41]

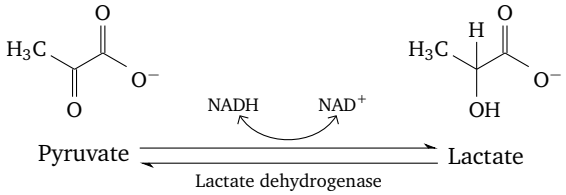

Figure 1.3: Reduction of pyruvate to lactate.[39] This reaction helps to recycle NADH back into NAD^+ for use in further glycolysis.

It is this reduction in glucose metabolism that leads RBCs to consume only $3\,$mmol to $4\,$mmol of glucose over the course of a 42-day storage period.[42] An empirically-derived model of RBC glucose utilization has been developed by the Spence laboratory and is shown in Equation (1.2).[41] This model can be integrated with respect to time, resulting in a model for total glucose consumption as shown in Equation (1.3). Note that $\lim_{day\to 0} consumption(day) = 0\,\text{mmol}\,\text{L}^{-1}$.

$$\text{Glucose utilization}\left[\text{mmol}\,\text{L}^{-1}\,\text{day}^{-1}\right] = -0.2228\ln(day) + 0.9355 \qquad \forall\ day > 0 \tag{1.2}$$

$$\text{Glucose consumption}\left[\text{mmol}\,\text{L}^{-1}\right] = day(1.1583 - 0.2228\ln(day)) \quad \forall\ day > 0 \tag{1.3}$$

These models are plotted for $day \in (0, 42]$ in Figure 1.4, though glucose utilization is limited to $day \geq 1$. This is due to the asymptotic nature of the logarithmic model, which does not correlate well with empirical data. Fortunately, this has minimal impact on the model for total glucose consumption, which will be relevant in Chapter 2.

After 42 days of storage, Equation (1.3) suggests that there will be glucose drop of approximately $13.67\,\text{mmol}\,\text{L}^{-1}$. This is certainly significant, however, it does not compensate for the excessively hyperglycemic environments in RBC storage. Table 1.3 shows estimated glucose concentrations for a unit of RBCs using common anticoagulants and additive solutions. These calculations are based on a donor blood glucose of $5\,\text{mmol}\,\text{L}^{-1}$ and packed RBC volume of $230\,$mL (not including the additive solution). All additive solutions in this example are paired with CPD, with the exception of AS-3, which is paired with CP2D as previously discussed.

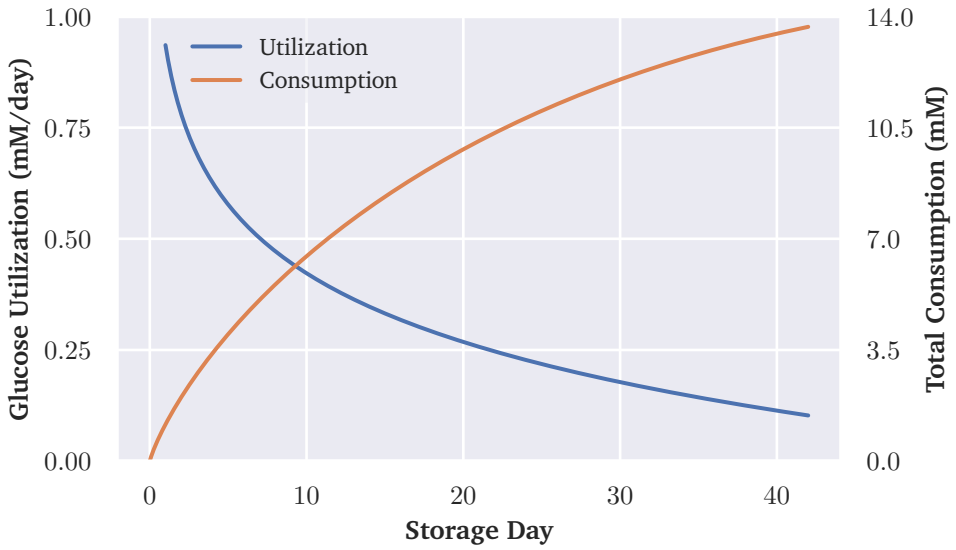

Figure 1.4: Average glucose utilization and consumption of a unit of RBCs throughout 42 days of cold storage.

Table 1.3: Modeled glucose concentrations in a unit of stored RBCs for various additive solutions. Extreme hyperglycemia is present throughout storage in all cases.

Day	Glucose ($\mathrm{mmol\,L^{-1}}$)			
	AS-1	AS-3	AS-5/SAGM	AS-7
0	49.6	42.2	28.2	39.6
7	44.5	37.1	23.1	34.5
14	41.6	34.2	20.2	31.6
21	39.5	32.1	18.1	29.5
28	38.0	30.6	16.6	28.0
35	36.8	29.4	15.4	26.8
42	35.9	28.5	14.5	25.9

Even AS-5 and SAGM, the least hyperglycemic additive solutions, result in a final glucose concentration in excess of $14\,\mathrm{mmol\,L^{-1}}$. This is approximately threefold greater than physiological glucose levels and has been referred to in the literature as a "quasi-diabetic" storage condition.[43,44] Unsurprisingly, there is considerable overlap between aspects of the RBC storage lesion and damages found in RBCs of type 1 diabetes (T1D) patients (e.g., reduced cellular deformability, reduced ATP release, increased oxidative stress, increased endothelial adhesion, increased cellular glycation, and morphological changes).[37,45–50]

In addition to the Embden-Meyerhof-Parnas glycolytic pathway, glucose 6-phosphate may also go through the pentose phosphate pathway.[51] This pathway, shown in Figure 1.5, is critical in

maintaining cellular antioxidant mechanisms.[51] While it generally only contributes to 8 % of glucose metabolism under normal conditions, it is the only source of reduced nicotinamide adenine dinucleotide phosphate (NADPH) for RBCs.[52–56]

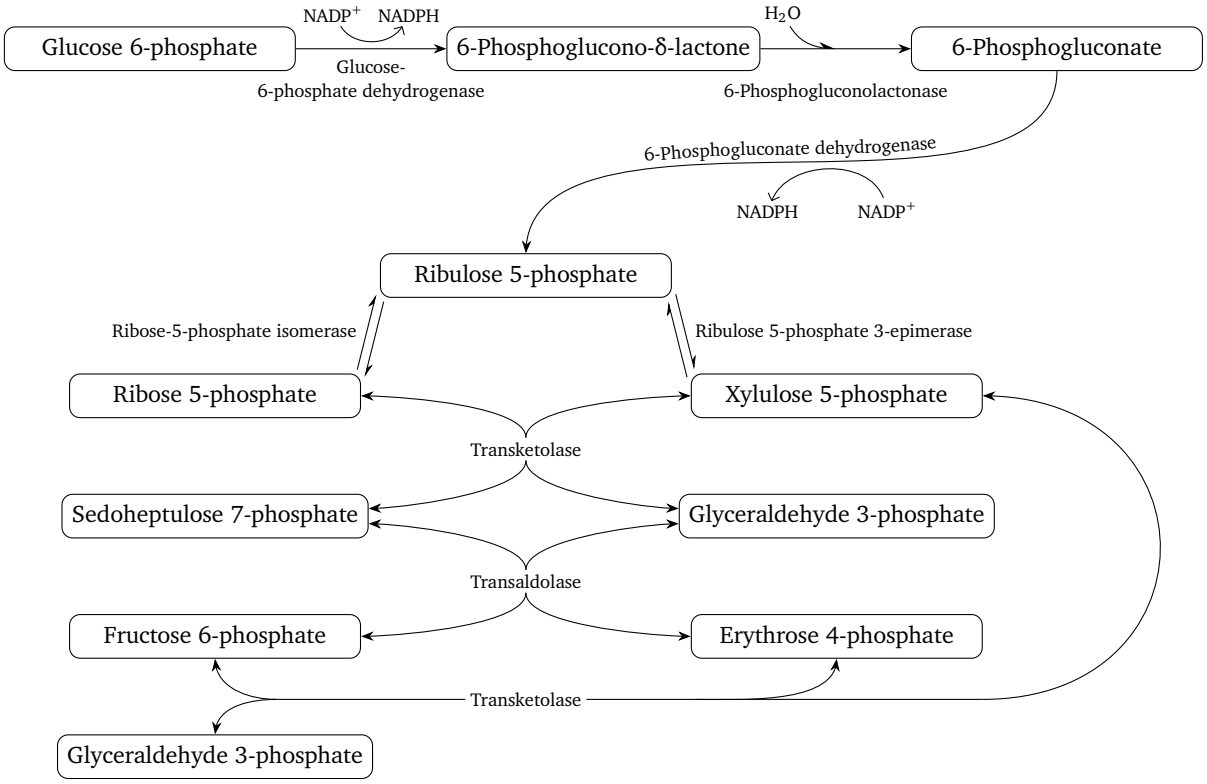

Figure 1.5: Pentose phosphate pathway.[57] This pathway runs in parallel to glycolysis and generates NADPH, a reducing agent necessary for the regeneration of the antioxidant glutathione.

The NADPH generated in the pentose phosphate pathway is required to maintain antioxidant mechanisms in RBCs.[58] During times of increased oxidative stress, glucose flux through the pentose phosphate pathway increases dramatically, accounting for up to 90 % of glucose metabolism in RBCs.[59,60] This may be explained, in part, by inhibition of glycolytic enzymes by oxygen-dependent conformational changes in hemoglobin.[61] Glucose metabolism has been shown to shift toward the pentose phosphate pathway throughout RBC storage, although overall glucose flux decreases through both pathways.[62–64]

1.2.2 Leukoreduction

As previously discussed, packed RBCs are often forced through a leukofiltration device before being transferred to the additive solution.[10] This generally removes over 99.9 % of white blood cells from the final product, resulting in fewer than 5 million leukocytes per unit of RBCs.[10,65] Leukocytes

have a tendency to lyse during hypothermic storage, resulting in RBC damage through the released proteases and lipases.[11,66] This damage manifests as increased hemolysis, ATP depletion, osmotic fragility, and decreased post-transfusion RBC recovery.[67,68]

Clinically, transfusions of leukoreduced blood have been found to decrease the odds of post-transfusion infection by approximately $50\,\%$ relative to non-leukoreduced RBCs.[69] Transfusions using leukoreduced RBCs have also been shown to result in less immunomodulation compared to non-leukoreduced RBCs.[70] Despite this, other reviews and meta-analyses have found little improvement in patient outcomes using leukoreduced blood.[46,71,72] Due to the increased costs associated with leukoreduction, approaching an extra $\$30$ per unit, there is some debate as to whether it is warranted.[72]

Despite this, Canada, France, Germany, Italy, and the UK have now implemented requirements that stored RBCs be universally leukoreduced.[66] It is also widely practiced in the United States, despite not being required by the FDA.[73] These universal leukoreduction requirements may be justified, as they have been demonstrated to reduce the incidence of alloimmunization by $50\,\%$.[74]

1.2.3 The Storage Lesion

The *storage lesion* is a collection of detrimental biochemical and physical changes that occur to RBCs throughout storage.[38] These changes can be broadly categorized as oxidative damages or metabolic impairments.[37] The storage lesion contributes to both the degradation of RBCs themselves and clinical complications that stem from transfusions.[37]

As storage time increases, accumulation of metabolic waste products such as lactate reduce the pH of the RBCs.[40] This, in turn, decreases the rate of glucose flux through both glycolysis and the pentose phosphate pathway by inhibiting the enzymes phosphofructokinase and glucose-6-phosphate dehydrogenase, respectively.[37] The result of this is a depletion in both ATP and NADPH, two molecules that are key in maintaining RBC health.[64,75–77] It is this reduction in NADPH levels that precipitates much of the oxidative damage that occurs during storage.[78]

RBCs have robust antioxidant mechanisms to protect against oxidative damages *in vivo*.[55] One of the most important and abundant reducing agents in RBCs is glutathione (GSH).[55,58] GSH is responsible for detoxifying nearly all H_2O_2 generated by the autoxidation of hemoglobin to methemoglobin.[58,79] Through this mechanism, GSH is converted to glutathione disulfide (GSSG)

through glutathione peroxidase.[58] GSSG is then used to regenerate GSH through glutathione reductase and the NADPH generated in the pentose phosphate pathway.[57,58] This regeneration cycle is shown in Figure 1.6.

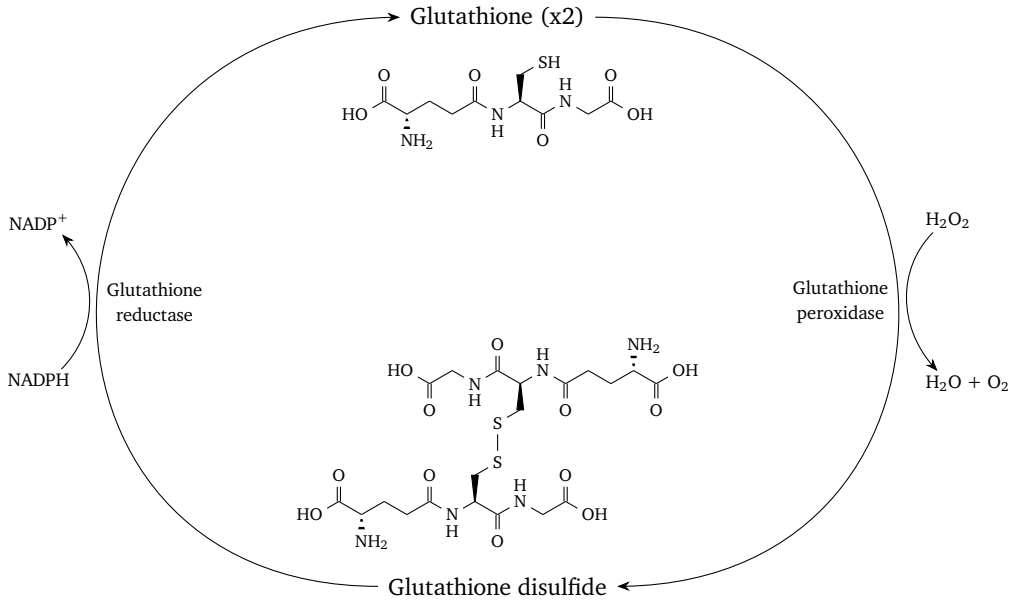

Figure 1.6: Glutathione reducing H_2O_2 and subsequently being regenerated.[58] This potent antioxidant is critical to RBC survival. This cycle consumes one molecule of NADPH.

Furthermore, NADPH is also consumed by the polyol pathway, shown in Figure 1.7.[80] The extreme amounts of glucose in additive solutions saturate typical glycolytic pathways, resulting in conversion to sorbitol instead.[81] This depletion of NADPH and concomitant decrease in the ratio of GSH to GSSG results in the buildup of destructive oxidizing agents.[82]

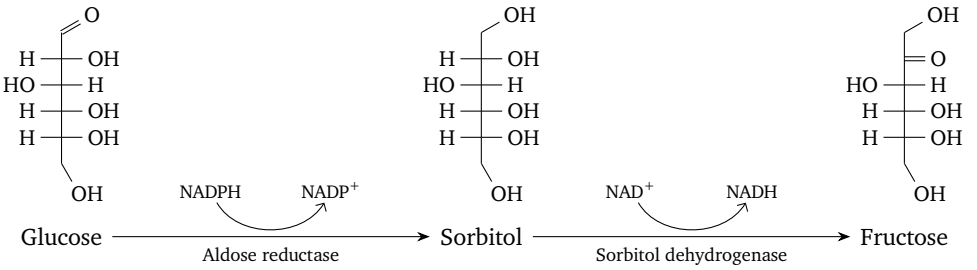

Figure 1.7: Polyol pathway.[80] Glucose flux through this pathway is significantly higher in the presence of hyperglycemia.[81] This pathway consumes one molecule of NADPH.

An increase in ROS leads to the oxidation of proteins and lipids in RBCs.[83] Oxidized proteins have been shown to bind to the RBC cytoskeleton, resulting in reduced deformability and morphological changes.[84,85] Lipid peroxidation contributes to increased osmotic fragility, hemolysis, and

phosphatidylserine exposure (a marker for apoptosis).[86,87] The resulting morphological alterations and increased phosphatidylserine exposure have been implicated in the microvesiculation of RBC membranes, contributing to transfusion-related clinical sequelae.[87]

Morphological alterations to RBCs in storage can be extreme. In 1973, scanning electron microscopy allowed for more detailed evaluation and classification of pathological RBC morphologies.[88] This classification can be used to calculate a morphological index to semi-quantitatively evaluate the degree of morphological change.[89,90] An example of various RBC morphologies and their associated morphological indices is shown in Figure 1.8.

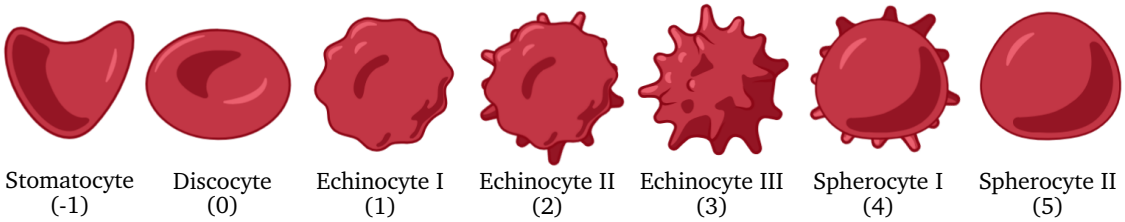

Figure 1.8: RBC morphologies and morphological indices.[13,88,89] Bulk classification of stored RBCs and averaging of morphological indices can be used to compare morphological states and as an indicator for progression of the storage lesion.[90]

The depletion of ATP also contributes to increased cellular oxidation as it further impairs glucose flux through the pentose phosphate pathway. Additionally, ATP-dependent enzymes such as sodium-potassium ATPase and calcium ATPase are impaired by both the decrease of available ATP and temperature in cold storage.[91,92] This results in cation leakage across RBC membranes and subsequent increases in both intracellular sodium and calcium and decreased intracellular potassium.[62,68,86,87,91–96] Potassium leakage inhibits flippase activity, eventually resulting in increased phosphatidylserine exposure.[97] Calcium influx is similarly known to increase phosphatidylserine exposure, as well as promote morphological changes and microvesiculation of RBCs.[68,96]

Increased phosphatidylserine exposure increases RBC adhesion to the endothelium and promotes platelet activation post-transfusion.[96,98,99] This can lead to impaired blood flow and thrombosis, potentially even occluding perfusion through the microvasculature.[96,98,99] In addition to increased adhesion, stored RBCs also exhibit increased aggregation to themselves, often forming stacked discs known as rouleaux.[99–101] Mechanistically, this increase in aggregation can be explained by the reduction in cell surface charges, and therefore repulsive electrostatic forces, due to the loss of sialic acid during RBC storage.[99,100,102]

RBCs are capable of producing nitric oxide (NO) via an isoform of endothelial NO synthase.[103–105] The activity of this enzyme, and therefore synthesis of NO, decreases significantly throughout storage.[105] Furthermore, scavenging of NO by RBCs, microvesicles, and free hemoglobin increases during storage, leading to an overall diminished post-transfusion bioavailability of NO.[106] This is implicated in the reduced deformability of stored RBCs and potentially contributes to post-transfusion inflammation and circulatory dysfunction.[105,106]

Beyond lactate accumulation and ATP depletion, further metabolic impairments involve the depletion of 2,3-diphosphoglycerate (2,3-DPG).[94,107–110] This molecule is produced by the Luebering-Rapoport shunt, a branch of glycolysis, as shown in Figure 1.9.[111] It binds to hemoglobin in RBCs, competing with oxygen and thus shifting the oxyhemoglobin dissociation curve to the right.[108,112] This results in an enhanced oxygen offloading and delivery, allowing RBCs to better oxygenate tissues.

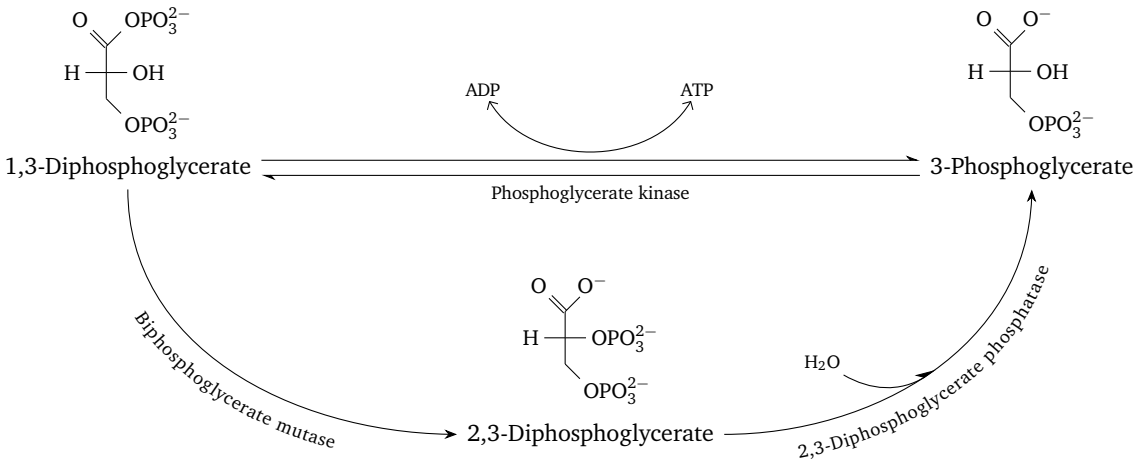

Figure 1.9: Luebering-Rapoport shunt.[111] Typically, 1,3-diphosphoglycerate generated during glycolysis is typically converted to 3-phosphoglycerate. In this pathway, it alternatively can be converted to 2,3-DPG.

As storage progresses, the acidification of blood products results in a significant decline in the activity of biphosphoglycerate mutase.[37] Near complete depletion of 2,3-DPG within the first few weeks of storage follows.[110] Ultimately, this shifts the oxyhemoglobin dissociation curve far to the left, resulting in deficient oxygen delivery from the stored RBCs.[94] Post-transfusion, 2,3-DPG levels start to recover, reaching physiologically normal concentrations within 72 hours.[113] Clinically, there is evidence to suggest that 2,3-DPG regeneration acts quickly enough that stored RBCs are just as effective in reversing oxygenation deficits as fresh blood.[114]

As previously discussed, glucose concentrations in stored RBCs remain extremely high even after 42 days of storage. In such hyperglycemic environments, glycation, the deleterious and irreversible non-enzymatic covalent attachment of sugars to various proteins and lipids, occurs more readily.[115] In T1D, medium-term glycemic control is measured by the ratio of glycated hemoglobin (HbA1c).[43] This strategy can also be applied to stored RBCs as a proxy measure of overall damaging cellular glycation. Indeed, increases in HbA1c of stored blood have been well-documented, although the extent of increase is quite variable.[45,116–118] Some investigators argue that these increases in HbA1c are clinically insignificant, although this may neglect to consider both glycation of other cellular components and baseline HbA1c levels of the transfusion recipient.[118,119] A potential explanation for the relatively moderate increases in HbA1c during storage involves the slowing of glycation reaction kinetics due to decreases in temperature and pH, although more investigation is warranted.

Beyond increases to HbA1c, advanced glycation end-products (AGEs) develop throughout RBC storage.[82,116,120,121] These AGEs are implicated in impaired RBC deformability and ATP release.[82] Surface AGEs have also been shown to interact with AGE receptors on endothelial cells, increasing ROS generation.[82,121] This is one proposed mechanism of post-transfusion damage to the endothelium, inflammation, and lung injury.[121]

1.3 Clinical Applications

1.3.1 Indications for Transfusion

The objective of an RBC transfusion is to increase *in vivo* oxygen delivery in patients experiencing (or at high risk of experiencing) symptomatic anemia. Transfusions of packed RBCs are generally indicated when blood hemoglobin levels fall below $7\,\mathrm{g\,dL^{-1}}$.[122,123] The normal range for males is $14\,\mathrm{g\,dL^{-1}}$ to $18\,\mathrm{g\,dL^{-1}}$, while that of females is $12\,\mathrm{g\,dL^{-1}}$ to $16\,\mathrm{g\,dL^{-1}}$.[124] Each transfused unit should lead to an increase in hemoglobin of $1\,\mathrm{g\,dL^{-1}}$ and an increase in HCT of $3\,\%$.[122,123]

There are a number of causes of anemia.[125–127] Broadly, these include hemolytic anemias, blood loss anemias, hypoproliferative anemias, and hemoglobinopathies.[127] While many anemias can be managed pharmacologically, certain conditions inevitably require transfusion.[125–127] Conditions such as glucose-6-phosphate dehydrogenase deficiency, sickle cell anemia, and aplastic anemia may require occasional transfusions.[126,127] Meanwhile, people with β-thalassemia major, a form of hemoglobinopathy, are dependent on frequent transfusions for survival.[126,127] Other causes of

transfusion include cancer and cancer treatments, trauma, and surgical procedures that result in extreme blood loss.[128,129]

Approximately $40\,\%$ of critically ill patients will receive a transfusion.[129] On average, two to five units of RBCs will be transfused to raise hemoglobin levels to post-transfusion targets.[129] This is approximately $20\,\%$ to $25\,\%$ of the average blood volume of an adult.

1.3.2 Blood Typing

Correctly pairing donor and recipient blood types is a critical aspect of transfusion medicine. While there are now 45 recognized human blood group systems, the ABO and Rh groups are of primary relevance for transfusion.[130,131] Transfusion of incompatible blood types leads to severe hemolytic reactions with fatality rates around $10\,\%$.[131] All donated blood is therefore routinely tested for ABO and Rh antigens.[132] Table 1.4 shown the compatibilities of different blood types in the ABO and Rh systems. Note that type O− is considered the "universal donor" and type AB+ is considered the "universal recipient".

Table 1.4: ABO and Rh blood group compatibility table.

		Donor							
		O+	A+	B+	AB+	O−	A−	B−	AB−
Recipient	O+	✓				✓			
	A+	✓	✓			✓	✓		
	B+	✓		✓		✓		✓	
	AB+	✓	✓	✓	✓	✓	✓	✓	✓
	O−					✓			
	A−					✓	✓		
	B−					✓		✓	
	AB−					✓	✓	✓	✓

The distribution of blood types varies widely around the world, with significant variation among different ethnicities.[133] It can therefore be difficult for blood banks to predict both the supply and demand distributions of blood types. Table 1.5 shows the blood type distribution of over three million blood donors in the United States.[133] Effective donor recruitment is key in maintaining appropriate supplies of each blood type. In emergency situations where the blood type of the recipient may not be known, O− blood can be used. Because of this and its relative scarcity, O− donors are in high demand.

Table 1.5: Donor distribution of ABO and Rh blood groups in the United States.[133]

Rh	Phenotype			
	O	A	B	AB
+	39.8	31.5	10.6	3.5
−	6.9	5.6	1.5	0.6

Even in cases where ABO and Rh blood types are matched, transfusion-related immune reactions can occur.[131,134] This may be due to alloimmunization from previous transfusions or an incompatibility in other blood groups.[131,134] This can further strain donor-recipient matching, as additional antigen screening may be necessary.[131] In some cases, chronically transfused patients may generate so many alloantibodies that finding compatible donors becomes impossible.[134]

1.3.3 Iatrogenic Complications

Transfusion is not without risk, and there are serious complications that give rise to the restrictive hemoglobin threshold. Complications can be categorized as acute or delayed, and either category can include both infectious and noninfectious complications.[122] Generally, acute complications will occur within 24 hours of transfusion, while delayed complications can develop over the course of years in some cases.[122]

While transfusion-transmitted infections are rare, the risk is always present.[122,123] Improvements to donor screening and blood product testing have resulted in a significant reduction in infection risk relative to the 1980s; some estimate a 10^4-fold change.[135] Table 1.6 highlights the current risk of transfusion transmission of select pathogens, however, estimates vary widely.

Table 1.6: Risk of transfusion transmission of select pathogens.[122,123,136,137] Note that estimates vary widely and include all blood components, so risk of transmission via RBC transfusion may differ.

Pathogen	Transfused units per transmission
Cytomegalovirus	3,000,000
Hepatitis B	500,000
Hepatitis C	800,000
Human immunodeficiency virus	1,000,000
Human T-lymphotrophic virus	3,000,000
Sepsis-causing bacteria	1,000,000

Noninfectious acute transfusion reactions include acute hemolytic reactions, febrile nonhemolytic reactions, transfusion-associated circulatory overload (TACO), transfusion-related acute lung injury

(TRALI), allergic reactions, and sepsis.[122] Noninfectious delayed transfusion reactions include delayed hemolytic reactions, iron overload, transfusion-associated graft-versus-host disease, and transfusion-related immunomodulation (TRIM).[122] All of these except for TACO and sepsis are immune-mediated transfusion reactions.[123]

Both acute and delayed hemolytic transfusion reactions are caused by excessive lysis of transfused RBCs.[138] This may be due to transfusion of incompatible RBCs eliciting an immune response, or storage lesion associated damages to the RBCs.[138] In the latter case, mechanical, thermal, and osmotic fragility may contribute to hemolysis.[123]

TACO is the leading cause of transfusion-related deaths in the United States, per the FDA.[139] It is caused by rapid transfusion of a large volume and presents with, among other symptoms, respiratory distress, hypoxemia, and pulmonary edema.[122,140] TRALI presents similarly, and differentiation between the two can be difficult.[140] In contrast to TACO, TRALI seems to involve neutrophil activation and immune-mediated damage to the pulmonary endothelium.[123,140] The mechanisms behind TRALI are still not fully understood.[140]

Alloimmunization and TRIM are both immune-system related transfusion reactions. In TRIM, immune function is suppressed, leading to increased infection risk and cancer recurrence.[125] The mechanism for this is also still under investigation.[125,141] Frequent transfusions can lead to alloimmunization, increasing the risk of hemolytic reactions from future transfusions.[134] This can lead logistical issues in donor-recipient matching, particularly for chronically transfused patients.[134]

Additional complications with etiologies implicating the storage lesion include insufficient nitric oxide bioavailability (INOBA) and non-transferrin bound iron (NTBI).[19,37,142,143] As discussed in Section 1.2.3, major aspects of the RBC storage lesion include increased hemolysis and microvesiculation and decreased cellular deformability. Both free hemoglobin and RBC-derived extracellular vesicles (EVs) are NO scavengers, as are RBCs with impaired deformability.[37,144] Collectively, this leads to a decrease in bioavailable NO post-transfusion, resulting in impaired tissue perfusion.[142]

Furthermore, the hemolysis in stored RBCs results in the transfusion of iron metabolites.[37] These are generally sequestered by transferrin to prevent ROS generation, but in the case of transfusion, this protein can become saturated, ultimately leaving NTBI circulating.[37] This can lead to iron overload, a toxic condition associated with widespread organ damage and increased morbidity and mortality.[143]

1.4 Improving Blood Storage

1.4.1 Clinical Implications

Modern day transfusions are relatively safe and effective. Yet, these life-saving interventions can still be improved to ameliorate some of the remaining risks. By increasing their efficacy, the same therapeutic benefit may be realized with smaller transfusion volumes. This can be accomplished by further mitigating the progression of the storage lesion.

The benefits of reduced transfusion volume are threefold. By reducing the total volume added to the circulatory system, the incidence of the leading cause of transfusion-related mortality, TACO, may be diminished. Transfusion-transmitted infection risk is directly proportional to the number of units transfused, so the incidence of pathogen transmission will likewise be reduced. Finally, the demand for blood products will wane, alleviating some burden on the supply chain for these valuable therapeutics.

As discussed in Section 1.2.2, leukoreduction of packed RBCs is one demonstrated way of improving transfusion outcomes. In addition to reducing the storage lesion, this process has been found to reduce both the accumulation of RBC EVs and their propensity to cause inflammation post-transfusion.[145,146] Leukoreduction also reduces TRIM, post-transfusion nosocomial infections, alloimmunization, febrile nonhemolytic transfusion reactions, and cytomegalovirus transmission.[69,70,74,123]

There is some debate regarding the effect of RBC storage duration on transfusion outcomes.[129] Some large-scale studies have concluded that transfusion of older RBCs is associated with increased risk of complications, organ dysfunction, and death.[147–149] Conversely, other large-scale studies have concluded there is no association between RBC storage duration and recipient organ dysfunction and mortality.[150–152] A 2018 Cochrane review states they observed “no clear difference in the risk of death at different time points between transfusion of blood stored for a shorter duration versus blood stored for a longer duration” based on a meta-analysis of 22 randomized controlled trials.[153]

These studies are not without issue themselves, however. One criticism that has been discussed in the literature is the vague delineation between “fresh” and “old” units of RBCs.[129] Likewise, the ability of these studies to fully control for comorbidities in an already-ailing population has been called into question.[37] Finally, progression of the storage lesion even in the “fresh” RBCs has been posited as a confounding factor.[19] It is worth noting that although RBCs can be stored for up to 42

days, the age of the average transfused unit is only 18 days.[129]

1.4.2 Alternative Storage Processes

Rejuvenation of stored RBCs to reverse various aspects of the storage lesion is possible. In 1997, Rejuvesol was approved by the FDA for this purpose.[96] Rejuvesol restores ATP and 2,3-DPG in stored RBCs, and is an aqueous solution of sodium pyruvate, inosine, adenine, dibasic sodium phosphate, and monobasic sodium phosphate.[154]

Rejuvesol is approved for use with RBCs stored in non-leukoreduced CPD and CPDA-1 or in leukoreduced CPD/AS-1 and CP2D/AS-3.[154] To rejuvenate these cells for immediate use, $50\,\mathrm{mL}$ of Rejuvesol is transferred into the storage bag and thoroughly mixed.[154] The bag is then incubated for 1 hour in a $37\,^{\circ}\mathrm{C}$ water bath before the RBCs are washed to remove any excess solution prior to transfusion.[154]

Although this process is somewhat labor-intensive, the rejuvenation process can successfully reverse multiple aspects of the storage lesion. Rejuvenated RBCs has been shown to have significantly increased levels of ATP and 2,3-DPG.[155] These RBCs demonstrate decreased cellular fragility and endothelial cell adhesion, increased deformability, and improved hemoglobin/oxygen affinity.[96,155,156] Clinically, this results in reduced organ dysfunction and increased tissue perfusion and post-transfusion RBC recovery.[155,157] However, Rejuvesol is primarily used in blood banking research as the time and labor requirements involved render it unpractical in most clinical settings.[158].

In contrast with simple hypothermic storage, cryopreservation of RBCs allows for storage durations exceeding 10 years.[159] However, cryogenic storage of RBCs results in the formation of ice crystals, leading to total hemolysis unless stored in a cryoprotectant.[160] The most commonly used cryoprotectant is glycerol, first described as effective in RBC cryostorage in 1950.[161]

The glycerol used in RBC cryopreservation leads to post-transfusion osmotic lysis, and thus RBCs must be fully deglycerolized after thawing.[162] Beyond being a labor-intensive process requiring specialized equipment, there is a resulting loss of approximately $15\,\%$ of the RBCs.[160] This, along with the equipment required to maintain cryogenic temperatures, has led to the use of cryopreserved RBCs primarily only in military settings.[160,163]

Cryopreservation of RBCs is effective, however. Under these extremely low temperatures, essentially all physiological activity stops.[164] This halts progression of the storage lesion, resulting

in a product that is generally unaffected by storage with post-transfusion RBC recovery rates over $80\,\%$.[160,165] In the United States military, deglycerolized cryopreserved RBCs are considered equivalent to fresh RBCs for all transfusion requirements.[163]

One emerging yet promising technique is hypoxic storage of RBCs. The role of oxidative stress in the development of the storage lesion has been well-discussed, leading to the investigation of hypoxia as a mechanism to combat RBC damage.[166] Anaerobic storage of RBCs has been shown to prevent the depletion of ATP, decrease hemolysis, free hemoglobin, and EV production, and maintain acceptable post-transfusion RBC recovery rates even after 9 week of storage.[167]

In 2023, the FDA approved a new container system designed for the hypoxic storage of RBCs.[168] This product has been demonstrated to improve ATP production, 2,3-DPG levels, glutathione homeostasis, membrane remodeling, and post-transfusion RBC recovery.[169] While no large-scale studies have yet taken place, these results (using AS-3) are quite promising. If they are repeatable in further randomized clinical trials, and correlate with improvements in patient outcome, it is likely hypoxic storage of RBCs will become a new standard practice.

