University of Alberta

The Deformability of Hypothermically Stored Red Blood Cells

by

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Abstract

Transfusion medicine is dependent upon the ability to hypothermically preserve red blood cell (RBC) function *ex vivo*. Recent evidence suggests that the deformability of hypothermically stored RBCs may be compromised, potentially leading to microvasculature occlusion and tissue hypoxia in transfused patients. The main objectives of this thesis were to develop an ektacytometric technique suitable for detecting RBC deformability changes, establish whether deficits in RBC deformability due to hypothermic storage are occurring, and investigate liposomes as a biopreservation tool to modify the function of RBC membranes. This thesis demonstrates that hypothermic storage leads to RBC deformability impairments which can be detected by ektacytometry. In addition, this thesis has shown that the membrane characteristics and deformability of hypothermically stored RBCs can be differentially modified by treatment with liposomes of varying chemical composition. Through these investigations, this thesis has contributed to the advancement of the fields of transfusion medicine and biopreservation science.

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List of Abbreviations

ACD	acid citrate dextrose
ATP	adenosine triphosphate
CBS	Canadian Blood Services
CPD	citrate-phosphate-dextrose
CPDA-1	citrate phosphate dextrose adenine
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DMPE	1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine
DMPS	1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOPS	1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine
DPPC	1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPE	1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine
DPPS	1, 2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine
G6PD	glucose-6-phosphate dehydrogenase
H_2O_2	hydrogen peroxide
Hb	hemoblobin
Hct	hematocrit
HiCN	cyanmethemoglobin
HK	hexokinase
HS	hereditary spherocytosis
ICU	intensive care unit

LUV	large unilamellar vesicles
LORCA	Laser Assisted Optical Rotational Cell Analyzer
MβCD	methyl β-cyclodextrin
MCV	mean corpuscular volume
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MLV	multi-lamellar vesicles
MW	molecular weight
NADH	nicotinamide adenine dinucleotide
netCAD	Network Centre for Applied Development
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PIPA	pyruvate-inosine-phosphate-adenine
pRBC	packed red blood cell
PS	phosphatidylserine
PVP	polyvinylpyrrolidone
RBC	red blood cell
SAGM	saline-adenine-glucose-mannitol
SM	sphingomyelin
SRA	specific research aim
t-BuOOH	*tert*-butyl-hydroperoxide
Tm	gel-liquid crystal transition temperature
TRALI	transfusion related lung injury
TS	thalassemia

2,3 DPG	2,3 diphosphoglycerate

Chapter 1

Introduction

1.1 Hypothermic Preservation

As the use of cellular therapies to treat disease has dramatically increased in the past half century, the ability to successfully preserve cellular viability has become increasingly important. One of the most investigated and perhaps simplest approaches to biopreservation is hypothermic storage. In the contexts of cellular biopreservation, hypothermic conditions can be defined as those which are below normal physiological temperature *in vivo,* but above the freezing point of a storage solution [1, 2]. The rationale behind hypothermic preservation is based on the fact that cellular metabolism slows as temperatures are decreased. A decrease in the rate of metabolic reactions occurring in the cell slows the depletion of important cellular metabolites as well as the accumulation of any waste products that may be deleterious to the cell, allowing the cell to be preserved *ex vivo* for extended periods of time. For cellular applications, hypothermic storage is usually carried out in a preservative solution designed to modulate the cell's response to hypothermic conditions and minimize hypothermia-induced injury [1]. These solutions provide nourishment for the cells, buffers for pH maintenances, as well as additives to enhance cell survival during hypothermic conditions. Although hypothermic preservation has been used for a variety of cells and tissue types including platelets, corneas, hepatocytes, pancreatic islets, skin, and whole organs, perhaps no better example of the clinical application of hypothermic storage techniques exists than the preservation of red blood cells (RBCs) [1, 3-8]. Presently, the vast majority of the over 16 million RBC units transfused in Canada

and the USA every year are preserved hypothermically, thus demonstrating the value of hypothermic storage in clinical contexts [1]. The transfusion of hypothermically preserved RBCs to improve oxygen delivery to the tissues is used for the treatment of numerous conditions including hemorrhage due to trauma, aplastic anemias, hemoglobinopathies, thalassemias, and hemolytic anemias[2]. Without effective preservation strategies to maintain RBC structure and viability *ex vivo*, the use of transfusion for the treatment of various diseases would not be possible.

1.2 RBC Hypothermic Storage

1.2.1 RBC Physiology

Due to the fact that RBC hypothermic preservation techniques strive to maintain normal RBC metabolism and biochemistry, it is necessary to gain an understanding of the basic physiology of RBCs. The *in vivo* development and maturation of RBCs is referred to as erythropoiesis, and proceeds from pluipotent stems cells in the bone marrow [9]. The process includes 6 morphologically unique developmental stages: rubriblast, prorubricyte, rubricyte, metarubricyte, reticulocyte, and fully mature RBCs. As the developmental stages proceed there is a progressive reduction in cell volume, chromatin condensation, loss of nucleoli, decrease in cytoplasmic RNA and mitochondria, as well as an increase in the synthesis of hemoglobin [9]. At the metarubricyte stage, the nucleus is eventually extruded from the cell, leading to the release of reticulocytes into the peripheral

circulation. These reticulocytes subsequently mature over 1 to 2 days into fully functional RBCs (erythrocytes).

Fully mature RBCs are approximately 6-8 μm in diameter, 1.5-2.5 μm thick and have a mean cell volume of around 90 fL [9]. They exhibit bi-concave disc morphology and when stained with Wright's stain they appear as a circular cell with well defined margins and a region of central pallor[9]. This bi-concave character maximizes the surface area to volume ratio of the cell and is essential for proper RBC function. The cell membrane of RBCs is composed of various phospholipids, cholesterol, as well as numerous integral membrane proteins. Additionally, the cell membrane is supported internally by a complex membrane cytoskeleton which functions to maintain normal RBC shape, stability and deformability [9]. The major components of the cytoskeleton matrix include spectrin, ankyrin, protein 4.1, actin, and adducin [9]. Together, these proteins form a hexagonal lattice that is tethered to the inner leaflet of the RBC membrane. Although mature RBCs lack a nucleus and organelles and are therefore incapable of protein synthesis, they are capable of limited metabolism to sustain cellular processes while traversing the vasculature and delivering oxygen from the lungs to the tissues of the body. Despite being a relatively simple cell, RBCs have strong mechanical demands placed upon them, and are required to withstand shear rates and deformations if entry to the microvasculature and the subsequent delivery of oxygen to the tissues is to occur[9]. The *in vivo* lifespan of RBCs is approximately 120 days and changes in metabolism, deformability, and surface antigens during RBC senescence eventually lead to the removal of RBCs from the

circulation via macrophages associated with the reticuloendothelial system [9]. By the end of its 120 day lifespan, a typical RBC travels 200 to 300 miles through the vasculature and is subject to countless deformations [9]. In the contexts of cellular biopreservation, these heavy functional requirements become increasingly important, as the preservation of functionally viable and structurally intact RBCs is paramount for the successful practice of transfusion medicine.

1.2.2 Brief History of Blood Storage

As with numerous medical advancements, the development of RBC preservation strategies was truly a product of war. With the increased demand for blood ever pressing during World War I, vein to vein transfusion became impractical and methods to separate patients and donors in time and space were required [10]. Coagulation of whole blood during liquid storage was the first hurdle to overcome and in 1915 Rous and Turner developed the first RBC liquid storage solution (a mixture of citrate (anticoagulant) and dextrose (nutrient)) for preserving rabbit RBCs [11]. This technique was later applied to human RBCs by Oswald Robertson in 1915, leading to the first transfusions of stored RBCs in humans, as well as the establishment of the world's first blood bank in France during World War I [12]. Although Robertson had demonstrated that mixtures of citrate and dextrose could be used to store blood up to 26 days, the United States Army Medical Corps favored the collection of blood into glass bottles containing only citrate due to the fact that dextrose caramelizes at the autoclave temperatures used to sterilize the storage vessels [10]. This greatly diminished the longevity of

blood storage and the collection of whole blood into glass bottles containing 3.8% sodium citrate with a subsequent 5 day maximum storage duration on ice became standard for RBC hypothermic preservation. Improvements to these techniques were slow to arrive, with the first major advance occurring during the Second World War. Loutit and Mollison demonstrated that the acidification of citrate-dextrose solutions allowed autoclaving to take place without dextrose caramelization [13]. The inclusion of dextrose into the storage bottles resulted in the extension of storage duration to 21 days, meaning whole blood could be preserved and shipped around the world before expiry. The use of acid citrate dextrose (ACD) as a storage solution became the foundation for national blood systems in both the British Commonwealth and USA during and after the Second World War [10].

Further advances to the liquid storage of RBCs have been incremental and gradual. As the understanding of RBC cellular metabolism increased, sodium phosphate was added into preservation solutions to offset the loss of intracellular phosphate observed during storage duration [14, 15]. These citrate phosphate dextrose (CPD) solutions increased the recovery of RBCs after 3 weeks of storage and became the standard anticoagulant solution for RBC liquid preservation. Nakao *et al.* demonstrated in the early 1960's the importance of adenosine triphosphate (ATP) for maintaining RBC viability during hypothermic storage and subsequently, adenine was added to storage solutions resulting in the development of citrate phosphate dextrose adenine (CPDA-1)[16].The inclusion of adenine in storage solutions shifts the equilibrium conditions of RBC metabolism toward

ATP production. This resulted in better maintenance of ATP levels and subsequently, the ability to store whole blood for up to five weeks, a full two weeks longer than previously permitted [17, 18]. As these solutions were being investigated, the paradigm of whole blood storage and transfuion shifted to the production and storage of cellular components, and the transfusion of packed RBCs (pRBCs) rather than whole blood became the norm. Due to the higher hemtocrits associated with red cell concentrates, the pRBC units were much more viscous than the previously utilized whole blood units, making the administration of transfusions difficult when under time constraints [10]. This led to the development of additive solutions designed to provide additional volume and nutrients to pRBC units, resulting in longer storage durations and decreases in viscosity [10, 19]. These additive solutions are generally used as components of a collection system. Whole blood is drawn from donors into a primary collection bag containing anticoagulant solution (CPD or CPDA), which following collection is centrifuged resulting in the separation of RBCs, the buffy coat (white cells and platelets), and the plasma [10]. After separation from the other components, pRBC are then suspended in the additive solution and stored hypothermically at 1-6 °C prior to transfusion [10]. The original additive solutions consisted of saline, adenine, and glucose (SAG) and improved RBC storage by decreasing pRBC viscosity, providing nourishment for RBCs in the form of glucose, increasing the availability of adenine for maintenance of intracellular ATP levels, as well as decreasing the amount of hemolysis occurring during storage [20]. A further reduction in RBC hemolysis was shown to be achievable

by the addition of mannitol (membrane stabilizer) into the additive media, leading to the development of saline adenine glucose mannitol (SAGM) [21, 22]. SAGM additive solutions or similar derivatives (AS-1, AS-3, AS-5) are now the standard licensed additive solutions used in Europe, Canada, and the USA.

The implementation of leucoreduction has also led to improvements in the quality of hypothermically stored RBCs [23]. The breakdown of any white blood cells present in RBC units leads to the release of various proteases and lipases that are detrimental to the health of the RBC membrane during storage [10, 24]. The removal of leucocytes via buffy coat removal or leucofiltration techniques has been shown to increase RBC recovery and decrease hemolysis [25]. Currently, leucoreduction is practiced universally in some European countries and Canada, and is widely used in numerous locations in the United States [10].

1.2.3 Current RBC Hypothermic Storage Status

As a result of the extensive research occurring over the past 90 years, RBCs can currently be preserved hypothermically for a maximum of 5 to 7 weeks at 1-6 °C, depending on the additive solution utilized [26]. In general, the standards for red cell storage require that at the end of the approved storage period at least 75% of the infused RBCs must remain in the circulation 24 hours after infusion and the hemolysis in the preserved units must be less than 1% (0.8% in Canada and Europe) [27]. Although new variants of the currently licensed additive solutions have been shown to preserve RBCs for longer periods of time while still meeting the required standards of at least 24 hours circulation and less

than 1 % hemolysis, these solutions have yet to be put into clinical practice [10, 26, 27]. However, the current standards for RBC storage are routinely met in blood banks across the world. It has been postulated by Hess that more than 90% of collected units eventually find a recipient, thus demonstrating the utility of current hypothermic storage techniques at providing RBCs for clinical use[10].

1.3 Shortcomings of RBC Hypothermic Storage

1.3.1 The Hypothermic Storage Lesion

Despite being the earliest and most widely investigated method to preserve RBC viability, hypothermic preservation is not without its shortcomings [2]. Although decreases in temperature slow metabolic processes in the cell that potentially lead to the accumulation of harmful waste products, hypothermic storage does not halt cellular metabolism completely. In the case of RBCs, this leads to the accruement of an array of changes to the cell, known collectively as the RBC 'hypothermic storage lesion'. Hypothermia-induced injury is the result of perturbations to the normal processes responsible for maintaining cellular homeostasis. This type of injury have been investigated extensively in RBCs and numerous reviews describing aspects of the hypothermic storage lesion are available [2, 10, 23, 24, 27-29]. The changes to RBCs induced by hypothermic storage can be broadly categorized into biochemical and biomechanical lesions. However, these are only loose categories, as both types of lesions are intimately related to each other.

Biochemical Changes

The two major biochemical changes occurring during RBC hypothermic storage are decreases in intracellular 2, 3-diphosphoglycerate (2, 3-DPG) and ATP concentrations [24]. It is not the biochemical changes themselves that are deleterious to the proper functioning of RBCs, but rather the downstream cellular processes that are modified as a result of these disturbances. 2,3-DPG is the major allosteric effector of RBC O_2 affinity with normal concentrations ensuring that RBCs are able to unload oxygen to the peripheral tissues [24]. Decreases in intracellular 2, 3-DPG concentrations result in RBCs with increased O_2 affinity, potentially compromising the ability of RBCs to releases oxygen. Although it has been shown that 2, 3-DPG levels are restored naturally upon transfusion, this restoration may take 24-48 hours which may be significant in critical situations where the efficient delivery of oxygen is of paramount importance [2, 30, 31].

ATP depletion during storage is associated with a number of changes to RBCs including but not limited to the shutdown of cationic ion pumps important for regulating osmotic balance (Na^+/K^+, Ca^{2+} ATPase), reduction in the phosphorylation of cellular kinases, modifications to the cell membrane and cytoskeletal structure, and as a consequence, a loss of normal RBC morphology [32]. It has been postulated that nearly all aspects of the storage lesion are attributed to ATP depletion, however the irreversibility of some storage induced changes following the restoration of ATP levels with RBC rejuvenation solutions suggests that ATP depletion results in secondary mediators of injury important for

maintaining normal RBC function [32]. In addition to 2,3-DPG and ATP depletion, other biochemical changes include increases in intracellular calcium (which may be associated with RBC morphology changes), decreases in intracellular potassium, decreases in pH, as well as hypothermia-induced metabolic modulation [24].

Biomechanical changes

The biomechanical component of the RBC storage lesion is primarily localized to the RBC membrane and cytoskeleton and includes a loss of membrane phospholipid (via microvesiculation), abnormal phospholipid distribution within the membrane, oxidation of membrane and cytoskeletal proteins, lipid peroxidation, as well as gross changes in RBC morphology [24].

The only known mechanism for phospholipid loss from RBC membranes during hypothermic storage is microvesiculation, as first described in hypothermically stored RBCs by Rumsby *et al.* in 1977 [33]. These small vesicles (50 -250 nm in diameter) have been shown to contain phospholipids, transmembrane proteins, cytoskeletal proteins, as well as hemoglobin, and the blebbing of microvesicles off of the cell membrane is thought to contribute to the changes in RBC morphology observed during hypothermic storage [24, 26, 33-36]. In addition to causing changes in RBC morphology, microvesicles themselves have been shown to be immunogenic, possibly leading to clinical complications in transfusion recipients [34]. Given that many transfusion

recipients may already have a burden on the immune system, the transfusion of potentially immunogenic microvesicles is undesireable.

RBCs are characterized by an asymmetric distribution of phospholipids between the outer and inner leaflets of the membrane. Typically, phosphatidylcholine (PC) and sphingomyelin (SM) are most common in the outer leaflet, while phospatidylserine (PS) and phosphatidylethanolamine (PE) are found almost exclusively in the inner leaflet of the plasma membrane [37]. A translocation of PS to the outer leaflet of the membrane signals RBC senescence *in vivo* and facilitates the removal of RBCs via the reticuloendothelial system [9, 37-39]. This is a normal response as RBCs reach the end of their natural lifespan of around 120 days. The externalization of PS is also observed during extended hypothermic storage and is a possible indicator of accruing cellular damages in aging cells. Transfusion of RBCs with increased PS is likely to result in the clearance of infused RBCs from the circulation, leading to an increased burden on the RES as well as increased vascular resistance due to exposed PS facilitating adhesion of RBCs to endothelial cells in the microvasculature [40-42].

Oxidative injury to RBCs during storage is also a concern and has been illustrated in numerous studies [43-46]. Sites of oxidative injury include the hemoglobin, cytoskeletal proteins, as well as membrane phospholipids [43, 45-49]. Although the effects of oxidative injury on RBCs are not completely understood, it has been demonstrated that oxidation of cytoskeletal proteins as well as lipid peroxidation in the membrane disrupts the association of the plasma membrane with the cytoskeleton scaffold [46]. This has been postulated to be a

possible mechanism for the increase in microvesiculation observed during hypothermic storage[46, 50].

As a culmination of the described biochemical/biomechanical effects, gross changes in RBC morphology also occur as hypothermic storage duration increases. Changes to the RBC membrane and cytoskeleton lead to a progressive loss of bi-concavity, characterized by membrane loss and the development of echinocytic spicules [44, 51, 52]. Progressive spheroechinocytosis results in significant decreases in the normally large surface area to volume ratio of RBCs, as well as increases in the mean cell hemoglobin concentration[24].

In addition to the well characterized biochemical/biomechanical aspects of the RBC hypothermic storage lesion, changes in RBC rheology have also been implicated in liquid blood preservation [23, 41, 53-60]. RBC rheology can be defined as the study of how RBCs flow in the body, which is influenced primarily by RBC aggregation, deformability, as well as adhesion to endothelial cells. These functional characteristics of RBCs are of increasing interest in RBC biopreservation contexts as they are perhaps better indicators of *in vivo* viability compared to more traditional RBC quality measures. In recent years studies have shown that hypothermically stored RBCs have deformability impairments which may affect their ability to enter the microvasculature and participate in gas exchange [40, 53, 54, 56]. However, many of these studies focused primarily on non-leukoreduced RBCs. In addition to deformability deficiencies, hypothermically stored RBCs have also been shown to adhere to endothelial cells,

potentially leading to vascular occlusion and/or increases intravascular pressure [10, 29, 53, 61].

1.3.2 Clinical Consequences of the RBC Hypothermic Storage Lesion

It is inarguable that the transfusion of RBCs saves countless lives every year around the world and improves the quality of life for many chronically ill patients. Without the achieved improvements in RBC liquid storage over the last century the field of transfusion medicine could not have developed to the point it has today. The ability to sufficiently preserve RBCs for 5 to 7 weeks has maximized the utilization potential of donated blood, resulting in significantly fewer wasted units compared to the past when RBCs could be preserved for only 3 weeks[27]. Although there is no question that increasing the acceptable storage duration of RBCs eases inventorial and logistical strains, the vast array of cellular changes that characterize the hypothermic storage lesion bring the quality of hypothermically stored RBCs into question. Mounting clinical evidence suggests that RBC transfusion is perhaps not as safe as previously thought. Deaths have been associated with cold growing bacteria in older RBC units, the infusion of aged units containing high levels of extracellular potassium, the infusion of units with high levels of hemolysis, as well as with transfusion related acute lung injury (TRALI) from oxidation –induced lysophospholipids [27, 62] In addition, numerous retrospective epidemiological studies have indicated that the transfusion of older RBC units is associated with increased morbidity and mortality in critically ill patients, increased risk of multiple organ failure,

increased risk of post operative pneumonia, increased intensive care unit (ICU) length of stay, as well as overall increased hospital length of stay[23, 63-72]. However, it is worth mentioning that biases were present in many of these studies due specifically to the fact that 'sicker' patients often received more transfusions and were therefore more likely to receive older units. In addition, RBC units are not issued randomly from blood banks [27]. Perhaps the most noted clinical study investigating the safety of blood was the TRICC (transfusion requirements in critical care) study published by Hebert *et al.* in 1999[73]. The results of the study suggested that a more restrictive transfusion regiment leads to decreased mortality in critically ill patients compared to liberal transfusion strategies. Presently, two large prospective studies (The ABLE study and the Red Cell Storage Age Study) are being planned in Canada and the U.S.A respectively, which will hopefully shed more light on the safety of hypothermically stored blood in terms of the effect of red cell age as well as transfusion strategies in critically ill patients[27].

1.3.3 Current Predictors of Hypothermically Stored RBC Quality

Considering the clinical data which suggests that the transfusion of older RBC units (>15 days) leads to adverse clinical outcomes, a closer look at the standards for RBC hypothermic storage is warranted. The guidelines on acceptable RBC quality following hypothermic storage are vague and likely poor indicators of.RBC *in vivo* viability. In fact, both universal standards of preserved RBC quality (greater than 75% of transfused RBCs in circulation 24 hours after infusion and less than 1% hemolysis) are not indicative of RBC viability by any

means. Hemolysis can be defined as the rupture of the RBC membrane resulting in the release of the cellular contents, specifically the hemoglobin, to the surrounding solution. This can therefore be classified as an end stage quality measure, as a variety of changes which are detrimental to RBC functioning *in vivo* (such as those exemplified by the hypothermic storage lesion) are likely to occur well before the RBC actually ruptures. Surprisingly, no mention of these more subtle changes in RBC quality are found in current RBC minimal quality standards [24]. The requirement that 75% of transfused RBCs must remain in the circulation 24 hours after infusion also conveys little about actual red cell function. The simple fact that transfused RBCs are present in the vasculature does not indicate that effective oxygen transfer to the tissues is occurring. In addition, the removal of a quarter of the transfused RBCs may put undue pressure on the reticuloendothelial system, especially when transfusing critically ill patients that may already have compromised immunity. Considering these arguments, it seems better predictors of RBC viability *in vivo* should be utilized when examining the quality of hypothermically RBCs *ex vivo.*

1.3.4 Hemorheological Factors as Predictors of Hypothermically Stored RBC Quality

As previously mentioned, RBC rheology is altered in hypothermically stored RBCs which has been demonstrated by several studies investigating the effect of hypothermic preservation on RBC deformability and endothelial adhesion [40, 53, 55, 59, 61]. The efficient and unimpeded delivery of oxygen

from the lungs to tissues of the body is highly dependent upon both of these hemorheological parameters.