1.4.3 Normoglycemic Blood Storage

As discussed in Section 1.2.1, various aspects of the storage lesion bear a striking similarity to the RBC dysfunction observed in diabetes. While investigators have questioned the implications of these "quasi-diabetic" storage conditions, there is a relative dearth of research on the effects of reducing hyperglycemia in storage on the storage lesion.[44] Understanding this will help to inform the constituents of the next generation of additive solutions, potentially contributing to the reduction of RBC damages throughout storage.

To this end, the Spence laboratory has developed an experimental anticoagulant, normoglycemic citrate phosphate dextrose solution (CPD-N), and additive solution, normoglycemic Additive Solution 1 (AS-1N).[170] The constituents of both solutions are identical to that of their hyperglycemic equivalents, except with significantly reduced glucose concentrations of $5.5\,\mathrm{mmol\,L^{-1}}$. This has the additional effect of reducing the hyperosmolarity of both the anticoagulant and additive solution. Table 1.7 recapitulates the composition of the traditional solutions alongside their normoglycemic counterparts.

One major consideration of normoglycemic storage is the depletion of glucose due to cellular

metabolism. This necessitates periodic "feeding" of stored RBCs to maintain physiological glucose levels throughout storage. As RBC membrane maintenance is ATP-dependent, hypoglycemic storage conditions quickly led to significant hemolysis.[170]

Initial studies used "micro-units" of RBCs stored in 2 mL PVC bags.[170] These bags were prepared in-house, and notably did not contain DEHP plasticizer.[170] Feeding was accomplished by opening the bags and dropping in small quantities of $200\,\mathrm{mmol\,L^{-1}}$ glucose in saline before resealing them.[170]

Table 1.7: Comparison of traditional CPD and AS-1 versus normoglycemic CPD-N and AS-1N.

	Component ($\mathrm{mmol\,L^{-1}}$)			
	CPD	CPD-N	AS-1	AS-1N
Trisodium citrate	89	89	–	–
Citric acid	16	16	–	–
Monosodium phosphate	16	16	–	–
Dextrose	129	5.5	111	5.5
Sodium chloride	–	–	154	154
Adenine	–	–	2	2
Mannitol	–	–	41	41
pH	5.2–6.2	5.6	5.2–6.2	5.6
Osmolarity ($\mathrm{mOsm\,L^{-1}}$)	533	409.5	462	356.5

These studies found that CPD-N/AS-1N stored RBCs had significantly improved ATP release.[170] Lactate continued to accumulate throughout the entire storage period, indicating metabolic activity slowed to a lesser extent than in the hyperglycemic condition.[170] Investigation of the ability for these cells to elicit NO production was achieved with the use of a microfluidic device containing bovine pulmonary artery endothelial cells.[170] During the first three weeks of storage, the normoglycemic cells elicited significantly more NO production from the endothelial cells under flow conditions.[170] A follow-up study using a novel 3D-printed microfluidic chip confirmed that ATP release from RBCs stored in normoglycemic conditions was significantly higher throughout the first week of storage, although longer storage durations were not tested.[171]

Further experimentation compared normoglycemic and hyperglycemic versions of AS-1, AS-3, and AS-5.[172] All normoglycemic additive solutions were paired with CPD-N, while AS-1 and AS-5 were paired with CPD and AS-3 was paired with CP2D.[172] These studies utilized similar bags prepared in-house, but in 1 mL volumes instead.[172]

In this comparison study, the ability of normoglycemic storage to improve ATP release was confirmed for all three additive solutions.[172] Likewise, all normoglycemic additive solutions resulted in much less generation of sorbitol relative to their hyperglycemic counterparts, indicating reduced oxidative stress.[172] The osmotic fragility of normoglycemic RBCs was only significantly improved during the first two weeks of storage, however.[172] Interestingly, there was relatively little difference in the hyperglycemic additive solutions despite their varying compositions, although that comparison was not a direct objective of this study.

More recent work has continued to replicate maintenance of flow-induced ATP release in normoglycemic storage.[173] To investigate the implications of this metabolic improvement on RBC membrane health, cellular deformability was also tested in these studies. This was accomplished using a bulk-filtration method in which RBCs, nominally 8 µm in diameter, are forced through a membrane with 5 µm pores.[173] The fraction of the RBCs that are able to deform through the membrane can be used as a relative measure of cellular deformability. This study reports RBC deformability is maintained throughout the storage period that under normoglycemic conditions, while it becomes significantly impaired under hyperglycemic conditions.[173]

One drawback of these studies is the need to open the storage bags to feed the normoglycemic RBCs. The FDA does not permit the sterility of stored blood bags to be breached, and so this method of feeding would not translate to actual practice. To increase the rigor of future experiments, a new feeding technique was developed.[174] This utilized a 3D-printed "piggyback" system, using new in-house prepared storage bags with an injection port connected to a feeding solution reservoir.[174] This system included a valve that could be manually actuated to dispense feeding solution into the storage bag as necessary.[174]

While this system was able to successfully maintain normoglycemia for up to 28 days, it is also not without limitations.[174] Since the valve is manually actuated, dispensing repeatable volumes may be difficult. It also required the use of custom storage bags, although there may be already-approved storage bags that are also compatible. Finally, the increased labor requirement of manually feeding every bag is infeasible for a commercial blood bank containing many units. Together, these detriments limit the likelihood of translating this technology to blood banking praxis.

In order to overcome some of these limitations, an automated version of this system was later developed.[41] While similar in principle, this system used a microcontroller-actuated solenoid valve

to dispense the feeding solution, increasing repeatability.[41] The glucose utilization curve shown in Equation (1.2) was used to automate the actuation of the solenoid valve, decreasing manual intervention significantly.[41] This system was able to successfully maintain normoglycemia for up to 37 days.[41]

Blood stored using this system confirmed the reduction in sorbitol accumulation in normoglycemic storage, as well as the reduction in osmotic fragility up to week three of storage.[41] The sterility of blood stored using this system was confirmed by investigating bacterial growth on blood agar after six weeks of storage.[19,82] Studies using this system also confirmed the increase in total AGEs for both normoglycemic and hyperglycemic storage, although total AGE formation was greater in the hyperglycemic condition.[82]

These studies show there is clear potential for normoglycemic storage to improve the health of stored RBCs. However, *in vivo* studies will be required to demonstrate whether there is an improvement in clinical outcomes. Though the work accomplished by the Spence laboratory thus far provides a significant impetus for translation to these *in vivo* studies, there are a number of *in vitro* refinements that can still be made. The next two chapters of this dissertation will attempt to move these investigations toward this translational goal.

First, development of a second-generation RBC feeding system will be discussed. This system incorporates upgrades that increase the translational potential of normoglycemic storage by allowing compliance with regulatory requirements and enhancing usability. Additionally, an improved dosing algorithm and sterile pumping mechanism are employed to improve experimental control and rigor.

Next, this system will be used to further demonstrate the benefits of normoglycemic storage. The efficacy of this system and effects of an improved RBC feeding solution will be investigated. Finally, rejuvenation of stored RBCs within this context to reverse various aspects of the storage lesion will be explored.

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CHAPTER 2

DEVELOPMENT OF A SEMI-AUTONOMOUS GLUCOSE FEEDING SYSTEM FOR NORMOGLYCEMIC BLOOD STORAGE

2.1 Introduction

Over 10.5 million units of red blood cells (RBCs) were transfused in the United States in 2021, per the National Blood Collection and Utilization Survey.[1] Despite the ubiquity of these life-saving procedures, they are not without risk. Transfusion-transmitted infections, circulatory overload, lung injury, hemolytic reactions, immunomodulation, and iron overload are all potential side effects of every transfused unit.[2–7] Because of these risks, restrictive thresholds on hemoglobin levels have been implemented in transfusion indications.[8] Normal hemoglobin levels range from $12\,\mathrm{g\,dL^{-1}}$ to $18\,\mathrm{g\,dL^{-1}}$, while transfusions are only indicated when hemoglobin falls below $7\,\mathrm{g\,dL^{-1}}$ in most cases.[8,9]

These restrictive thresholds have been shown to be at least as effective as more liberal hemoglobin thresholds, but with significantly decreased in-hospital mortality.[10] Mortality rates were likewise significantly lower using restrictive thresholds in patient groups who were less acutely ill or below the age of 55.[10] It is clear that reducing the number of transfusions has a clinically relevant impact on patient outcomes. Similarly, when transfusions are warranted, reducing the number of units transfused will reduce the risk of iatrogenic complications.

Improving transfusion efficacy is key to reducing transfusion volume. This has the added benefit of reducing strain on the beleaguered supply chain of blood products, a persistent issue around the world.[11–13] By mitigating development of the storage lesion in RBCs, transfusion efficacy will improve. One approach to reducing the storage lesion involves modification of additive solutions to reduce the extreme hyperglycemia to which stored RBCs are exposed.

To this end, the Spence laboratory has conducted significant research into evaluating the impact of normoglycemic storage on RBC health. In 2013, a microfluidic device was used to demonstrate RBCs stored using normoglycemic Additive Solution 1 (AS-1N), described in Chapter 1, stimulated over $25\,\%$ more nitric oxide production in endothelial cells *in vitro* than RBCs stored in hyperglycemic Additive Solution 1 (AS-1).[14] This study, along with two others, also confirmed AS-1N stored RBCs release significantly more adenosine triphosphate (ATP) compared to RBCs stored under hyperglycemic conditions.[14–16] One explanation for this was posited in a 2022 paper showing significantly impaired

cellular deformability when RBCs are stored in hyperglycemic conditions, as membrane deformation precipitates ATP release.[17]

A major drawback of these studies was the need to manually feed the RBCs under normoglycemic storage. As hypoglycemic conditions lead to extreme lysis, and glucose metabolism continues throughout storage, this is necessary to keep the stored RBCs alive throughout a full storage period.[14] In addition to being labor-intensive, this process also breaks sterility of the stored blood, negating any possibility of regulatory approval. A custom-made storage bag with an integrated feeding solution reservoir was first described in 2017.[18] However, this system continued to use a manually actuated valve; this only modestly reduces the labor requirements of normoglycemic storage and is potentially error-prone and inconsistent.

Recently, an automated version of this valve-based system that is compatible with commercial storage bags was developed.[19] This consists of a reservoir of feeding solution connected to a solenoid valve and suspended above the blood storage bags. The output of the solenoid valve is connected to tubing leading into the blood bag. Glucose utilization throughout storage was measured and used to empirically derive the curve given by Equation (1.2), and integrating this curve yields a model for glucose consumption as shown in Equation (1.3). Evaluating this for a given storage window provides an estimate for glucose depletion over that time span, $\Delta Glucose$. If the volume of a storage bag is known, the concentration of glucose in a feeding solution and target glucose setpoint can be used to find the required volume of feeding solution by Equation (2.1).

$$\text{Feed volume} = \frac{V_{bag}\Delta Glucose}{[Glucose]_{feed} - Setpoint} \tag{2.1}$$

This first-generation feeding system was an excellent proof-of-concept that worked well throughout multiple RBC storage periods. However, several drawbacks existed which may have impacted system performance and translational potential. Much like the previous valve-based bags, the feeding solution was gravity-fed. By Bernoulli's principle, flow through the system is dependent on the relative elevation of the feeding solution reservoir. This means calibration of the system is dependent on a fixed geometry, reservoir, and starting volume of feeding solution. Additionally, since the reservoir is depleted throughout storage, the calibration curve of volume dispensed versus valve timing will almost certainly be nonlinear.

The use of valves in this system necessitates their direct contact with the feeding solution. While

care was taken to clean these valves, they could not be autoclaved or easily be disassembled for sterilization. Chemical disinfection was used, but resulted in corrosion of internal components, inevitably contaminating the feeding solution with metal oxides. It is worth noting that blood agar swabs following initial storage trials showed no signs of bacterial contamination, however.[20] Despite this, regulatory concerns over possible contamination are valid.

Finally, the system did not individually manage each storage bag. A general feeding schedule was established with delivery volumes scaled based on initial bag volume. This accounts for increases in volume due to feeding, but not reductions due to sampling for experimentation. It also does not allow for glucose feedback, preventing the system from compensating in the case of hypoglycemia or hyperglycemia. As storage duration was not tracked, separate systems were required for each set of blood bags in storage.

Further improvements could be made to increase experimental control, rigor, and flexibility, as well as system usability and translational potential. Peristaltic pumps could be used in lieu of gravity-fed valves to make system calibration both easier and more robust. This style of pump does not directly contact the working fluid, allowing the entire system to remain closed and sterile. This is a Food and Drug Administration (FDA) requirement for all blood storage systems.[21]

With sufficient motors and pump heads, much greater resolution in dispensing volume is attainable. This would allow for more continuous feeding, allowing the glucose to diffuse without large concentration gradients occurring near the delivery site. It also enables more concentrated feeding solutions to be used, negating the need to supplement them with sodium chloride to maintain isotonicity (a requirement of the first-generation system). This may itself be beneficial in reducing the storage lesion, as feeding solution containing chloride anions may contribute to detrimental morphological changes.[20]

An improved dosing algorithm will also contribute to better glycemic control of each blood bag throughout storage. The accuracy of Equation (2.1) can be improved by accounting not just for volume added to the bags during feeding, but also the volume of samples removed for experimentation and glucose testing. By factoring actual glucose feedback into the value for $\Delta Glucose$, corrective measures can be taken if the bag glucose is straying from the setpoint. This level of individual bag management enables dynamic starting and stopping of storage bags with customizable parameters. Using one centralized system with a user interface enhances usability and is capable of scaling to

manage many blood bags, limited only by the number of pumps in the blood bank. Figure 2.1 shows a graphical representation of this second-generation feeding system, where any WiFi-enabled device can be used to interact with the feeding system to maximize experimental control.

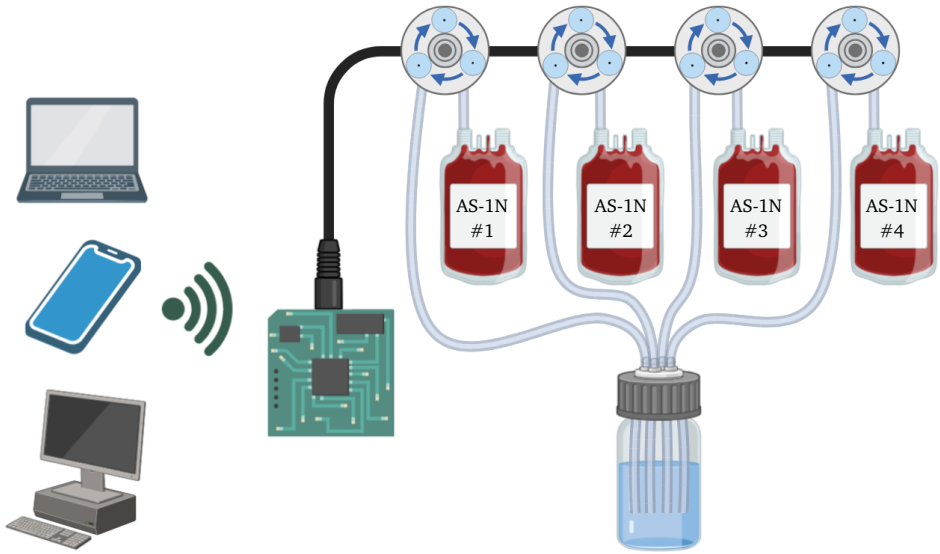


Figure 2.1: Graphical overview of a second-generation automated feeding system for normoglycemic RBC storage.[22] Peristaltic pumps are used rather than a gravity-fed approach. Blood bank management and glucose feedback over Wi-Fi allows for enhanced experimental control.

2.2 Control Server

The first-generation feeding system utilized an open-source single-board microcontroller (SBM) (Arduino Uno R3) to actuate the solenoid valve. These development boards are inexpensive, simple to operate, and allow for rapid prototyping when interfacing with hardware. For these reasons, it was decided the second-generation feeding system would also use this platform for controlling the peristaltic pumps.

Unfortunately, these SBMs have insufficient computing power for the planned software upgrades. Thus, an open-source single-board computer (SBC) (Orange Pi Zero2) was selected to act as a control server. This SBC can run the software and communicate commands to the SBM, enabling simple hardware control with sophisticated software. The SBC also is capable of acting as a wireless access point, allowing easier access to the user interface.

2.2.1 Software

The SBC software can be broken down into four modules, each handling a separate set of functions for the application. This software runs in a virtualized container on the Docker platform,

enabling easy deployment and upgrades. It also takes advantage of container restart policies to ensure the application is always running, even after an SBC reboot or power failure. The entirety of the application is written in the Python programming language.[23]

The first module runs the control loop that calculates the volume of feeding solution to be dispensed into each bag (see Equation (2.1)). This control loop is executed at a user-defined interval; for these studies, it was run every five minutes. For each bag currently in the system, individual bag parameters are calculated for maximum dosing accuracy and experimental flexibility. Figure 2.2 shows a graphical overview of the control loop logic and calculations.

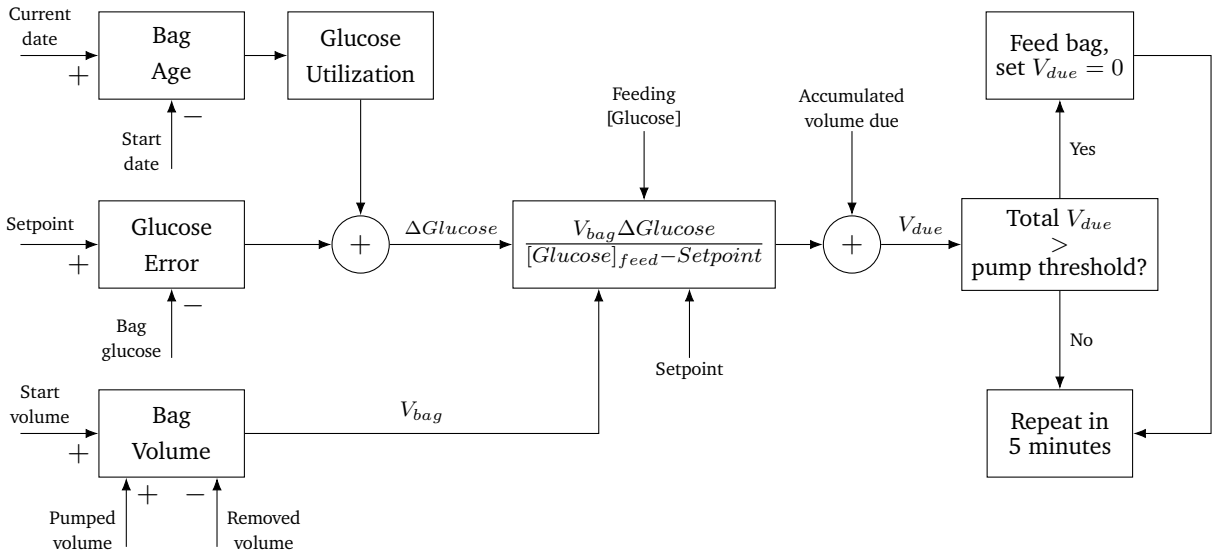


Figure 2.2: Block diagram of the algorithm used by the control loop of the semi-autonomous feeding system. This calculates the volume of feeding solution to be delivered to each bag every five minutes. Bag parameters are tracked individually for "personalized" feeding, which occurs when the feed volume exceeds the pumping threshold.

The second module handles object-relational mapping to read and write from a persistent database. For this application, a SQLite database is used to store application settings and experimental data.[24] This is a popular embedded database that does not require a separate database server. The database is stored as a single file outside the application container, allowing for simple data backups and restorations. Other modules of the application use an object-oriented approach to handling data; this module serves as a translator to read and write the objects to and from the database.

The third module handles asynchronous communications between the SBC and SBM. This is accomplished via serial communication over a Universal Serial Bus (USB) cable. The control server

sends commands over USB, which are acted upon by the SBM. A timestamped return communication to the control server is logged in the database indicating if the command was successfully executed.

The fourth module is a web user interface for managing the bags being stored, written with the NiceGUI Python package.[25] This displays the current status of each blood bag currently in storage, as well as any data associated with them. An archive of previously stored blood bags is also available, as is the ability to download a copy of the database. Individual pump settings are available on another page of the interface, along with manual pump control options for calibration and priming. Finally, an error log is available to view any exceptions that occur in the application. Figure 2.3 shows what the home screen of this interface may look like with four bags in storage.

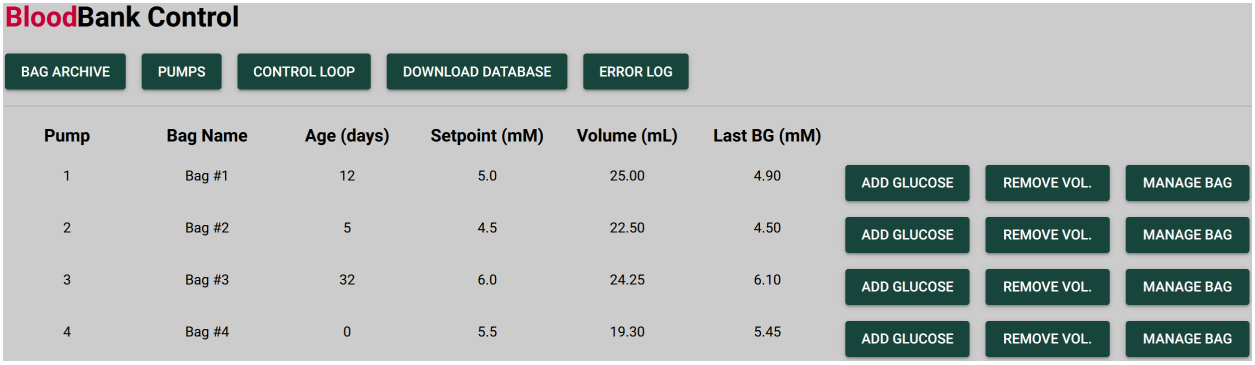


Figure 2.3: Home screen of the web user interface for the blood banking software. An overview of bags currently in storage is show. Buttons link to other pages of the interface.

2.2.2 Networking

In order to connect to the web user interface hosted on the control server, clients must be connected to the same network as the SBC. Fortunately, the SBC can easily be configured to act as a Wi-Fi host, broadcasting its own network to other devices. This negates the need to connect the SBC and client devices to an uncontrolled third-party network and simplifies communications between them. The tradeoff of this approach is the additional networking configuration required for the initial setup.

To this end, the dnsmasq software package is used.[26] This provides lightweight and easily configurable Domain Name System (DNS) and Dynamic Host Configuration Protocol (DHCP) servers that can run on the SBC. Briefly, the DHCP server assigns an Internet Protocol (IP) address to the client device and, among other information, provides IP addresses the client should use for DNS requests. The DHCP server used here is configured to provide the IP address of the SBC itself as the

DNS server.

DNS servers translate human-readable website domain names to the IP addresses used by computers. Thus, a client device connected to the SBC Wi-Fi will request the IP address of a website from the SBC itself. Here, the DNS server is configured to provide the IP address of the blood banking software web server in response to any query. In other words, when connected to the SBC network, navigating to any website in a web browser will redirect you to the application interface.

2.3 Custom Pumping System

Much like the first-generation feeding system, development of this pumping system aimed to use inexpensive and readily-available components that are easily interchangeable. This is complemented by the exclusive use of open-source software to enhance the extensibility of this project. The modularity of this design allows for flexibility and easy iteration in the prototyping process. While certain components did use more advanced manufacturing techniques, these are quality-of-life improvements that are not inherently required.

To this end, the first iteration of the second-generation feeding system used simple brushed direct current (DC) motors to drive peristaltic pump heads. These motors are inexpensive and easy to control using relatively basic circuitry; their simplicity allows for easy drop-in replacement or substitution. They are also quite common, and can be procured to many different specifications.

2.3.1 Microcontroller Firmware

Pump commands between the SBC and SBM were communicated using the Firmata protocol. Specifically, the pyFirmata2 Python package was used on the control server and the Firmata library was used on the microcontroller.[27,28] This allows for simple control of general-purpose input/output (GPIO) pins on the SBM over a USB connection. No further modifications to the firmware were necessary.

2.3.2 Motor Control Board

The GPIO pins on the SBM can only provide $20\,\text{mA}$ of current at $5\,\text{V}$, which is insufficient to drive the $12\,\text{V}$ motors. To overcome this, the $12\,\text{V}$ power source used by the SBM was also used to power the DC motors through the input voltage (V_{in}) header on the SBM. The SBM GPIO pins can then be used to switch the power supplied to the DC motors via an NPN-type bipolar junction transistor (BJT). A schematic of the circuit for a single DC motor is shown in Figure 2.5A.

The speed of the DC motor and the duration it runs will affect the output volume of the peristaltic pump. As the SBM can switch the power supplied to the motor, controlling the duration it runs is simple. However, the speed of the motor is governed by the supply voltage. While in this case the supply voltage is fixed, there is still a way to directly control the speed of each motor.

Pulse-width modulation (PWM) is the practice of modulating the duty cycle of a load by switching the power supplied at a sufficiently high frequency. The SBM used here has six PWM-capable GPIO pins that operate at $490\,\text{Hz}$. These pins are used to control the speed of the DC motors to enable better control of the pumped volume. Figure 2.4 shows ideal waveforms for the outputs of the PWM pins at various duty cycles.

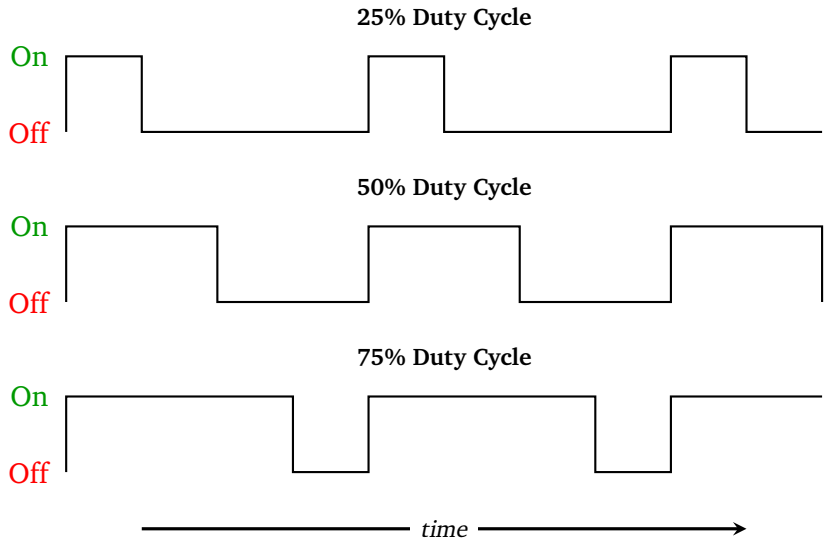


Figure 2.4: Examples of rectangular waves with modulated duty cycles. Digital outputs from a microcontroller can use this pulse-width modulation to vary the average power supplied to a load.

While the breadboard-based circuitry used in the first-generation feeding system worked well, utilizing all six PWM pins on the SBM would quickly become convoluted. To make the control circuitry of the motor control board more compact and robust, a custom printed circuit board (PCB) was designed using electronic design software (Autodesk Fusion).[29] The GPIO pin headers on the SBM allowed this to be mounted directly on top of the microcontroller board. A schematic of the PCB is shown in Figure 2.5B, and the assembled system is shown in Figure 2.5C.

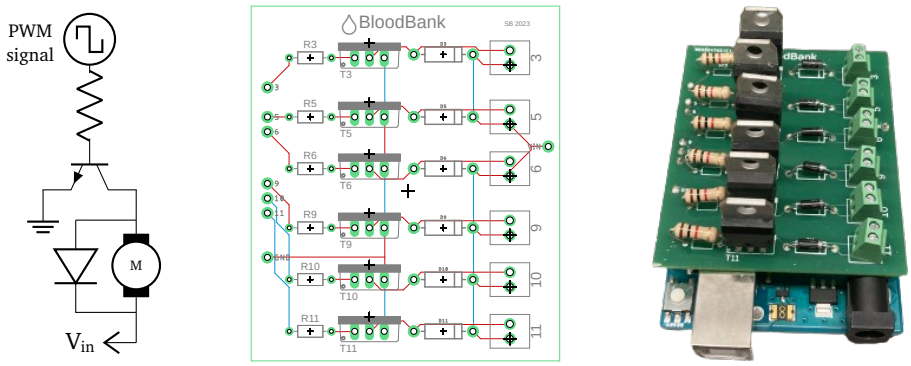


Figure 2.5: (A) Circuit controlling the DC motor of a peristaltic pump. (B) Schematic drawing of the PCB, including the circuit in sextuplicate. (C) Assembled PCB mounted on the SBM.

Briefly, each control circuit starts with a resistor to limit the current supplied to the BJT. This BJT acts as a low-side switch, only allowing current to flow through the motor coils when the PWM signal is high. A flyback diode is placed in parallel with the motor to prevent damage to the BJT from voltage spikes caused by the inductance of the DC motor. Each circuit ends with screw terminals so motor leads can be easily connected to the control board.

2.3.3 Planetary Gearbox

The first peristaltic pumps used in the second-generation feeding system were directly coupled to inexpensive 12 V DC motors (Gikfun 12V DC Dosing Pump). As discussed, PWM was used to control the speed of these motors. To better control the volume of fluid being pumped, motor speed was minimized as much as possible. Unfortunately, PWM modulates torque as well, and the DC motors would begin to stall even at relatively high duty cycles.

To remedy this, gearing can be used to increase the output torque of the motors. This has the added benefit of reducing the output speed, which further increases the precision of volume delivery. To this end, a gearbox was developed to enable further reduction of the DC motor output.

In order to keep the gearbox compact while maintaining a reasonable transmission ratio, a planetary architecture was used. This has the added benefit of a coaxial input and output, further simplifying the assembly of the DC motor, gearbox, and peristaltic pump head. In planetary gearing, also known as epicyclic gearing, there are three components: an outer ring gear, an inner sun gear, and one or more planet gears between them. If the ring gear is grounded, the planet gears can act as a reduced output relative to the sun gear, which can be driven by the DC motor shaft. In this

configuration, the transmission ratio is given by Equation (2.2) where z is the number of gear teeth.

$$\text{Gear ratio} = 1 + \frac{z_{ring}}{z_{sun}} \tag{2.2}$$

There are a number of important design considerations when developing a planetary gear train. For the gears to mesh properly, they all must have the same gear module, m. This is defined by z and the diameter of the gear pitch circle, d_{pc}, per Equation (2.3). The pressure angle, α, of a gear tooth is related to the width of the base of the tooth, and therefore its overall strength. A pressure angle of 20° is typical. The minimum number of teeth needed to avoid interference is determined by α as shown in Equation (2.4).

$$m = \frac{d_{pc}}{z} \tag{2.3}$$

$$z_{minimum} = \frac{2}{\sin^2 \alpha} \tag{2.4}$$

For epicyclic gearing, spacing of the planet gears must also be considered. Equidistant spacing is generally desired to reduce unbalanced forces, and is possible when the sum of teeth on the sun and ring gears is evenly divisible by the number of planets, N. Equation (2.5) shows this constraint mathematically.

$$\frac{z_{sun} + z_{ring}}{N} \in \mathbb{N} \tag{2.5}$$

With these concepts in mind, a three-planet gearbox was designed using computer-aided design (CAD) software (Autodesk Fusion).[29] A gear ratio of 5 and gearbox diameter of 50 mm were targeted. However, maximizing the gear ratio and minimizing the gearbox size are two competing design objectives. To ensure the gear teeth were robust, the module and pressure angle were set to 1 and 20°, respectively.

Applying Equation (2.4) when $\alpha = 20^\circ$ leads to a $z_{minimum}$ of 17. However, this would require the ring gear to have a d_{pc} of 68 mm. To compromise, a small amount of backlash was added to reduce interference. This allowed for a small, albeit nonideal, reduction in z_{sun} while maintaining the target transmission ratio. The details of the gear train are described in Table 2.1.

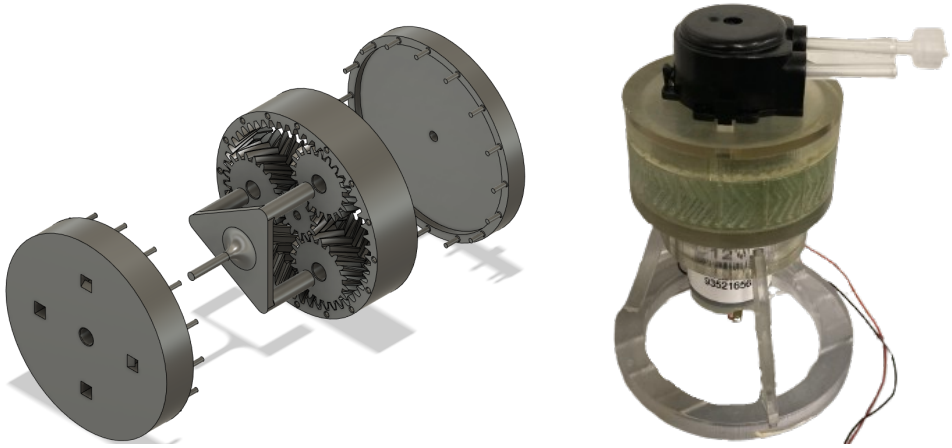
Table 2.1: Design parameters of the planetary gearbox.

Parameter	Value
α	20°
m	1.00
N	3
z_{sun}	13
z_{ring}	53
z_{planet}	20
Gear ratio	5.08

As the gearbox was to be fully 3D printed, proper design allows for it to be conveniently "printed in place" nearly fully-assembled. Further, the use of herringbone teeth allows the gear train to be locked together by constraining axial movement. This tooth pattern is composed of counter-oriented helical teeth forming a "V"-shaped pattern in the middle of each tooth. Note that in this design, the transverse module is 1 while the normal module is 0.866. This is due to the 30° helix angle, β; the relation is shown in Equation (2.6).

$$m_{transverse} = \frac{m_{normal}}{\cos\beta} \tag{2.6}$$

Mounting plates for the front and rear of the gearbox were also created, as well as a planet carrier with output shaft. Figure 2.6A shows an exploded view of this assembly. A high-resolution PolyJet 3D printer (Stratasys J750; Stratasys, Ltd.; Eden Prairie, MN) was used to manufacture the gearbox. The components were printed in an acrylic-like resin (VeroClear; Stratasys, Ltd.; Eden Prairie, MN) with a soluble support material (SUP706B; Stratasys, Ltd.; Eden Prairie, MN). The completed peristaltic pump assembly is shown in Figure 2.6B. A small amount of polytetrafluoroethylene grease was applied to reduce friction in the gearbox (Magnalube-G; Magnalube LLC; Arlington, TX).



(A) **(B)**

Figure 2.6: (A) Exploded CAD assembly of the planetary gearbox. (B) Assembled planetary gearbox mounted on a DC motor with a peristaltic pump and 3D-printed mount.

2.4 Commercial Component Pumping System

Initial testing and use of the custom gearbox paired with a 12 V DC motor was successful. Despite this, they were ultimately abandoned in favor of a system with higher precision and increased reliability. The second iteration of the second-generation feeding system uses stepper motors to drive peristaltic pump heads (Kamoer KPHM100; Kamoer Fluid Tech Co., Ltd.; Shanghai, China).

These motors offer several advantages over the first iteration of the pumping system. Although they are more expensive, this is balanced by their ability to be precisely controlled without the need for a gearbox. Similarly, they are more complex to drive, yet can be paired with inexpensive stepper driver boards. This helps simplify the electrical components of the system without designing a custom PCB or using convoluted circuitry.

2.4.1 Microcontroller Firmware

As stepper motors are significantly more complex to drive, more sophisticated firmware was required. The control server communications were upgraded to use the asynchronous Telemetrix-AIO Python library, which includes native support for stepper motors.[30] The SBM likewise used the Telemetrix4Arduino server firmware.[31] However, undocumented limitations were discovered when this firmware was used on an Arduino Uno R3. Mainly, the memory of the microcontroller was insufficient to operate four stepper motors simultaneously.