The diameter of a typical RBC is 6-8 μm which is greater than the intraluminal diameter of much of the microvasculature. To successfully traverse the capillary beds and deliver oxygen to the tissues, a red cell must retain the ability to reversibly deform, which is primarily a function of cell membrane and cytoskeletal characteristics [57, 74]. Significant changes to both the cell membrane and cytoskeleton are associated with the hypothermic storage lesion [24]. Similar to losses in deformability, the adhesion of RBCs to the endothelial cells that line the vasculature is not conducive to efficient oxygen delivery, and may lead to vascular occlusion. The interactions mediating this adhesion have been postulated to involve the externalization of PS to the outer leaflet of the RBC membrane, a process which is associated with the hypothermic storage lesion [61].

A uniting factor of both these hemorheological parameters is their mutual involvement of the RBC membrane. As described previously in this review, gross changes to the RBC membrane are a major component of the hypothermic storage lesion including the loss of lipid via microvesiculation, perturbations of the protein interactions between the membrane and underlying skeleton, as well as the abnormal translocation of lipids between the inner and out leaflets of the membrane[24]. Changes in RBC morphology during storage are also indicators of poor membrane health. Given that both of these rheological parameters are important for normal RBC functionality, they are likely stronger predictors of

RBC viability *in vivo* than currently accepted measures of RBC quality. Therefore, the monitoring of these parameters in blood bank quality control programs could give significant insights into the viability of hypothermically stored RBCs as well as suggest new preservation strategies to improve post hypothermic storage membrane function.

From a diagnostics standpoint, RBC deformability is likely easier to assess for a number of reasons. Firstly, numerous techniques for measuring RBC deformability exist, including filtration methods, rheoscopes, optical tweezers, and ektacytometers [75]. Measurement of RBC adhesion to endothelial cells is generally limited to the use of parallel plate flow chamber systems which are tedious and time consuming to use. Secondly, in addition to actually measuring RBC adhesion, extensive cell culture maintenance is required to perform measurements, adding to the time and cost of adhesion analysis [40, 53]. In contrast, ektacytometric analysis of RBC deformability requires very little time, is well characterized, and has good reproducibility [75]. Despite the reported effectiveness of this technique for assessing RBC deformability changes associated with chemical treatments and various RBC pathologies, few studies have utilized ektacytometry as a technique to monitor the deformability of RBCs during hypothermic storage [41, 54-56, 76-78].

1.4 Thesis Approach

In light of recent studies implicating that the transfusion of older blood leads to increases in adverse clinical outcomes including higher rates of morbidity and mortality, methods to assess the quality of hypothermically stored RBCs that are more reflective of *in vivo* viability are needed. In contrast to the current standards of > 75% infused RBCs remaining in the circulation 24 hours post transfusion and < 1 % hemolysis at the end of the storage period, assessments of RBC hemorheological properties are perhaps more indicative of RBC quality during hypothermic storage. The establishment of techniques to better assesses the *in vivo* viability of hypothemically stored RBCs may potentially lead to improvements in the quality and safety of the current RBC supply in blood banks, as well as shape the next generation of preservative strategies striving to improve the quality of banked blood. The focus of this thesis will be twofold: Firstly, the development and application of ektacytometry as a potential method to determine if current RBC hypothermic storage strategies in Canada sufficiently preserve RBC deformability will be undertaken. The development of an effective method to assess RBC deformability may lead to improvements in blood bank quality control programs, resulting in more reliable measures of *in vivo* RBC viability and ultimately a safer blood supply.

Given that changes in RBC deformability associated with hypothermic storage are almost certainly related to the health of the RBC membrane and cytoskeleton, the second focus of this thesis will be the investigation of liposomes as a possible tool to alter the characteristics of the RBC membrane. Liposomes are spherical synthetic vesicles composed of an intact lipid bilayer surrounding an

aqueous core. Although originally used as models of biological membranes, the use of liposomes has largely expanded in the last 25 years. Currently, liposomes are used most prolifically as carrier/delivery vessels for a variety of molecules including peptides, proteins, drugs, and DNA [79-82]. In addition, liposomes have also been used in biopreservation contexts as membrane stabilizers and delivery vehicles [83-86]. Given that a loss of RBC membrane lipid via microvesiculation is thought to play a significant role in RBC deformability decreases observed during hypothermic storage, stabilization of the RBC membrane via liposome treatment may result in RBC deformability improvements. Therefore, by the development and investigation of methods to assess and alter deformability in hypothermically stored RBCs, this thesis strives to contribute and advance the sciences of biopreservation and transfusion medicine.

1.5 Hypothesis and Thesis Objectives

This thesis will test the following hypotheses: (1) Ekatcytometry is an effective method for detecting deformability changes in RBCs occurring during hypothermic storage; (2) The deformability of hypothermically stored RBCs can be modified by liposome treatment. The major goals of this thesis are to develop an effective method to assess RBC deformability as well as investigate liposome treatment as a technique to enhance the deformability of RBCs during *ex vivo* preservation. This thesis is made up of three experimental studies consisting of three specific research aims (SRAs):

SRA (1): *To establish ektacytometry as an effective method to assess the deformability of RBCs. (Chapter 2)*

SRA (2): *To determine if hypothermic storage leads to decreases in RBC deformability by applying the ektacytometric technique established in SRA (1).Specifically, the relationship between ATP levels, membrane damage, and deformability changes will be explored (Chapter 3)*

SRA (3): *To investigate the use of liposomes as a potential tool to modify the deformability of hypothermically stored RBCs. (Chapter 4)*

Following the investigation and discussion of each specific SRA, a broad discussion of the project as a whole will be presented, including the significance of the results and proposed future research (Chapter 5).

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Chapter 2*

Eadie-Hofstee analysis of red blood cell deformability

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2.1 Introduction

The ability of RBCs to deform is essential for their successful navigation through the microvasculature and subsequent delivery of oxygen to the tissues of the body. Impairments in RBC deformability have been described in a diverse number of hematological disorders such as hereditary spherocytosis, thalassemia, sickle cell disease, and malaria. Although the pathologies of these disorders vary widely, the decreases in RBC deformability associated with each may lead to decreased tissue perfusion and splenic destruction of erythrocytes [1]. Due to the clinical importance of RBC deformability, effective methods to assess this parameter are desirable from both a clinical diagnostic and basic research perspective. This is especially apparent in transfusion medicine, where maintenance of erythrocyte viability is essential for the delivery of quality RBC products to patients.

There are three main factors that contribute to the deformability of RBCs. The first is cytoplasmic viscosity, which is due primarily to the intracellular hemoglobin concentration. An increase in hemoglobin concentration due to osmotic factors or a loss of cell volume will lead to increased cytoplasmic viscosity and a subsequent decrease in cellular deformability. The second is the RBC membrane, including the membrane viscoelastic properties (degree of stretching possible), membrane lipid composition and fluidity, and dynamic interactions between the membrane and underlying cytoskeletal scaffolding

structure [1,2]. The third is the surface area to volume ratio of an RBC. The characteristic biconcave shape permits a high degree of deformation and a loss of this biconcave character due to a loss of membrane lipids or rearrangements of cytoskeletal networks will invariably lead to a decrease in deformability [1, 3]. This type of morphological change has been associated with various hematological pathologies as well as the RBC hypothermic storage lesion [1]. It is important to note that RBC deformability is not attributed to any one of these factors alone, but rather results from all three factors acting synergistically.

Although numerous methods to study erythrocyte deformability have been investigated, ektacytometry has been shown previously to be an effective method with good reproducibility and precision [4]. Despite the merits of this technique, there has not yet been a standard method established to interpret ektacytometric RBC deformability data. Previously, elongation index values were typically analyzed at single shear stresses, allowing samples to be defined by elongation indexes at a few chosen shear stresses the investigator found relevant. In most cases, samples were analyzed at high shear stresses (30 Pa or greater) to elucidate changes in 'maximum' RBC deformability and at relatively low shear stresses (0-5 Pa), which were thought to demonstrate RBC elongation at physiologically relevant shear stresses. This appeared to negate the need to compare complex deformability curves between samples and simplified data interpretation. However, it dismissed much of the data in the RBC deformability curve, failing to give a complete picture of RBC deformability.

In recent years, RBC deformability curve data transformations have been employed to simplify the comparison of multiple samples [5-9]. Data transformations aim to derive a set of global parameters to describe the RBC elongation curve in its entirety without computing differences at each individual shear stress. Historically, linear data transformations have been used for the determination of the enzyme parameter V_{max} (maximum reaction rate) and K_m (substrate concentration at which the reaction rate reaches half of its maximum), in contexts where simple Michaelis-Menton enzyme kinetics can be assumed. Condon *et al.* [5] were the first to linearize RBC deformability curves in a similar manner, utilizing the Lineweaver-Burk linearization method (double reciprocal plot) to achieve this. In doing so, two parameters describing RBC deformation were described, EI_{max} and K_{EI}, which can be defined respectively as the maximum elongation index predicted at an infinite shear stress and the shear stress required to elongate to ½ of the EI_{max} [5]. Although Lineweaver-Burke analysis has been used in several studies, it is not without disadvantages. Due to the reciprocal nature of the plot, emphasis is unevenly weighted to deformability measurements at small shear stresses (further away from the y-axis). In addition, a back-extrapolation through the upper left quadrant of the plot is required to calculate $K_{EI.}$ Extrapolations are subject to greater uncertainty, as one must assume that the established relationship holds true once the range of the data set is exceeded. Due to this uncertainty, it is beneficial to limit the extent of any extrapolations as to minimize any errors if the investigated relationship does not hold constant beyond the range of the data set. When examining RBC deformability curves via the

Lineweaver-Burke method, it is important to consider that all plotted data is in the upper right quadrant. Therefore, it would be beneficial to employ a linearization method that is contained within the upper right quadrant, thus minimizing any errors due to lengthy extrapolations.

In this chapter, we wish to introduce an alternative linearization technique, the Eadie-Hofstee transformation, as a method to linearize the RBC deformability curve and examine RBC deformability data in its entirety. *In vitro* induced changes to the RBC membrane and cytoskeleton are used to examine the utility of the Eadie-Hofstee transformation in dissecting RBC membrane injury. Oxidative damage is induced via *tert*-butyl-hydroperoxide (*t*-BuOOH) or hydrogen peroxide (H_2O_2) treatment. The effect of these two agents on RBC deformability has not been previously investigated. In addition, methyl β-cyclodextrin (MβCD) treatment is employed to investigate the effect of cholesterol depletion on RBC deformability. Furthermore, RBC deformability in samples from patients with hematological pathologies known to affect the RBC cytoskeleton and hemoglobin (hereditary spherocytosis and α-thalassemia), is assessed via ektacytometry with subsequent Eadie-Hofstee data linearization to investigate the utility of this technique in a clinical setting.