After reviewing the source code, a simple solution was found. In order to free up memory, unused features of the firmware could be safely deleted and the reduced firmware could be flashed to the SBM.

As the firmware contained several features not needed here, this was a relatively straightforward endeavor. Once completed, the SBM worked as intended.

2.4.2 Stepper Driver Board

Stepper motors have excellent rotational precision, but require considerably more power and are more complex to drive. An open-source, commercial off-the-shelf stepper motor driver board was purchased rather than designing a custom PCB as before (Protoneer Arduino CNC Shield). This board is similarly compatible with the SBM.

The stepper driver board has pin headers for up to four stepper motor drivers. Paired with a $12\,\mathrm{V}$ power supply capable of up to $5\,\mathrm{A}$, it also manages power distribution to both the stepper drivers and the SBM through the V_{in} pin. The board includes selectable microstepping for each individual stepper driver, allowing for increased precision. Four stepper motor drivers (A4988) were installed on the board and tuned to limit the current through them to approximately $750\,\mathrm{mA}$ each. Each driver was also set to use $1/8$ microstepping, which will be further discussed in the following section.

2.4.3 Stepper Motors

Stepper motors have impressive open-loop position control, often featuring 200 steps per rotation or 1.8° per step. This level of precision negates the need for any gearbox when driving small peristaltic pump heads. However, tradeoffs of switching to stepper motors come in the form of increased cost, complexity, and power consumption.

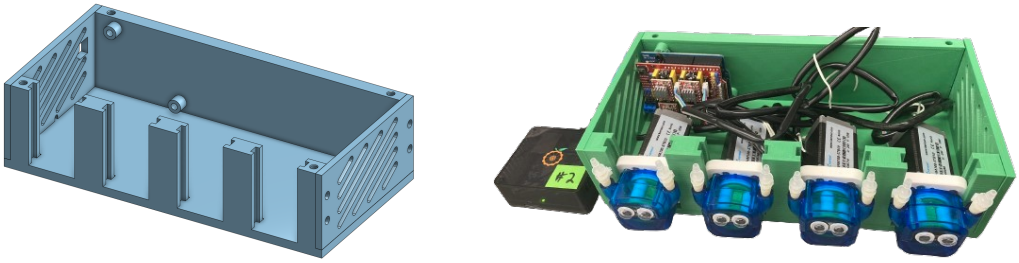
As previously discussed, driving these motors is not as trivial as applying a DC voltage across two wires. Generally, dedicated driving boards are used to simplify this process, as precisely timed voltage pulses on two motor poles is required. An adequate external power supply is also needed to maintain current flow through at least one of the motor windings at all times. For these reasons, the commercial off-the-shelf solution described previously was used.

One major benefit of using dedicated stepper motor drivers is their ability to microstep the motor by approximating phase-shifted sinusoidal currents in each winding. Thus, resolution is further increased by subdividing each step. Here, $1/8$ microstepping was used, resulting in 1600 microsteps per revolution or 0.225° per step. This technique does reduce the incremental torque of each step per Equation (2.7), where μ is the step divisor. However, a relatively small amount of torque is required

to drive the peristaltic pump heads used here.

$$\tau_{\mu} = \tau_{full} \sin\left(\frac{\pi}{2\mu}\right) \tag{2.7}$$

A housing for this system was also fabricated. Using CAD software (PTC Onshape), the model shown in Figure 2.7A was created.[32] This was 3D printed on a fused-deposition 3D printer (Lulzbot TAZ 6) using polylactic acid filament (Polymaker PolyLite; Polymaker LLC; Shanghai, China). The assembled system is shown in Figure 2.7B.



(A) **(B)**

Figure 2.7: (A) CAD model of the stepper motor and microcontroller housing. (B) Assembled pumping system with control server (left).

2.5 System Performance Comparison

The custom pumping system proved to work quite well during initial testing. However, variations in the DC motors, planetary gearbox efficiencies, and assembly process necessitated a multi-variable calibration. The first step of the calibration process is to determine the lowest PWM duty cycle that prevents stalling of each motor to drive the peristaltic pumps as slowly as possible. Only once the slowest smooth rotation possible was established could calibration of pumped volume versus time occur. This two-factor optimization was not only time-consuming, but also resulted in pump assemblies with varying resolutions. Despite this, proper calibration resulted in acceptable performance. Multiple pumps were able to achieve resolutions of $50\,\mu\text{L}$ with coefficients of variation less than $10\,\%$.

To test the experimental performance of these pumps, they were used to feed two bags of packed RBCs that were stored for six weeks under normoglycemic conditions. Details of the methodology are discussed in Chapter 3. Glycemic control was well-maintained near $5\,\text{mmol}\,\text{L}^{-1}$ throughout storage as shown in Figure 2.8. However, shortcomings with this pumping system quickly became apparent. While performance at room temperature was satisfactory, operation in cold storage at $4\,^{\circ}\text{C}$ was poor.

Likely due to temperature-induced contraction of the ring gears, binding of the gearbox was

a frequent issue. This resulted in inconsistent pumped volumes, often times requiring manual intervention to ensure proper feeding. This ranged from remeasuring and resubmitting glucose values to manually injecting the bags with feeding solution. A total of 12 manual interventions were required across both bags throughout the storage period.

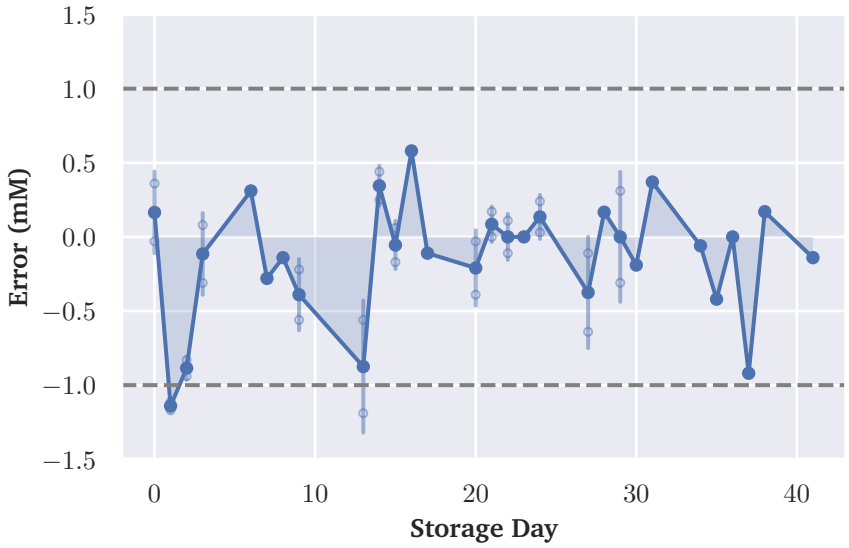


Figure 2.8: Glucose error in stored blood bags throughout a storage period using a custom pumping system (mean $\pm$ SD, $n = 1 - 2$). Manual intervention to ensure adequate feeding was required a total of 12 times across both bags.

Following this somewhat unsuccessful trial, the decision to use more reliable commercial components was made. In contrast, calibration of the stepper motor system is significantly easier. Since the angular displacement of the peristaltic pumps can be controlled very precisely, calibration of pumped volume versus rotation angle is simple. Figure 2.9 shows calibration curves for four of these pumps.

Although the calibration response of these pumps is highly linear, there is a large amount of variation in the triplicate measurements. The coefficients of variation for each of the pumps are shown in Figure 2.10. Note that the variation in pumped volume is very low when the angular displacement is an increment of $1/3$ of a rotation. This is because the peristaltic pump heads have three rollers; this will be elaborated on in the next section.

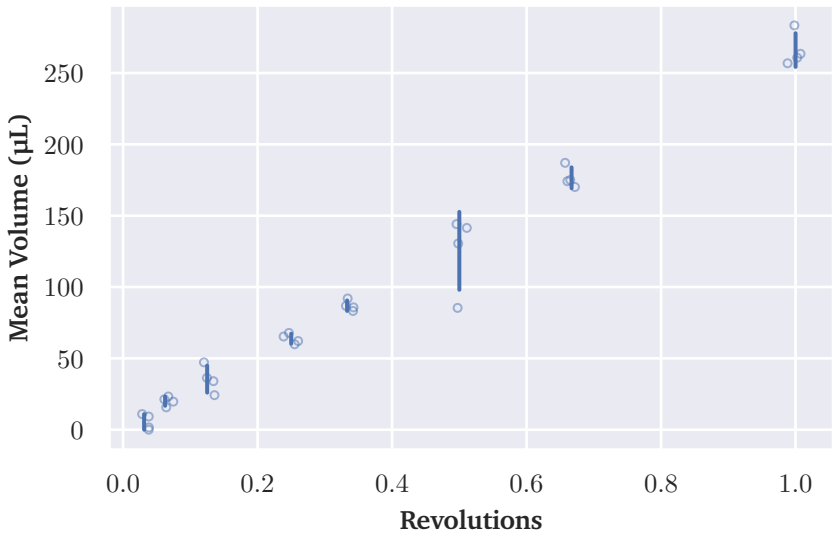


Figure 2.9: Volume dispensed versus rotation for each of the stepper motor peristaltic pumps of the feeding system (mean ± SD, $n = 4$). Each pump was measured in triplicate.

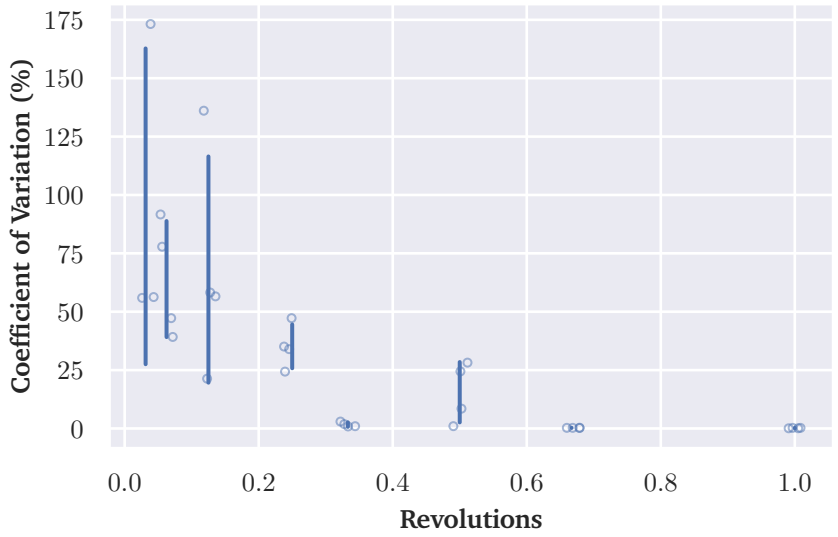


Figure 2.10: Coefficient of variation of volume dispensed versus rotation for each of the stepper motor peristaltic pumps of the feeding system (mean ± SD, $n = 4$). Each pump was measured in triplicate.

2.6 Discussion

In general, development of the second-generation pumping system was a success. The first iteration used a 3D printed, custom designed pumping system. This worked well and succeeded as a prototype. However, its performance in experimental conditions was underwhelming. This led to the development of a second iteration utilizing commercial components. Although the stepper motors

are more complex, their precision and reproducibility outweighs this detriment. The experimental performance of the second iteration of this second-generation feeding system will be further discussed in Chapter 3.

As noted, there is a large variation in pumped volume whenever the rotation angle is not a multiple of $120°$. All standard peristaltic pumps have a pulsatile flow due to the nature of their operation; as each roller passes over the output section of tubing, the flow is briefly interrupted.[33] The frequency of these pulsations is determined by the number of rollers in the pump head. Figure 2.11 shows an example of this pulsatile flow.

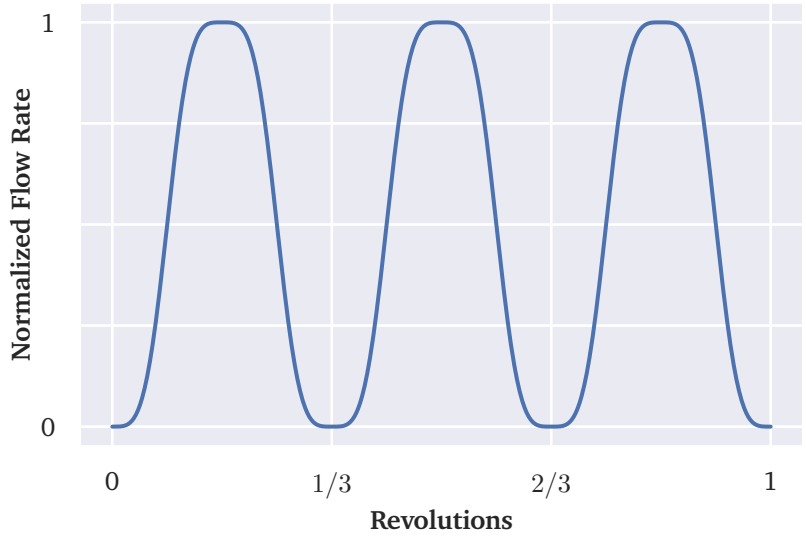


Figure 2.11: Example pulsatile flow rate from a peristaltic pump with three rollers.

In this system, the three-roller pump heads result in a pulsatile period of $1/3$ of a rotation. In other words, rotation angles that are a multiple of $120°$ will always pump consistent volumes. However, all other rotations will be highly variable due to phase shifting of the pulsations.

This leads to two possible pumping paradigms: discrete boluses of a full $1/3$ rotation, or "continuous" infusion limited only by motor resolution. While the discrete approach allows for much higher precision in metering the exact volume being pumped, it is worth considering that this precision is far greater than necessary. The volume of each stored blood bag is known only to within approximately $500\,\mu L$, while the error is known pumping volume is limited to far less than $95\,\mu L$.

In contrast, the "continuous" approach allows for much slower infusion of the feeding solution, allowing the glucose to diffuse more consistently throughout the stored blood bag. Larger boluses of

feeding solution may lead to a strong glucose gradient near the tip of the dispensing needle, exposing RBCs in that region to significant hyperglycemia. As the total volume required over a six-week storage period is many times greater than $95\,\mu L$, the detriments of this approach may be insignificant as overall accuracy will remain high.

In Chapter 3, both approaches are tested. Both were successful in maintaining normoglycemia during six weeks of storage, and neither resulted in significantly better glycemic control than the other. For these reasons, and the potential benefits of "continuous" infusion, it is recommended that the pumping system be limited only by the resolution of the stepper motors rather than the precision of the peristaltic pump heads. If greater volumetric precision is required, more rollers or smaller tubing may be used with a discrete pumping strategy.

Overall, the second-generation feeding system was able to significantly improve on the initial concept. The switch to peristaltic pumps results in a much more robust system that better aligns with the regulatory requirements for blood storage set by the FDA. Stepper motors with higher quality pump heads has lead to much better dispensing resolution, as well. The valve-based system could only dispense down to approximately $175\,\mu L$, while this system can achieve high-precision dosing down to approximately $95\,\mu L$.[19] While precision suffers, "continuous" pumping can also be used with nominal resolutions down to nearly $160\,nL$.

Using a centralized control server also presents several benefits. It allows for the addition of more pumps to scale the system as needed and includes individual bag management to improve experimental control and flexibility. In addition to providing a user interface for management of the blood bank, an improved dosing algorithm can include glucose feedback to compensate for setpoint error. These improvements not only greatly improve the usability of the system for *in vitro* studies, but provide a path toward commercial adaptation by improving translational potential and likelihood of regulatory approval.

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CHAPTER 3

NORMOGLYCEMIC BLOOD STORAGE AND STORED RED BLOOD CELL REJUVENATION

3.1 Introduction

Blood transfusions are important procedures necessary for managing various anemias, stabilizing patients during major surgeries, and replacing blood loss due to trauma.[1–4] With two in every five critically ill patients receiving a transfusion, over 10.5 million units of red blood cells (RBCs) are transfused in the United States each year.[4,5] While transfusions are life-saving interventions, they are also accompanied by risks. Transfusion-transmitted infections, immune-mediated reactions, sepsis, and transfusion-associated circulatory overload (TACO) are all relevant complications that must be considered.[6,7] The leading cause of transfusion-related death in the United States, TACO, is caused by rapid transfusion of large volumes leading to hypervolemia.[8,9] On average, each transfusion recipient will receive two to five units, or $700\,\text{mL}$ to $1750\,\text{mL}$, of RBCs.[4]

Transfusion efficacy is measured by post-transfusion recovery of RBCs. This is generally measured by radiolabeling an aliquot of a unit of RBCs and determining the fraction of labeled cells that remain in the circulation 24 hours later. The Food and Drug Administration (FDA) mandates all RBC storage systems maintain *in vitro* hemolysis below $1\,\%$ and *in vivo* 24 hour post-transfusion recovery over $75\,\%$ at the end of a storage period.[10] The development of detrimental biochemical and physical changes in RBCs throughout storage negatively impacts post-transfusion recovery and clinical outcomes.[11,12] These RBC damages are collectively referred to as the *storage lesion*.

The storage lesion includes metabolic, oxidative, and physiological components.[13] As storage progresses, glycolytic waste products drive the pH of the blood down to around 6.5.[13] This decreases the activity of important metabolic processes within the RBC.[12] Ultimately, reduced glucose flux through glycolysis and the pentose phosphate pathway results in decreases in production of the key reducing agent reduced nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP).[13] This prevents regeneration of the potent antioxidant glutathione, increases sorbitol production through the polyol pathway, and inhibits 2,3-diphosphoglycerate (2,3-DPG) formation through the Luebering-Rapoport shunt (which is required for proper oxygen-hemoglobin dissociation).[12] Together, this ensemble of dysfunction causes oxidative damage to proteins and lipids which impair RBC function and reduce viability post-transfusion.

Physiological changes to RBC throughout storage are stark. Microvesiculation causes membrane loss that, with oxidative damages and ATP depletion, impairs cellular deformability.[12–14] Membrane maintenance is an ATP-dependent process, and as ATP is depleted severe morphological alterations occur.[12,15,16] Structural changes through oxidative damages and loss of membrane surface area contribute to this process, known as echinocytosis.[12,13] Healthy RBC morphology, a biconcave disk, degrades over time forming first bumpy then spiky projections until membrane loss results in spheroid cells called spherocytes. These physiological changes have detrimental effects on blood rheology, increasing viscosity and decreasing flow in the microvasculature.[13]

By reducing the impact of the storage lesion and improving transfusion efficacy, smaller transfusion volumes may be used to attain similar therapeutic benefits. In turn, this may help to reduce both iatrogenic complications of blood transfusion and reduce the occurrence of transfusion-related death due to TACO. Throughout the history of transfusion medicine, large improvements in blood storage have come about primarily through the development of improved additive solutions.[17] These solutions help to preserve RBCs throughout storage by providing them with nutrients and enable easier transfusion by reducing the viscosity of stored RBCs.[18] However, current FDA-approved additive solutions contain extreme amounts of glucose (up to $111\,\mathrm{mmol\,L^{-1}}$) which have been implicated in the development the storage lesion.[19]

Previous reports from the Spence laboratory have demonstrated beneficial effects when storing RBCs in normoglycemic additive solutions. In flow-based microfluidic platforms, extracellular ATP release from RBCs is significantly increased relative to standard hyperglycemic additive solutions.[20–23] Downstream, this effect has been shown to increase nitric oxide (NO) production from endothelial cells exposed to this *in vitro* blood flow.[21] Interestingly, intracellular ATP is not increased in normoglycemic storage.[21] This is further corroborated by equivalent levels of lactate production in both storage conditions, indicating equivalent rates of glycolysis and thus ATP production.[21,24] However, filtration-based assays have shown normoglycemic storage increases RBC deformability, potentially explaining this increase in ATP release.[23]

Additionally, normoglycemic storage results in much lower levels of sorbitol accumulation within RBCs.[22,24] This is a marker of both oxidative stress and osmotic stress which may play a role in the development of the storage lesion.[22] It follows then that hyperglycemic storage may result in RBCs prone to osmotic lysis, which has also been reported by the Spence laboratory.[22,24]

Beyond normoglycemic storage, rejuvenation of store RBCs is also an area of ongoing investigation. There is one FDA-approved rejuvenation solution, though its clinical application is rare due to a laborious protocol (thus it is primarily used in blood banking research).[25] Regardless, efforts into improving the quality of RBCs for transfusion include improved rejuvenation methods.

There is sufficient evidence to show that washing of RBCs pre-transfusion improves aspects of the storage lesion.[26] Though further investigation is required, preliminary studies show clinical improvements in the form of mitigated transfusion-related acute lung injury and decreased mortality.[26] Interestingly, washing with buffers containing serum albumin has shown to reverse echinocytosis by a considerable amount.[27,28] The mechanism behind this effect is still under investigation, though washing in buffers without serum albumin seemingly does not improve RBC morphology.[26,28]

With significant improvements in stored RBC quality demonstrated both with normoglycemic storage and after washing in an albumin-containing buffer, it naturally follows that combining these approaches may show further improvements. Here, we combine these methods to investigate the effects of RBC rejuvenation in the context of normoglycemic blood storage. Physiological, functional, and biochemical changes throughout storage were assessed.

To enable these studies, the Spence laboratory has developed a semi-autonomous, remotely-controlled feeding system for normoglycemic RBC storage. This iteration improves on previously published work to enable better experimental control, allowing for more rigorous study of low-glucose blood storage. With higher volumetric precision to previous systems, feeding solutions with increased glucose concentration may be used. In addition to diluting the stored RBCs less, this also enables an isotonic feeding solution to be tested without NaCl supplementation. This may itself play a role in reducing echinocytosis, as it has been suggested feeding solutions containing chloride anions may contribute to detrimental morphological changes.[29]

3.2 Methods

All reagents were procured from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

3.2.1 Storage and Semi-Automated Glucose Feeding

All blood was collected via forearm venipuncture from informed and consenting donors following procedures approved by the Biomedical and Health Institutional Review Board at Michigan State University. Approximately 7 mL of whole blood was collected into untreated 10 mL glass Vacutainer

tubes (BD; Franklin Lakes, NJ) containing 1 mL of in-house made citrate phosphate dextrose solution (CPD) or normoglycemic citrate phosphate dextrose solution (CPD-N) anticoagulants (see Table 1.7). The solutions were sterilized via autoclave at $121\,^{\circ}$C and 2.1 MPa for 30 minutes.

Six tubes of each condition were collected from all donors and allowed to sit at room temperature for 30 minutes with inversion every 5 minutes. Blood components were then separated by centrifugation at $2000\,\mathrm{gravitationalforceequivalent}(g)$ for 10 minutes, followed by aspiration of the plasma, buffy coat, and top 2 mm of packed RBCs. For each donor, the remaining RBCs were pooled by condition in a 50 mL conical tube and added in a $2{:}1$ ratio to either Additive Solution 1 (AS-1) or normoglycemic Additive Solution 1 (AS-1N) additive solutions (see Table 1.7) likewise sterilized by autoclave. The RBCs were then transferred into sterile 100 mL veterinary blood storage bags (Animal Blood Resources International, catalog number BG-DD-100-BAG; Stockbridge, MI). All steps following centrifugation took place in a biosafety cabinet to maintain sterility.

RBCs undergoing normoglycemic storage were then connected to the feeding system described in Chapter 2. All tubing was sterilized via ultraviolet germicidal irradiation and/or chemically disinfected using a $10\,\%$ bleach solution followed by a thorough flush using $18.2\,\mathrm{M\Omega\,cm}$ water. Sterilized 1.5 inch, 18-gauge blunt-tip needles were connected to the tubing and the entire system was primed with feeding solution. This solution contained either $100\,\mathrm{mmol\,L^{-1}}$ glucose with $100\,\mathrm{mmol\,L^{-1}}$ NaCl ($n = 4$) or $300\,\mathrm{mmol\,L^{-1}}$ glucose ($n = 8$) in $18.2\,\mathrm{M\Omega\,cm}$ water and was filter sterilized using a $0.22\,\mu$m pore Stericup system (MilliporeSigma; Burlington, MA). The needle was used to pierce the spike port and inserted fully into the bag, forming a tight seal between the port collar and the needle base. All RBC units were kept in cold storage at $2\,^{\circ}$C to $6\,^{\circ}$C for six to eight weeks.

3.2.2 Sampling of Stored Red Blood Cells

Blood was sampled periodically to monitor glucose concentrations in the bags and weekly to perform various experiments. A 1 mL syringe was used to remove aliquots of blood from each bag in storage via its sampling port. The graduations on the syringe were used to estimate the volume of each aliquot and were entered into the feeding system interface as previously described to maintain an accurate bag volume for feeding solution dosing. Blood glucose measurements generally required only a few microliters to be removed while weekly experimentation required approximately $300\,\mu$L to $400\,\mu$L.

3.2.3 Red Blood Cell Washing for Rejuvenation

Once drawn, larger samples were divided for further processing. Namely, one aliquot was to remain unwashed, one aliquot was to be washed in phosphate buffered saline (PBS), and one aliquot was to be washed with PBS supplemented with $0.4\,\%$ human serum albumin (PBS-A). The pharmaceutical-grade human serum albumin was purchased from CSL Behring (King of Prussia, PA). In further experiments, these solutions were substituted for a physiological salt solution (PSS) (in mmol L^{-1}: KCl, 4.7; $CaCl_2$, 2.0; NaCl, 140.5; $MgSO_4$, 1.2; Tris, 21.0; glucose, 5.5 and $5\,\%$ human serum albumin at pH 7.4) and a solution of PSS with $20\,\text{nmol}\,L^{-1}$ $ZnCl_2$ and $20\,\text{nmol}\,L^{-1}$ C-peptide. Washing consisted of diluting the aliquot to a hematocrit (HCT) of $1\,\%$ to $2\,\%$ in the washing buffer, incubating at room temperature for 10 minutes, and centrifuging at $2000\,g$ for 10 minutes. The supernatant was then aspirated off, and the packed RBCs were resuspended to $60\,\%$ HCT in their respective buffer.

3.2.4 Blood Glucose, HCT, and HbA1c Measurements

Glucose measurements were made approximately every three days to provide feedback to the feeding system. A commercial glucometer (AimStrip Plus; Germain Laboratories; San Antonio, TX) was used to measure glucose levels in the stored blood bags. However, due to the increased HCT of the stored blood (around $60\,\%$ versus approximately $42.5\,\%$ in whole blood), the glucometer was reporting values biased approximately $30\,\%$ lower than expected. Thus, a correction factor was developed and is shown in Equation (3.1).

$$\text{Glucose}_{actual} = \frac{0.575\,\text{Glucose}_{meter}}{1 - HCT} \tag{3.1}$$

HCT measurements were taken using a hematocrit centrifuge and microcapillary reader (StatSpin; HemoCue America; Brea, CA). The ratio of glycated hemoglobin (HbA1c) in the RBCs was measured using an FDA-approved analyzer (DCA Vantage; Siemens Healthineers; Erlangen, Germany).

3.2.5 Hemolysis Quantification

The degree of lysis in the storage bags was measured via spectroscopy using a microplate reader (SpectraMax M4; Molecular Devices; San Jose, CA). An aliquot of stored blood was centrifuged at $2000\,g$ for 15 minutes. Approximately $80\,\mu\text{L}$ of supernatant and $20\,\mu\text{L}$ of the cell pellet were collected. The supernatant was diluted tenfold and the packed RBC pellet was diluted 1000-fold in Drabkin's reagent. Absorbance was measured at $540\,\text{nm}$ and quantified against six standards prepared from

lyophilized human hemoglobin reconstituted and diluted in Drabkin's reagent to concentrations ranging from $0\,\mathrm{g\,L^{-1}}$ to $1\,\mathrm{g\,L^{-1}}$. Lysis percentage can then be calculated using the Equation (3.2).

$$\text{Lysis}(\%) = 100 \cdot \frac{[\text{Hb}_{supernatant}](1 - HCT)}{[\text{Hb}_{supernatant}] + [\text{Hb}_{cell\,pellet}]} \tag{3.2}$$

3.2.6 Stored RBC Morphology Assessment

Cellular morphology was assessed by calculating a morphological index (MI) in the method of Ferrell and Huestis based on established RBC shape classifications from Bessis.[30,31] RBC samples were drawn from each storage bag and diluted to $12.5\,\%$ HCT in AS-1 or AS-1N. Each sample was then fixed by drop-wise addition of $100\,\mu\mathrm{L}$ of $4\,\%$ glutaraldehyde in $100\,\mathrm{mmol\,L^{-1}}$ sodium phosphate buffer. Following a incubation for 60 minutes at room temperature, aliquots of fixed RBCs were pooled from each donor by storage condition to a final volume of $500\,\mu\mathrm{L}$. This pooling was necessary for logistical reasons, and classified RBCs are presumed to be a representative sample of all donors.

Scanning electron microscopy (SEM) slides were then prepared. First, a small amount of $1\,\%$ poly-L-lysine was placed in a dish with a glass coverslip and allowed to incubate for 10 minutes. The coverslip was then removed and gently rinsed with water. While still wet, two drops of the fixed RBCs were placed on the side of the coverslip treated with polylysine and allowed to settle for 10 minutes. The coverslip was again gently rinsed with water followed by submersion in a graded ethanol series ($25\,\%$, $50\,\%$, $75\,\%$, $95\,\%$) for 10 minutes each. This was followed by three 10 minute incubations in $100\,\%$ ethanol. A critical-point drier (EM CPD300; Leica Microsystems GmbH; Wetzlar Germany) was then used to dry each sample, followed by chemical vapor deposition of approximately $20\,\mathrm{nm}$ of osmium (NeoC-AT osmium coater; Meiwafosis Co., Ltd.; Shinjuku, Japan). SEM imaging at a 1200x magnification was performed using a $5.0\,\mathrm{kV}$ beam voltage with a working distance of $12\,\mathrm{mm}$. RBCs in each image (approximately 160 cells) were classified and assigned values as follows: stomatocyte $= -1$, discocyte $= 0$, echinocyte I $= 1$, echinocyte II $= 2$, echinocyte II $= 3$, spherocyte I $= 4$, spherocyte II $= 5$. See Figure 1.8 for a graphical representation of each morphology. Morphological indices were calculated by bootstrapping RBC classification values $10{,}000$ times to determine MI mean and $95\,\%$ confidence intervals for each sample.

3.2.7 Comparison of Relative RBC Bulk Deformability

Bulk deformability of RBCs was measured by filtration rate using a method adapted from Mataseje, et al.[32] Relative deformability between samples can be established by the fraction of RBCs that pass

through a membrane with pores smaller than $8\,\mu m$, the average diameter of an RBC.[33] To this end, centrifugal filters with a 5-micron pore size were used (Ultrafree-MC; MilliporeSigma; Burlington, MA). Samples were diluted in their respective buffers to $0.01\,\%$ HCT and $500\,\mu L$ of each aliquot was added to the filter tubes followed by centrifugation at $150\,g$ for 5 minutes. The filtration cups were then removed from each tube and the RBCs that passed through the filter were resuspended by repeated pipetting. Aliquots of $200\,\mu L$ of both filtered and unfiltered sample were transferred to a 96-well microplate. A flow cytometer (Accuri C6; BD; Franklin Lakes, NJ) was used to count RBCs in the pre- and post-filtration suspensions with agitation between samples to prevent cell settling. The filtration rate can be calculated by Equation (3.3) and compared to other samples as a measure of relative bulk deformability.

$$\text{Filtration rate}\,(\%) = 100 \cdot \frac{[\text{RBC}_{filtered}]}{[\text{RBC}_{unfiltered}]} \tag{3.3}$$

3.2.8 RBC Relative Size, Dispersity, and ζ-Potential Measurement

Dynamic light scattering (DLS) was used to quantify relative changes in size and dispersity of RBCs over time. A Zetasizer Nano ZS with folded capillary cuvettes was used (Malvern Panalytical; Worcestershire, United Kingdom). Refractive indices were set at 1.43 for the sample (phospholipids) and 1.33 for the diluent (water). Measurements were made at room temperature.

Samples were diluted to $1.2\,\%$ HCT in their respective buffers and $900\,\mu L$ aliquots were pipetted into cuvettes. Samples were stoppered and inverted several times directly before they were run. Size and dispersity measurements of RBCs were taken followed directly by ζ-potential measurements via electrophoretic light scattering using the same instrument and samples.

3.2.9 RBC Extracellular Vesicle Size and Dispersity Measurement

RBC-derived extracellular vesicles (EVs) released during storage were also measured using DLS. Absolute size and dispersity of normoglycemic and hyperglycemic samples was determined. The same instrument, capillaries, and parameters described above were used.

Approximately $1\,mL$ of blood was sampled from each storage bag and centrifuged at $2200\,g$ for 10 minutes using slow deceleration. A sample of $250\,\mu L$ of supernatant was removed and centrifuged again with the same parameters. Another aliquot of $200\,\mu L$ of supernatant was removed and diluted in $400\,\mu L$ of PBS. Samples were thoroughly mixed, pipetted into DLS cuvettes, and run as previously described.

3.2.10 Immunogenicity in an *In Vitro* Transfusion

Immunogenicity of stored RBCs was investigated by simulating an autologous transfusion and measuring the innate immune response from leukocytes. After a full six-week storage period, fresh whole blood was drawn from each original donor into heparinized Vacutainer tubes (BD; Franklin Lakes, NJ). A sample of autologous RBCs was drawn from the corresponding stored RBCs.

Stored RBCs were mixed with fresh whole blood from the same donor *in vitro* mimicking the ratio of an average RBC transfusion. Briefly, $200\,\mu L$ of stored RBCs were added to $800\,\mu L$ of fresh whole blood. The mixture was allowed to incubate at $37\,^{\circ}C$ for 1 hour.

The samples were then centrifuged at $500\,g$ for 5 minutes. Approximately $500\,\mu L$ of supernatant was collected and split into two aliquots. Each aliquot was used to quantify an immune response biomarker mediated by white blood cells: interleukin 6 (IL-6) and tumor necrosis factor (TNF). These were assayed using commercial enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen; Waltham, MA).

3.2.11 Intracellular RBC ATP Quantification

Intracellular ATP of stored RBCs was quantified using a standard luciferin/luciferase luminescence assay. ATP standards were prepared from ATP disodium hydrate in $18.2\,M\Omega\,cm$ water via serial dilution. Firefly lantern extract and D-luciferin (Gold Biotechnology; St. Louis, MO) were used to prepare a luciferin/luciferase solution in $18.2\,M\Omega\,cm$ water.

RBCs ($10\,\mu L$) were lysed and diluted by the addition of $3.99\,mL$ of $18.2\,M\Omega\,cm$ water. Aliquots of $100\,\mu L$ each were added in triplicate to a 96-well microplate along with the ATP standards. One well at a time was tested by the addition of $50\,\mu L$ of luciferin/luciferase solution. A microplate reader (SpectraMax M4; Molecular Devices; San Jose, CA) was used to quantify luminescence for each sample after a fixed reaction time period.

3.3 Results

3.3.1 General Blood Measurements

Standard measurements such as glucose level, HbA1c, and HCT were made weekly. Glucose monitoring allowed for assessment of feeding system performance, incorporation of feedback to correct setpoint error, and supervision to ensure normoglycemia was maintained throughout storage. Due to the high concentration of glucose in AS-1, it was expected there may be a modest increase in

HbA1c over six weeks. Measurement of HCT was necessary for various experimental protocols and acted as a metric to check feeding system volume deliveries were approximately correct.

The second-generation RBC feeding system proved to work quite well. As shown in Figure 3.1, average blood glucose values were well-maintained throughout storage. While there were some individual units that experienced excursions outside the target window of $4\,\mathrm{mmol\,L^{-1}}$ to $6\,\mathrm{mmol\,L^{-1}}$, these were corrected and each value returned to the normoglycemic range.

The total deviation in glycemic control, termed here *absglycemia*, during the storage period can be quantified by integrating the setpoint error. This is shown mathematically in Equation (3.4). For the twelve units of RBCs shown here, the feeding system absglycemia was $-2.21\,\mathrm{mmol\,week\,L^{-1}}$.

$$\text{Absglycemia} = \int_0^t \text{error}_{setpoint}\,\mathrm{d}t \tag{3.4}$$

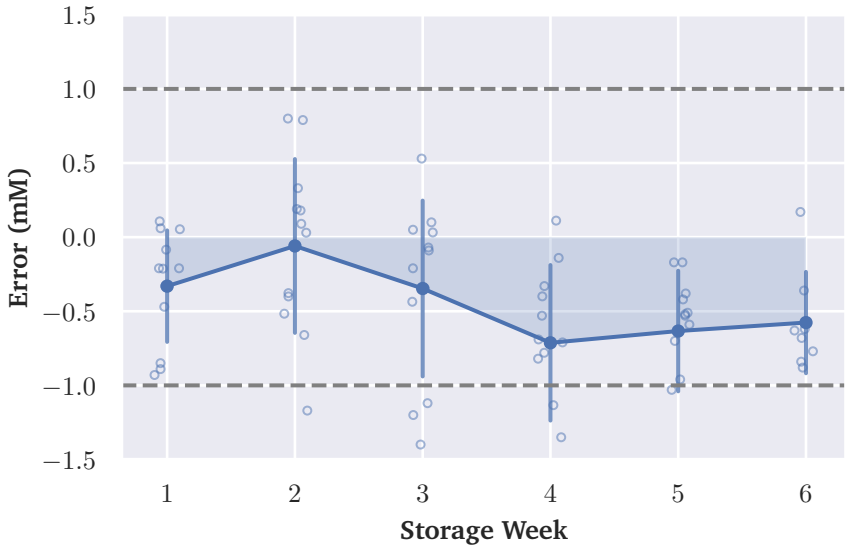


Figure 3.1: Setpoint error of glucose levels of AS-1N blood bags throughout storage (mean ± SD, $n = 12$). The target window (± 1 mM) is outlined in dashed grey. Average glucose levels in the bags were well-maintained for all six weeks of storage.

This normoglycemia was beneficial in controlling the increase in HbA1c versus RBCs stored in AS-1. Figure 3.2 shows that there was no significant change in HbA1c for normoglycemic RBCs. In contrast, there was a statistically significant difference between storage conditions after the third week of storage. This indicates that RBCs stored in AS-1 experience cellular glycation which may contribute to storage lesion-associated dysfunction.

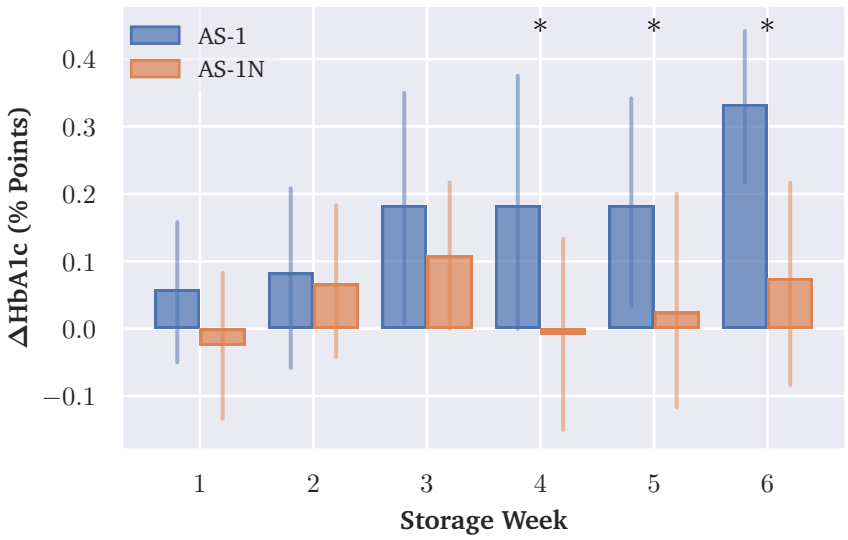


Figure 3.2: Changes in percent HbA1c between AS-1 and AS-1N blood bags throughout storage (mean $\pm$ 95 % CI, $n = 12$). Statistically significant differences between conditions and within weeks are indicated by $*$, where $p < 0.05$ with Holm–Šidák correction.

The HCT of each storage condition was also monitored throughout storage. As RBCs stored in AS-1 were unaltered during storage, no appreciable difference in HCT is expected. Indeed, Figure 3.3 verifies RBC concentration within the AS-1 storage units remained consistent. The variation may be explained by measurement error, as the technique used was rather imprecise.

In contrast, RBCs stored in AS-1N were administered feeding solution throughout storage. This causes dilution of the HCT, which can be estimated based on the glucose utilization curve shown in Equation (1.2). The degree of this dilution is dependent on the glucose concentration of the feeding solution. As two different feeding paradigms were tested here, two different models are shown in Figure 3.4.

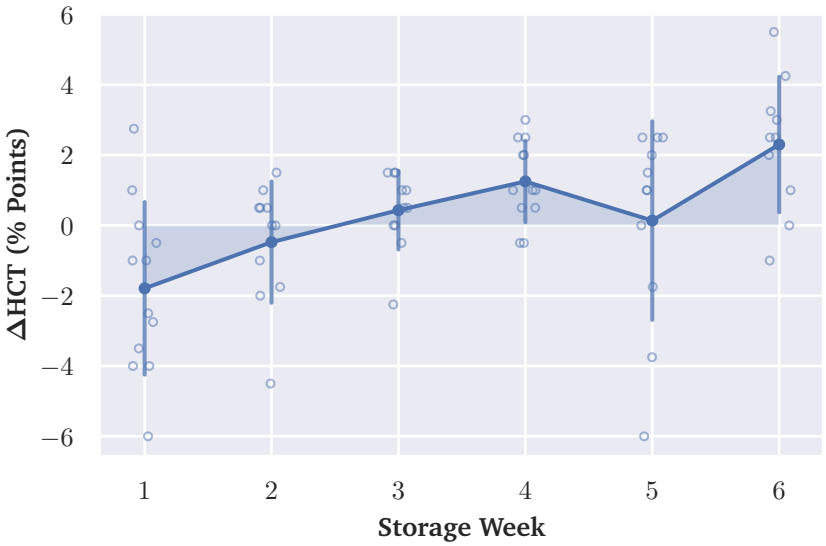


Figure 3.3: Change in percent HCT of AS-1 blood bags throughout storage (mean ± SD, $n = 12$).

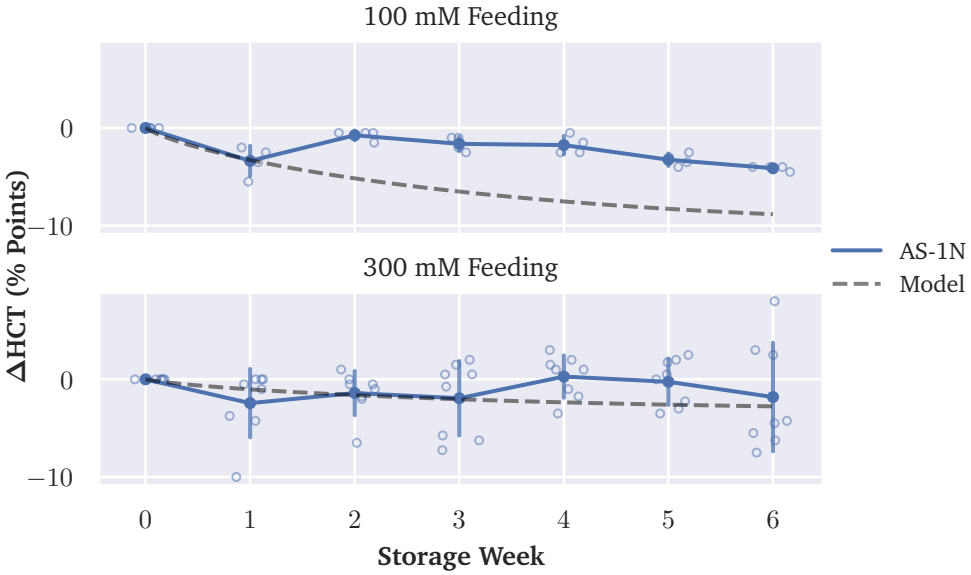


Figure 3.4: Changes in hematocrit of AS-1N blood bags throughout storage with two different feeding regimens (mean ± SD, $n = 4$ or $n = 8$, respectively). Models of expected ΔHCT are shown in the dashed grey lines and are based on the glucose utilization curve in Equation (1.2). The total volume delivered to each bag is inversely proportional to the glucose concentration in the feeding solution.

Similarly to absglycemia, the error in the HCT models can be quantified. A more well-known metric, the root mean squared error (RMSE), is shown in Equation (3.5). The RMSE for the $100\,\mathrm{mmol\,L^{-1}}$ glucose feeding solution was 4.55 percentage points. For the $300\,\mathrm{mmol\,L^{-1}}$ glucose feeding solution, it was 1.60 percentage points. Thus, the model was more accurate for the more concentrated feeding

solution, although it was relatively accurate in the other case as well.

$$\text{RMSE} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(y_{i,\,observed} - y_{i,\,model})^2} \tag{3.5}$$

As the average glucose utilization of RBCs is known, the expected glucose drop for RBCs stored in AS-1 can also be calculated. Both the expected and observed data are shown in Figure 3.5. The RMSE for this model was 2.44 mmol L^{-1}, showing the glucose utilization model is quite accurate at least in the case of hyperglycemic RBCs.

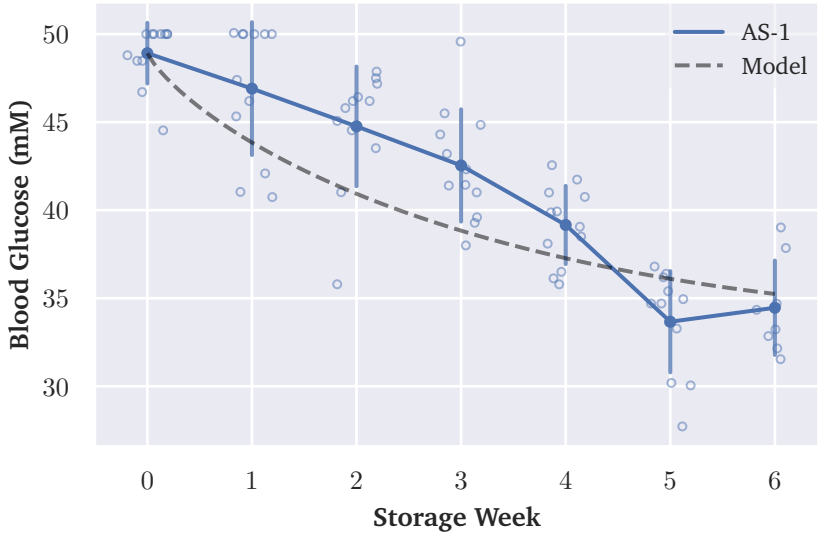


Figure 3.5: Change in glucose level of AS-1 blood bags throughout storage (mean ± SD, $n = 12$). A model of the expected glucose drop is shown in dashed grey. Glucose values are clipped at 50 mmol L^{-1} due to measurement limitations.

3.3.2 Normoglycemic Storage Hemolysis

As previously discussed, the FDA mandates that all blood storage systems must maintain an end-point hemolysis level below 1 %. This is necessary to prevent transfusion of a large amount of free hemoglobin, which may lead to iron overload and other serious complications. The lysis of RBCs stored in AS-1N has previously been shown to meet this standard.[24] However, these measurements were made using the first-generation RBC feeding system described elsewhere.[24]

Thus, these measurements were made again during one round of storage. This FDA requirement was again confirmed for AS-1N in these studies with the second-generation feeding system as shown in Figure 3.6. Extended storage of RBCs has always been a goal in the development of AS-1N. Thus,

this round of stored RBC units extended storage to eight weeks rather than the FDA-approved six weeks used in AS-1 storage.

Though there was no significant difference between storage conditions, hemolysis did increase during the last two weeks of storage. However, even with an additional week of storage the hemolysis level was not statistically greater than 1 %. It is well-known that hemolysis as a storage endpoint is highly donor-dependent.[10] Therefore, more studies are required to determine if extended storage with AS-1N is feasible.

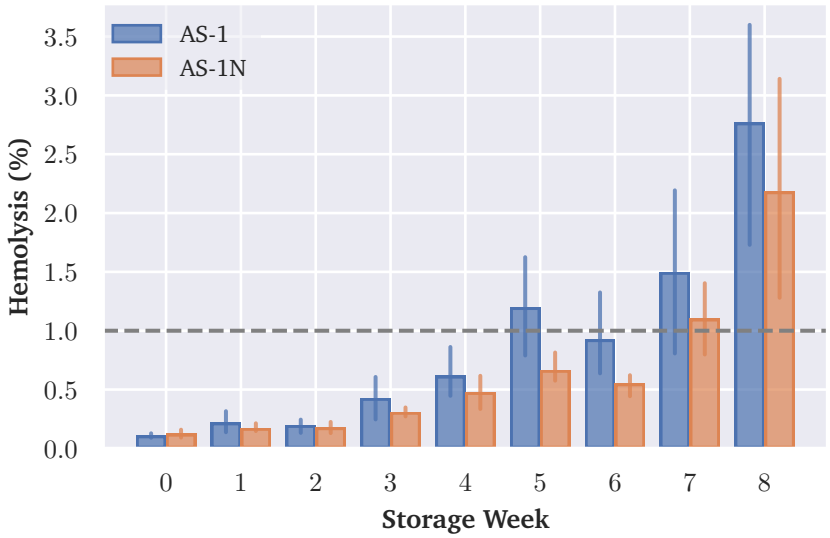


Figure 3.6: Hemolysis percentage within each storage condition (mean $\pm$ 95 % CI, $n = 4$). The FDA threshold (1 %) is shown in dashed grey. RBC lysis in AS-1N was controlled through a full six-week storage period but not in extended storage. There were no statistically significant differences between storage conditions.

3.3.3 RBC Morphological Indices

Measurement and comparison of MI is an important aspect in demonstrating the benefits of normoglycemic storage. Previous reports have shown storage in AS-1N has lower mean MI throughout storage compared to AS-1, though not statistically significant in most cases. A large discrepancy in the proportion of discocytes between AS-1 and AS-1N has been reported as well. Confirmation of these findings, as well as investigation into the effects of rejuvenation in normoglycemic storage, was the main impetus for these studies.

Here, there was no difference in MI between AS-1 and AS-1N solutions, though AS-1N did frequently show small but insignificant reductions in MI relative to AS-1N. In both conditions, significant

increases were observed as storage duration increased indicating progression of echinocytosis. This comparison is shown in Figure 3.7, while the average MI of both conditions at the beginning and end of storage is shown in Figure 3.8.

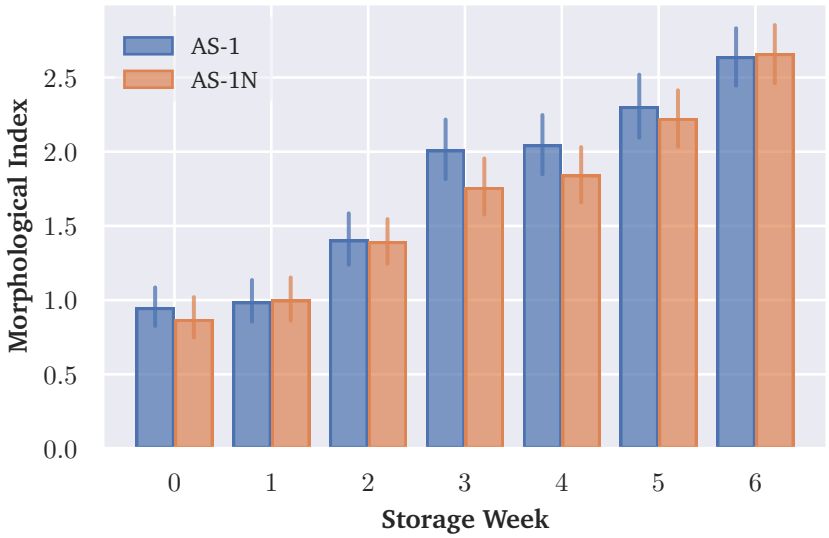


Figure 3.7: Morphological indices of RBCs stored in AS-1 and AS-1N over time (bootstrapped mean $\pm$ 95 % CI, $n = 8 - 12$).

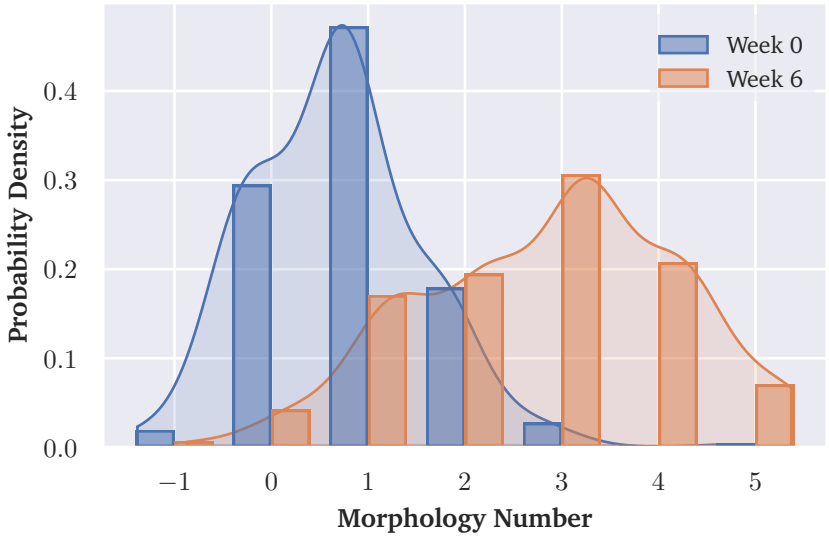


Figure 3.8: Probability histogram of morphological classification of RBCs at the beginning and end of a storage period ($n = 8$). A KDE is overlaid on each histogram to show the potential continuous population distribution.

Figure 3.8 also shows a kernel density estimate (KDE) of the morphology histograms representing a more continuous distribution. As the classification system is discrete but cellular morphology

changes continuously, this illustrates a smoother distribution of RBC morphologies. In other words, the KDE estimates a histogram with bin widths approaching zero to demonstrate a more granular characterization.

Next, rejuvenation of stored RBCs to reverse echinocytosis by washing in a buffer containing serum albumin was investigated. Figure 3.9 shows the results of these studies. Seemingly any washing of RBCs improved MI, though the effect is limited when the wash buffer does not contain serum albumin. When washed using a buffer supplemented with human serum albumin, the improvement in MI is significantly improved. Example SEM images (magnified to show detail) are given in Figure 3.10 showing the stark reversal of echinocytosis in Figure 3.10C.

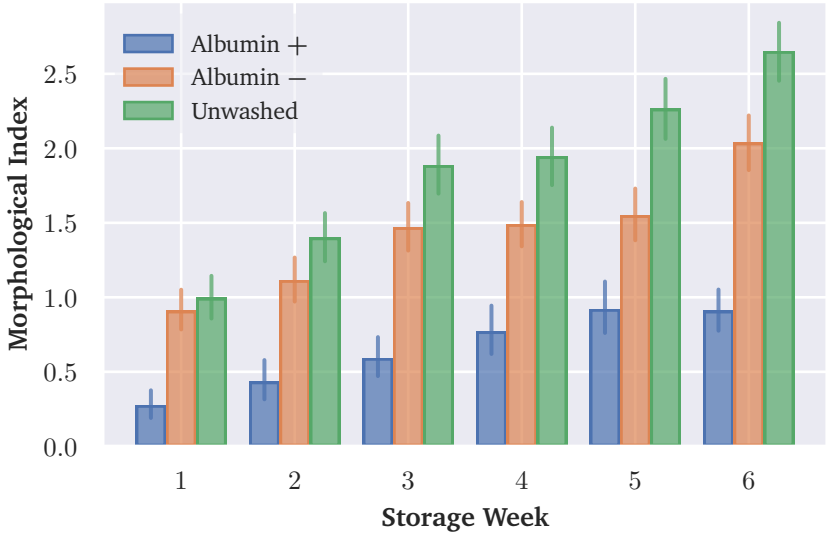
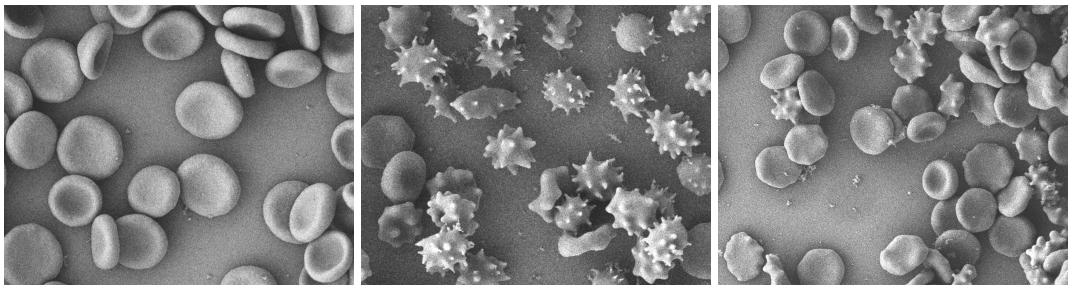


Figure 3.9: Morphological indices of RBCs either unwashed or washed in a buffer with or without albumin over time (bootstrapped mean $\pm$ 95 % CI, $n = 4 - 12$).



(A) **(B)** **(C)**

Figure 3.10: SEM images of RBCs showing the variation in cellular morphology. (A) Fresh control RBCs. (B) RBCs stored in AS-1 for six weeks without rejuvenation. (C) RBCs stored in AS-1 for six weeks after rejuvenation with a buffer containing human serum albumin.

One important finding from previous investigations of AS-1N was the preservation of discocytes during early storage. Figure 3.11 shows this was not replicable in these studies. Unfortunately, further investigation has found no significant differences in discocyte proportion between either condition throughout six weeks of storage. However, washing stored RBCs in a buffer containing serum albumin does have a significant impact on discocyte proportion as shown in Figure 3.12. Interestingly, in the absence of albumin discocyte proportion is only marginally improved.

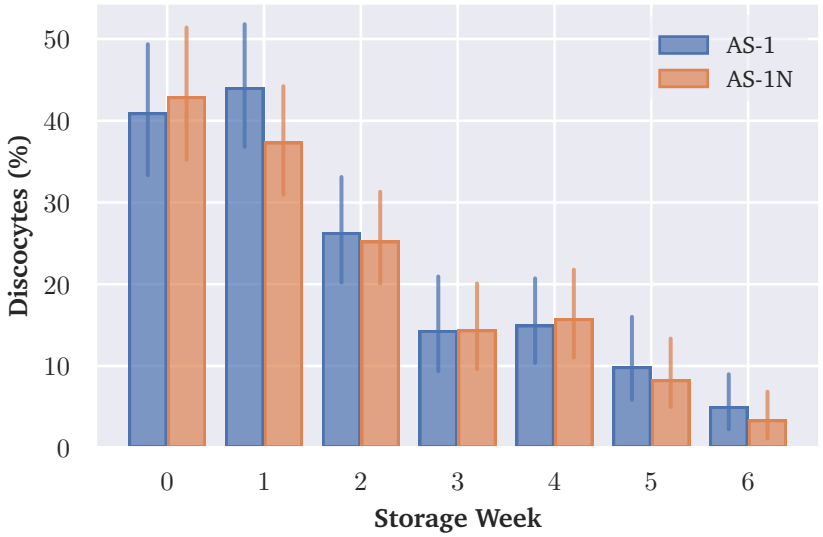


Figure 3.11: Discocyte percentage of each RBC storage condition over time (bootstrapped mean $\pm$ 95 % CI, $n = 8$).

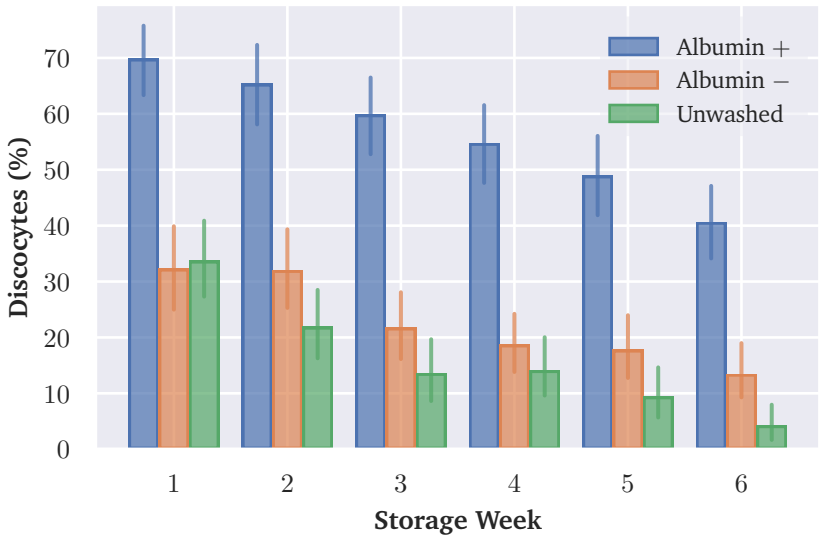


Figure 3.12: Discocyte percentage of RBCs either unwashed or washed in a buffer with or without albumin over time (bootstrapped mean $\pm$ 95 % CI, $n = 4 - 8$).

While it was hopeful removing NaCl from the feeding solution would improve MI, Figure 3.13 shows this was not the case for either storage condition. It is possible there was some effect during the first week of storage, though more investigation would be required to confirm this. Further into storage, no differences between condition or feeding solution were observed.

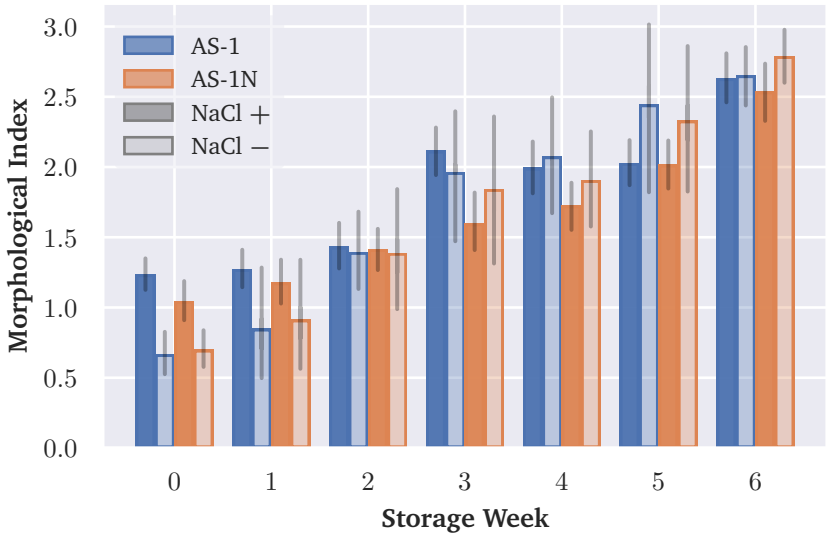


Figure 3.13: Morphological indices of RBCs by both additive solution and feeding solution (bootstrapped mean $\pm$ 95 % CI, $n = 4 - 8$).

3.3.4 Stored RBC Deformability

Previously, it has also been reported storage in AS-1N improves RBC deformability compared to AS-1.[23] These studies were again unable to replicate these findings. While the methodologies between those studies and the centrifugal filtration method used here are similar, there are some differences that may contribute to the contrasting results.

Figure 3.14 shows no statistically significant differences between storage conditions. However, rejuvenation of stored RBCs does indeed significantly improve RBC deformability as measured by bulk filtration rate. This effect is seemingly independent of the presence of serum albumin in the washing buffer (preliminary data not shown).

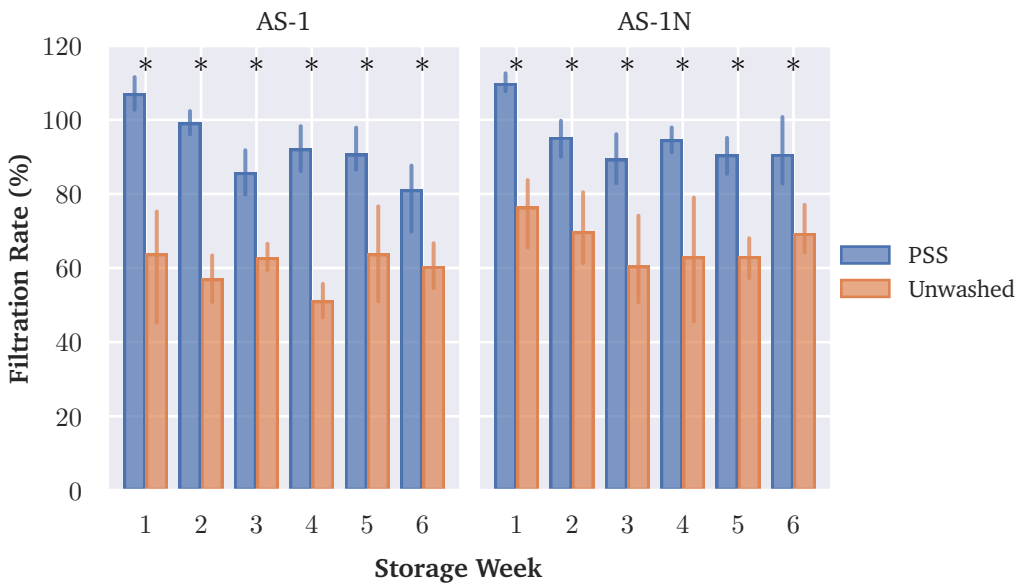


Figure 3.14: Cellular deformability throughout storage as measured by bulk filtration rate (mean $\pm$ 95 % CI, $n = 4$). AS-1 stored cells are shown on the left and were either unwashed or washed with PSS. AS-1N stored cells are shown on the right with similar washing conditions. Statistically significant differences are indicated by $*$, where $p < 0.05$ with Holm–Šidák correction.

3.3.5 RBC Size and Dispersity

Relative size and dispersity of RBCs throughout storage was measured by DLS. This measurement technique reports hydrodynamic size presuming a spherical particle, thus, RBC morphology will result in anisotropic responses. This being the case, and RBCs being around the maximum size the instrument can accurately measure, all measurements were normalized to the baseline when storage began.

RBC volume is not expected to change drastically during storage, though small changes may occur as membrane is lost. A caveat to this measurement technique, as stated above, is the anisotropy of measurement when the analyte is not a sphere. Thus, as echinocytosis progresses, it is possible the accuracy increases over time.

Similarly, the polydispersity index (PDI) is expected to change as RBC morphology shifts toward spherocytes. Regardless, these measurements, when normalized, may still be useful for making relative comparisons. Both size and dispersity, indicating homogeneity of RBC morphology, are shown in Figure 3.15 and Figure 3.16, respectively.

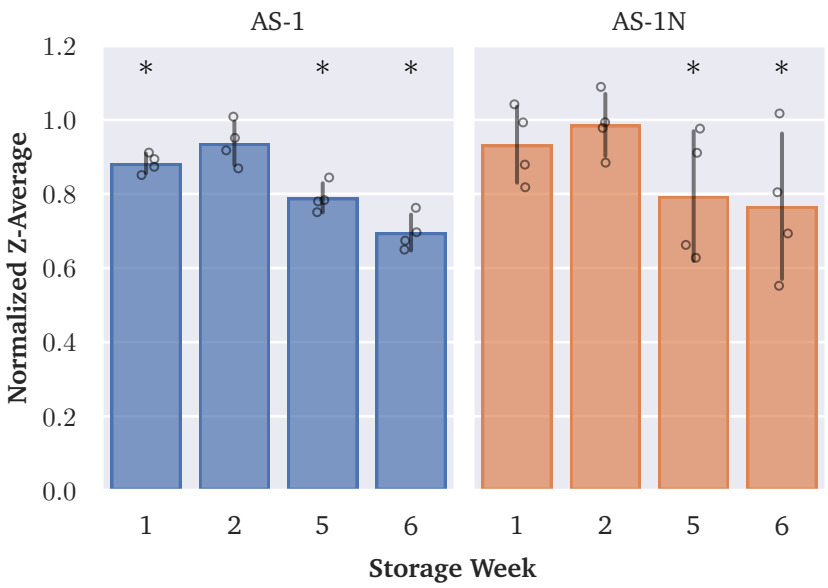


Figure 3.15: Normalized RBC size throughout storage as measured by DLS (mean ± SD, $n = 4$). AS-1 stored cells are shown on the left while AS-1N stored cells are shown on the right. Statistically significant differences relative to baseline are indicated by $*$, where $p < 0.05$ with Holm–Šidák correction.

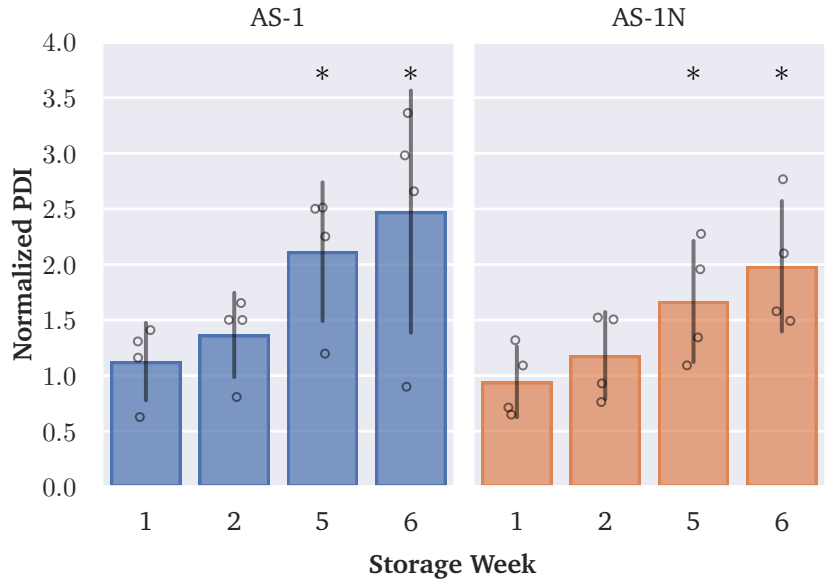


Figure 3.16: Normalized RBC PDI throughout storage as measured by DLS (mean ± SD, $n = 4$). AS-1 stored cells are shown on the left while AS-1N stored cells are shown on the right. Statistically significant differences relative to baseline are indicated by $*$, where $p < 0.05$ with Holm–Šidák correction.

3.3.6 RBC ζ-Potential

The DLS instrument was also used to measure RBC ζ-potential via electrophoretic light scattering. This has implications in cellular aggregability and hemorheology. Briefly, as ζ-potential decreases, electrostatic repulsive forces also decrease, allowing aggregation.

The results of these measurements are shown in Figure 3.17. Reports indicate RBCs in hyperglycemic storage have a decreased ζ-potential.[34] However, this again was not able to be replicated in these studies. There were no statistically significant differences between storage conditions or with rejuvenation.

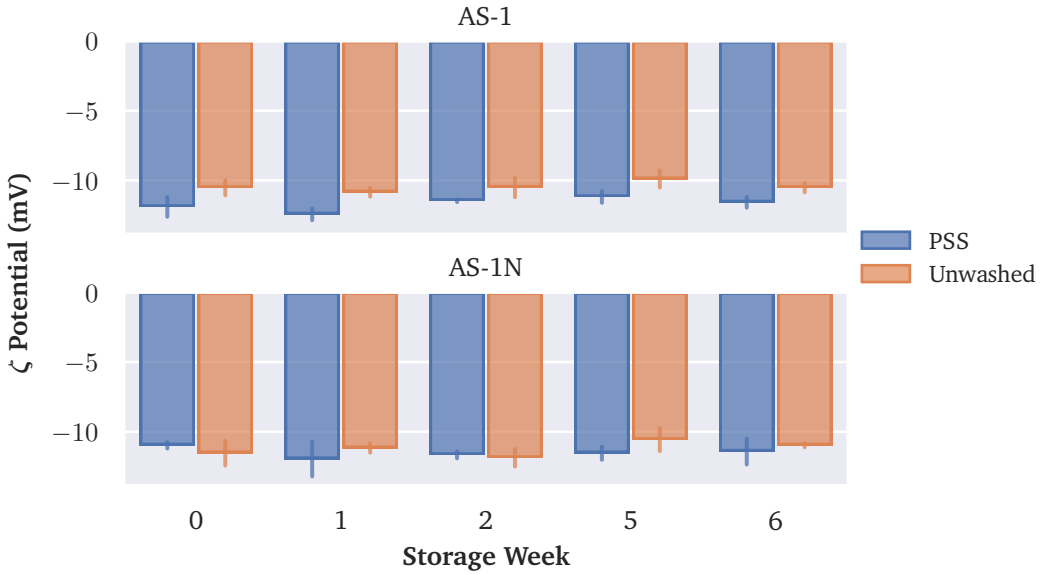


Figure 3.17: RBC ζ-potential throughout storage as measured by ELS (mean ± SD, $n = 4$). AS-1 stored cells are shown on the top while AS-1N stored cells are shown on the bottom. There are no statistically significant differences between storage or washing conditions.