2.2 Materials and Methods

2.2.1 RBC Processing

Leukoreduced CPD-SAGM RBC units (n=10) were obtained from the Network Centre for Applied Development (netCAD), Canadian Blood Services (CBS), Vancouver, Canada. Collections were performed using standard blood bank phlebotomy procedures. All RBC units were stored at 1 to 6 ºC for less than five days before sampling. Approximately 35 mL of RBCs were drawn aseptically from packed RBC units and washed twice with phosphate buffered saline (PBS) (HyClone, Logan, UT). Following the second wash, the hematocrit of each sample was determined by micro-hematocrit centrifugation. Briefly, this technique requires the drawing of a RBC suspension into a small diameter capillary tube which is subsequently sealed at one end. The capillary tubes are then centrifuged for 5 minutes to separate the RBC fraction from the supernatant. The ratio of the height of the RBC fraction from the sealed end of the tube versus the height of the RBC fraction and supernatant fraction combined defines the hematocrit. Samples were then diluted appropriately with PBS buffer in preparation for chemical incubation. Ethics approval was obtained from both CBS and the University of Alberta Research Ethics Board prior to study commencement.

2.2.2 Chemical treatments

All chemical treatments were performed at a hematocrit of 15%. RBCs were first treated with 1 mM sodium azide (EMD, Damstadt, Germany) for 30 minutes at 21 ˚C on a nutating shaker (VWR International, West Chester, PA)

prior to H_2O_2 treatment. This step was omitted for the *t*-BuOOH and MβCD treated samples. After initial processing, samples were treated with either *t*-BuOOH or H_2O_2 at final concentrations of 1 and 2 mM and incubated for 15 minutes at 37 ˚C on a nutating shaker. MβCD treated samples were incubated at final concentrations of 5 and 10 mM for 1 hour at 37 ˚C on a nutating shaker. Following chemical incubation, samples were washed twice with PBS and the RBC pellet was resuspended in PBS buffer to a hematocrit of 15% in preparation for ektacytometric analysis. All chemical agents were purchased from Sigma Aldrich, St. Louis, MO.

2.2.3 Ektacytometric Analysis

Ektacytometry was performed using the Laser Assisted Optical Rotational Cell Analyzer (LORCA) (Mechatronics, The Netherlands). Figure 1 illustrates the geometry of the instrument. RBCs are diluted in the high molecular weight polymer polyvinylpyrrolidone (PVP) and inserted into the gap between the static inner cylinder and the outer rotating bob of the instrument (Fig 2.1). An internal laser beam is then directed through the RBC suspension, resulting in a characteristic diffraction pattern that is projected onto a small screen. By rotating the outer cylinder, shear stress can be imparted upon the red cells, causing their major axis to elongate. The magnitude of this shear stress is related to the shear rate, which can be estimated by:

$$\gamma = \frac{4\pi \cdot r_b \cdot r_c}{60 \cdot (r_c^{\,2} - r_b^{\,2})} \cdot N \qquad (2.1)$$

Where:

γ = shear rate (1/s)
r_b = Outer radius of the bob (mm)
r_c = Inner radius of the cup (mm)
N = Revolution speed of the cup (rpm)

By using the calculated shear rate, the shear stress exerted up the RBCs can be calculated by:

$$\tau = \eta \cdot \gamma \qquad (2.2)$$

Where:

τ = shear stress (Pa)
η = medium viscosity (mPa·s)
γ = shear rate (1/s)

As the shear stress imparted upon the RBCs increases, the diffraction pattern detected on the projection screen transitions from circular to elliptical (Fig 2.2). From these diffraction patterns, the computer calculates an elongation index (EI), which is defined as:

$$EI = \frac{A - B}{A + B} \qquad (2.3)$$

Where:

A = major elongation axis
B = minor elongation axis

As the diffraction pattern becomes more elliptical, the major elongation axis increases while the minor axis invariably decreases. This leads to higher elongation indexes at higher shear stresses. For this and all subsequent studies, RBCs were suspended in high molecular weight PVP (MW~30 000) and subjected to shear stresses ranging from 0.95 to 30 Pa at a temperature of 37 ºC. The resultant RBC deformability curves were then linearized via the Eadie-Hofstee linear data transformation.

2.2.4 Eadie-Hofstee Linear Data Transformation

RBC deformability curves were linearized via the Eadie-Hofstee technique to obtain two RBC deformation kinetic parameters; EI_{max} and $K_{EI.}$ EI_{max} can be defined as the maximum elongation index predicted at an infinite shear stress while K_{EI} can be defined as the shear stress required to achieve half of EI_{max} (Fig 2.3). The transformation requires plotting the measured elongation index vs. elongation index / respective shear stress (Fig 3). The y-intercept of the plot corresponds to the theoretical EI_{max} while the slope is equivalent to $-K_{EI}$. Figure 3 depicts a representative RBC deformability curve (inset) when RBCs are subjected to increasing shear stress while the same data is expressed following Eadie-Hofstee linearization. For each chemical treatment, EI_{max} and K_{EI} values were determined and compared to determine the suitability of this linear data transformation for detecting and resolving *in vitro* induced changes in RBC

deformability. All EI_{max} and K_{EI} values are expressed as means ± standard error (SEM).

2.2.5 Pathologic RBC Samples

Two relevant RBC pathologic samples (hereditary spherocytosis (HS) and α-thalassemia major (TS)) were obtained from the Special Hematology Laboratory at the University of Alberta Hospital (Department of Laboratory Medicine and Pathology). Blood was collected via standard phlebotomy in EDTA anti-coagulated vacutainer tubes and stored for less than 48 hours at 1-6 ˚C prior to ektacytometric analysis. RBC deformability curves were obtained for both pathologies and analysed via Eadie-Hofstee linearization as described previously. RBCs from a healthy volunteer which were aged accordingly served as a reference for analysis of the pathologic samples. Approval from the University of Alberta Hospital and Health Research Ethics Board was obtained prior to study commencement.

2.2.6 Statistical Analysis

The data were analyzed using commercial statistical software (SPSS Version 12.0, Lead Technologies,Charlotte, NC). Due to the small sample size (n=10), nonparametric statistical analysis was performed. The Mann-Whitney U test was used to examine the differences in calculated RBC deformation

parameters. Probabilities less than 0.05 were considered significant. Due to the availability of only one of each pathologic sample, no statistical analysis was performed.

2.3 Results

2.3.1 Chemical treatments

The chemical treatment of RBCs with all agents tested resulted in detectable changes in deformability following Eadie-Hofstee linearization. With respect to the oxidizing agents, *t*-BuOOH and H_2O_2, the K_{EI} parameter was most drastically affected. As illustrated in figures 2.4 and 2.5, dose dependant increases in K_{EI} were observed with both oxidizers. However, the increase was more pronounced in *t*-BuOOH treated RBC samples. Treatment with 1 mM *t*-BuOOH resulted in a 1.5 fold increase in K_{EI} while 2 mM treatment resulted in a 2.7 fold increase. Both increases were statistically significant compared to untreated controls ($p < 0.001$). K_{EI} increases following H_2O_2 treatments were 1.2 fold for 1 mM H_2O_2 and 1.6 fold for 2 mM H_2O_2 which were both statistically significant compared to untreated controls ($p < 0.001$) (Fig 2.5). Although the reported increase in K_{EI} observed with *t*-BuOOH treatment was nearly twice that of H_2O_2, the result was not statistically significant ($p = 0.062$) (Fig 2.4 and 2.5).

In contrast to induced K_{EI} changes, EI_{max} values following RBC oxidation remained relatively constant (Fig 2.4 and 2.5). For H_2O_2 treated samples, no

statistically significant difference was detected for 1 mM or 2 mM doses compared to control ($p = 0.19$, $p = 0.13$)(Fig2.3). For *t*-BuOOH samples, treatment with 1 mM resulted in no statistically significant change ($p = 0.88$), however, treatment with 2 mM *t*-BuOOH resulted in a statistically significant decrease compared to control ($p = 0.019$) (Fig 2.4).

The treatment of RBCS with MβCD resulted in an entirely different effect on RBC deformability (Fig 2.6). Unlike the oxidizing agents tested (Fig 4 and 5), MβCD treatment led to pronounced decreases in EI_{max} values. At 5 mM MβCD, the EI_{max} had decreased to 0.492 ± 0.032 from 0.587 ± 0.012 in control samples which was statistically significant ($p = 0.002$). Following treatment with 10 mM MβCD, the EI_{max} decreased to 0.273 ± 0.025 from 0.587 ± 0.012 in control samples which was statistically significant ($p < 0.001$). RBC K_{EI} values in response to MβCD treatment also differed compared to that of RBCs subjected to oxidative stress. At 5 mM MβCD, no statistically significant change in K_{EI} was observed compared to control ($p = 0.15$). However, following treatment with 10 mM MβCD, K_{EI} levels decreased to 1.53 ± 0.09 which was a statistically significant decrease compared to both untreated control ($p < 0.001$) and 5 mM treated samples ($p = 0.001$).

2.3.2 Pathologic RBC Samples

In addition to detecting deformability changes in *in vitro* modified RBCs, the analysis of pathologic RBC samples also demonstrated contrasting

deformability profiles (Fig 7). The EI_{max} and K_{EI} of the normal RBC control sample was of similar magnitude to the untreated controls used in the chemical incubation portion of the study (EI_{max} = 0.586, K_{EI} = 2.36). The HS sample had a markedly decreased EI_{max} value of 0.293 which was only half that of a normal control. The observed K_{EI} value was also lower than that of a normal control (1.00 vs. 2.36). The TS sample had a depressed EI_{max} value compared to the healthy control, however this decrease was not as dramatic as with the HS sample. In contrast, the K_{EI} of the TS sample was higher than that of HS and healthy control samples (3.87, 1.00, and 2.36 respectively).

2.4 Discussion

Eadie-Hofstee deformability analysis was capable of detecting RBC deformability changes induced by both oxidative stress and membrane cholesterol manipulation *in vitro.* Furthermore, the employed technique was able to detect deformability changes associated with two hematological disorders, hereditary spherocytosis and α–thalassemia. Although RBC deformability curves have been previously linearized via the Lineweaver-Burke method, this study is the first to apply the Eadie-Hofstee transformation to RBC deformability data.

By transforming ektacytometric RBC deformability curves to reveal EI_{max} and K_{EI} values, we are able to gain significant insight into RBC deformability changes which would be otherwise difficult to obtain by analyzing deformability plots at single shear stresses. EI_{max} values illustrate how far a population of RBCs

can be physically stretched and thus, are an indicator of overall RBC deformability. In contrast, K_{EI} values illustrate how much force is required for RBCs to reach their maximal stretching capacity, and are therefore an indicator of RBC rigidity. The utility in calculating these parameters via linearization is twofold: firstly, EI_{max} and K_{EI} values can be used to describe the RBC deformability curve in its entirety. Previously presented methods considering RBC elongation at 'snapshot' shear stresses [3, 10-12] fail to do this. Secondly, by calculating the two parameters, one can investigate the kinetics of RBC deformation and elucidate possible mechanisms for the decrease in RBC deformability associated with various hematological conditions.