3.3.7 RBC EV Size and Dispersity

RBC EVs were similarly measured by DLS. These microvesicles are irrevocably removed directly from the RBC membrane and thus have important implications in echinocytosis. Size and dispersity over time are shown in Figure 3.18 and Figure 3.19, respectively. While size increased throughout time, corroborating the literature, dispersity decreased. This indicates the size of RBC EVs becomes more homogeneous as it increases. This trend can be seen in Figure 3.20.

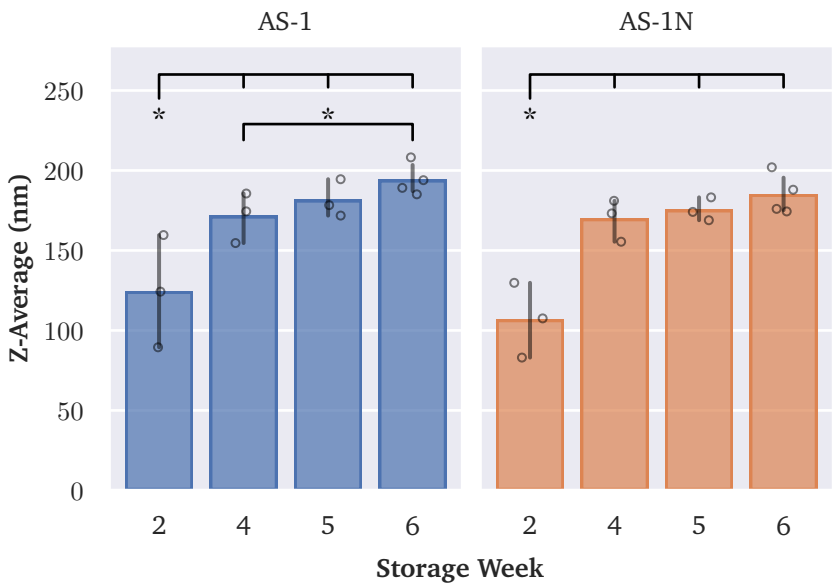


Figure 3.18: Z-average diameter of RBC EVs throughout storage as measured by DLS (mean ± SD, $n = 3 - 4$). EVs from AS-1 stored cells are shown on the left while those from AS-1N stored cells are shown on the right. Statistically significant differences are indicated by $*$, where $p < 0.05$ with Holm–Šidák correction.

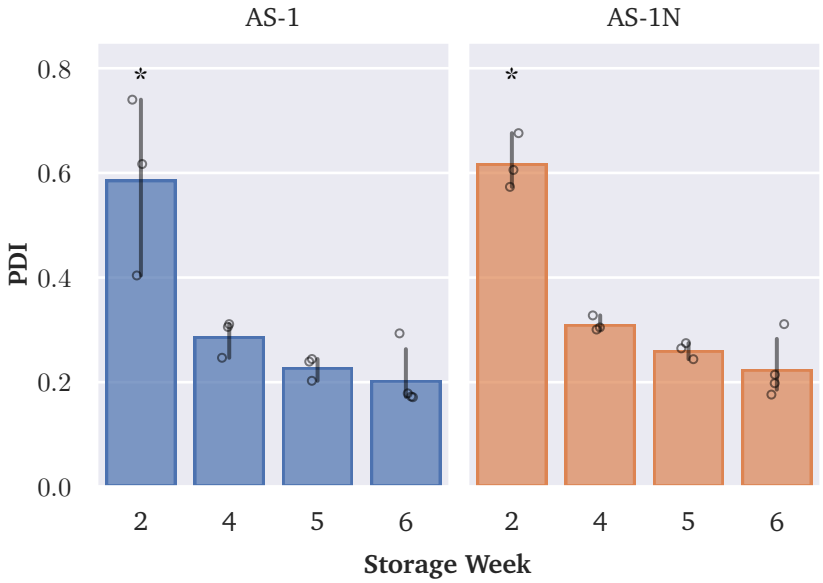


Figure 3.19: PDI of RBC EVs throughout storage as measured by DLS (mean ± SD, $n = 3 - 4$). EVs from AS-1 stored cells are shown on the left while those from AS-1N stored cells are shown on the right. Statistically significant differences to all other weeks are indicated by $*$, where $p < 0.05$ with Holm–Šidák correction.

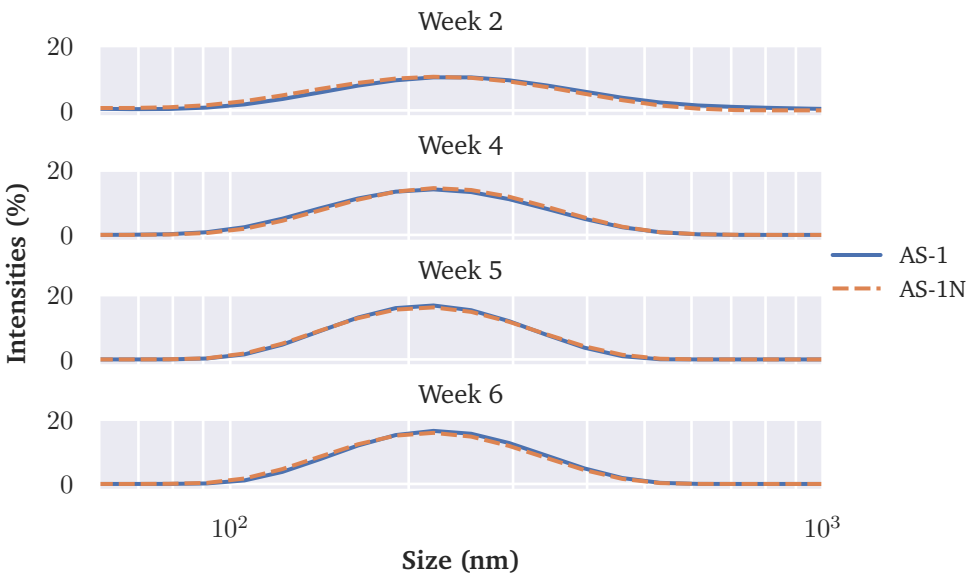


Figure 3.20: Log-scale distribution of the Z-average diameter of RBC EVs throughout storage as measured by DLS (mean $\pm$ SD, $n = 3 - 4$). Note the increase in positive kurtosis over time. This is indicative of the observed decrease in PDI as the EVs become more uniform in size.

3.3.8 Stored RBC Immunogenicity

In other studies using a feeding system to maintain normoglycemia in AS-1 storage, sterility of the stored blood was tested via blood agar smears. No bacterial contamination has been detected, and these studies followed all the same precautions. Here, the innate immune response to stored RBCs was tested in an *in vitro* transfusion as described. The results of these investigations are shown in Figure 3.21. After baseline correction, responses of both biomarkers assayed were well below the lower limit of quantification (LLOQ) of the ELISAs. This suggests the RBCs remained sterile throughout the storage period.

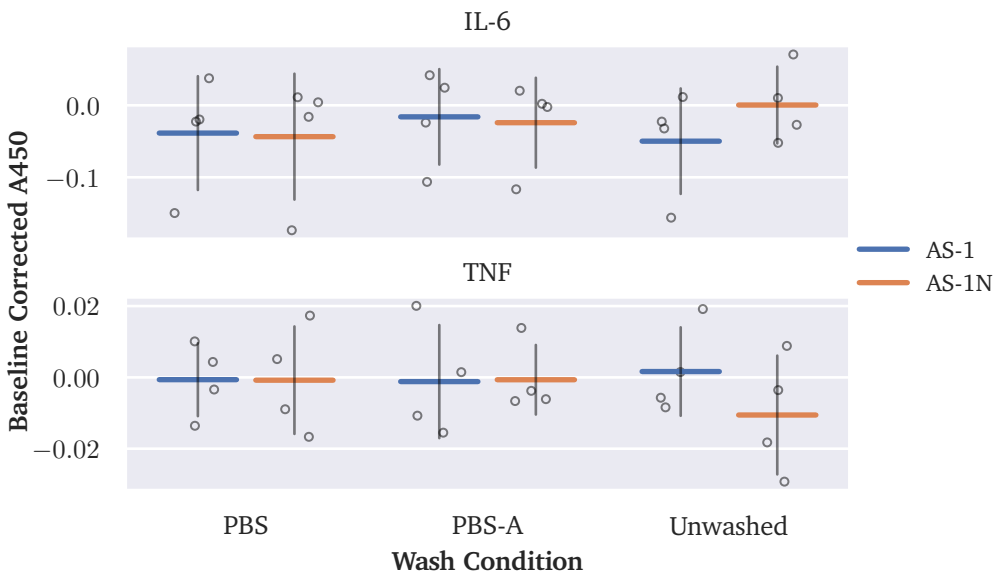


Figure 3.21: Baseline corrected A450s of IL-6 and TNF ELISAs following an *in vitro* autologous transfusion (mean ± SD, $n = 4$). Absorbance is shown as values are below the LLOQ of the assays. This indicates, in this context, no immune response was elicited.

3.3.9 RBC ATP Quantification

Intracellular ATP was quantified to determine if RBCs stored in AS-1N were able to better utilize glucose. Normalization to the unwashed AS-1 condition each week was used to control biological variation. As shown in Figure 3.22, there was no difference in intracellular ATP between storage conditions. Intracellular ATP was significantly reduced in both AS-1 and AS-1N as storage duration increased. Both of these findings agree with previously published results.

This technique was also used to determine if rejuvenation improves ATP production. Figure 3.23 shows the results of these studies. Here, each condition is normalized to the corresponding unwashed condition each week. When stored RBCs are washed, intracellular ATP increases. However, there are no differences between storage conditions or between washing buffers.

Note that flow-induced RBC ATP release was not investigated as part of this study. While intracellular ATP may be equivalent between hyperglycemic and normoglycemic storage, the ability of these cells to release ATP in response to mechanical shear may differ. Indeed, this has previously been suggested as the mechanism behind observations from the Spence laboratory.[21] In those experiments, RBCs stored in AS-1N were able to release significantly more ATP under flow conditions compared to those stored in AS-1 despite no differences in intracellular ATP concentration.

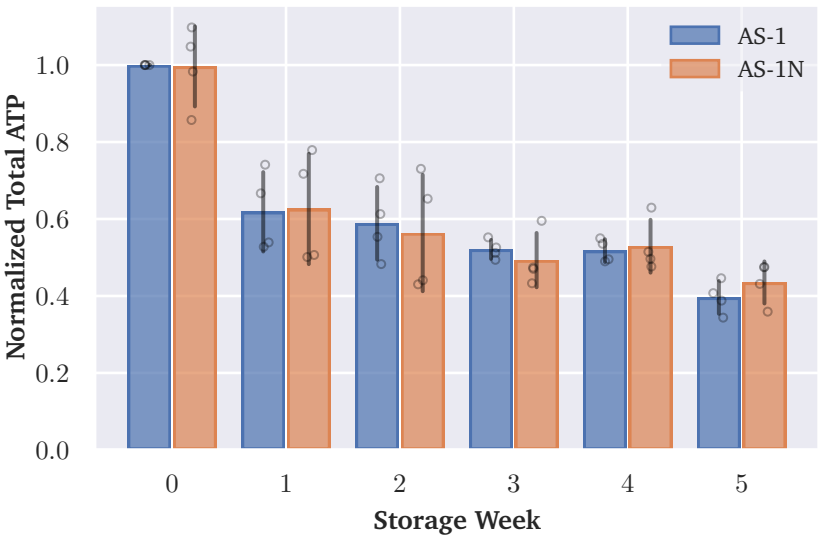


Figure 3.22: Intracellular ATP of RBCs throughout storage, normalized to AS-1 baseline (mean ± SD, $n = 4$). Both storage solutions resulted in decreased ATP production over time, however, there are no statistically significant differences between storage conditions.

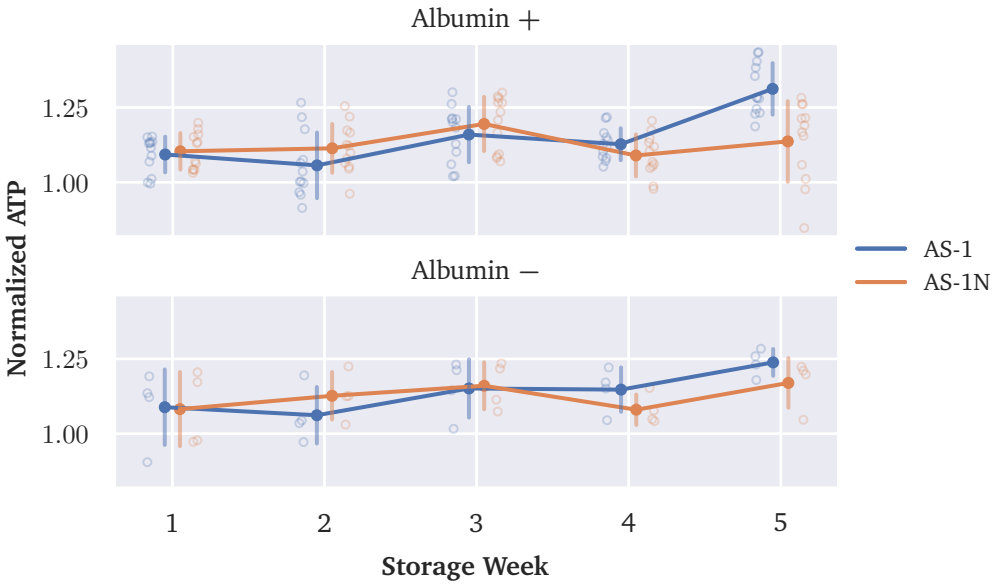


Figure 3.23: Total ATP of rejuvenated RBCs throughout storage, normalized each week to the unwashed condition (mean ± SD, $n = 4 - 12$). Both rejuvenation with and without albumin resulted in increased ATP production, however, there are no statistically significant differences between them.

3.4 Discussion

3.4.1 RBC Feeding System Performance and General Blood Measures

The second-generation RBC feeding system developed by the Spence laboratory for these studies has now succeeded in three sets of RBC storage. As shown in Section 3.3.1, total absglycemia for twelve

donors was fairly minimal at -2.21 mmol week L^{-1}. For a six-week storage period, this is equivalent to an average glucose value around 0.37 mmol L^{-1} below the setpoint of 5 mmol L^{-1}, or 4.67 mmol L^{-1}. This indicates the glucose utilization curve given in Equation (1.2) slightly underestimates the metabolic requirements of stored RBCs, leading to underfeeding. However, this can easily be rectified by incorporation of these values into the utilization curve to further refine our model and improve maintenance of normoglycemia.

The normoglycemic condition clearly had an impact on the glycation of RBC proteins, as highlighted by the changes in HbA1c shown in Figure 3.2. After the halfway point of storage, the difference between AS-1 and AS-1N storage conditions became statistically significant. While RBCs stored in AS-1 ended with changes in HbA1c greater than zero, those stored in AS-1N did not. This is only one measure of cellular glycation but provides evidence for overall formation of advanced glycation end-products (AGEs) and oxidative stress in hyperglycemic storage.

The HCT of both storage conditions was within expected ranges. As no feeding solution was delivered to RBCs stored in AS-1, there should be no change in HCT. Small variations were observed, though this is likely explained by error in the measurement method rather than significant changes in RBC volume. In contrast, delivery of feeding solution to the AS-1N condition was expected to dilute the RBCs somewhat, decreasing HCT over time. This was noted in both feeding paradigms, although the 100 mmol L^{-1} glucose solution did not decrease as much as the model would suggest. It is unclear if this is due to measurement error, or if delivery of a feeding solution supplemented with NaCl did have some effect on the RBCs. The change in HCT for the 100 mmol L^{-1} glucose solution followed the model quite well. The RMSE for each of these was 4.55 percentage points and 1.60 percentage points, respectively.

Similarly, the RMSE for glucose drop in the AS-1 condition was only 2.44 mmol L^{-1}. While there were some disparities between expected and observed values throughout storage, the endpoint was very similar as shown in Figure 3.5. Reconciling this with the underestimation of glucose demands in normoglycemic RBC will require further study. It is possible that normoglycemic storage results in different rates of glucose metabolism compared to hyperglycemic storage, although indicators of glycolysis suggest this is not the case. However, the glucose utilization model did indeed underestimate glucose consumption toward the end of storage, which was noted for RBCs stored in AS-1N as well.

3.4.2 Hemolysis in AS-1N Stored RBCs

The FDA requires all RBC storage systems to maintain hemolysis levels below $1\,\%$ at the end of storage. Excessive hemolysis of stored RBCs leads to potentially dangerous levels of free hemoglobin that may be deleterious post-transfusion. The ability of a feeding system to meet this requirement for RBCs stored in AS-1N has been previously reported by the Spence laboratory.[24] However, this was using the first-generation RBC feeding system that was only compatible with a $100\,\mathrm{mmol\,L^{-1}}$ glucose solution supplemented with $100\,\mathrm{mmol\,L^{-1}}$ NaCl. To confirm this did not have any effect on hemolysis in normoglycemic storage, these studies were repeated with the second-generation feeding system using an alternative feeding solution containing only glucose.

Figure 3.6 shows that for a six-week storage period, AS-1N hemolysis values never exceeded this threshold. In contrast, increased levels of hemolysis were noted in the AS-1 condition by week five. While extended storage resulted in increased hemolysis levels for both conditions, one additional week of storage resulted in a hemolysis level not statistically greater than $1\,\%$ for AS-1N only. Thus, it is possible with further testing extended RBC storage may prove feasible using current FDA standards.

It is worth noting that hemolysis of stored RBCs is highly dependent on biological variation in the donors.[10] This may help to explain differences in observations made in these studies compared to previous reports.[24] Further study of hemolysis in normoglycemic storage is warranted to ensure donor-dependent variations are accounted for.

3.4.3 RBC Morphological Assessment

The morphology of stored RBCs is certainly of significant interest. Degradation of the cellular membrane, oxidative stress, and depletion of intracellular ATP are all implicated in the gross malformations observed in echinocytosis. Quantification of these changes is therefore important in assessing overall RBC health and *in vivo* viability post-transfusion.

Section 3.3.3 shows an in-depth analysis of these changes across both storage condition and rejuvenation methods. As shown in Figure 3.7, unrejuvenated RBCs show no difference in MI regardless of storage in AS-1 or AS-1N. Both conditions do have significant increases in MI over time however, indicating echinocytosis cannot be improved by reduction in hyperglycemia alone. The degree of this increase is demonstrated in Figure 3.8. Here, histograms of RBC classifications (combining both conditions) is shown at the beginning and end of storage. Again, these classifications followed the

methods of Bessis with morphological numbers assigned per Ferrell and Huestis (stomatocyte = -1, discocyte = 0, echinocyte I = 1, echinocyte II = 2, echinocyte II = 3, spherocyte I = 4, spherocyte II = 5). A KDE for each histogram is overlaid, and indicates what the continuous population of RBC morphologies may look like. Were these classifications more granular, the histograms may approach the smoothed KDE distributions shown.

Rejuvenation of stored RBCs, shown in Figure 3.9, results in a significant improvement in MI. Storage condition was not relevant to this phenomenon, thus this figure shows average values for both storage conditions under different rejuvenation schemes. In contrast to previous reports, washing in a buffer that does not contain serum albumin did result in improved MI values.[26] However, when serum albumin is present, an even greater benefit is realized. The significant improvement in morphology can be seen in the magnified SEM images shown in Figure 3.10.

As these data were bootstrapped to determine average MI and confidence intervals, no formal hypothesis tests were conducted. However, separation of the $95\,\%$ bootstrapped confidence intervals is indicative of statistically significant differences. Thus, it is safe to say rejuvenation in an albumin-containing buffer significantly improves MI relative to unwashed RBC and RBC washed using a buffer that does not contain albumin. Rejuvenation without the use of serum albumin may be beneficial in late storage, but improvement in MI do not seem to be statistically significant in early storage. The mechanisms behind these results are yet to be determined, though W. Reinhart, et al. and S. Reinhart, et al. suggest serum albumin may be able to remove echinocytotic molecules from the outside of RBC membranes.[28,35]

Previous data from the Spence laboratory has indicated improved proportions of discocytes during early storage in AS-1N. However, the studies here were unable to replicate this as shown in Figure 3.11. These data indicate no significant differences in the discocyte population of either storage condition. As expected, washing in a buffer containing serum albumin does have a significant impact on discocyte proportion; this is shown in Figure 3.12

The inclusion of NaCl to increase the tonicity of the previously used $100\,\mathrm{mmol\,L^{-1}}$ glucose feeding solution perhaps has cellular effects itself. This has been suggested by Soule to be an echinocytotic agent.[29] Mechanistically, this can be understood as a shift in the Gibbs-Donnan equilibrium ratio resulting in a corresponding increase in inward-facing Band 3 protein as shown in Equation (3.6).[36] This integral membrane protein is responsible for the electroneutral exchange of chloride and

bicarbonate ions across the RBC membrane, which is key in carbon dioxide transport.[37] An increase in the inward-facing conformation of Band 3 is associated with echinocytosis; this conformation promotes folding of spectrin, contracting the RBC membrane.[36]

$$\frac{\text{Chloride}^-_{extracellular}}{\text{Chloride}^-_{intracellular}} = \frac{\text{Band 3}_{inward}}{\text{Band 3}_{outward}} \tag{3.6}$$

As shown in Figure 3.13, this does not appear to be the case. In unrejuvenated RBCs, there is no apparent trend in MI relative to the storage condition or presence of NaCl in the feeding solution. While the presence of this salt potentially increased MI during the first week of storage, this effect virtually disappeared later on. It is worth noting that those data were among the first SEM images collected and classified, and so experimental error may play some role in artificially increasing MI at those time points.

3.4.4 RBC Bulk Deformability

The deformability of RBCs is key to their *in vivo* function. It both allows them to traverse the microvasculature and induces ATP release to stimulate vasodilation.[33,38] Rigid RBCs are also unable to pass through the splenic pulp, resulting in their removal from circulation.[33]

As noted in Section 3.3.4, previous reports from the Spence laboratory indicate RBCs stored in AS-1N show improved deformability relative to those stored in AS-1.[23] Here, we observed no such difference. While the experimental methods of previous reports varied from those employed here, the basic principle (bulk filtration of stored RBCs) remained the same. It is unclear why the results from those studies were not able to be replicated. However, further investigation into stored RBC deformability under normoglycemic conditions using more sensitive techniques may help elucidate whether deformability is indeed improved under these conditions.

Notably, deformability of RBCs stored in both AS-1 and AS-1N was significantly improved after washing. This contradicts some reports stating rejuvenation of RBCs via washing does not increase cellular deformability.[28] Again, more sensitive measurements of RBCs deformability are warranted to determine the effects of rejuvenation on deformability.

3.4.5 Changes in RBC Size and Homogeneity

Measurement and dispersity of RBC size were quantified through DLS. As discussed in Section 3.3.5 the results of these studies are limited to relative comparisons only. Though this is the

case, these measures are still useful to identify differences in RBC characteristics between storage conditions.

Figure 3.15 shows that there was no difference in normalized size between storage conditions. In both cases, statistically significant decreases were observed toward the end of storage. Though RBC volume doesn't change drastically throughout storage, this technique reports the average hydrodynamic size of a spherical particle. Thus, it can be concluded the radius of a sphere enveloping RBCs decreased over time. This aligns with literature reporting spherocyte radii around $5.6\,\mu m$ compared to normal discocyte widths of $8\,\mu m$.[13]

The normalized PDI of each storage condition increased throughout storage as shown in Figure 3.16. This can be interpreted as a widening of the DLS size distribution over time. This aligns with the observed widening of the cell morphology distribution highlighted in Figure 3.8, indicating a more heterogeneous population of RBC sizes.

3.4.6 Changes in RBC ζ-Potential

The ζ-potential of RBCs was measured via electrophoretic light scattering following size and dispersity DLS measurements. While not often an investigated aspect of the storage lesion, there is evidence to suggest reduction in ζ-potential during RBC storage.[34] This is believed to be caused by oxidative damage to the RBC membrane.[34] As ζ-potential decreases, the electrostatic repulsive forces between individual cells decreases as well. Thus, ζ-potential may be a useful measure to predict the aggregability and adhesion of stored RBCs.

Measurements were made to both washed and unwashed RBCs in both storage conditions. Reported values were in agreement with the literature, though Figure 3.17 shows no significant changes throughout storage.[34] There was also no difference between storage condition or with rejuvenation. Therefore, we are unable to confirm that the ζ-potential of stored RBC decreases during storage in these studies.

3.4.7 Changes in EV Size and Homogeneity

Microvesiculation of RBC membranes during storage results in the accumulation of hemoglobin-filled EVs. In addition to this membrane loss contributing to echinocytosis, transfusion of EVs may be associated with proinflammatory and prothrombotic responses.[13,16] DLS was again used to measure both size and dispersity of these RBC-derived microvesicles. As these spheroid particles are much

more suited to DLS measurements, normalization was not required.

Figure 3.18 shows the increase in average EV diameter. Indicative of the RBC progressive membrane loss seen throughout storage, this shows advancements in echinocytosis. Similarly, the vast reduction in PDI shown in Figure 3.19 is associated with increasing homogeneity of EV size. This is visualization in the size distributions plotted in Figure 3.20. There are no differences between storage conditions in either of these measures.

3.4.8 Immunogenicity of Stored RBCs

As mentioned in Section 3.3.8, previous studies in the Spence laboratory have confirmed our normoglycemic storage protocols with automated RBC feeding maintain sterility. Though these procedures have not changed, improvements to the feeding system have altered potential infection vectors. Ostensibly, these changes would reduce any risk of infections. However, to be sure, immunogenicity of stored RBCs as measured by leukocyte innate immune response was tested via an *in vitro* autologous transfusion.

Two biomarkers derived from white blood cells were assessed before and after the transfusion of stored blood. The pro-inflammatory cytokines IL-6 and TNF were assayed in whole blood incubated with aliquots of stored blood in transfusion-relevant ratios. Figure 3.21 shows that neither of these analytes had increased responses following transfusion and incubation of stored RBCs. Note that these values are baseline-subtracted; all donors had measurable basal levels of both analytes, but with no increase observed in these studies.

3.4.9 Intracellular ATP Levels in Stored RBCs

Intracellular ATP is relevant to several aspects of the storage lesion and stored RBC health. First, it acts as a measure of glucose metabolism via glycolysis and the pentose phosphate pathway. Second, as ATP is critical to RBC membrane maintenance, it provides insight into the progression of echinocytosis. Finally, RBC-derived ATP serves important functions *in vivo* such as the stimulation of NO production in endothelial cells. As intracellular ATP levels decrease throughout storage, RBC functionality may become impaired.

The Spence laboratory has previously reported that intracellular ATP levels in normoglycemic storage are similar to those in hyperglycemic storage.[21] In agreement with that is the observation that glycolytic end products remain at similar levels between storage conditions.[24] Section 3.3.9

shows two relevant findings. As seen in Figure 3.22, intracellular ATP indeed is statistically equivalent between RBCs stored in AS-1 and those stored in AS-1Ns. Additionally, Figure 3.23 shows that ATP production is seemingly improved after rejuvenation. This effect is independent of both storage condition and the presence of serum albumin in the rejuvenation buffer. In all cases, rejuvenated RBCs show increased intracellular ATP levels between approximately $110\,\%$ to $125\,\%$ of the unwashed condition.

3.5 Experimental Limitations

While these studies were conducted as rigorously as possible, there are always limitations due to measurement error and logistical constraints. The glucometer used to measure glucose values could not report values above $50\,\mathrm{mmol\,L^{-1}}$. This is relevant for the early-storage supervision of AS-1 glucose levels only. Glucose readings during the first week of storage were therefore artificially clipped at this value as shown in Figure 3.5.

Additionally, this glucometer reported plasma-equivalent glucose values presuming physiological HCT. The glucometer seemingly corrects its reading to $42.5\,\%$ HCT, far below that of stored RBCs. Thus, an empirically-derived correction had to be determined (see Equation (3.1)) and applied to each measurement. This further incorporates measurement error from the determination of sample HCT.

Similarly, the precision of HCT measurements is limited to, roughly, $\pm 2\,\%$. Not only is there imprecision with the instrument itself, but operator error is involved as well as aspects of the measurement are somewhat subjective. Attempts were made to minimize this bias by having only one individual make these measurements each week. However, logistical challenges prevented this from being the case every time. Measurement of HCT during week 1 of testing with the $100\,\mathrm{mmol\,L^{-1}}$ glucose was particularly affected by this, as can be seen Figure 3.4.

Due to logistical considerations beyond our control, SEM imaging of every sample was not feasible. Therefore, a pooled approach was used as described in Section 3.2.6. Traditional data analysis techniques could therefore not be performed, and bootstrapping was used to generate confidence intervals about mean MIs.

Classification of RBCs was itself rather subjective. With seven different categories, including three categories of echinocytes and two categories of spherocytes, there were many cases where RBC

morphology could justifiably be classified differently. As all MI measurements were to be compared relative to others, bias was reduced by having one individual classify an entire storage set ($n = 4$). Ideally, future studies will utilize multiple experts to individually classify every image and their average response used instead. Blinding of each image condition, rejuvenation status, and time point would additionally increase the rigor of these classifications.

DLS measurements of RBCs, as discussed, resulted in highly variable data due to the morphology of the cells. Again, normalization and relative comparisons of this data prevented this from being problematic. Use of a different instrument for RBC assessment, such as a hematology analyzer, would be ideal. Similarly, an ektacytometer would allow for much more precise and physiologically relevant RBC deformation studies. However, no such instruments were available at the time of the study.

While intracellular measurement of ATP is an important aspect in assessing the storage lesion, ATP release from RBCs is also critical. Previous studies have used microfluidic platforms to measure flow-induced ATP release from stored RBCs. Replicating these studies would provide additional information into the effects of both normoglycemic storage and rejuvenation.

Finally, due to the limited sample size, the donor population was fairly homogeneous. During selection, sex was split evenly for all three sets of storage. However, age and ethnicity were fairly uniform across all donors. Further studies that include wider variations in these characteristics will provide more rigorous data.

3.6 Conclusion

These studies provide ample evidence to show the efficacy of the second-generation feeding system described in Chapter 2. Normoglycemia was well-maintained throughout multiple sets of storage, leading to improvements in RBC health. The glucose utilization curve employed in this system was validated through empirical evidence and the accuracy of models for HCT change and glucose drop in the hyperglycemic storage conditions. Further, immunogenicity testing via a novel *in vitro* transfusion method demonstrated RBCs stored using this system elicit no measurable immune response, showing no major contamination. Contrary to the speculation, feeding RBCs with a solution containing NaCl seemed to have no detrimental effects on cellular morphology.

Benefits of normoglycemic storage were also confirmed in these studies. RBCs stored using a novel low-glucose additive solution did not experience significant glycation of hemoglobin during

storage. In contrast, RBCs stored in traditional FDA-approved AS-1 had an increase in HbA1c over 0.3 percentage points. This is indicative of overall cellular glycation and oxidative stress, though *in vitro* studies will be required to confirm if this difference is clinically significant. In late-stage and extended storage, hemolysis in the normoglycemic condition was also lower compared to the hyperglycemic condition, although without statistical significance.

However, intracellular ATP and RBC morphology is not improved in normoglycemic storage, nor is the proportion of discocytes. This contrasts with previous data collected by the Spence laboratory showing an increased proportion of discocytes during early storage with AS-1N. There is also no difference between AS-1 and AS-1N in regard to cellular deformability as measured by bulk filtration rate, or RBC or EV size and dispersity as measured by DLS. Also contrasting with literature, no reduction in RBC ζ-potential was measured in either storage condition by electrophoretic light scattering.

Cellular rejuvenation proved to be beneficial in both storage conditions, with albumin-containing rejuvenation buffers resulting in the strongest improvements to RBC health. Particularly, rejuvenation was effective in reversing echinocytosis and impaired RBC deformability. Rejuvenated RBCs showed increases in bulk filtration rate around $50\,\%$. Similarly, RBCs rejuvenated in a buffer containing serum albumin had MI values at six weeks of storage equivalent to unrejuvenated RBCs at just one week of storage.

Both normoglycemic storage and RBC rejuvenation seem promising in their abilities to reduce various aspects of the storage lesion. In these multifaceted studies, combining these techniques continued to show improvements in RBC health, however, no more so than individually. Regardless, there is evidence that both approaches improve blood storage. Further studies combining and improving upon these methodologies may elucidate additional benefits combating the storage lesion.

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CHAPTER 4

CONCLUSION TO BLOOD BANKING AND THE STORED RED BLOOD CELL REJUVENATION POTENTIAL OF C-PEPTIDE

4.1 Improving the Health of Stored Red Blood Cells

The Spence laboratory has thoroughly demonstrated the benefits of normoglycemic red blood cells (RBCs) storage over the last decade.[1–6] Here, further investigation using a more rigorous normoglycemic storage technique has demonstrated some of these key effects. Additionally, a second-generation RBC feeding system was validated in three separate storage sets (total $n = 12$). Euglycemia was maintained with few excursions outside the target window of $4\,\mathrm{mmol\,L^{-1}}$ to $6\,\mathrm{mmol\,L^{-1}}$. The dosing precision was further shown to be accurate by modeling and assessment of hematocrit drop due to increased storage bag volume. Further, hemolysis in RBC units fed by this system was within Food and Drug Administration (FDA) limits throughout a full six-week storage period. These RBCs were also shown to be nonimmunogenic in a novel *in vitro* autologous transfusion, indicating sterility was maintained during storage.

The degree of cellular glycation, measured by glycated hemoglobin (HbA1c), was statistically equivalent to zero when RBCs are stored in normoglycemic Additive Solution 1 (AS-1N). In contrast, hyperglycemic storage in an FDA-approved additive solution, Additive Solution 1 (AS-1), resulted in an increase in HbA1c around 0.3 percentage points after six weeks of storage. Based on the conversion formula between HbA1c and estimated average glucose used by the American Diabetes Association, this is equivalent to an increased average blood glucose of $0.48\,\mathrm{mmol\,L^{-1}}$.[7] While this difference is fairly minimal, HbA1c is only one measure of cellular glycation. This increase indicates hyperglycemic storage may result in accelerated formation of advanced glycation end-products (AGEs) and is associated with increased levels of oxidative stress. Further study is warranted to determine the clinical significance of these findings.

RBCs stored in AS-1N also had consistently lower levels of hemolysis compared to those stored in AS-1. In these studies, the difference was not statistically significant. However, the endpoint hemolysis level in any given unit of stored RBCs is highly donor dependent.[8] Thus, further investigation may reduce the variability of this data to determine whether there is a significant improvement in hemolysis using normoglycemic storage. Notably, even with an additional week of extended storage, hemolysis in AS-1N stored condition was very close to the FDA threshold of $1\,\%$.

Cellular rejuvenation of stored RBCs has been reported to be effective in reducing certain aspects of the storage lesion.[9–11] The work presented here confirms this rejuvenation is effective at reducing the degree of echinocytosis in stored RBCs. In particular, albumin-containing solutions show tremendous potential for reducing detrimental morphological changes in these cells. When rejuvenated with such a solution, RBCs morphological indices are equivalent after six weeks of storage to unrejuvenated RBCs after just one week of storage. In contrast to previous reports, the deformability of washed stored RBCs showed marked improvement as measured by bulk filtration rate.[10] Though large-scale *in vivo* studies of rejuvenated RBCs have not yet taken place, these techniques show great potential *in vitro* to improve the health of transfused RBCs.

Though RBC rejuvenation in the context of normoglycemic storage did not show any further improvements, the benefits of combining both techniques may maximize RBC health. Beyond this, other advancements to additive solutions and storage conditions may prove worthwhile. For example, Additive Solution 7 (AS-7) is a basic additive solution that was recently approved by the FDA. The alkalinity may serve to buffer storage bag pH, extending the time in which glycolysis dominates stored RBC glucose metabolism. This may help maintain adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) levels, reducing oxidative stress and improving membrane maintenance. Despite these improvements, AS-7 still contains $80\,\mathrm{mmol\,L^{-1}}$ glucose, thus continues to result in hyperglycemic storage.

Hypoxic storage of RBCs is another promising avenue. One storage bag system, the Hemanext ONE, has been designed to maintain hypoxic RBC storage and was also recently approved by the FDA. While the clinical impacts of this technology are yet to be proven, *in vitro* reports show significant reductions in various aspects of the storage lesion. For example, studies report hypoxic storage improves post-transfusion RBC recovery, ATP production, and hemolysis.[12,13]

One publication has reported an attempt to store RBCs with additional serum albumin, rather than using post-storage washing for rejuvenation.[9] Though stored RBCs will surely have some residual serum albumin, the addition of excess albumin may provide some beneficial effect. While the study reported no difference in RBC morphology with albumin-supplemented storage, we did attempt to reproduce this experiment using AS-1 with $5\,\%$ bovine serum albumin. The preliminary data shown in Figure 4.1, although conducted with only one donor, demonstrates a marked increase in morphological index (MI). This may be explained by the use of bovine serum albumin rather than human serum

albumin, though the two have been shown to function similarly by both the Spence laboratory and others.[14] However, given the literature using human serum albumin reported no improvements with this technique, further investigation into albumin supplementation was discontinued.

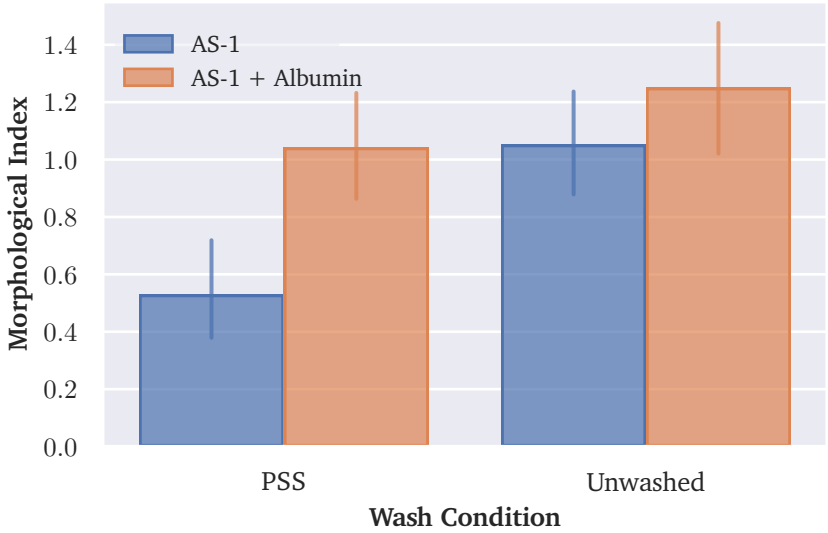

Figure 4.1: Morphological indices of RBCs stored in AS-1 with and without 5 % bovine serum albumin (bootstrapped mean ± 95 % CI, $n = 1$). Samples were either unwashed or rejuvenated with PSS.

Still, the potential impact of both alkaline additive solutions and hypoxic storage seem high. The possibility of combining these techniques with normoglycemic storage is feasible. Perhaps a normoglycemic, alkaline, hypoxic storage system could mitigate even more aspects of the storage lesion, leading to healthier stored RBCs and advancing the field of transfusion medicine.

4.2 Future Work in Normoglycemic Storage

As discussed in Section 3.5, improving donor heterogeneity will be an important aspect of future studies. Representing a variety of populations is critical to gathering robust data to support clinical studies. Along with this pursuit, there are also a multitude of additional measures of RBC health that may be considered.

For example, the aggregation of RBCs is clinically relevant and impacted by storage, and may be determined with a simple erythrocyte sedimentation rate test. Similarly, a hematology analyzer could be used to assess RBC mean corpuscular volume and improve hematocrit measurements in various normoglycemic and rejuvenated conditions. Percoll gradients may be used to semi-quantitatively determine differences in stored RBC density which, with cellular volume, can be used to assess RBC

swelling during storage.

Ektacytometry is an instrumental technique for functional RBC analysis.[15] These analyzers enable RBC deformability measurements as a function of shear stress. Additionally, shear-induced deformability curves in oxygen and osmotic gradients can be generated and RBC aggregation, disaggregation, and rouleaux formation can be quantified. Thus, these instruments serve as an excellent platform for assessing the mechanics and interactions of stored RBCs.

Osmotic fragility is an important aspect of RBC health. However, little attention has been given to the mechanical fragility of stored RBC. A platform to measure this using an *in vitro* transfusion could elucidate differences in post-transfusion hemolysis and splenic clearance. For example, stored RBCs could be radiolabeled with ^{51}Cr and added to autologous whole blood circulating through microcapillaries at physiologically relevant shear rates. This radionuclide binds hemoglobin, is not toxic to RBCs, and is already commonly used to measure post-transfusion recovery.[16] By quantifying free ^{51}Cr over time, hemolysis of the transfused RBCs can be determined. In a similar scenario, forcing the cells through a membrane to replicate filtration in the spleen may be helpful in determining if impaired RBC deformability will result in removal from the circulation.

Measurement of storage-associated AGEs may be useful in determining the degree of cellular glycation. The Spence laboratory has recently reported on a method to quantify two common AGEs, carboxymethyl-lysine and carboxyethyl-lysine.[17] Using mass spectrometry, these can be identified and quantified on stored RBCs. Better assessing these detrimental modifications to RBC proteins and lipids may reveal new correlations between hyperglycemic storage and the storage lesion.

Contemporary technology may allow more efficient and less biased classification of RBC morphology. The use of machine learning for classification of pathological cells is a popular area of investigation. One such tool, published in 2023, is able to classify RBCs as stomatocytes, discocytes, or echinocytes with 78 % balanced accuracy.[18] An attempt was made to use this tool for the scanning electron microscopy (SEM) images collected in the studies presented here, although it was unsuccessful. The pre-trained model used optical microscopy images of blood smears with a monolayer of RBCs. Meanwhile, our SEM images vary somewhat in cellular appearance and often have overlapping cells, causing issues with segmentation and feature extraction.

Finally, measuring flow-induced ATP release will allow for a better assessment of stored RBC function *in vivo*. Previous work from the Spence laboratory included these measurements, but they

were not logistically feasible for these studies. However, preliminary data from the last storage set is shown in Figure 4.2. This indicates that although intracellular ATP is equivalent between normoglycemic and hyperglycemic storage, RBCs stored in AS-1N are able to release more ATP in response to deformation.

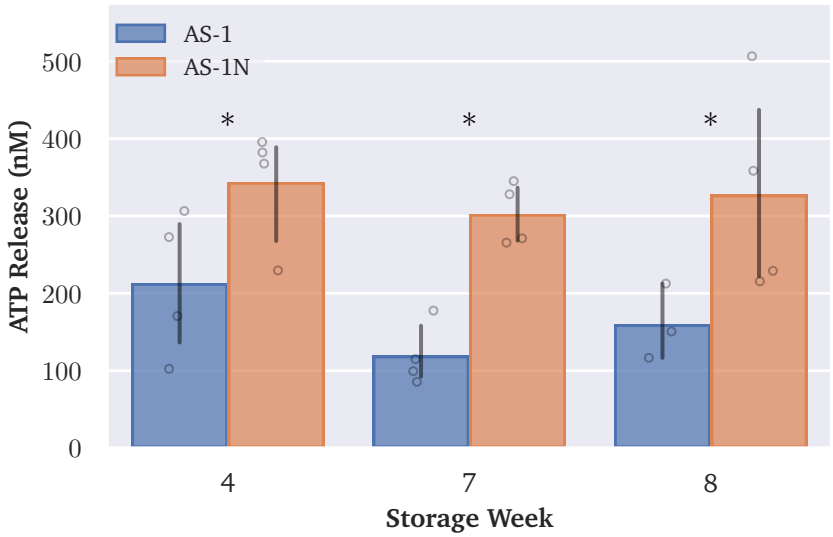

Figure 4.2: Flow-induced ATP release from RBCs in each storage condition (mean $\pm$ 95 % CI, $n = 4$). Statistically significant differences between conditions and within weeks are indicated by $*$, where $p < 0.05$ with Holm–Šidák correction.

4.2.1 Improvements to the Automated Feeding System

The second-generation feeding system discussed in Chapter 2 has functioned very well for multiple storage periods. However, improvements can continue to be made. Based on the data shown here, the glucose utilization curve used in the feeding algorithm consistently underestimates the demands of RBC metabolism. By incorporating new data into the data used to originally generate the curve, refinements can be made to prevent underfeeding in future studies.

Additionally, the sterility of the system should continue to be verified. The novel *in vitro* transfusion discussed in Section 3.3.8 shows RBCs stored using this system are nonimmunogenic. However, additional confirmation of RBC sterility should be confirmed throughout future studies.

Modifications could be made to the pump heads to allow easier exchange of the peristaltic tubing. This would allow all components that interact with the working fluid, including fittings, to be replaced for each storage period. Alternatively, as peristaltic pumps are compatible with any deformable tubing that fit into the rollers, it may also allow an entirely closed multi-bag system to be used. In this

configuration, a small feeding reservoir could be incorporated directly into the storage bag system and connected via a short length of peristaltic tubing to prevent any external connections to the pumps.

4.2.2 Alternative Feeding Strategies

Despite efforts to allow regulatory compliance and reduce the labor involved with an RBC feeding system, it is possible adoption of such a system may face opposition. As promising as normoglycemic storage is, failure to move forward to clinical studies due to the feeding system would be undesirable. Fortunately, there may be a solution, albeit a challenging one, to avoid such a scenario.

Data from the Spence laboratory has shown the pH decreases approximately linearly with storage time using AS-1N.[6] Thus, it may be feasible to develop a pH-sensitive coating that could be applied to the interior of the storage bags that releases glucose in response to acidic conditions. Development of this may be difficult, as the response would have to be tuned such that glucose release approximates the metabolic needs of the RBCs per the glucose utilization curve. Further, such a system would surely require extensive validation and confirmation that it is safe for both the RBCs and transfusion recipients.

4.2.3 *In Vivo* Transfusion Studies

Translation from *in vitro* studies to *in vivo* studies will likely require an animal model. Normoglycemic storage will have to be proven both safe and efficacious in these models to warrant further investigation. The Spence laboratory has made one attempt at an animal model using sheep with the goal of assessing post-transfusion recovery following normoglycemic storage. All work followed protocols approved by the Institutional Animal Care & Use Committee at Michigan State University.

Unfortunately, these studies were not able to proceed as planned. As shown in Figure 4.3, the ovine RBCs were unable to be stored without significant lysis. Transfusion of RBCs with such high levels of hemolysis would be inappropriate. The mechanism behind this extensive lysis is currently unknown, though sheep do maintain lower blood glucose levels than humans and their RBC glycolytic activity is only around $20\,\%$ to $30\,\%$ that of a human.[19] Before translation into human studies, a more appropriate animal model must be investigated. Soule has suggested that a canine model may be used, as dog RBCs metabolize glucose at rates similar to human RBCs.[20]

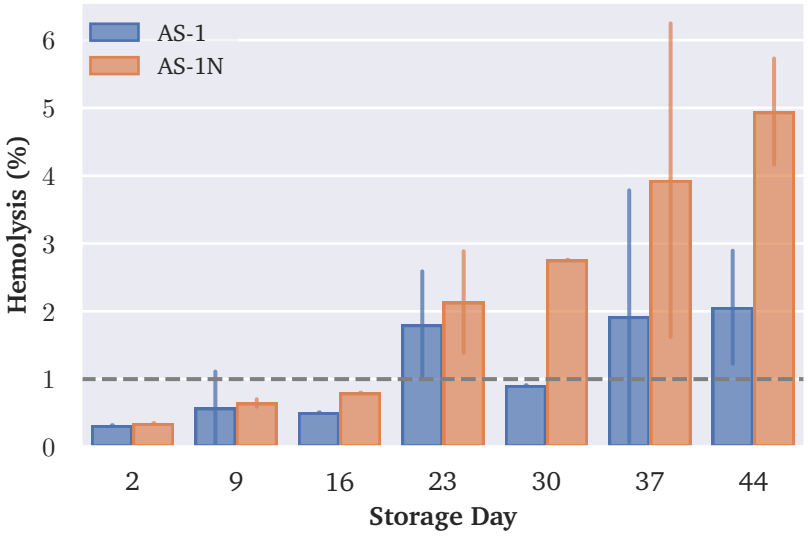

Figure 4.3: Hemolysis percentage of stored sheep blood (mean ± SD, $n = 1 - 2$). The FDA threshold (1 %) is shown in dashed grey.

4.3 C-Peptide as a Rejuvenating Agent

In addition to improving RBC storage for transfusion medicine, the Spence laboratory has also conducted a considerable amount of research into the biological effects of C-peptide. This 31-amino acid peptide hormone derived from proinsulin is produced and secreted by pancreatic β-cells in equimolar amounts to insulin.[21] Our research has demonstrated that C-peptide, when accompanied by Zn^{2+} and delivered by serum albumin, binds specifically to RBCs.[22–24] Importantly, while RBCs are unaffected by insulin, this complex seems to have an insulin-like effect on these cells. Measurable levels of the GLUT1 glucose transporter increase and glucose uptake improves by around 31 %.[22,24]

We suggest this metal-activated C-peptide complex has potential applications in blood banking in addition to the treatment type 1 diabetes (T1D).[5] In 2022, the Spence laboratory reported incubation of stored RBCs in an albumin-containing buffer with C-peptide and Zn^{2+} increased both flow-induced ATP release and cellular deformability.[5] Interestingly, these effects maintain ATP release and deformability levels equivalent to baseline for the entire duration of storage in AS-1N. However, the effects were limited to normoglycemic storage only. RBCs stored in AS-1 showed the typical degradation in both these metrics; it is possible this is due to enhanced glycation of these cells.

Thus, we propose that normoglycemic storage of RBCs, in combination with rejuvenation using a solution containing serum albumin, C-peptide, and Zn^{2+}, may result in significant improvements to

the storage lesion. This proposed rejuvenation solution, containing albumin, should also be successful in reversing echinocytosis of these stored RBCs. Given the data shown here and previously reported by the Spence laboratory, investigation of this potential method of rejuvenation should be a high priority in future studies.

In Chapter 5, a comprehensive introduction to T1D will be presented. This will provide background for the motivations behind the Spence laboratory's investigations into an auxiliary therapeutic for T1D. Ending with a brief review of the therapeutic potential of C-peptide, the impetus behind the continued pursuit of a functional C-peptide formulation will be discussed. Chapter 6 will then detail the development, both by the Spence laboratory and others, of the scientific corpus on the biological and therapeutic effects of C-peptide. Following that will be a description of recent activity within our laboratory on this project, leading to the reappraisal of C-peptide as a rejuvenating agent for blood banking.

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CHAPTER 5

INTRODUCTION TO TYPE 1 DIABETES MELLITUS

Portions of this chapter are adapted from previous unpublished work with permission.[1]

5.1 Background

Type 1 diabetes (T1D) is an autoimmune disease characterized by the near-total destruction of pancreatic β-cells.[2] The primary function of these cells is the production and secretion of insulin, the hormone responsible for regulating glucose transport into skeletal muscle, cardiac muscle, and adipose tissue.[3] While insulin also has some role in the brain, particularly hippocampal memory processes, its relation to cerebral glucose transport is still under investigation.[4,5] Despite only involving a few cell types, insulin-mediated glucose uptake is responsible for metabolism of over $70\,\%$ of all ingested glucose.[6,7]

Though not a universal subdivision, some literature differentiates T1D into types 1A and 1B.[8] Type 1A is diagnosed when insulin insufficiency is due to β-cell destruction by a cell-mediated autoimmune response.[9] In contrast, type 1B is a result of idiopathic β-cell destruction leading to insulin deficiency.[9] While the cause of this is ostensibly non-immune mediated, it is possible the process involves autoantibodies that are either not tested for or not yet identified.[10] It is also possible these are misdiagnoses of rarer monogenic forms of diabetes mellitus, such as neonatal diabetes mellitus or maturity-onset diabetes of the young.[10,11]

Within the pancreas, β-cells reside within clusters of endocrine cells known as the islets of Langerhans.[12] They are the sole producers of insulin, which is packaged into secretory granules in concentrations around $118\,\mathrm{mmol\,L^{-1}}$.[13] Within these granules, insulin forms hexamers with two Zn^{2+} ions and one Ca^{2+} ion.[14] These hexamers have low solubility within the granule due to the acidic pH around 5.5 to 6.0, causing crystallization that greatly increases their stability.[14,15] As blood sugar levels rise, glucose influx into β-cells stimulates release of these insulin granules, where the increased extracellular pH results in dissolution back to monomers.[15]

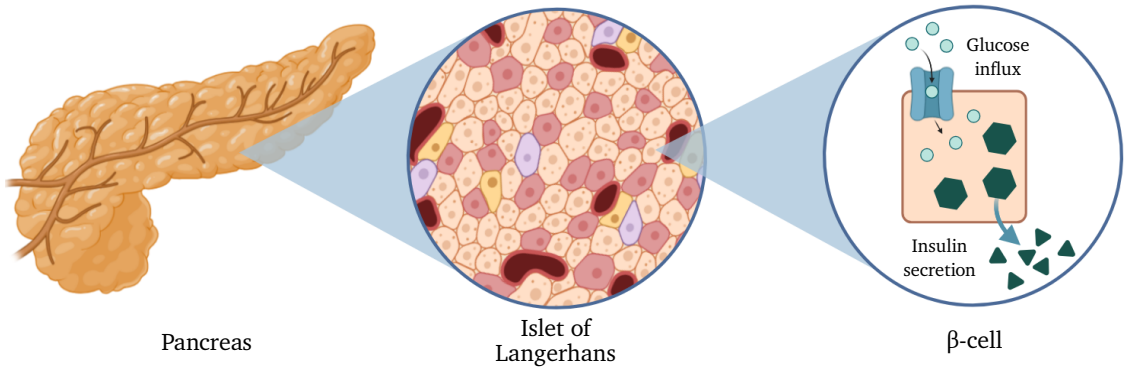

Figure 5.1: β-cells, located within the islets of Langerhans of the pancreas, secrete insulin in response to glucose influx.[12,16]

Secreted insulin diffuses throughout the body and binds to the insulin receptor, a tyrosine kinase.[17] This stimulates translocation of GLUT4, the only insulin-dependent glucose transporter, to the cell membrane.[18] GLUT4 is generally sequestered in intracellular vesicles, but through a complex signaling cascade from the insulin receptor, these vesicles fuse to the cell membrane where GLUT4 is inserted.[19] The increase in transmembrane GLUT4 enables increased glucose transport into the cell.[4]

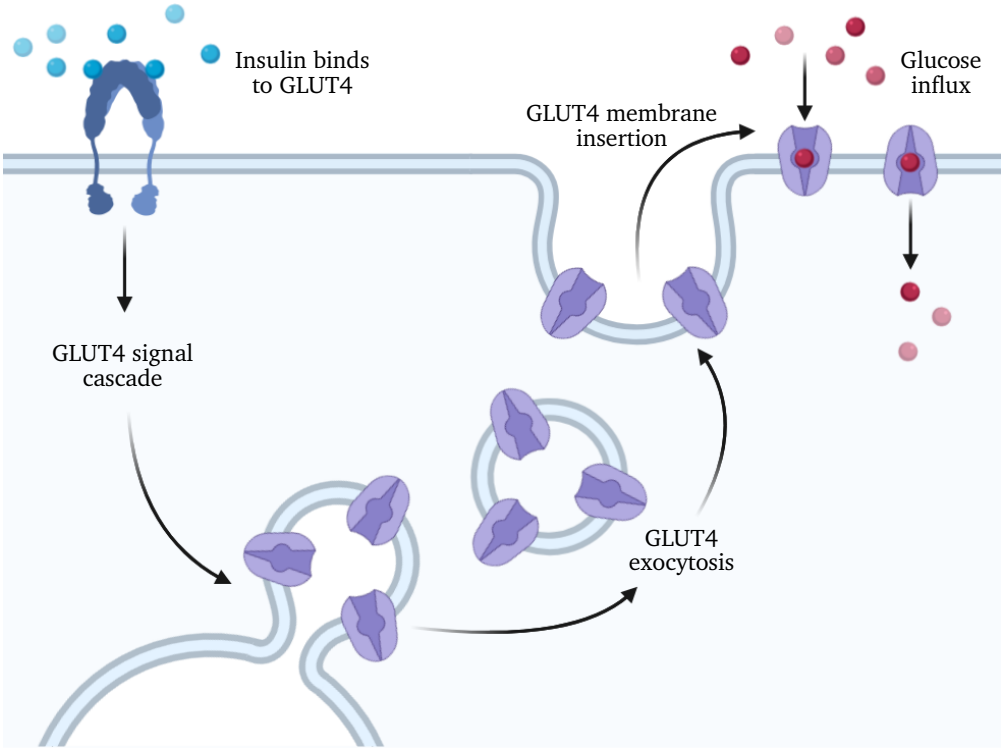

Figure 5.2: Insulin stimulated the translocation of the GLUT4 glucose transporter to the cell membrane.[18,20]

5.1.1 Clinical Manifestation

In T1D, the lack of insulin production prevents adequate glucose metabolism in GLUT4-containing cells, hindering their ability to sustain metabolic activities and resulting in a buildup of excess glucose in the body.[21] This insulin insufficiency and the accompanying severe hyperglycemia can quickly result in life-threatening conditions such as diabetic ketoacidosis (DKA) and hyperosmolar hyperglycemic state (HHS).[22] In DKA, energy production switches to non-insulin mediated pathways. Primarily, increased lipolysis liberates free fatty acids that can converted to acetyl coenzyme A through β-oxidation.[22] This can further be metabolized in the Krebs cycle to produce energy and, when produced in sufficient quantities, contribute to a process known as ketogenesis.[22] Products of ketogenesis include the ketone bodies acetone, acetoacetate and β-hydroxybutyrate; the latter two are acidic, and result in acidosis as they accumulate.[22] Concurrently, rates of endogenous glucose production through gluconeogenesis and glycogenolysis increase.[22] This leads to further hyperglycemia, ultimately causing excessive osmotic diuresis leading to severe dehydration and electrolyte imbalance.[22] While more common in type 2 diabetes, HHS presents similarly with respect to hyperglycemia and excessive osmotic diuresis, but without ketosis and the accompanying acidosis.[23] It is generally characterized by extreme hyperglycemia, high serum osmolality, and hypovolemia and is associated with mortality rates between $10\,\%$ to $20\,\%$, roughly an order of magnitude higher than DKA.[22,23]

5.1.2 Diagnosis

Diabetes is diagnosed when fasting blood glucose concentrations are $\geq 7.0\,\mathrm{mmol\,L^{-1}}$, random blood glucose concentrations are $\geq 11.1\,\mathrm{mmol\,L^{-1}}$ with symptoms present, or when an oral glucose tolerance test show blood glucose concentrations $\geq 11.1\,\mathrm{mmol\,L^{-1}}$.[24] If no symptoms are present, hyperglycemia must be present on at least two different occasions.[21] Finally, a diagnosis of diabetes can also be made if glycated hemoglobin (HbA1c) concentrations exceed $6.5\,\%$.[24] Normal HbA1c values should be below $5.7\,\%$, with values between $5.7\,\%$ to $6.4\,\%$ considered "prediabetic".[24]

Differentiation between T1D and type 2 diabetes can at times be difficult. This is particularly an issue in adult diagnoses where processes from both disease states may be present, leading to a mistaken diagnosis of type 2 diabetes.[10,25] While traditionally T1D was considered a juvenile disease, as many as $50\,\%$ of T1D diagnoses may occur during adulthood.[21] Of adults diagnosed with

type 2 diabetes, $5\,\%$ to $15\,\%$ may have T1D-associated autoantibodies present.[25] This suggests the true number of individuals with T1D may be much greater than clinical diagnoses suggest.[2]

Around $90\,\%$ of patients diagnosed with T1D present with diabetes-associated autoantibodies.[26] Commonly assayed autoantigens include glutamic acid decarboxylase, insulin, and insulinoma-associated protein 2.[27] In any case, immune response to these autoantigens leads to the development of anti-islet pathogenic T cells.[26] This disease state results in the destruction of β-cells such that exogenous insulin is required for survival.

The "gold standard" for T1D diagnosis remains the measurement of C-peptide levels, a marker for endogenous insulin production.[28] C-peptide and insulin are secreted in equimolar amounts from pancreatic β-cells, but the half-life of insulin is only around 5 minutes while that of C-peptide is around 30 minutes.[29] Further, insulin may be cleared from circulation at a variable rate, while C-peptide is cleared at a constant rate.[30] Thus, measurement of serum C-peptide levels can accurately determine β-cell function, perhaps better than measuring insulin itself.[31] An immunoassay for detecting blood C-peptide levels was first developed in 1969, and such assays continue to be the main diagnostic criteria used in assessing the state of β-cell destruction.[32] Physiologically normal fasting plasma C-peptide levels range from $300\,\mathrm{pmol\,L^{-1}}$ to $600\,\mathrm{pmol\,L^{-1}}$, while levels below $200\,\mathrm{pmol\,L^{-1}}$ are associated with T1D.[30]

5.1.3 Epidemiology and Economic Impact

In 1986, Eisenbarth suggested a six-stage model to the development of T1D: genetic susceptibility, environmental triggering, autoimmunity, progressive loss of glucose-stimulated insulin secretion, overt diabetes, and complete β-cell destruction.[33] This is still the generally prevailing model used today.[2,8,21,34] A number of risk factors for developing T1D have been identified. Age is a major factor, with peak incidence occurring between 10 years and 14 years old.[34] Ethnicity may play a role as well, with large variations in prevalence of T1D among youth in five ethnic groups in the United States.[35] Certain genotypes and childhood environmental factors are also associated with the development of T1D.[34]

Contemporary staging of T1D was proposed in 2015.[36] Stage 1 is characterized by the development of two or more T1D-associated autoantibodies but with maintained euglycemia.[36] This progresses to stage 2 once β-cell function has degraded to the point of dysglycemia, but only when

the individual remains asymptomatic.[36] Finally, stage 3 represents symptomatic, overt T1D that can be diagnoses by American Diabetes Association (ADA) standards.[36]

Globally, T1D accounts for $5\,\%$ to $10\,\%$ of all diabetes diagnoses, though both the incidence and prevalence of T1D vary geographically (albeit with universal increases in incidence of $2\,\%$ to $3\,\%$ per year).[2,21,34] In the United States, the Centers for Disease Control and Prevention estimated over two million Americans had T1D in 2021.[37] For 2022, the American Diabetes Association estimated diagnosed diabetes had a direct cost of $306.6 billion with an additional $106.3 billion burden in indirect costs.[38] However, this study does not differentiate between T1D and other forms of diabetes.

Two studies have attempted to separate these diseases in an economic analysis and found T1D costs approach $14.4 billion to $14.9 billion per year in the United States.[39,40] These studies are over a decade old, and estimates focus on a population of approximately one million patients; this number has doubled since their publication, suggesting annual costs have far exceeded the initial results. A more recent study from 2020 estimated the healthcare resource utilization among patients with T1D was nearly $19,000 per patient per year.[41] This corresponds to about a 2.4-fold increase relative to healthy individuals.

5.2 Insulin Production and Secretion

Insulin production in β-cells is a multistep process. First, preproinsulin, a precursor molecule consisting of proinsulin and a signal peptide, is synthesized in the cytosol.[42] Each β-cell can synthesize around 6000 molecules of preproinsulin per second.[42,43] After being guided to the rough endoplasmic reticulum, the preproinsulin signal peptide is cleaved off, leaving proinsulin.[3] This insulin precursor consists of an “A” chain and a “B” chain with a connecting “C” peptide.[31] Within the rough endoplasmic reticulum, this C-peptide assists in guiding the folding of proinsulin and formation of three disulfide bonds.[31]

Transport to the Golgi apparatus follows folding of proinsulin.[3] Here, proinsulin is packaged into immature secretory granules rich in divalent zinc and calcium ions.[14,31] It is at this stage where hexamers form with two Zn^{2+} ions and one Ca^{2+} ion.[14,44] As these granules mature, ATP-dependent proton pumps cause acidification of the lumen.[44] This in turn activates proprotein convertases 1 and 2 as well as carboxypeptidase E, which contribute to the removal of C-peptide and cleaving

of C-terminal residues, respectively.[3,31,44] The result is equimolar amounts of mature insulin and C-peptide within the acidic granule. The insulin hexamer is much less soluble than the proinsulin hexamer at this pH, so crystallization quickly takes place.[14,44]

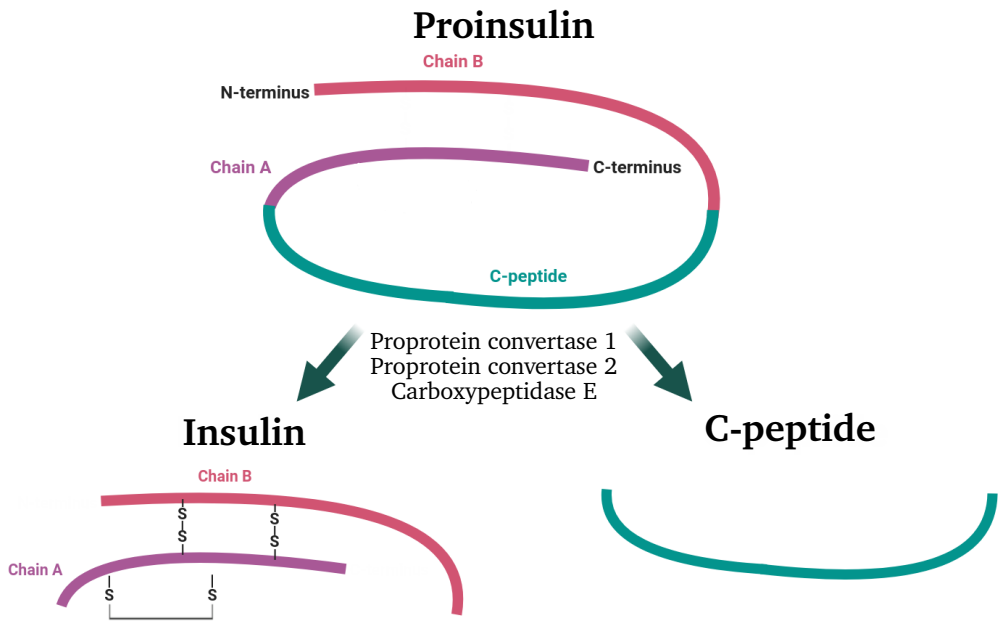

Figure 5.3: Proinsulin folding is facilitated by C-peptide, which is then cleaved off the mature insulin molecule.[3,16,31,44]

A major stimulus of insulin secretion is a rise in blood glucose levels, leading to increased glucose influx in β-cells.[3,13,44] As this is metabolized, there is a corresponding increase in the adenosine triphosphate (ATP) to adenosine diphosphate (ADP) ratio.[3,13,44] The shift in this ratio triggers ATP-sensitive potassium channels to close, depolarizing the cell membrane.[3,13,44] Voltage-gated calcium channels open in response to this depolarization, leading to an increase in intracellular calcium ions which ultimately stimulate exocytosis of insulin granules.[3,13,44] The increased pH of the extracellular milieu causes the dissolution of insulin hexamers back to monomeric form as they join the circulation.[15] This process is shown in Figure 5.4.

There is some debate as to which glucose transporter is responsible for glucose-stimulated insulin secretion.[45] Often, GLUT2 is cited as the transporter through which rising blood glucose triggers insulin secretion.[44,46] However, while this may be true in murines, human β-cells predominately express GLUT1 and GLUT3.[46,47] Despite more recent literature suggesting GLUT1 is the primary glucose transporter in human β-cells, there is evidence to suggest the relatively small amount of

GLUT2 present may still have a functional role.[3,13,48] At physiological glucose levels, GLUT1 and GLUT3 may already be at their maximum glucose transport velocities, while low-affinity GLUT2 will continue to increase glucose transport proportionally to blood glucose levels.[45] Yet, *in vivo* studies show transgenic GLUT1 alone can restore normal glucose-stimulated insulin secretion in GLUT2-knockout mice.[49] Further, Rorsman and Ashcroft note that the primary signals of insulin release *in vivo* are neurotransmitters and incretins, not hyperglycemia.[13] It is evident that despite all that has been elucidated about β-cell physiology, there remain gaps in our knowledge.