Eadie-Hofstee analysis has several advantages over the more commonly used Lineweaver-Burke analysis [5-7, 9] . As previously mentioned, Lineweaver-Burke analysis requires a significant back extrapolation through the upper left quadrant of the linear plot to calculate K_{EI} values. This extrapolation is likely to magnify any errors in the linear fit, thus leading to inaccuracies in the calculated K_{EI} value. Eadie-Hofstee linear analysis requires no such extrapolation as both deformability parameters are calculated within the upper right quadrant of the plot in close proximity to the plotted values (Figure 3). The unequal weighting of data points in Lineweaver-Burk analysis also presents a problem. Due to the reciprocal nature of the plot, the elongation index obtained at the lowest shear stress is located furthest away from the plot origin in the upper right quadrant. Therefore, this data point has the greatest influence on slope of the linear best fit line and subsequently the EI_{max} and K_{EI} values. As with historic enzyme kinetic plots, the

variability in the data of this point is generally the greatest, thus leading to possible inaccuracies in the calculated deformability parameters [13] . The Eadie-Hofstee plot minimizes the unequal weighting of data points by plotting each measured elongation index relative to the shear stress it was obtained at (Figure 3). This results in data that is uniformly distributed across the upper right quadrant, thus minimizing the chance of inaccurate calculations of the deformability parameters EI_{max} and K_{EI}. Although both methods are effective in calculating EI_{max} and K_{EI} values, Eadie-Hofstee analysis is a more robust method with respect to data inaccuracies and errors due to lengthy extrapolations.

The effects of oxidizing agents on the RBC membrane and cytoskeleton have been investigated extensively in previous studies [14-19] . Although many of the oxidative targets in RBCs have been established (hemoglobin, membrane lipids, cytoskeleton proteins), the effect of oxidative damage on RBC deformability has not been thoroughly investigated [14, 19] . In this study we have shown that treatment with either *t*-BuOOH or H_2O_2 leads to impairment of RBC deformability. Eadie-Hofstee analysis demonstrated that K_{EI} values were most affected, and increased significantly after treatment with *t*-BuOOH and H_2O_2. This is indicative of an increase in cellular rigidity. EI_{max} values were less affected, indicating that although oxidative treatment resulted in more rigid cells, the RBCs do retain the ability to elongate but require more shear force to achieve it. It is then reasonable to conclude that oxidative damage resulted in modifications to some component of the cytoskeleton/membrane assembly without detrimentally affecting overall RBC ultra-structure [5] . The fact that

RBCs treated with *t*-BuOOH appeared to have a decreased capacity to deform compared to those treated with H_2O_2 is not surprising considering the cellular targets of each agent. H_2O_2 in the cell is normally eliminated via inherent antioxidants native to RBCs such as glutathione and catalase (hyrdroperoxidases) [19]. This was the rational for treating the RBCs in this study with sodium azide prior to H_2O_2 as sodium azide is a known inhibitor of cellular hyrdroperoxidases [20]. If not eliminated from the cell, H_2O_2 is known to react with the hemoglobin of the cell, resulting in a cascade of reactions that can lead to heme degradation and the production of strong oxidizing agents [19, 21, 22]. The details of theses reactions are diverse and beyond the scope of this study. Production of these oxidizing species has been linked with lipid peroxidation as many are hydrophobic in nature, allowing them to easily interact with lipids in the RBC membrane [19]. Due to the close association and dynamic interaction of the RBC membrane with the underlying cytoskeleton, deformability impairment following treatment with H_2O_2 is unavoidable.

Similar to H_2O_2, *t*-BuOOH is known to quickly penetrate the RBC membrane and induce a cascade of events involving hemoglobin oxidation, the production of various strong oxidizing agents, and subsequent lipid peroxidation in the plasma membrane [14]. Furthermore, *t*-BuOOH is known to interact with components of the cytoskeleton directly, namely the horizontal junctional sites composed of spectrin, actin, protein 4.1, as well as integral membrane proteins [18]. These junctional sites are key components of the cytoskeleton scaffold and oxidative damage is likely to affect the gross organization of the cytoskeleton as

well as dynamic interactions with the plasma membrane. It has also been shown that the oxidation of spectrin plays a key role in the release of microvesicles from the membranes of RBCs [23]. A loss of membrane lipid will potentially decrease the surface area to volume ratio of the red cell and adversely affect RBC deformability. Accruement of these oxidative injuries is evident by the observed decrease in EI_{max} values at higher doses of *t*-BuOOH which were absent in H_2O_2 treated samples. This is indicative of gross alterations to the structure of the RBC membrane rather than changes in only membrane rigidity. Taking all these factors into consideration, the fact that *t*-BuOOH treatment elicited a more pronounced effect on RBC deformability compared to H_2O_2 treatment is not surprising.

Cholesterol is an essential component of all mammalian membranes and makes up about 30% of the lipid portion of RBC membranes [24]. The presence of cholesterol in the RBC membrane affects the overall membrane fluidity as increasing amounts of cholesterol allow for the increased packing efficiency of membrane lipids [25]. This tighter packing of phospholipids results in an overall decrease in membrane fluidity. Therefore, the modification of membrane fluidity can be achieved by altering cholesterol proportions. Extraction of membrane cholesterol using cyclodextrins has been previously described and well characterized [25-31]. Following treatment with MβCD, Eadie-Hofstee analysis resulted in dose dependant decreases in EI_{max} values, accompanied by a decrease in K_{EI} values at higher doses of MβCD (10 mM). This deformability profile is opposite to that observed previously with the oxidizing agents. The sharp EI_{max} decreases suggest an inability of these cells to deform, even at exceedingly high

shear rates. In contrast, the decrease in K_{EI} observed at higher doses of MβCD indicates a decrease in rigidity. This is likely due to the increase in membrane fluidity associated with depleting membrane cholesterol. It is intuitive to assume that an increase in membrane fluidity would have the effect of enhancing RBC deformability; however, this is not the case. Interestingly, studies in which RBC membranes were enriched with cholesterol have also reported a decrease in filterability indicating a decreased capacity of these cells to deform [25]. Morphology of these RBCs following cholesterol enrichment was also reported to be abnormally characterized by gross surface contours, resulting in changes to the normal surface area to volume ratio of the erythrocytes. In the current study, the depletion of membrane cholesterol likely resulted in a decrease in surface area to volume ratio, which may partially explain the decrease in EI_{max} values observed. It is known that cholesterol is often located within micro-domains in the membrane knows as 'lipid rafts' which in many cell types are important for cell signalling functions [24]. Perhaps disruption of these rafts in RBCs, either by cholesterol enrichment or extraction, alters the association of the membrane with the cytoskeleton, resulting in the decreases in deformability observed in both cases. These alterations in membrane cholesterol appear to illuminate how important the maintenance of a normal surface area to volume ratio is for successful RBC deformation.

In addition to detecting deformability changes in RBCs chemically modified *in vitro*, we were able to demonstrate contrasting deformability profiles of RBCs affected by hereditary spherocytosis (HS) and α–thalassemia major (TS).

HS is a genetic disorder characterized by the appearance of spherical RBCs that lack the normal biconcave morphology usually observed in healthy RBCs. This defect is due to mutations in one or more of the cytoskeletal proteins resulting in abnormal and unstable cytoskeletal networks and associations with the plasma membrane [32]. Due to these abnormalities, RBCs are often less deformable and are prematurely removed by the spleen resulting in anemia. In the current study, EI_{max} values calculated via the Eadie-Hofstee linearization method were heavily depressed, being only half that of control RBCs. As previously mentioned, large decreases in EI_{max} values are generally indicative of gross structural abnormalities in the RBC cytoskeleton / membrane complex; both of which are hallmark to HS pathophysiology. Alpha thalassemias are genetic disorders resulting in decreased production or complete absence of the α–globin chains [33]. If this deficiency is severe, abnormal and unstable hemoglobins may be formed, resulting in decreased erythrocyte lifespan and subsequently anemia. Previous studies have demonstrated decreases in TS RBC deformability [34]. We have also demonstrated a marginal decrease in EI_{max} values in TS affected RBCs using the Eadie-Hofstee method. K_{EI} values were also elevated indicating more rigid RBCs compared to control RBCs and HS RBCs. The presence of abnormal hemoglobins in TS may change the cytoplasmic viscosity, possibly explaining the elevation of observed K_{EI} levels. It is also possible that the marked decrease or absence of alpha chains may lead to hemoglobins that are more susceptible to oxidative injury, thus leading to changes in the cytoskeleton or membrane resulting in decreased RBC deformability.

2.5 Conclusion

Cellular deformability is a powerful indicator of RBC viability as it is essential for the optimal functioning of RBCs *in* vivo. Therefore, the ability to detect and resolve deformability changes in RBCs could prove useful not only in a basic research setting, but also in clinical settings, especially when prevention of tissue hypoxia is of paramount importance. This chapter has demonstrated the utility of Eadie-Hofstee analysis in dissecting RBC deformability changes induced chemically *in vitro* as well as in pathologic RBCs *in vivo*. With further research, there is potential for this technique to be utilized in clinical hematology laboratories as well as in blood banks to ensure blood products delivered to patients are of the highest standard possible. The linearization technique investigated in this chapter will be used to assess RBC deformability in all subsequent chapters.

Figure 2.1: LORRCA Geometry and principal of operation. This illustration was modified from the Laser-assisted Optical Rotational Cell Analyzer User's Manual, *Version 2.1.[110]*

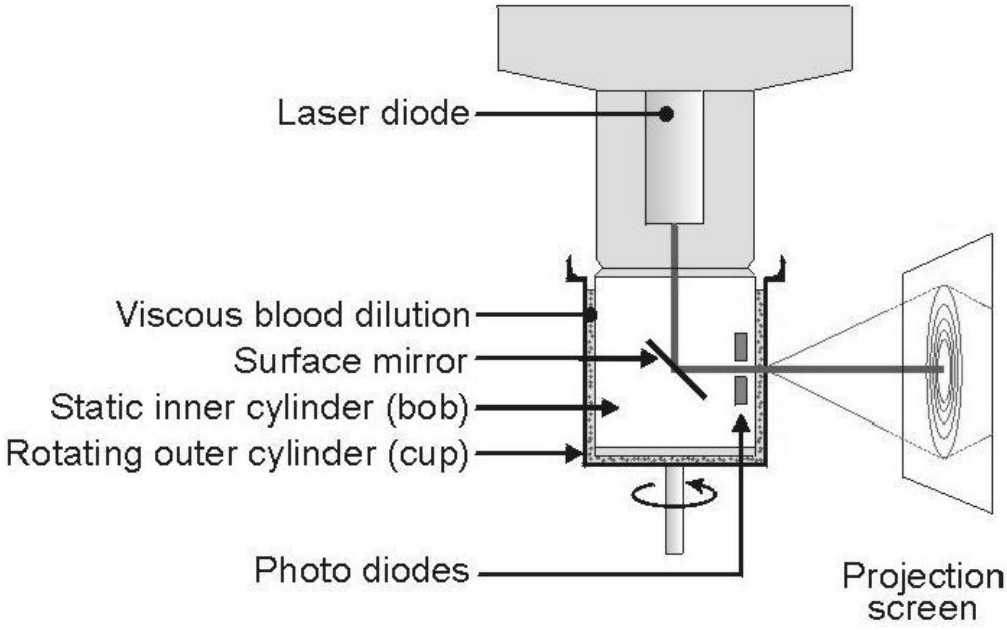


Figure 2.2: RBC deformability curve. As shear stress increases, the diffraction pattern of the RBCs becomes increasingly elongated.