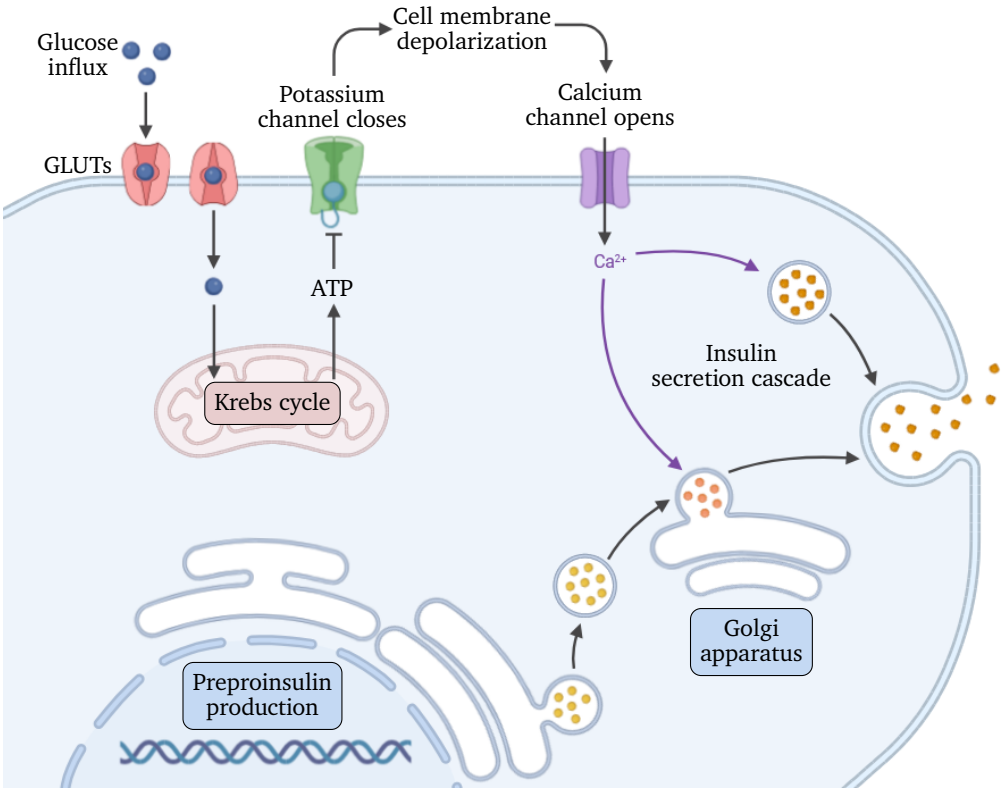

Figure 5.4: Higher blood glucose levels lead to more glucose metabolism within β-cells, increasing cytosolic ATP content.[3,13,44] This closes ATP-dependent potassium channels, depolarizing the cell membrane and opening voltage-gated calcium channels.[3,13,44] The influx of calcium ions triggers exocytosis of insulin granules.[3,13,44,50]

5.3 Insulin Therapy and Glucose Management

Descriptions of the disease we call diabetes mellitus today have been found dating back as far as circa 1550 BCE in an Ancient Egyptian medical papyrus.[51] The term *diabetes*, meaning "to pass through" in reference to the characteristic polyuria, was coined in Ancient Greece with the addition of *mellitus*, meaning "honey-sweet", in 1675 due to the excess sugar in the urine of patients.[52,53]

The distinction of two forms of diabetes and the implication of pancreatic origin came in the mid- to late-1800s.[53,54] The primary treatment, ineffective as it was for those with T1D, was a well-managed diet. In the early 1900s, even "starvation dieting" was recommended by the foremost diabetologists in America, Frederick Allen and Elliott Joslin.[55] Unfortunately, most all patients with T1D perished within months of the onset of symptoms.[52,54]

Luckily, in the early 20th century Frederick Banting hypothesized that administration of exogenous insulin could be used to treat T1D.[56] Banting's hypothesis was proven correct in 1922 when a dying diabetic child, Leonard Thompson, became the first human to be administered a therapeutic injection of insulin.[56] Today, human insulin analogues, often produced recombinantly in *Saccharomyces cerevisiae* yeast or *Escherichia coli* bacteria, dominate diabetes care.[57]

These insulins are key to effective management of T1D through intensive glucose control. The Diabetes Control and Complications Trial (DCCT) was a landmark controlled clinical trial starting in 1983 that aimed to compare the differences in conventional insulin therapy and intensive insulin therapy that kept blood sugars as close to the non-diabetic range as possible.[58] This trial enrolled 1441 patients and followed their glycemic control and development of diabetic complications for an average of 6.5 years.[58] The intensive therapy cohort showed significant reductions in rates of myriad chronic complications, although did also experience an increased risk of severe hypoglycemia.[58] This has led the ADA to set glycemic targets aligned with those of the intensive therapy DCCT cohort. As HbA1c correlates well with a three-month rolling mean blood glucose, the current ADA recommendation is to maintain an HbA1c of 7 % or less.[59]

The Epidemiology of Diabetes Interventions and Complications study (EDIC), an observational follow-up to the DCCT, has continued to investigate the long-term outcome of 88 % of the original DCCT participants.[60] Despite the conventional therapy group being trained on intensive therapy in the interim between the DCCT and EDIC, resulting in mean HbA1c values of 8.0 % for both cohorts, 18 years of follow-up have shown a "metabolic memory" among participants.[60] That is, despite losing separation of glycemic control, the initial intensive therapy cohort continues to have a substantial reduction in severe complications relative to the initial conventional therapy cohort.[60–63] Thus, both early and sustained intensive therapy is the most effective way to reduce chronic complications of T1D.[60]

It is worth noting that insulin requirements can vary drastically among individuals depending on

a variety of factors including age, sex, duration of disease, body mass index, physical activity, diet, method of insulin delivery, and presence of other comorbidities, risk factors, and pharmaceutical treatments.[64] During the initial treatment of T1D, some patients may even experience a partial remission in β-cell function, referred to as the "honeymoon phase", while euglycemia is maintained.[65] Interestingly, even decades after diagnosis some individuals with T1D may exhibit a small amount of residual β-cell function, which is associated with longer lifespan and reduced complications.[10,66,67] There is evidence to suggest these benefits may be due to both secretion of insulin and secretion of C-peptide.[68,69]

Two major advancements to T1D management are the insulin pump and the continuous glucose monitor. When using multiple daily injections, a long-acting insulin formulation must be taken once or twice each day to maintain basal insulin levels (to counter gluconeogenesis and glycogenolysis) along with up to several boluses of fast-acting insulin to correct hyperglycemia and counteract any consumed carbohydrates. In contrast, the continuous subcutaneous infusion offered by insulin pumps requires only fast-acting insulin which can be dosed much more precisely than with a manual injection, allowing dose delivery that fulfills the role of both basal and bolus injections. Meanwhile, continuous glucose monitors can sample glucose levels in interstitial fluid every few minutes and correlate the results to recent plasma glucose values, providing constant feedback and enabling preemptive action against rising or falling blood sugar levels. When pairing contemporary insulin pumps and continuous glucose monitors, some of which even work in concert to offer hybrid closed-loop algorithms to adjust insulin dosing, effective glycemic control is easier than ever before.[70] Usage of an insulin pump has been associated with a decrease in HbA1c percentage points of around $0.5\,\%$ to $1.0\,\%$, and use of real-time continuous glucose monitor is similarly associated with reductions in HbA1c and healthcare utilization.[71–74]

Despite this, consistent euglycemia is difficult to maintain for many patients with T1D. In the United States, data from 2018 shows insulin pump and continuous glucose monitor usage among individuals with T1D around $54.4\,\%$ and $45.9\,\%$, respectively.[75] Yet, that year only $21\,\%$ of adults with T1D in the United States achieved HbA1c values at or below the ADA recommended $7\,\%$.[76] It is clear that improvements in treatments and management strategies can continue to improve glycemic control for a large number of individuals living with T1D.

5.4 Chronic Complications

Hyperglycemia leads to increased oxidative stress through generation of reactive oxygen species and advanced glycation end-product (AGE) formation.[77–79] Furthermore, hyperglycemia results in increased glucose flux through the polyol pathway which inhibits innate antioxidant mechanisms as discussed in Section 1.2.3.[80,81] Both oxidative stress and AGEs themselves are implicated in the development of diabetic complications.[77–79]

Vascular damage and circulatory dysfunction are major pathophysiologies in T1D.[82,83] AGE receptors in the endothelium may lead to their accumulation in the subendothelial space, where they can impair production of the potent vasodilator nitric oxide (NO).[79,84] Additionally, AGEs have been shown to increase endothelial adhesion, leading to thrombosis, and promote atherogenesis, leading to vascular thickening and loss of elasticity.[78,79,84] Hyperglycemia itself can also lead to vasoconstriction via activation of protein kinase C through the *de novo* synthesis of diglyceride from intermediate products of glycolysis.[82,85]

Unfortunately, impaired perfusion due to T1D-mediated processes can occur even in individuals with excellent glycemic control.[86] This leads to both microvascular and macrovascular complications, some of which are summarized in Table 5.1.[86,87] These complications can have broad negative impacts on patient quality of life and contribute considerably to morbidity and mortality in T1D.[87]

Table 5.1: Perfusion-related complications in T1D.[37,88–91]

Complication	Prevalence in T1D (%)	Possible Effect
Macrovascular		
Cardiovascular disease	9.50	Heart failure
Peripheral artery disease	8.70	Lower extremity amputation
Stroke	4.00	Disability and death
Microvascular		
Nephropathy	39.2	Renal failure
Neuropathy	32.7	Varied nerve damage
Retinopathy	27.4	Progressive vision loss

5.4.1 Pathophysiologies

In patients with T1D, macrovascular complications tend to occur earlier in life with accelerated courses and increased mortality.[87] In general, the risk of cardiovascular disease and death is between twofold and eightfold greater in T1D than in the normal population.[10,92] Coronary artery disease

is the most common form of cardiovascular disease in T1D, resulting in accelerated atherosclerosis.[61,93–96] Predominately influenced by poor glycemic control and renal impairment, people with T1D have around a fourfold increased risk of heart failure compared to healthy individuals.[97,98] Similarly, the risk of stroke in T1D is about 3.7-fold higher for men and 4.8-fold higher for women.[99] Though the data are older and therefore may be exaggerated relative to contemporary figures, one study found an 85-fold increased risk of lower-extremity amputation by age 65 in people with T1D.[100] These complications have become more prevalent as management of T1D has improved and patient lifespan has increased.[2] However, strict glycemic control is associated with a significant reduction in the incidence of cardiovascular disease.[62,63]

Diabetic nephropathy is the leading cause of end-stage renal disease.[101] It is often caused by renal fibrosis due to hemodynamic dysfunction within the kidney causing glomerular hypertension and inflammation from oxidative stress.[102] Nephropathy is generally diagnosed on the basis of increased albumin concentrations in the urine, a condition referred to as albuminuria.[87] Advanced albuminuria presents with hypertension, hyperlipidemia, and a decrease in glomerular filtration rate.[103] Thus, diabetic nephropathy is highly associated with increased cardiovascular risk in patients with T1D; this condition results in a tenfold increase in risk of developing cardiovascular disease.[103]

Diabetic neuropathy is an umbrella term encompassing both sensorimotor and autonomic neuropathies that can be focal or diffuse.[101] Distal symmetric polyneuropathy is the most common of these disorders and results from damage to small sensory and large motor nerve fibers in the extremities.[104] This form of neuropathy is symptomatic approximately $50\,\%$ of the time and can result in sensory dysfunction (such as numbness, tingling, and burning sensations) and loss of vibration, touch, and joint position senses and tendon reflexes.[86,104] Autonomic neuropathies can vary widely in nature, but may manifest with tachycardia, orthostatic hypertension, gastroparesis, impaired neurovascular function, and hypoglycemia-associated autonomic failure.[101] Neuronal loss cannot be reversed, but can be slowed by improving glycemic control.[101]

Diabetes is also associated with bladder dysfunction, which may have a large impact on patient quality of life.[105] Termed cystopathy in the context of diabetes, the implication of autonomic neuropathy in its etiology suggests relation to neurogenic bladder dysfunction.[105,106] The prevalence of diabetic cystopathy is related to duration of disease, occurring in over $50\,\%$ of patients who have had diabetes for 45 years or longer.[106] It ultimately manifests as impaired bladder emptying, reduced

filling sensation, and loss of contractility.[107] Polyuria, a common symptom of hyperglycemia, may contribute to initial increases in bladder wall compliance, while changes to the extracellular matrix affects late-stage decompensation.[108] The remodeling of the bladder wall seen in cystopathy results from a reduction in collagen synthesis and increase in elastin synthesis.[109]

Diabetic retinopathy is one of the leading causes of blindness in adults aged 20 to 74 in the United States.[101] Perhaps one of the most debilitating complications of T1D, it occurs in four distinct stages.[87,110] The first three, mild, moderate, and severe nonproliferative retinopathy, start with microaneurysms and lead to progressive occlusion of retinal blood vessels.[87] These ischemic vessels upregulate vascular endothelial growth factor in response to hypoxia, leading to the final stage of diabetic retinopathy.[111] This stage, proliferative retinopathy, is characterized by angiogenesis with vessels growing along the retina.[87,110] However, these new vessels are thin-walled and prone to leaking, resulting in vision loss as blood pools in the center of the eye.[87,110] While vision loss due to retinopathy generally cannot be reversed, laser photocoagulation surgery can significantly reduce the risk of vision degeneration in cases of proliferative retinopathy.[87,101]

As briefly discussed in Section 1.2.1, there are a number of changes in the red blood cells (RBCs) of people with T1D as well. These are similar to many of the detrimental impacts of hyperglycemic RBC storage, collectively known as the "storage lesion", which are discussed in Section 1.2.3. Included in these changes are reductions in cellular deformability, membrane fluidity, ATP release, and stimulation of NO production, as well as increases in oxidative stress, aggregability, endothelial adhesion, hemolysis, and morphological changes.[112–115]

5.4.2 Mortality

Historically, T1D all-cause mortality has been significantly higher than the general population.[92,97,116–118] This excess mortality is best explained by acute complications such as DKA and hypoglycemia in young patients, while chronic cardiovascular conditions are the predominate factor as age and duration of disease increases.[97,119–121] The relationship between glycemic control and incidence of life-threatening complications has been clearly established.[58,60–63,97,98,118] As there have been significant advances in the management of T1D in recent decades, it is hopeful that the incidence of this morbidity and mortality will continue to decrease. Yet, there is still a large opportunity for advancement in the treatment of T1D to further improve glycemic control and

mitigate these issues.

5.5 Emerging Therapies

There are a number of new strategies to both combat and manage T1D that will hopefully come to fruition within the coming years. Some of these focus on screening for and delaying the onset of β-cell destruction, while others focus on pharmacological and technological innovations to enhance glycemic control and mitigate the development of chronic complications. While investigation of these innovations is still ongoing, there is also considerable effort into curing T1D by restoring endogenous insulin production.

Population-based screening programs have been piloted in several countries to assess genetic risk of T1D and presence of autoantibodies.[53,122] In some cases, these programs have been shown to reduce the incidence of DKA at the time of diagnosis by $75\,\%$ due to the anticipatory guidance they provide.[53,123] These programs can also identify high-risk individuals that may benefit from interventions to reduce autoimmunity.[53] Teplizumab, an anti-CD3 antibody, can delay the onset of stage 3 T1D by around 2.5 years in individuals with stage 2 T1D who receive a two-week treatment.[53,124,125] This first-in-class drug was approved by the Food and Drug Administration (FDA) in November 2022.[126]

The Bacillus Calmette-Guérin vaccine, typically used for tuberculosis, has also shown some promise in reducing HbA1c after long-term use.[127,128] Mechanistically, this may be caused by shifting the glucose metabolism of lymphoid cells from oxidative phosphorylation to aerobic glycolysis, which uses far more glucose.[129] However, there is debate over the efficacy of this treatment, and more randomized controlled trials are required.[130]

Transplantation of pancreatic islets has long been a subject of diabetes research, first attempted in 1893 in the United Kingdom.[131] In 2000, Shapiro et al. developed the *Edmonton protocol*, the first feasible clinical procedure for pancreatic islet transplantation.[132,133] This protocol has proven to be safe and moderately effective; while most patients are able to achieve insulin independence, one trial shows only around $10\,\%$ remained so after 5 years, though approximately $80\,\%$ had some residual C-peptide production and significantly reduced HbA1c.[134,135] There are also a number of investigations into the use of both human embryonic stem cells and induced pluripotent stem cells to replace β-cells.[133] The main advantage of this approach is the possibility of transplantation without

immunosupression and without relying on compatible donor tissue.[133]

While insulin is a critical component of glucose homeostasis, its action can only lower blood sugar levels. A counterregulatory hormone, glucagon, is secreted by pancreatic α-cells and acts to promote gluconeogenesis and glycogenolysis.[136] However, in T1D, glucagon release in response to hypoglycemia is impaired.[137] Thus, a more complete *ex vivo* system would include both hormones to better mimic *in vivo* glucose regulation. To this end, significant effort has gone into the development of bihormonal insulin/glucagon pumps that can act to both lower and raise blood glucose levels.[138] Such systems would enable much better closed-loop control of blood sugar levels, and their efficacy and safety has been demonstrated in small-scale studies.[139,140] Several hurdles stand in the way of clinical translation of these systems, such as the instability of aqueous glucagon, so none have yet succeeded in commercialization.[138]

In addition to improved insulin pumps, development of methods for transdermal insulin delivery is being investigated as well.[141] Chemical enhancement and encapsulation, iontophoresis and electroporation, ultrasound, jet injection, and microneedles are all possible approaches.[141] Recently, feasibility of a glucose-responsive microneedle insulin patch was demonstrated.[142] These were able to correct hyperglycemia and maintain near-normal blood glucose levels in diabetic models of both mice and minipigs.[142]

Similarly, glucose-responsive “smart insulins” are also being investigated by several research groups.[143,144] These may be able to circulate throughout the body with modulated activity proportional to blood glucose levels.[143,144] Beyond these, improvement of traditional insulin analogues is always an objective.[53] Changes to pharmacokinetics may enable even faster rapid-acting insulins or ultra-long lasting multi-day basal insulins.[53] Alternative delivery methods, such as oral and inhalable insulins, continue to be developed as well.[145,146]

5.5.1 C-peptide as an Auxiliary Therapeutic

C-peptide itself may play a yet-unproven role in T1D management. Contrary to the initial belief that this peptide is simply a byproduct of insulin production, it has been shown to be bioactive in numerous ways.[147] Demonstrated effects include specific binding to multiple cell types leading to increased intracellular calcium uptake, sodium-potassium pump activity, endothelial NO production, and blood flow, among others.[147,148] This has led some to posit that T1D is a two-hormone disease,

involving dysfunction of more than just insulin.[148] C-peptide replacement therapy has potential clinical benefits that could address pathophysiologies directly implicated in the development of many chronic complications of T1D.[149–151]

Of particular interest is the effect of C-peptide on glucose metabolism and blood flow *in vivo*.[152,153] Two studies published in 1992 found that infusing T1D subjects with physiological concentrations of C-peptide increased glucose utilization by $25\,\%$ and blood flow by $27\,\%$.[153,154] Supraphysiologic concentrations had no further effect, suggesting binding site saturation around $900\,\mathrm{pmol\,L^{-1}}$.[148,154]

Following successful animal studies, a year-long randomized controlled clinical trial was conducted to investigate the beneficial effects of C-peptide replacement therapy on peripheral neuropathy in humans.[155] This trial utilized a form of C-peptide modified to increase circulation time by attaching a $43\,\mathrm{kDa}$ molecule of polyethylene glycol to the N-terminus.[155] While the drug, known as Ersatta (a Swedish word meaning "to replace"), proved safe, it was not efficacious.[155] The trial was stopped at phase IIb and the sponsoring company, Cebix, shut down.

After the failure of Ersatta, interest in C-peptide research waned.[156] Despite decades of promising *in vitro* and *in vivo* research, the failure to translate C-peptide replacement to human therapy seemingly discouraged additional investigation. Perhaps one of the largest obstacles impeding additional research is the lack of an identified receptor for C-peptide.[156,157] Yet, not all have lost hope for the potential of this peptide, and important information continues to be published.[158–161]

While a receptor is still yet to be identified, years of thorough investigation by the Spence laboratory have elucidated a wealth of information regarding the function of C-peptide. Notably, the formation of a complex with albumin and a divalent or trivalent transition metal seems to be required for C-peptide to be delivered to and act on RBCs.[162,163] This has critical implications for the ability of C-peptide to act as a therapeutic for T1D. In the next chapter, a comprehensive update on the Spence laboratory's efforts into developing a functional C-peptide formulation will be provided.

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CHAPTER 6

PRECLINICAL DEVELOPMENT OF AN AUXILIARY THERAPEUTIC FOR TYPE 1 DIABETES

Portions of this chapter are adapted from previous unpublished work with permission.[1,2]

6.1 Introduction

Type 1 diabetes (T1D) is an autoimmune disease resulting in the destruction of pancreatic β-cells and subsequent deficiency in insulin production.[3] However, insulin is not produced, packaged, and secreted by itself. Its precursor, proinsulin, consists of both the "A" and "B" chains of mature insulin as well as a connecting "C" peptide that assists in protein folding before being cleaved from the parent hormone.[4] This results in an equimolar amount of insulin and C-peptide being released into the circulation from β-cell secretory vesicles.[5] Section 5.2 provides a detailed description of the synthesis and secretion of these hormones.

Human C-peptide is a 31-amino acid peptide hormone with a mass of $3020\,\mathrm{Da}$.[6] It is a random coil peptide and does not form any tertiary structure under normal conditions.[7] It contains five acidic amino acids residues but no basic residues and therefore has a negative charge under physiological conditions.[6,7] The isoelectric point of C-peptide is around pH 3.0, well below what is found even in the acidic lumen of mature insulin granules.[6,8] The typical fasting C-peptide plasma concentration ranges from $300\,\mathrm{pmol\,L^{-1}}$ to $600\,\mathrm{pmol\,L^{-1}}$ with postprandial peaks in the $1\,\mathrm{nmol\,L^{-1}}$ to $3\,\mathrm{nmol\,L^{-1}}$ range.[9]

The C-terminal pentapeptide, EGSLQ, appears to be the binding segment of C-peptide and is necessary for proper function.[10,11] Substitution of the pentapeptide glutamic acid residue (E27) results in complete loss of biological activity and impairs cellular membrane binding.[11,12] Interestingly, while the sequence of C-peptide is varied among mammals, the glutamic acid residue at position 27 is well-conserved (as are residues 1, 3, 6, 11, 12, 21, and 31).[9] The mechanism behind C-peptide functionality and membrane binding is still unknown, and the form of receptor is debated. G-protein coupled receptors appear to play some role in C-peptide activity, as both binding and functional effects are inhibited by pertussis toxin, a known modifier of receptor-coupled G proteins.[7,10,11] However, it is likely other interactions are involved in C-peptide binding, as not all effects of C-peptide are inhibited in this way.[11] Further, there is evidence a complex forms between C-peptide and serum albumin and therefore a receptor for C-peptide alone may not exist.[13] The fact that a receptor is

yet to be identified after decades of study continues to be an obstacle in C-peptide research.[14,15]

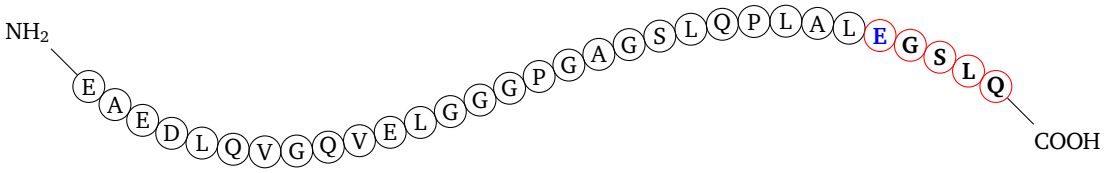

Figure 6.1: The sequence of C-peptide (A=alanine, D=aspartic acid, E=glutamic acid, G=glycine, L=leucine, P=proline, Q=glutamine, S=serine, V=valine). Note the pentapeptide EGSLQ motif (red) near the C terminus. The glutamic acid at position 27 (blue) is particularly important for functionality.

While C-peptide was often thought to be biologically inactive once cleaved from proinsulin, data from the Diabetes Control and Complications Trial (DCCT) showed a positive correlation between residual C-peptide production and clinical outcomes even after controlling for initial glycated hemoglobin (HbA1c) levels.[16] Additionally, contemporary understanding of C-peptide has shown it to be bioactive beyond just participating in the proper structural folding of insulin.[17] For example, C-peptide has been shown to bind to renal cells, endothelial cells, fibroblasts, neutrophils, and red blood cells (RBCs) with varying effects.[11,18–23] Through this, C-peptide improves microvascular function and dilation, sodium-potassium pump activity, endothelium-derived nitric oxide (NO) production, and insulin hexamer dissociation.[17,20,24] The specific binding between C-peptide and RBCs leads to increased RBC deformability and release of adenosine triphosphate (ATP), a known stimulus of production of the potent vasodilator NO.[14]

Despite the massive success of insulin analogues in managing blood glucose levels in T1D, myriad chronic complications remain prevalent.[25] One major pathophysiology of T1D is impaired blood flow in the microvasculature.[26] Data from the DCCT suggest intensive therapy may reduce microangiopathy, though it can occur even in individuals with excellent glycemic control.[27] Notably, these circulatory issues are an implicated etiology in many of the complications of diabetes.[28] While insulin itself may have some effect on blood flow, T1D may not be a single-hormone disease.[21,29]

Destruction of the pancreatic β-cells results in disruption of the production of both insulin and C-peptide. Of particular importance is the potential role of C-peptide in promoting vasodilation of and therefore perfusion through the microvasculature.[30,31] Despite this, there have been few attempts in delivering replacement C-peptide to restore blood flow in T1D. Development of a C-peptide-based auxiliary therapeutic for T1D that can mitigate the incidence of perfusion-related complications and

improve glucose distribution and metabolism would have a large impact on the long-term health of the diabetic population.

The proposed mechanism for such a therapeutic is fairly straightforward. C-peptide, when delivered by serum albumin, a globular carrier protein abundant in the bloodstream, binds specifically to RBCs.[13] When a divalent zinc ion is present in this purported complex, there is a significant increase in measurable levels of the GLUT1 glucose transporter within the RBC membrane.[14] Other divalent or trivalent transition metals seem to be congeners for zinc in this complex, as Fe^{2+}, Cu^{2+}, and Cr^{3+} have also been reported to interact with C-peptide to elicit a cellular response.[32,33] However, due to the high levels of zinc present within β-cell granules ($10\,\mathrm{mmol\,L^{-1}}$ to $20\,\mathrm{mmol\,L^{-1}}$), this ion is preferred in studies conducted by the Spence laboratory.[34]

With the increase in measurable GLUT1 comes an increase in glucose flux into the RBC, reportedly increasing glucose uptake by $31\,\%$.[33] As this glucose is metabolized through glycolysis (see Figure 1.2 for an overview of the pathway) more ATP is produced. Concomitant with this is a significant increase in deformability of the RBC membrane, a cell known to be more rigid in T1D.[22,35–37] RBCs release ATP in response to the mechanical shear that is often induced by flow through restricted blood vessels, a process which is inhibited by increased cellular rigidity.[38] Together, these effects of C-peptide contribute to around a $50\,\%$ increase in ATP release from treated RBCs *in vitro*.[13]

Within the circulation, free ATP binds to P2Y purinergic receptors on vascular endothelial cells.[39–41] This leads to an increase in cytosolic Ca^{2+} from intracellular stores, which binds to calmodulin to modulate the activity of endothelial nitric oxide synthase (eNOS).[42,43] The stimulated eNOS, along with cofactor tetrahydrobiopterin, produces NO through the conversion of L-arginine to L-citrulline as shown in Equation (6.1).[44]

$$2\,\text{L}-\text{arginine} + 3\,\text{NADPH} + 3\,\text{H}^+ + 4\,\text{O}_2 \xrightarrow{\text{THB}} 2\,\text{L}-\text{citrulline} + 4\,\text{H}_2\text{O} + 3\,\text{NADP}^+ + 2\,\text{NO} \quad (6.1)$$

The small NO molecule can then diffuse out of the endothelial cells and, eventually, into the smooth muscle cells of the vasculature. Within the myocyte, NO activates soluble guanylate cyclase, catalyzing the conversion of guanosine triphosphate to cyclyic guanosine monophosphate.[45] This leads to the activation of myosin phosphatase, which dephosphorylates the myosin light-chain causing muscular relaxation.[46]

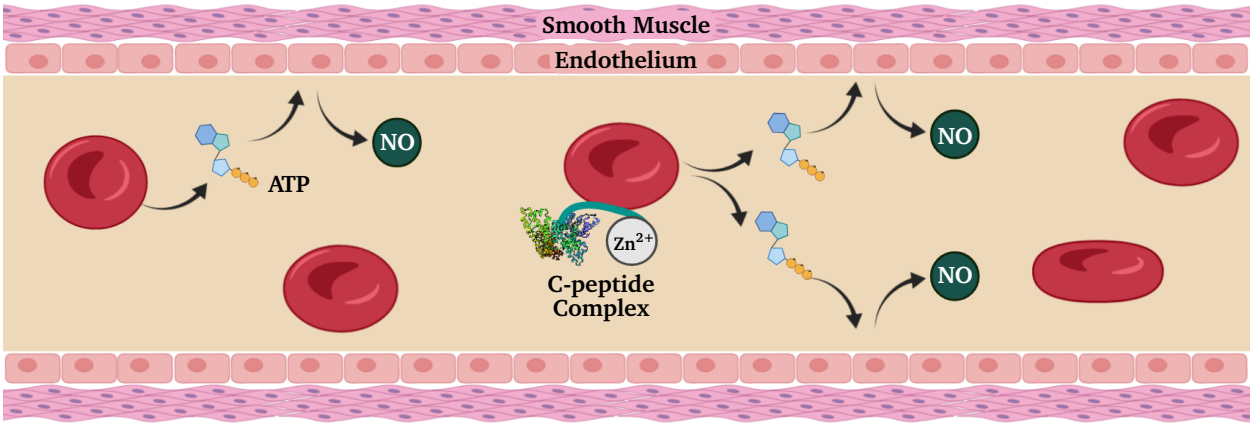

Figure 6.2: C-peptide increases ATP release from RBCs which stimulates endothelial NO production.[47]

6.2 C-Peptide as an Auxiliary Therapeutic

The persistence of chronic complications in T1D, despite the efficacy of contemporary insulin therapy in maintaining euglycemia, suggests there may yet be some "missing link" in management strategies. A wealth of information has shifted our understanding of the effects of C-peptide *in vivo* such that some propose T1D is indeed a dual-hormone deficiency disease.[11,20,21,48–50] Thus, there may be sufficient evidence to suggest C-peptide is this "missing link", and that replacement therapy along with insulin will improve long-term outcomes in T1D.[7,9,17,51,52]

Investigations of the beneficial effects of C-peptide have been well-documented by multiple authors.[7,9,17,23,50,52] For the sake of brevity, a full recapitulation of the literature will not be provided here. However, a brief chronology of pertinent publications is summarized in Table 6.1. Note that this is not intended to be an exhaustive list, but rather a glimpse into the landscape of therapeutic-oriented C-peptide literature over recent decades. A brief summary of the model used in each study is provided; generally, small-scale human or rat studies were used for *in vivo* work while donated human blood is used for *ex vivo* work. Cultured cells are used in some *in vitro* studies and include human umbilical vein endothelial cells or bovine pulmonary artery endothelial cells.

Table 6.1: Chronological summary of select publications elucidating the beneficial effects of C-peptide.

Year	Citation	Model	Main Findings
1992	[53]	Human, *in vivo*	Improved blood flow and capillary diffusion capacity. Augmented oxygen and glucose uptake.
	[54]	Human, *in vivo*	Reduced glomerular filtration rate and increased whole-body glucose utilization.
1993	[55]	Human, *in vivo*	Reduced glomerular permeability. Improved metabolic control.
1996	[31]	Murine, *ex vivo*	Active vasodilation of the microvasculature in skeletal muscle.
	[56]	Human, *in vivo*	Improved autonomic nerve function.
1997	[57]	Murine, *in vivo*	Prevention of vascular and neural dysfunction.
1999	[22]	Human, *ex vivo*	Improved RBC deformability.
2000	[58]	Human, *in vivo*	Improved renal function. Amelioration of autonomic nerve dysfunction.
2001	[59]	Murine, *in vivo*	Prevention and improvement of diabetic polyneuropathy.
2002	[60]	Human, *in vivo*	Improvement of myocardial function and perfusion.
2003	[61]	Murine, *in vivo*	Improved nerve function mediated by an NO-sensitive vascular mechanism.
	[62]	Bovine, *in vitro*	Stimulation of Ca^{2+}-sensitive eNOS.
	[63]	Human, *in vivo*	Increased sensory nerve conduction velocity. Improvement of vibration perception.
2004	[64]	Murine, *in vivo*	Prevention of nociceptive sensory neuropathy.
	[65]	Murine, *in vivo*	Corrected endoneurial blood flow.
2007	[66]	Human, *in vivo*	Improved sensory nerve function in early-stage neuropathy.
	[67]	Murine, *in vivo*	Improvement in diabetic polyneuropathy.
2008	[33]	Human, *ex vivo*	Increased RBC ATP release and enhanced glucose uptake.
2012	[68]	Human, *ex vivo* Bovine, *in vitro*	C-peptide + Zn^{2+} increases RBC ATP release leading to increased endothelial NO production.
2013	[69]	Human, *in vitro* Murine, *in vivo*	Prevention of hyperglycemia-induced endothelial cell apoptosis.
2015	[13]	Human, *ex vivo*	C-peptide + Zn^{2+} requires serum albumin for delivery to RBCs.
2021	[49]	Canine, *in vivo*	Enhancement of hypoglycemia-induced glucagon secretion.

6.2.1 Previous Attempts

The notion of administering exogenous C-peptide as a potential T1D therapeutic is not novel. To date, there have been four randomized double-blind placebo-controlled clinical trials investigating the safety, pharmacokinetics, and therapeutic effects of C-peptide in T1D. The first two studies, taking place in 2002 (Swedish Research Council project number 14x-4255) and 2003 (NCT00278980) in Sweden, administered unmodified C-peptide.[63,66] The third and fourth studies, taking place in 2011 and 2012 (NCT01293461 and NCT01681290, respectively), were performed in the United States and utilized a modified C-peptide.[70] This modification "PEGylated" the C-peptide by attaching a 43 kDa molecule of polyethylene glycol to the N-terminus to increase circulation time.[70]

The initial 2002 study administered either a placebo or 1.8 mg of C-peptide daily to participants for three months, split into four subcutaneous injections throughout the day.[63] There were 49 participants enrolled in the study with 46 completing the protocol ($n = 20$ placebo, $n = 26$ treatment).[63] Relative to the placebo group, the treatment group had a statistically significant increase in their sensory nerve conduction velocities and vibration perception.[63] There was no significant change in temperature perception, and no adverse events or drug reactions were observed.[63]

The follow-up 2003 study by Creative Peptides Sweden included both a low dose of $1.5\,\mathrm{mg\,day^{-1}}$, equivalent to physiological replacement, and a high dose of $4.5\,\mathrm{mg\,day^{-1}}$.[66] The C-peptide doses (or placebo) were administered to 161 participants as four subcutaneous injections per day for six months.[66] At the conclusion of the study, 139 subjects had completed the protocol ($n = 47$ placebo, $n = 53$ low dose, $n = 39$ high dose).[66] In both treatment groups, improvements were seen in sensory nerve conduction velocities, neuropathy impairment assessment scores, and vibration perception relative to the placebo control group.[66] No adverse events or reactions occurred during the trial for either treatment group.[66]

In 2011, after acquiring Creative Peptides Sweden, California-based Cebix sponsored clinical trials for their long-acting PEGylated C-peptide formulation, Ersatta (CBX129801). Cebix also sponsored toxicokinetic studies using primate models.[71] In these studies, healthy cynomolgus monkeys received weekly subcutaneous injections of either a placebo or PEGylated C-peptide for either 26 weeks or 39 weeks.[71] A subset of subjects was also given a seven-week recovery period prior to necropsy while the remainder were immediately examined.[71] PEGylated C-peptide doses were provided to the treatment groups at supraphysiologic dosages of $0.4\,\mathrm{mg\,kg^{-1}\,week^{-1}}$, $1.33\,\mathrm{mg\,kg^{-1}\,week^{-1}}$, or

$4.0\,\text{mg}\,\text{kg}^{-1}\,\text{week}^{-1}$, resulting in plasma C-peptide concentrations far exceeding physiological ranges (by up to three orders of magnitude for the highest dose).[71] All dosages were well-tolerated with no evidence of systemic toxicity, adverse effects, immunogenicity, or atherogenesis in any treatment group.[71]

In the 2011 phase I trial, three cohorts of 10 participants each were enrolled in a multiple ascending dose escalation study.[72,73] Subjects received either a placebo ($n = 2$) or PEGylated C-peptide ($n = 8$) by subcutaneous injections.[72,73] Cohorts A and B received an initial dose of $0.3\,\text{mg}$ or $1.0\,\text{mg}$, respectively, followed by a three-week waiting period, followed by four more weekly injections.[72,73] Cohort C followed a similar protocol, but with $3.3\,\text{mg}$ doses and only three weekly injections following the waiting period.[72,73] Finally, a fourth cohort was enrolled, consisting of 42 participants ($n = 13$ placebo, $n = 29$ treatment).[73] This cohort D received an initial loading dose of $2.0\,\text{mg}$ of PEGylated C-peptide, followed by a one-week waiting period, followed by eleven weekly $0.8\,\text{mg}$ doses.[73] PEGylated C-peptide was found to be safe and well-tolerated in the study participants, with C-peptide plasma concentrations being approximately dose-proportional.[72,73]

The 2012 phase II trial by Cebix enrolled 250 participants to receive weekly subcutaneous injections of PEGylated C-peptide.[70] The subjects received a placebo, $0.8\,\text{mg}$ doses, or $2.4\,\text{mg}$ doses.[70] At the conclusion of the study, 198 subjects had completed the protocol ($n = 85$ placebo, $n = 53$ low dose, $n = 60$ high dose).[70] While neither treatment group showed improvements in sensory nerve conduction velocity or neuropathy assessments relative to the placebo group, vibration perception was significantly improved.[70] PEGylated C-peptide was well-tolerated and primarily nonimmunogenic, with similar incidences of adverse effects in all three treatment groups.[70]

Unfortunately, the 2012 clinical trial failed in phase IIb and development of Ersatta was discontinued. Despite Ersatta's failure to move forward to a phase III clinical trial, the studies performed by Cebix (and its precursor, Creative Peptides Sweden) provide valuable information on the potential of C-peptide as a therapeutic for T1D. C-peptide not only has shown to be clinically efficacious but has also been demonstrated to be safe, nontoxic, and well-tolerated in both animal models and human studies. The Spence laboratory has identified key weaknesses in the Ersatta trials believed to have contributed to their failure, and we are hopeful that the groundwork has been laid for another attempt at a C-peptide therapeutic.

6.2.2 Recent Developments

The Spence laboratory has made many reports on the biological activity of C-peptide and its requisite conditions to elicit cellular effects in recent years. In 2008, Meyer et al. reported that a complex of C-peptide with Fe^{2+} or $^{3+}$ is required to increase glucose uptake through GLUT1 and promote ATP release in RBCs.[33] A follow-up 2009 review from Medawala et al. elaborated on the requirement of metal-activation of C-peptide and addressed the potential for iron impurities in studies conducted by other research groups.[40] This review also suggested that divalent zinc may be the most likely candidate ion to complex with C-peptide *in vivo* due to the high concentrations within β-cell granules.[40] Notably, while such metal ions do exist in the bloodstream, it is unlikely they are bioavailable or will outcompete sodium or potassium for binding sites.[40]

A 2010 publication from Keltner et al. highlighted the importance of the C-terminal pentapeptide, EGSLQ, and confirmed substitution of the well-conserved glutamic acid residue (E27) resulted in a complete loss of biological activity.[12] Giebink et al. then used a microfluidic device to demonstrate zinc-activated C-peptide can stimulate RBC ATP release, which downstream can mediate endothelium-derived NO production in 2012.[68] Finally, the third required component of the C-peptide complex was confirmed by Liu et al. in a 2015 paper showing serum albumin is required to deliver C-peptide to RBCs.[13] This paper also suggests two C-peptide molecules can be carried by each molecule of human serum albumin.[13]

Other publications by the Spence laboratory have elucidated further potential *in vitro* applications of C-peptide. Lockwood et al. demonstrated in 2016 that significantly more C-peptide binds to RBCs from people with multiple sclerosis compared to controls or other neurological diseases.[74] Such a quantitative biomarker may have important implications as a potential *in vitro* diagnostic, as the current diagnostic process is lengthy and error-prone.[74] Additionally, Liu et al. suggested in 2022 that a solution containing C-peptide and Zn^{2+} may be beneficial for the rejuvenation of RBCs stored for transfusion medicine by improving cellular deformability and ATP release.[75]

Focusing on applications in T1D, Gieger et al. showed in 2020 that serum albumin enables specific and saturable binding of C-peptide to RBCs and, when Zn^{2+} is present, there is a significant increase in measurable membrane GLUT1 glucose transporter.[14] In 2022, Jacobs et al. reported that glycation of serum albumin impairs its ability to deliver C-peptide to RBCs.[76] As people with T1D often have higher levels of glycated serum albumin, this may help explain replicability issues with *in vivo*

studies involving C-peptide replacement therapy. Armed with all of this new information, the Spence laboratory is confident a properly formulated complex of C-peptide, serum albumin, and divalent zinc may finally succeed in delivering beneficial effects *in vivo* for people with T1D.

6.3 Development of an Activated C-Peptide Formulation

A novel auxiliary therapeutic for T1D consisting of C-peptide, serum albumin, and Zn^{2+} could help to alleviate circulatory issues by improving microvascular tone. In doing so, it is expected the litany of chronic complications experienced in T1D may be reduced. This may also improve RBC health and overall glucose distribution and metabolism. These outcomes would lead to a marked reduction in the incidence of diabetic complications, improving patient quality of life and reducing a significant burden (both financial and physical) on the healthcare system. This in turn could shift the paradigm of diabetes treatment and prognosis, just as the advent of exogenous insulin once did over a century ago. If successful, such a therapy could be the most important innovation in diabetes care since the invention of insulin pumps and continuous glucose monitors.

An all-in-one C-peptide formulation must contain all three necessary components for activity: serum albumin, Zn^{2+}, and the C-peptide itself.[13] As both human serum albumin and zinc sulfate solutions are available as pharmaceuticals already approved by the Food and Drug Administration (FDA), a formulation of purified C-peptide compounded with these commercial products is proposed. An injectable, insulin-like solution will be the most convenient and familiar form for patients, and therefore the tonicity and sterility of the solution must also be considered.

To this end, *m*-Cresol, a common preservative in many insulins on the market today, will be included in the formulation to prevent microbial growth.[77] Concentrations ranging from $0.15\,\%$ to $0.315\,\%$ are found in various insulin formulations, indicating values within this window are both safe and above the minimum inhibitory concentration for any microbes of concern.[77] An *m*-Cresol concentration of $0.25\,\%$ will be used here. Additionally, sodium chloride will be included to ensure isotonicity of the formulation to prevent tissue damage at the injection site.

The proposed formulation is found in Table 6.2. Serum albumin is supplied in excess at a $2{:}1$ ratio relative to C-peptide, and zinc sulfate is similarly supplied in excess at a 2:1 ratio relative to serum albumin. The concentration of C-peptide aims to match the concentration of exogenous insulins. While there are multiple standardized concentrations of insulin, by far the most popular is the U-100

standard ($100\,\mathrm{IU\,mL^{-1}}$).[78] With 34.7 µg of insulin per iu, it follows that the concentration of U-100 insulin is $3.47\,\mathrm{g\,L^{-1}}$.[79] Insulin has a molar mass of $5808\,\mathrm{g\,mol^{-1}}$, leading to a molar concentration of $597\,\mathrm{\mu mol\,L^{-1}}$.[79]

Table 6.2: Proposed formulation of an activated C-peptide therapeutic.

Component	Molarity ($\mathrm{mmol\,L^{-1}}$)	Osmolarity ($\mathrm{mOsm\,L^{-1}}$)
C-peptide	0.597	0.597
$ZnSO_4$	2.39	4.78
Albumin	1.19	1.19
NaCl	135	270
m-Cresol	23.1	23.1
Total osmolarity ($\mathrm{mOsm\,L^{-1}}$)		300.

The basis for this C-peptide concentration is that, ultimately, a replacement strategy aims to mimic physiological concentrations. As C-peptide is secreted in equimolar amounts to insulin *in vivo*, it follows exogenous C-peptide should be supplied in an equimolar ratio to the exogenous insulin already being administered by patients.[5] In an isovolumetric insulin–drug dosing scheme this design ensures equimolar administration of insulin and C-peptide, mimicking the physiological secretion ratio. This will help to ensure accurate and convenient dosing and increase patient compliance using an "equal volume" dosing strategy, such that a patient can administer their insulin as usual before administering an equal volume of the formulation.

It is currently unknown how the formation of a C-peptide/serum albumin/Zn^{2+} complex will affect bioavailability and pharmacokinetics following a subcutaneous injection. This information is critical to accurately determine dosing as it is known some amount of the injected bolus will be lost to metabolism before reaching the circulation.[80] While it is already known endogenous C-peptide has a much longer half-life compared to insulin, it may be reasonable to begin by presuming similar bioavailabilities for exogenous C-peptide and insulin.[81] As equal plasma concentrations of insulin and the C-peptide complex are desired, a relative bioavailability can be characterized by Equation (6.2).

$$F_{relative} = \frac{AUC_{complex} D_{insulin}}{AUC_{insulin} D_{complex}} \tag{6.2}$$

Here, AUC is the area under a plasma drug concentration curve versus time per Equation (6.3) and D is the injected dose to which each curve is normalized. Thus, $F_{relative}$ will serve as a bioavailability

equivalence measure such that we can achieve equimolar dosing with insulin by adjusting dosing of the complex. If $F_{relative}$ is not near unity, dose compensation may be applied per Equation (6.4).

$$AUC = \int_0^\infty [drug]_{plasma}\,\mathrm{d}t \tag{6.3}$$

$$D_{adjusted} = \frac{D_{complex}}{F_{relative}} \tag{6.4}$$

Alternatively, the C-peptide concentration of the complex formulation may also be adjusted by a factor of $F_{relative}^{-1}$ to maintain equal-volume dosing. As the bioavailabilities of FDA-approved insulins are already well-studied, $F_{relative}$ can be determined relatively easily once the complex is approved for human use in preliminary studies. However, while this scheme may be ideal in terms of biomimetic replacement, supraphysiologic plasma levels of PEGylated C-peptide have been demonstrated to be safe in primate models.[71] Since all C-peptide binding sites are expected to be saturated at physiological concentrations, it may be easier to target any plasma concentration exceeding $900\,\mathrm{pmol\,L^{-1}}$.[17] Such a goal may have to be justified to regulators and clinicians, however.

Characterization of the stability and shelf life of the formulation will also be critical to gain FDA approval. These tests will follow stability testing guidelines published by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) in documents *Q1A*, *Q1B*, and *Q1E*.[82–84] The guidance provided by the ICH includes detailed descriptions of stability testing recommendations for various batches, temperatures, humidity ranges, lighting conditions, and more, as well as evaluation of this data to determine appropriate expiration dates and recommended storage conditions. Ideally, the formulation could be stored similarly to current insulin analogues on the market by being refrigerated for up to 90 days or used in non-refrigerated scenarios (e.g., in infusion pumps or injection pens) without changes in efficacy for several days. Preliminary data from the Spence laboratory suggests that the C-peptide/serum albumin/Zn^{2+} complex may be stable at refrigerated temperatures for many weeks.

C-peptide itself does not form disulfide bonds and participate in folding, rather, it remains unstructured at physiological pH.[85] This may prove beneficial for storage applications as functional changes due to denaturation are virtually nonexistent. As C-peptide seemingly does not require any particular conformation to bind to albumin or be delivered to RBCs, stability studies evaluating conformational changes should not be required.

The FDA maintains stringent requirements for the manufacturing, testing, and release of all drug products. This includes a set of Current Good Manufacturing Practice regulations covering everything from manufacturing to packaging of the product. By following guidance from the ICH published in document *Q6B*, quality control and release testing procedures will meet or exceed FDA requirements.[86] Similarly, ICH document *Q7* provides recommendations for manufacturing facilities, methodologies, and controls and will be used to confirm compliance with all regulatory requirements.[87] Additionally, direct consultation with the FDA Center for Drug Evaluation and Research will ensure compliance of all regulations pertaining to this investigational new drug.

Table 6.3: Examples of C-peptide formulation quality control and release specification testing.

Specification	Test Methodology
Appearance	Qualitative description must be compared to desired formulation.
Identity	Physicochemical characterization (amino acid sequence and molecular weight).
Purity	Mass spectrometry to verify impurity presence and abundance.
Quantity	Enzyme-linked immunosorbent assay to determine ingredient concentration.
pH	Measurement of pH with adjustments to 7.4 *pro re nata*.
Osmolarity	Osmometer measurements with adjustments to $300\,\mathrm{mOsm\,L^{-1}}$ *pro re nata*.

6.3.1 Preclinical Efficacy Testing

Although some improvements were seen in the Ersatta clinical trials, the overall efficacy was not sufficient to justify further investigation. However, the administration of solely PEGylated C-peptide may help explain these underwhelming results. In 2015, the Spence laboratory reported that serum albumin was required to deliver metal-activated C-peptide to RBCs.[13] Later, a 2022 paper reported that serum albumin glycated to levels seen in T1D is unable to effectively deliver C-peptide to these cells.[76] Therefore, it is possible the administered PEGylated C-peptide was unable to be delivered by endogenous serum albumin in the clinical trial participants. Furthermore, if any C-peptide was able to be delivered to the RBCs, there may not have been any metal activation of the complex and thus no downstream effect.

While the formulation proposed here will deliver both a metal for C-peptide activation and exogenous serum albumin with normal glycation levels, there is still some cause for concern regarding RBC glycation. As a receptor for such a complex, or C-peptide itself, has not been identified, RBC glycation could theoretically affect the ability of such a receptor to bind a C-peptide complex. Thus,

investigation into how increasing levels of RBC glycation may affect C-peptide delivery and efficacy is warranted.

To this end, studies into how RBC glycation affects C-peptide binding were initiated. Ideally, an array of donors with T1D and varied HbA1cs would be used for these studies. Unfortunately, due to an inability to consistently get these samples, *in vitro* glycation of healthy control RBCs was attempted instead.

All blood was collected via forearm venipuncture from informed and consenting donors following procedures approved by the Biomedical and Health Institutional Review Board at Michigan State University. Heparinized Vacutainer blood collection tubes (BD; Franklin Lakes, NJ) were used for collection. Whole blood samples were centrifuged at $2000\,\mathrm{gravitationalforceequivalent}(g)$ for 10 minutes and the plasma and buffy coat were aspirated off. The packed RBCs were then washed thrice in a physiological salt solution (PSS) buffer. Each wash was followed by further centrifugation and aspiration of the supernatant. After the third wash, only the packed and isolated RBCs remained.

Standard PSS was prepared as follows, in $\mathrm{mmol\,L^{-1}}$: KCl, 4.7; $CaCl_2$, 2.0; NaCl, 140.5; $MgSO_4$, 1.2; Tris, 21.0; glucose, 5.5. Bovine serum albumin was added to a concentration $5\,\%$ and the pH was adjusted to 7.4 before being filter sterilized. Alternative PSS solutions were also prepared using 10-fold, 50-fold, and 100-fold glucose concentrations. All reagents were purchased from Sigma-Aldrich (St. Louis, MO).

The RBCs were then subjected to *in vitro* glycation per the protocol proposed by Batista da Silva, et al.[88] Briefly, $500\,\mu\mathrm{L}$ aliquots from each donor were resuspended with $500\,\mu\mathrm{L}$ of PSS for each glucose concentration. These samples were then incubated at $37\,^{\circ}\mathrm{C}$ with orbital shaking at $300\,\mathrm{RPM}$ overnight (approximately 19 hours). The samples were then centrifuged at $500\,g$ for 5 minutes, the supernatant was aspirated off, and the packed RBCs were resuspended in normoglycemic PSS to around $50\,\%$ hematocrit (HCT).

Aliquots of each sample were then added to PSS containing $20\,\mathrm{nmol\,L^{-1}}$ each of $ZnCl_2$ (Sigma-Aldrich; St. Louis, MO) and human C-peptide (Peptide 2.0 Inc.; Chantilly, VA). The final volume of each sample was $1\,\mathrm{mL}$ and the final HCT was $7\,\%$. The samples were then incubated for 1 hour at $37\,^{\circ}\mathrm{C}$ with orbital shaking at $300\,\mathrm{RPM}$. Following incubation, each sample was centrifuged for 5 minutes at $2000\,g$ with soft deceleration. Approximately $700\,\mu\mathrm{L}$ of supernatant was removed from each sample and used to quantify unbound C-peptide. This was accomplished via colorimetric enzyme-linked

immunosorbent assay (ELISA). The ELISA kits used were FDA-approved human C-peptide assays (ALPCO; Salem, NH). The results of these studies are shown in Figure 6.3.

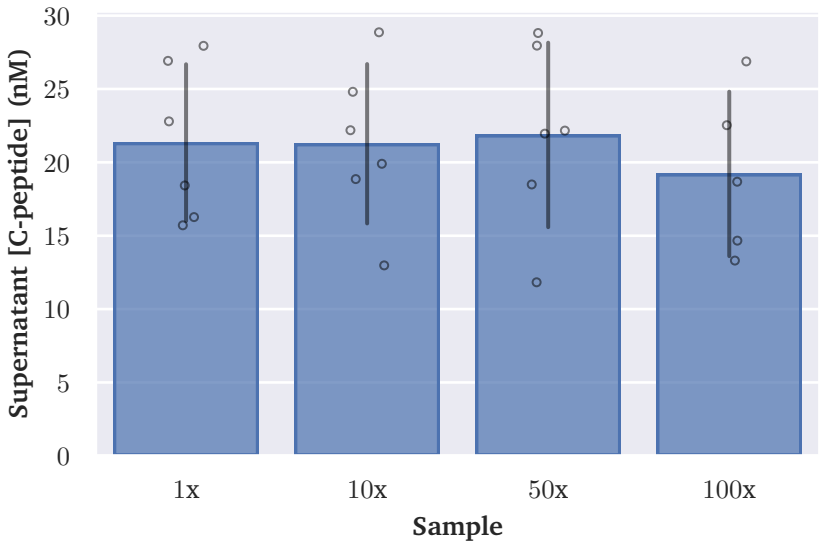

Figure 6.3: Unbound C-peptide remaining in sample supernatant (mean ± SD, $n = 6$). RBCs underwent *in vitro* glycation by incubation in PSS with 1x, 10x, 50x, or 100x glucose levels.

There are two notable issues with the data. First, although there was no C-peptide binding, several assays reported concentrations exceeding the initial $20\,\mathrm{nmol\,L^{-1}}$ added to each sample. Second, while these ELISA kits are generally quite precise, there is enormous variability in the results. The coefficient of variation for these samples ranged from $25.0\,\%$ to $29.2\,\%$. At this point, it was evident something was going wrong during these studies.

The first point may be resolved when considering the composition of the samples. Historically, the Spence laboratory has observed approximately $2\,\mathrm{pmol}$ of C-peptide binding following similar RBC incubations.[13,14] This is typically calculated by subtracting the ELISA-reported supernatant C-peptide concentration from the initial $20\,\mathrm{nmol\,L^{-1}}$. However, this does not account for the volume the RBCs themselves occupy in each $1\,\mathrm{mL}$ sample. As the HCT is $7\,\%$, the true volume of extracellular liquid is actually $930\,\mu\mathrm{L}$. Presuming no C-peptide binding, the nominal $20\,\mathrm{nmol\,L^{-1}}$ may actually be closer to $21.5\,\mathrm{nmol\,L^{-1}}$. Thus, the concentrations reported here may align quite well with the hypothesis that binding to RBCs was completely inhibited.

Despite this, there was no explanation as to why C-peptide was no longer binding to RBCs as expected. Similarly, the newfound imprecision of a standard assay contributed to the problem.

Following this, multiple senior members of the Spence laboratory attempted, and failed, to replicate over a decade of previous work demonstrating specific and saturable C-peptide binding to RBCs. The only success was in the confirmation of some issue with the reagents, the ELISA assays, or both.

6.4 Troubleshooting the C-Peptide Complex

As further work developing preclinical tests and procedures necessitated a functional peptide, efforts shifted toward resolving these new issues. The ability to precisely measure C-peptide concentrations is critical, so initial efforts first focused on rectifying the variability in measurements. In addition to the standard colorimetric ELISA kits (Alpco; Salem, NH), two alternatives were tested as well. One was also a colorimetric ELISA from a different manufacturer altogether (IBL America; Minneapolis, MN) while the other used a chemiluminescent reporter enzyme (Alpco; Salem, NH). However, several dozen tests by the Spence laboratory validated all assay performance. Matrix effects, manufacturing errors, inaccurate standards, alternative calibration methods, and different reporter types were all investigated. Although ELISAs are very sensitive to experimental error, precision was within kit specifications when using supplied standards and reference calibrators but not when using an in-house C-peptide.

Concurrently, whether C-peptide function was truly impaired was determined by assessing stimulation of RBC ATP release. Samples of RBCs were prepared and incubated in PSS with $20\,\mathrm{nmol\,L^{-1}}$ C-peptide and $ZnCl_2$ as previously described. Control samples were similarly prepared, but RBCs were incubated in PSS only (with no C-peptide or $ZnCl_2$). Each sample was then centrifuged for 5 minutes at $500\,g$ with slow deceleration and approximately $500\,\mu L$ of supernatant was collected. ATP standards were prepared via serial dilution of a stock solution prepared from ATP disodium hydrate (Sigma-Aldrich; St. Louis, MO). Firefly lantern extract (Sigma-Aldrich; St. Louis, MO) and D-luciferin (Gold Biotechnology; St. Louis, MO) were used to prepare a luciferin/luciferase solution. Luminescence from this solution is proportional to the ATP concentration, enabling precise quantification of ATP release from RBCs.

Aliquots of $100\,\mu L$ of the collected supernatant and each ATP standard were added in triplicate to a 96-well plate. A micropipette was used to add and mix $100\,\mu L$ of luciferin/luciferase solution one well at a time. After a set interval of 15 seconds, a microplate reader (FlexStation 3; Molecular Devices; San Jose, CA) was used to measure the luminescence from the well with an integration

time of 500 ms. This was repeated for every well individually before averaging the results of each replicate. Although these studies were repeated several times, ATP release from the treated and control RBCs were statistically equivalent. Therefore, it was concluded C-peptide binding was indeed being inhibited; simple quantification errors in binding measurements were not the sole cause of these abnormal results.

Once this was confirmed, attention turned to the reagents used in these studies. It was hypothesized some reagent had perhaps spoiled or been contaminated with something that was inhibiting the interactions between C-peptide, serum albumin, and RBCs. Eventually, all reagents involved were replaced and all stock solutions remade. Calibration of all equipment (pH meters, balances, and incubators) was checked as well. Yet, both binding and functionality assays showed no improvement.

While bovine serum albumin has been shown to function identically to human serum albumin, this too was substituted. Pharmaceutical grade human serum albumin (CSL Behring; King of Prussia, PA) and zinc sulfate solutions (American Regent; Shirley, NY) were procured for further studies. Commercially available human washed RBCs in phosphate buffered saline were purchased (Rockland Immunochemicals; Pottstown, PA) to ensure the in-house drawing and washing of blood was not involved. Unfortunately, none of these measures succeeded in restoring C-peptide functionality.

This narrowed the possible cause down to the C-peptide itself. Commercial C-peptide can be purchased at a relatively high purity by weight, yet this can translate to relatively high levels of contamination when considering the molecular mass of C-peptide relative to metal ions.[15] Therefore, the Spence laboratory purifies all C-peptide in-house via high-performance liquid chromatography (HPLC). Though previously thought unlikely to be responsible for these issues, by process of elimination the possibility of recent batches being ruined during purification had to be considered.

Once again, all reagents involved in these separations were replaced. A new HPLC column was purchased in case the previous column had somehow become fouled. Crude C-peptide was purchased from the previous source (Peptide 2.0; Chantilly, VA) as well as an alternative manufacturer (Bachem; Torrance, CA). After careful review of previous purification procedures, aliquots of these crude peptides were purified and lyophilized for future use. Despite the great care taken during these processes, functionality was not yet restored.

Investigations into the peptide itself then took place. Collaboration with the Mass Spectrometry and Metabolomics Core was used to assess the purity of C-peptide samples relative to past mass

spectra following in-house HPLC purification. A Xevo G2-XS quadrupole time-of-flight tandem mass spectrometer (Waters Corporation; Milford, MA) was used to confirm purified C-peptide was unadulterated.

Molecular interactions between C-peptide and serum albumin were checked at the Assay Development and Drug Repurposing Core. A Pioneer surface plasmon resonance (SPR) system (Pall FortéBio; Port Washington, NY) was used to investigate binding kinetics between these two molecules. First, bovine serum albumin was biotinylated in-house and applied to streptavidin-coated SPR chips. Various increasing concentrations of C-peptide in phosphate buffered saline were then injected into the chip and binding kinetics were recorded. While serum albumin is commonly used to block nonspecific binding in these chips, it could not be used here as it was acting as the ligand. Unfortunately, because of this and the size disparity between serum albumin and C-peptide, the signal-to-noise ratio was quite poor. To remedy this issues, biotinylated C-peptide was purchased (GenScript; Piscataway, NJ) and the SPR experiments were repeated. However, C-peptide was immobilized for these studies while increasing concentrations of unmodified serum albumin were injected. Binding events involved capture of the relatively large serum albumin molecules, leading to much greater changes in the angle of minimum reflection. Alas, this was still unable to overcome issues with nonspecific binding, and the quality of the data remained poor. SPR seemingly is incompatible with these experiments due to the involvement of serum albumin. Although a dissociation constant could not be determined, there was evidence to suggest some interaction between the C-peptide and serum albumin.

A great benefit of the scientific community is the ability to collaborate with other experts. Department of Chemistry Professor Liangliang Sun was contacted for assistance in verifying the sequence of the nonfunctional C-peptide. Specifically, Dr. Sun was able to use tandem mass spectrometry to confirm the peptide sequence, including the C-terminal pentapeptide (EGSLQ) required for binding. Later, Professor Marie Heffern of the Department of Chemistry at the University of California, Davis was contacted for consultation. Dr. Heffern's laboratory studies the interactions between and effects of C-peptide, divalent copper, and serum albumin. It was suggested that some issue may be occurring during the in-house purification or lyophilization of C-peptide. As changes to the purification process had been thoroughly investigated, troubleshooting efforts shifted to the lyophilization process.

6.4.1 Return of Function

Though C-peptide is unstructured, it is possible incomplete or inefficient lyophilization may cause some change that alters chemical interactions or stability in storage. For example, acetonitrile is used as an organic solvent and trifluoroacetic acid as an ion pairing agent during the HPLC purification of C-peptide. If the samples warm too quickly or are under an insufficient vacuum, they may not be effectively removed by lyophilization.

To test this hypothesis, a batch of C-peptide was purified in-house via HPLC as normal. However, the eluite was not freeze-dried using the same equipment as previous batches. This C-peptide was lyophilized using a different instrument offered by a collaborating research group. Once complete, an aliquot was reconstituted in water and a stock solution prepared as usual. Several *in vitro* binding assays have been performed using this batch of C-peptide following the protocols previously described. The results are consistent with previously reported values and suggest binding to RBCs has been restored as shown in Figure 6.4.[14]

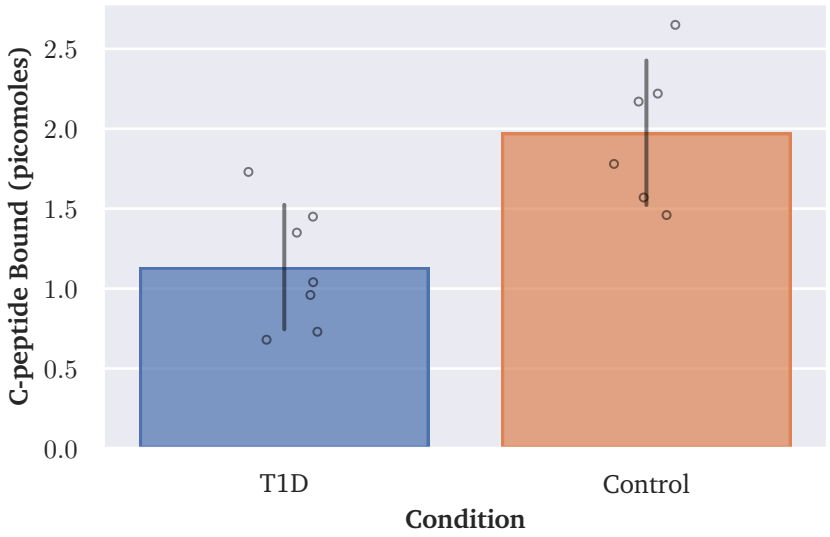

Figure 6.4: Bound C-peptide to T1D ($n = 7$) and control ($n = 6$) RBCs (mean $\pm$ SD). These values are consistent with previously reported data.[14]

The coefficient of variation of these measurements is still rather high. However, the results have so far been repeatable and align quite well with known binding values. Under these conditions, the Spence laboratory has previously reported C-peptide uptake of approximately 2 pmol by control RBCs and 1 pmol by T1D RBCs.[14] Additionally, the sensitivity of ELISA assays is well-known, such that

relatively small experimental errors can result in imprecise measurements.

As previously discussed, biological variability between donors (e.g., HbA1c) may also contribute to the variation in C-peptide uptake. It is that very relationship that was being studied when binding issues were noticed, precipitating an enormous amount of troubleshooting. In order to pick this study back up with a functional peptide, the HbA1c of all the RBCs used in the collection of the data shown in Figure 6.4 was measured using a DCA Vantage analyzer (Siemens Healthineers; Erlangen, Germany). The current results of this ongoing study are shown in Figure 6.3.

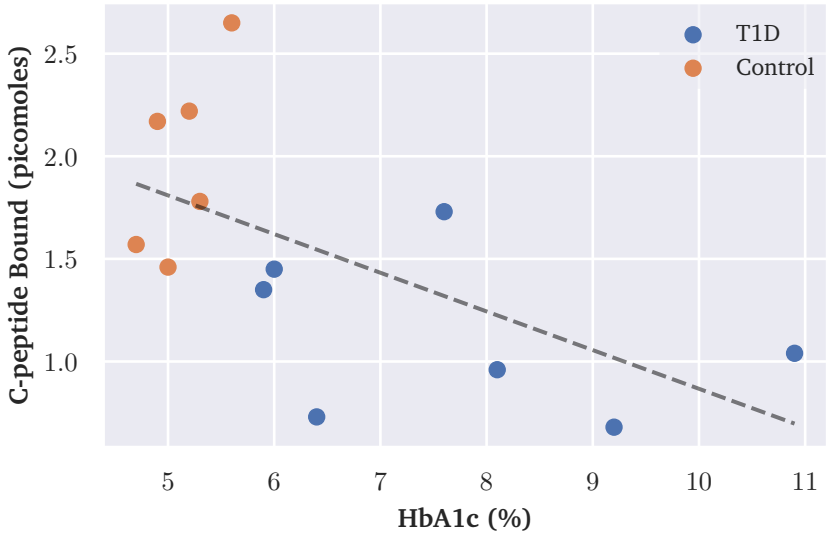

Figure 6.5: Bound C-peptide versus HbA1c for T1D ($n = 7$) and control ($n = 6$) RBCs. A linear regression is fit and shown in grey ($r^2 \approx 0.36$).

As previously reported, there is a clear difference in C-peptide binding between control RBCs and T1D RBCs. However, the relationship between HbA1c and C-peptide binding is less clear. The linear model shown does not fit very well ($r^2 \approx 0.36$), but perhaps more data will elucidate a different trend. It is also entirely possible there are other factors at play, and HbA1c is just one facet to a more complex model.

6.5 Discussion

C-peptide replacement therapy clearly has potential, under the correct conditions, to be beneficial in restoring microvascular function in T1D. Decades of *in vitro* experiments from numerous researchers have demonstrated the cellular effects of C-peptide. In some cases, animal models have shown positive results only for clinical trials to fail. This has caused some authors to question whether

diabetic animal models are truly useful as a predicitve tool for human clinical studies.[15,89]

The Spence laboratory has thus focused efforts on preclinical development of an activated C-peptide therapeutic for human use. During preparation for an Investigational New Drug application, persistent functionality issues plagued the project. After over a year of troubleshooting, the root cause of these issues appears to have been identified as improper lyophilization following in-house purification of crude C-peptide. Due credit should be awarded to Dr. Marie Heffern for her consultation which led to the resolution of this research impediment.

During the pause in *in vitro* C-peptide experiments, work proceeded on all other aspects of the project. This includes clinical proposal paperwork and completion of an Investigational New Drug application. The Spence laboratory, in collaboration with the Zinn laboratory, will soon meet with FDA liasons to recieve regulatory guidance and determine future steps.

With a functioning peptide, work may resume on laboratory preparations to begin small-scale human trials using the proposed C-peptide formulation. Importantly, investigations into the potential differences in C-peptide delivery to RBCs due to varying levels of cellular glycation may resume. This will help to inform expectations for dosing and observed effects during *in vivo* trials.

6.6 Conclusion

The therapeutic potential of C-peptide has been discussed for decades.[17] Over the last sixteen years, the Spence laboratory has been at the forefront of this research. Our laboratory has published major findings such as the metal activation of C-peptide and requirement of serum albumin to deliver it to RBCs.[13,33] Thus, the Spence laboratory, through rigorous *in vitro* investigation, suggests a complex of C-peptide, serum albumin, and Zn^{2+} may be required to elicit a biological response in RBCs.

Additionally, work from our laboratory has demonstrated cellular effects such as increased RBC deformability and ATP release *in vitro*.[33,75] Using microfluidic platforms to simulate *in vivo* microvascular blood flow, we have shown these effects stimulate downstream production of NO from endothelial cells.[13,68] Bioavailability of this potent vasodilator is known to be lower in T1D, potentially explaining circulatory issues that are implicated in the development of many long-term complications of this disease.[90]

There has been one previous attempt at development of a therapeutic for T1D based on C-

peptide.[70] Though some clinical improvements were seen in the study participants, the candidate drug ultimately failed during phase IIb clinical trials. However, the Spence laboratory believes there were multiple factors that caused insufficient efficacy. These studies administered solely a long-acting form of C-peptide, but we have demonstrated that metal activation is required for biological effects to occur. We believe the endogenous metals within the bloodstream would not be bioavailable in sufficient concentrations to activate C-peptide.[40] While there is certainly plenty of endogenous serum albumin available in the bloodstream, we have also demonstrated glycation of this albumin, as seen in T1D, impairs its ability to deliver C-peptide to RBCs.[76]

A therapeutic which comprises C-peptide, serum albumin, and Zn^{2+} may have a much better chance at succeeding in proving its efficacy. Therefore, we suggest C-peptide replacement therapy should be revisited by administering the entirety of the complex rather than just one component. Recombinant C-peptide is currently produced in large amounts as a byproduct of exogenous insulin production.[91] If this waste could itself be compounded into an effective therapeutic, it could shift the paradigm of T1D therapy.

Unfortunately, during preclinical development of this formulation, functionality issues were identified. The *in vitro* functionality of C-peptide has been well-studied by the Spence laboratory, yet previous standard experiments ceased yielding the expected results. Through a series of exhaustive investigations, the probable explanation for these problems was identified.

Though the exact mechanism behind the inhibited functionality remains a mystery, the lyophilization process of our in-house purification of C-peptide seemed to be causing the issues. This was rectified, and current batches of C-peptide are again functioning as expected. Thus, work may finally resume on the preclinical development of this therapeutic to mitigate chronic complications in T1D.

6.6.1 Future Work

The development of an all-in-one formulation comprising C-peptide, serum albumin, and Zn^{2+} should resume as described in Section 6.3. Particularly, efforts should be focused on the development of *in vitro* purification, testing, and release specification procedures. Additionally, current purification and lyophilization methods result in a C-peptide trifluoroacetate salt. Due to toxicity concerns, such salts are generally undesirable in peptides destined for therapeutic use.[92] Therefore, consideration should be given toward exchange of this trifluoroacetate salt to an acetate salt.

With the return of C-peptide function, investigation into the relationship between RBC glycation and C-peptide binding should also resume. It is already known that as glycation of serum albumin increases, its ability to deliver C-peptide to RBCs decreases.[76] Therefore, the possibility of increasing glycation of the RBCs themselves inhibiting C-peptide binding or its effects is not without justification. The importance of this information is twofold. First, it may help to explain the inconclusive results of previous clinical studies, as they did not control for HbA1c or any other marker of RBC health. Second, it could inform expectations for future *in vivo* studies; as HbA1c increases, the efficacy of a therapeutic may either be diminished, or it may require more time to take effect. Controlling for glycemic control in any future clinical studies may be important to gaining a full understanding of the outcomes.

The potential therapeutic described is suggested to be administered, like insulin, via subcutaneous injection. However, other methods of delivery are perhaps worth investigation as well. In 2019, Zashikhina et al. demonstrated delivery of C-peptide to RBCs via encapsulation in nanospheres.[93] As we suggest the entire C-peptide/serum albumin/Zn^{2+} complex be delivered together for maximum biological effect, a similar encapsulation of the compounded formula may be beneficial.

6.6.2 Potential Use in Blood Banking

As discussed in Section 4.3, C-peptide also has potential applications in the rejuvenation of stored RBCs. Indeed, this has previously been demonstrated by the Spence laboratory.[75] Detrimental changes in stored RBCs, referred to as the storage lesion, mirror the dysfunction observed in T1D RBCs.[94–100] In our previous studies, aspects of the storage lesion such as impaired RBC deformability and flow-induced ATP release were shown to be significantly improved after rejuvenation with an activated C-peptide complex.[75]

Should the development of the described C-peptide therapeutic succeed, it may well prove to be beneficial to blood banking as well. If rejuvenation of stored RBCs with C-peptide is able to consistently reverse aspects of the storage lesion and improve transfusion outcomes, the clinical applications of such a formulation would increase tremendously. With over 10.5 million units of RBCs transfused each year in the United States alone, the potential impact of such a product would be immense.[101]

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