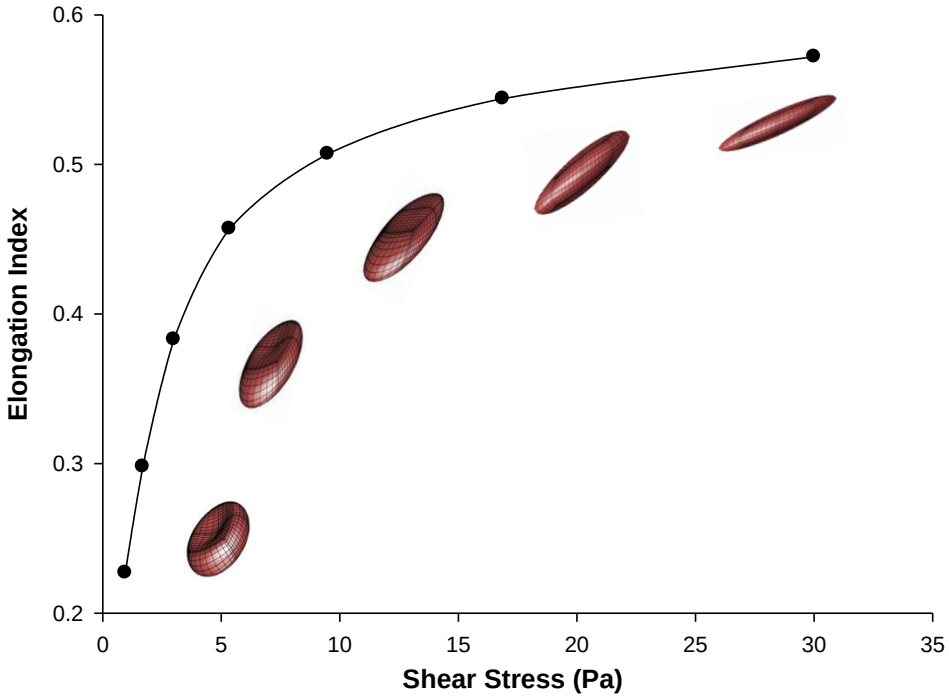


Figure 2.3: The Eadie-Hofstee linear data transformation applied to a typical RBC sample **Inset:** Deformability curve of a typical RBC sample obtained from LORCA prior to Eadie-Hofstee linearization.

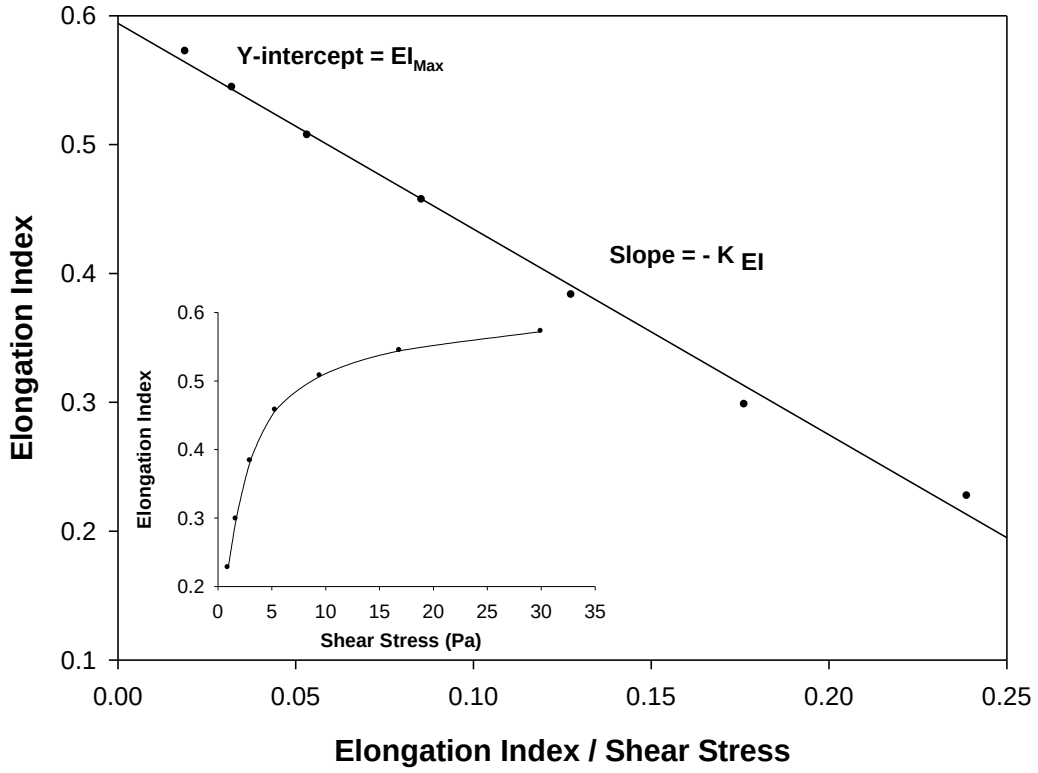


Figure 2.4: Deformability of RBCs treated with *t*-BuOOH. RBCs were treated with either 1 or 2 mM *t*-BuOOH and incubated for 15 min at 37 ˚C. EI_{max} and K_{EI} values were calculated via Eadie-Hofstee linearization.

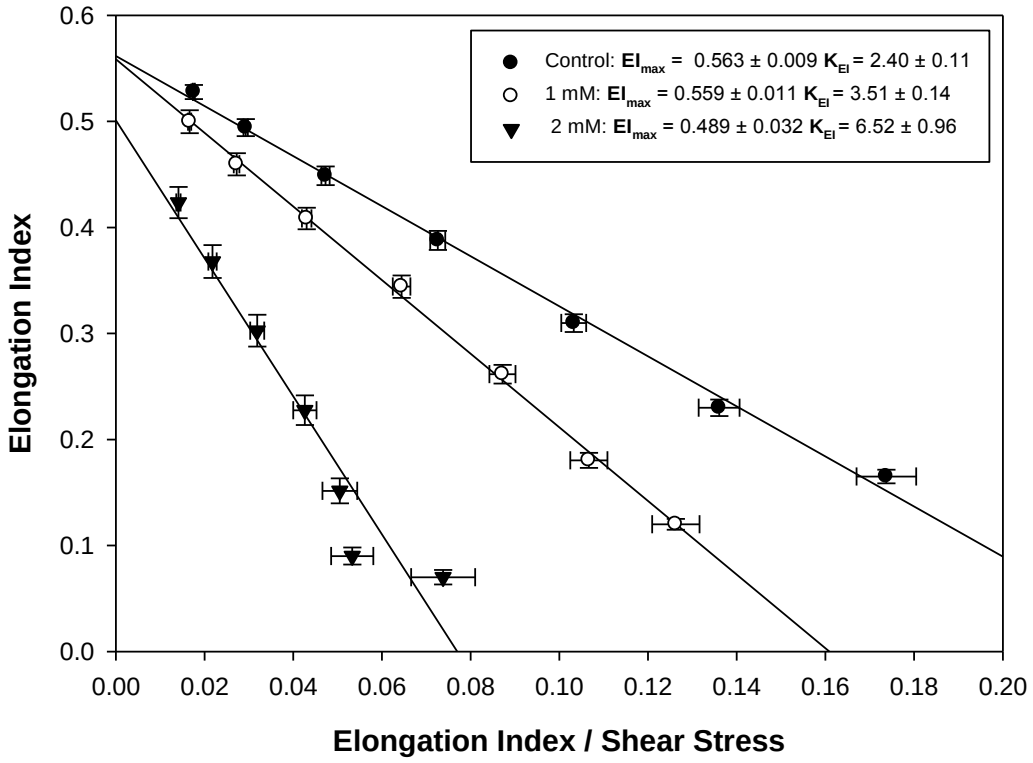


Figure 2.5: Deformability of RBCs treated with H_2O_2. RBCs were treated with either 1 or 2 mM H_2O_2 and incubated for 15 min at 37 ˚C. EI_{max} and K_{EI} values were calculated via Eadie-Hofstee linearization.

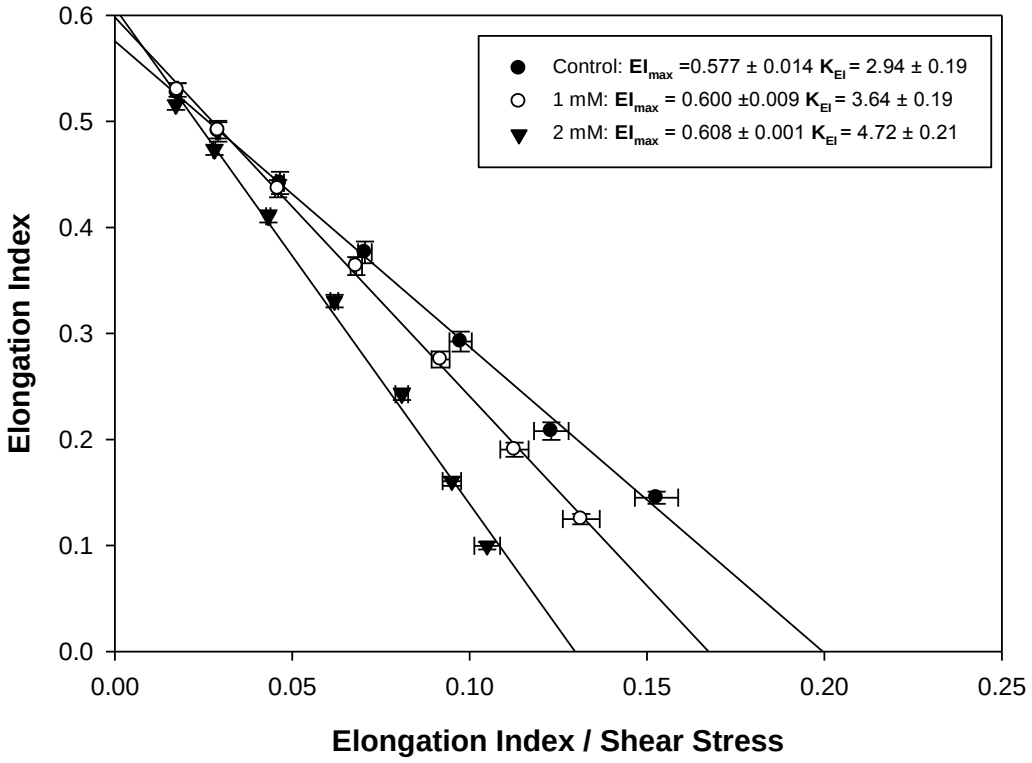


Figure 2.6: Deformability of RBCs treated with MβCD. RBCs were treated with either 5 or 10 mM MβCD and incubated for 1 hour at 37 ˚C. EI_{max} and K_{EI} values were calculated via Eadie-Hofstee linearization.

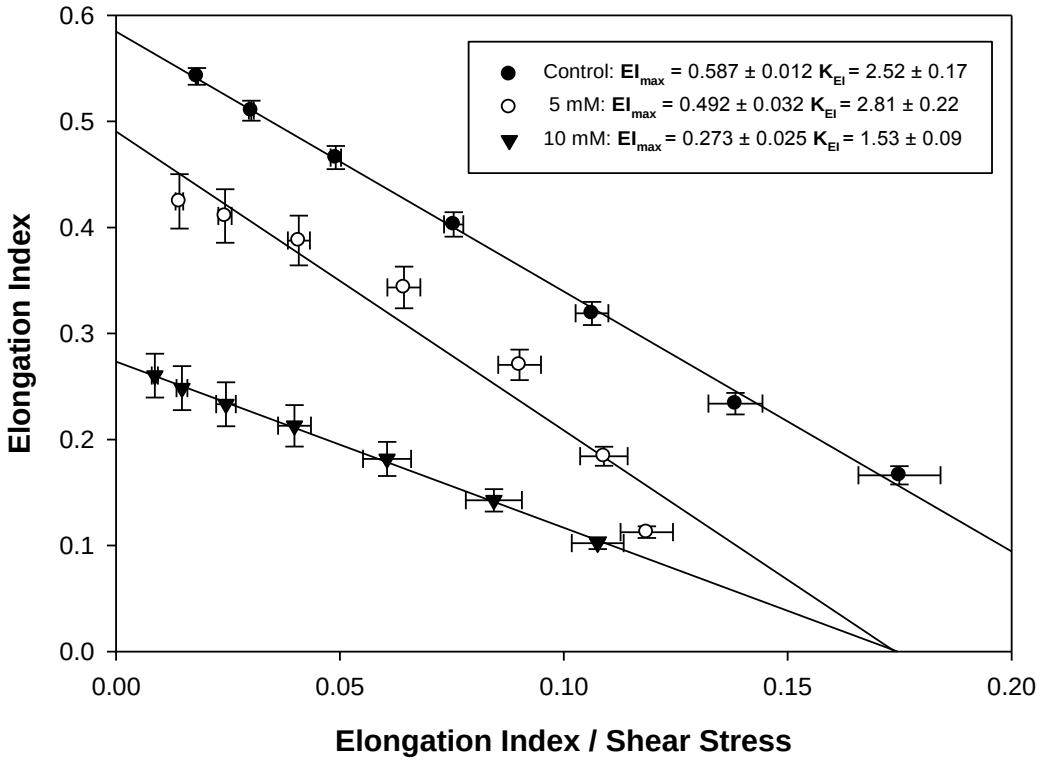
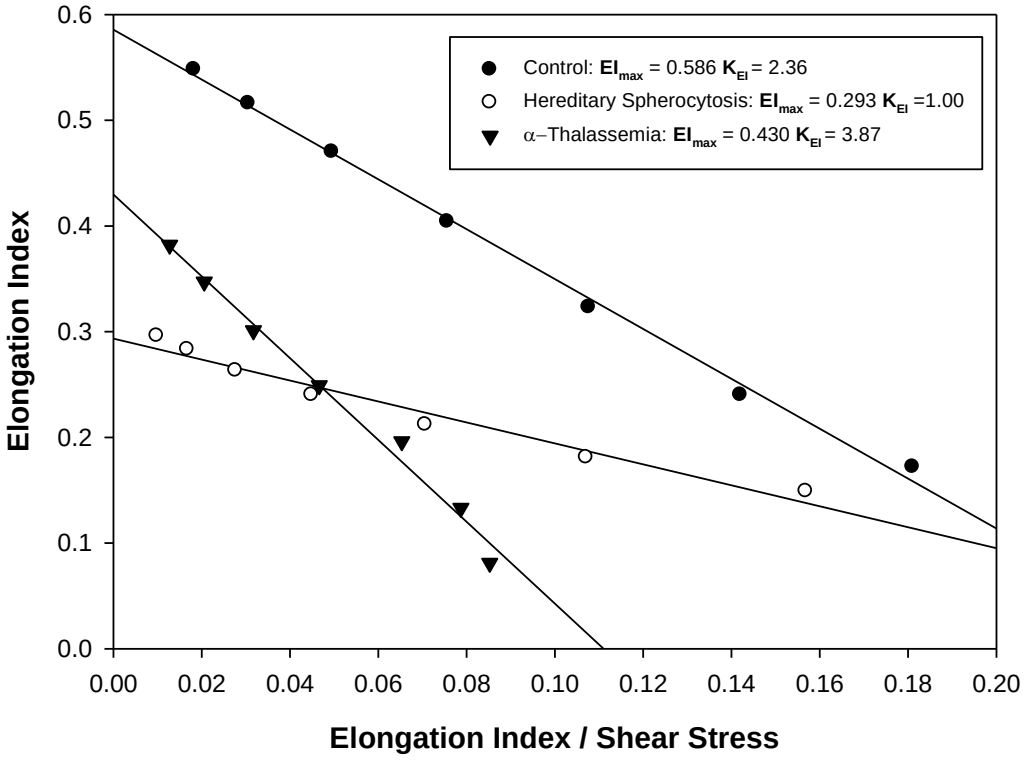


Figure 2.7: Deformability of pathologic RBCs. EI_{max} and K_{EI} values of RBCs isolated from patients with hereditary spherocytosis and α-thalassemia (single donors) were calculated using Eadie-Hofstee linearization. Control was isolated from a single donor and age-matched.



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Chapter 3

Investigation of red blood cell deformability changes during hypothermic storage

3.1 Introduction

The ability to successfully preserve RBC viability during *ex vivo* storage is essential for the practice of transfusion medicine. Currently, the majority of RBC units in blood banks are stored hypothermically at 1 to 6 ˚C in additive solution and anticoagulant. The rationale behind this type of storage is simple; the rate of any chemical reactions occurring in the cell are temperature dependant and will be depressed at lower temperatures, thus slowing the depletion of essential metabolites and reducing possible cellular injury [1]. Although cool temperatures do slow the rate of cellular metabolism, they do not halt it completely, leading to an array of biochemical and biomechanical changes in stored RBCs known collectively as the RBC 'storage lesion' [2]. A thorough summary of the changes can be found in the introductory chapter of this thesis, with some of the more characterized changes including the depletion of intracellular 2,3-DPG and ATP, changes in MCV, the loss of membrane lipid via microvesiculation, and gross changes in RBC morphology [1-5]. Although advances in the understanding of RBC physiology have led to increased knowledge regarding the biochemical nature of the RBC storage lesion, the effect of these biochemical changes on RBC rheological properties remains unclear. However, it has been previously suggested that many aspects of the hypothermic storage lesion are related to the significant decrease in intracellular ATP levels during storage [3].

The ability of transfused RBCs to successfully flow through the vasculature unimpeded is essential for the eventual delivery of oxygen to the tissues of the body [6]. It is therefore imperative that preservation strategies not

only address the biochemical changes associated with hypothermic storage, but also address any rheologic deficiencies. Recent publications have implied that the transfusion of older blood units can result in increased vascular resistance and decreased tissue oxygenation, leading to complications in critically ill patients such as an increased incidence of post-operative pneumonia [2, 7-9]. It has long been speculated that the hypothermic storage of RBCs results in less deformable cells, which has been demonstrated in a number of studies [2, 9-13]. As mentioned in Chapter 2, the ability of RBCs to deform is essential for proper RBC function as the diameter of RBCs is greater than the diameter of the capillaries [6]. In fact, a loss of deformability in senescent RBCs *in vivo* is thought to be one of the factors triggering the removal of older RBCs by the spleen [6]. Therefore, the transfusion of RBCs with impaired deformability may result in not only decreased tissue oxygenation, but also increased load on the reticuloenodthelial system . Because of this fact, the proposed use of restrictive transfusion protocols in critically ill patients is not surprising [10]. Despite the importance of RBC deformability for the proper functioning of RBCs *in vivo,* this parameter is not routinely monitored in blood banks or clinical laboratories. As discussed in Chapter 2, part of the reason for this may be that no standard method for determining RBC deformability or analyzing RBC deformability data has been established[14].

As with many aspects of the RBC hypothermic storage lesion, impairments in RBC deformability are thought to be related to decreases in intracellular ATP concentrations during the storage period [6]. It has been

previously shown that the maintenance of the biconcave shape of RBCs is an energy dependant process and that ATP depleted RBCs lose the ability to deform [15,16]. Furthermore, upon restoration of ATP levels in adenine solutions, losses in deformability occurring during RBC storage have been shown to be partially reversible [16]. However, deformability measurements in both of these studies were performed via filtration methods that have been superseded by modern techniques such as ektacytometry. In more recent years, the use of pyruvate-inosine-phosphate-adenine (PIPA) rejuvenation solutions has been shown to improve the quality of hypothermically stored RBCs as indicated by increases in intracellular ATP and 2,3-DPG concentrations, decreases in intracellular Ca^{++} concentrations, as well as improved RBC morphology [17-21]. In addition, it has been demonstrated that RBC rejuvenation reverses storage induced adhesion to cultured endothelial cells [18]. Although improvements in the biochemical properties of stored RBCs following rejuvenation are evident, no research has investigated the effect of rejuvenation on RBC deformability using ektacytometry. Given that these solutions are approved for clinical use in the Unites States, more investigations into the effect of PIPA rejuvenation on RBC rheologic properties are warranted.

The previous chapter of this thesis demonstrated that *in vitro* induced changes to RBC deformability are readily detectable using ektacytometry with subsequent Eadie-Hofstee analysis[14]. In the current study, we wish to determine the effect of hypothermic storage on RBC deformability in leukoreduced CPD-SAGM RBC units by applying the Eadie-Hofstee analysis technique investigated

in the previous chapter. In addition to deformability measurements, hematological measurements will also be recorded, including percent hemolysis, RBC indices, and ATP concentrations. Furthermore, the effect of RBC rejuvenation with PIPA solution will be investigated to determine if any detected deficiencies in RBC deformability during storage are reversible following ATP level restoration. The results of this study will determine if RBC deformability is indeed impaired during hypothermic storage and additionally demonstrate whether this impairment is due solely to ATP depletion or a combination of other cellular mechanisms.

3.2 Materials and Methods

3.2.1 RBC Processing

Whole blood (450 ± 50 mL) was collected from healthy volunteers in top-and-bottom collection systems using standard blood bank phlebotomy procedures (netCAD, CBS, Vancouver, Canada). Leukoreduced CPD-SAGM RBC units (B1)(n=6) were produced using the buffy coat production method [22,23]. Following collection, whole blood units were cooled rapidly to room temperature and held overnight [23]. On the following day, the units were centrifuged resulting in three layers: platelet poor plasma, the buffy coat, and RBCs. Following centrifugation, the whole blood was loaded into a component separator (Compomat G4, Fresenius-Kabi) which separated the plasma and RBCs into the top and bottom satellite bags of the collection set respectively [23]. After separation, SAGM (110 mL) was added to the RBC unit which was subsequently

leukoreduced by filtration and shipped to Edmonton. All units were received 2 days after collection and stored hypothermically at 1 to 6 ˚C for the duration of the study. RBC assays were performed immediately upon receiving the unit in Edmonton (referred to as week 0 in figures), as well as weekly for a total of 50 days (referred to as week 1, 2, 3, etc. in figures). At each test point, approximately 3 mL of RBCs were drawn aseptically by syringe from the units and stored hypothermically (1-6 ˚C) in microcentrifuge tubes for a maximum of 4 hours prior to assay commencement.

3.2.2 RBC Assays

In vitro RBC quality was assessed by conventional biochemical and biomechanical assays including percent hemolysis, ATP concentration, and the RBC indices [mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC)]. In addition to these conventional techniques, RBC deformability was assessed via ektacytometry with subsequent Eadie-Hofstee analysis which is described in section 2.2 of chapter 2.

3.2.2.1 Conventional measures of RBC quality

<u>Percent Hemolysis</u>

Hemolysis refers to the breakdown of the RBC membrane and subsequent release of hemoglobin to the surrounding environment. In this and the subsequent chapter, percent hemolysis was determined via the cyanmethhemoglobin (HiCN)) method, which is a spectrophotometric assay comparing the supernatant

hemoglobin concentration of a sample to the total hemoglobin concentration of a sample. A small volume of RBCs are diluted with Drabkin's reagent (0.61 mM Potassium ferricyanide, 0.77 mM Potassium cyanide, 1.03 mM Potassium dihyrdrogen phosphate, and 0.1 % v/v Triton X-100) resulting in the conversion of most forms of hemoglobin to HiCN. Subsequently, a two step reaction takes place converting hemoglobin to methemoglobin, followed by the conversion of methemoglobin to HiCN. HiCN absorbs light maximally at 540 nm with the measured absorbance being directly proportional to the hemoglobin concentrations present in the sample. The resultant hemoglobin concentration can be obtained from the following equation:

$$c = \frac{A_{540} \times M \times F}{\varepsilon_{540} \times l \times 1000} \tag{3.1}$$

Where:

c = concentration of hemoglobin (g/L)

A_{540} = absorbance of the solution at 540 nm

M = molecular mass of hemoglobin monomer (16114.5 mg/mmol)

F = dilution factor

ε_{540} = millimolar absorptivity of HiCN at 540 nm (11.0 $cm^{-1} \cdot mM^{-1}$)

l = light path (cm)

Following the calculation of the total hemoglobin and supernatant hemoglobin, the percent hemolysis in a sample can be determined by the following equation:

$$\%\ hemolysis = \frac{(100 - Hct) \times Hb_S}{Hb_T} \tag{3.2}$$

Where:

Hct = hematocrit (%)

Hb_S = supernatant hemoglobin concentration (g/L)

Hb_T = total hemoglobin concentration (g/L)

The Hct in the calculation is necessary to account for the volume of supernatant in the specimen. Tri-level hemoglobin control solutions are used to ensure the accuracy of the assay (Stanbio Laboratory, Boerne, USA).

ATP Concentration

RBC ATP concentrations were measured via an enzymatic method using a commercially available kit (Rolf Greiner BioChemica, Flacht, Germany). The chemical reactions occurring in the assay are summarized below:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glucose-6-phosphate} + \text{ADP} \tag{3.3}$$

$$\text{Glucose-6-phosphate} + \text{NAD}^+ \xrightarrow{\text{G6PD}} \text{6-phosphogluconate} + \text{NADH} + \text{H}^+ \tag{3.4}$$

RBC samples are treated with a trichloroacetic acid solution to precipitate out any RBC proteins present. The solution is then centrifuged to obtain a protein free supernatant. ATP in the supernatant is ultimately consumed in the enzymatic

conversion of glucose to 6-phosphogluconate and NADH by the enzymes hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PD)). The amount of NADH produced by the two reactions is directly proportional to the ATP concentration in the original test sample which can be measured spectrophotometrically at 340 nm. The following equation is required to calculate the original ATP concentration:

$$ATP(\mu mol/dL) = \frac{\Delta A \times V \times F \times 100}{\varepsilon_{340} \times v \times d} \qquad (3.5)$$

Where:

ΔA = (absorbance of the sample solution at 340 nm) – (absorbance of blank at 340 nm)

V = total volume of the sample

F = dilution factor of sample preparation

ε = extinction coefficient of NADH (6.3 @ 340nm)

v = sample volume used in ATP assay

d = light path (cm)

The units of μmol/dL are not commonly used for *ATP* concentrations and can be converted to μmol/gHb by:

$$ATP(\mu mol/gHb) = \frac{10 \times ATP(\mu mol/dL)}{Hct(L/L) \times Hb(g/L)} \qquad (3.6)$$

RBC Indices

RBC indices were determined via a Coulter automated cell counter (Coulter AcT, Beckman Coulter, New York, NY). Once submersed in the sample solution, an electric field is applied to the aperture of the instrument and the impedance between two electrodes in the aperture is measured. When RBCs pass through the sensing zone in the aperture they displace a volume of electrolyte equal to the cell volume, which momentarily increases the impedance within the aperture and creates a current pulse. The pulse size is directly proportional to the size of the cell [119]. MCV values are expressed as the average volume of cells crossing the aperture and are given in fL. A known volume of solution is passed through the aperture; therefore, the concentration of RBCs in solution can be calculated by dividing the number of pulses by the volume of the RBC suspension. The hematocrit is calculated by multiplying the MCV by the RBC count. The hemoglobin concentration (g/L) is calculated by the HiCN method and used to compute the MCH (pg) value by dividing the hemoglobin concentration by the RBC count (cells/L). The MCHC (mean percentage of hemoglobin within a cell) is expressed as a percent and is computed by dividing the hemoglobin content of the cell by the hematocrit of the sample.

3.2.2.2 RBC deformability assessment

RBC deformability was assessed via ektacytometry with subsequent Eadie-Hofstee analysis as previously described in Chapter 2, Section 2.2.3 .To summarize, at each successive sample point RBCs were suspended in PVP and analyzed with the LORCA at shear stresses ranging from 0.95 Pa to 30 Pa. The acquired RBC deformability curves were then linearized using the Eadie-Hofstee

linearization technique described in Chapter 2, Section 2.2.4 [14]. The resultant RBC deformability parameters EI_{max} and K_{EI} were then calculated for each week of storage and compared to initial values computed upon receipt of the RBC units. All EI_{max} and K_{EI} values are expressed as means ± standard error (SEM).

3.2.2.3 RBC Rejuvenation

Three mL of RBCs were drawn aseptically by syringe from six 45 day old CPD-SAGM RBC units (B1) stored hypothermically (1-6 ˚C) (units were obtained from netCAD, CBS, Vancouver, Canada), with each sample being split into 1.5 mL aliquots. All samples were subsequently centrifuged for 5 minutes at 1500 g to pellet the RBC fraction. In one set of samples, 800 uL of the supernatant was drawn off and replaced with an equal volume of PIPA solution (550 *mg* pyruvate, 1.34 *g* inosine, 34 *mg* adenine, 500 *mg* dibasic sodium phosphate, 200 *mg* monobasic sodium phosphate, in 50mL dH_2O, pH 7.0) followed by RBC resuspention by inversion. The other set of samples were resuspended following centrifugation without the addition of PIPA solution, thus serving as non-rejuvenated controls. All samples were then incubated in a 37 ˚C circulating water bath for 1 hour. Following incubation, ATP, RBC indices, and deformability measurements were performed on both rejuvenated and control RBCs within 1 hour, as described previously in the materials and methods section.

3.2.3 Statistical Analysis

The data were analyzed using commercial statistical software (SPSS Version 12.0, Lead Technologies, Charlotte, NC.) as well as Mynova (developed

b y Dr. Stephen PJ Brooks, 1992). Nonparametric (Mann-Whitney U test) as well as parametric (One-way ANOVA with SNK post-hoc) statistical analysis was performed to examine the differences in RBC *in vitro* parameters on a week to week basis. Unless otherwise specified, significant differences are expressed relative to initial measures (week 0 in figures). Probabilities less than 0.05 were considered significant.

3.3 Results

3.3.1 Conventional Measures of RBC Quality

The conventional measures of RBC quality are reported in Tables 3.1 as well as Figure 3.1 and 3.2. Percent hemolysis increased throughout storage duration with statistically significant differences becoming apparent at day 15 of storage ($p = < 0.05$ for both statistical tests) (Figure 3.1). The highest percent hemolysis values were observed in weeks 6 and 7 of storage (0.23 ± 0.04 in both cases). Although a statistically significant increase in hemolysis was observed, the maximum values are relatively low and are in accordance with values in the literature [4]. ATP levels dropped gradually throughout storage duration from a maximum of 4.9 ± 0.5 µmol/g Hb on day 2 of storage to 1.9 ± 0.2 µmol/g Hb after 7 weeks of storage (Figure 3.2). The decrease in ATP levels became statistically significant at week 4 (parametric statistics, $p < 0.05$) and week 5(nonparametric statistics, $p = 0.006$) of hypothermic storage and remained significantly reduced for storage duration . MCV measures fluctuated during storage, with a minimum

MCV of 94 ± 1 fL recorded on day 2 of storage and a maximum MCV of 98 ± 1 fL recorded after 7 weeks of storage. Although there was no statistically significant difference, MCV values appear to have an upward trend as displayed in table 3.1. MCV values remained within clinical reference ranges for the duration of storage (80-100 fL)[25]. MCH values fluctuated marginally throughout storage duration but remained close to 30 pg. Statistically significant differences in MCH compared to initial testing values were recorded during week1 and 3, however these values stabilized to within clinical reference ranges for the duration of the study (27 - 31 pg)(Table 3.1) [25]. MCHC values fluctuated throughout storage with statistically significant changes compared to initial testing evident in week 1, 3, 6, and 7 (Table 3.1). In addition, MCHC values were below clinical reference ranges for 5 out of the 7 weeks (weeks 1 and 3 excluded) (32 – 36 g/dL) [25].

3.3.2 RBC deformability assessment

RBC deformability curves from weeks 0, 2,5,6,7 are depicted in Figure 3.3 following Eadie-Hofstee linearization. There is downward shift in the weekly plots which was evident by week 2 of hypothermic storage and continued throughout storage duration (Figure 3.3). Although a downward shift in the plots is clearly evident, the slopes of the plots are very similar in each case (Figure 3.3). EI_{max} values are shown graphically in figure 3.4. Following an initial period with EI_{max} values remaining consistent, a downward trend becomes apparent beginning with week 4 of storage. Decreases in EI_{max} values became statistically significant

at five weeks of storage using nonparametric analysis ($p = 0.01$) and 6 weeks of storage using parametric analysis ($p < 0.05$). As indicated in figure 3.5, decreases in ATP concentration precede decreases in EI_{max}. In contrast to EI_{max} values, K_{EI} values remained constant throughout the storage period (Fig 3.6).

3.3.3 Post rejuvenation RBC assessment

RBC rejuvenation resulted in statistically significant differences in RBC indices values, ATP concentration, and deformability. As demonstrated in table 3.2, MCV values decreased significantly following RBC rejuvenation ($p < 0.05$ for both statistical methods), while MCHC values increased significantly ($p < 0.05$ for both statistical methods). No changes were observed in MCH concentrations. As expected, rejuvenation also resulted in statistically significant increases in ATP concentrations, as shown in figure 3.7 ($p < 0.005$ for both statistical methods). Deformability measurements are shown in figures 3.8. EI_{max} values decreased significantly following RBC rejuvenation as demonstrated in figure 3.7A ($p < 0.05$ for both statistical methods). In addition, K_{EI} values tended to increase, which bordered on being statistically significant (fig 3.7B) ($p = 0.06$ for both statistical methods).

3.4 Discussion

The delivery of oxygen to the tissues of the body is largely dependent on the rheological properties of RBCs, specifically their ability to deform. Therefore, the preservation of RBC deformability during *ex* vivo storage is essential for the

delivery of functional RBCs to transfusion patients. The purpose of this study was to apply the deformability analysis method established in the previous chapter to determine if hypothermically stored RBCs lose the capacity to deform[14]. In addition to deformability measurements, intracellular ATP concentrations were also monitored to investigate the relationship between ATP levels and RBC deformability. The results of this study demonstrate that decreases in the maximum deformability of hypothermically stored RBCs are detectable via ektacytometry with subsequent Eadie-Hofstee analysis. These changes were preceded by decreases in intracellular ATP concentration, suggesting ATP involvement in the maintenance of RBC deformability. However, decreases in RBC deformability were not reversible following ATP level restoration, indicating that ATP depletion is not solely responsible for decreases in RBC deformability occurring during hypothermic storage.

Previous studies have examined RBC deformability changes associated with hypothermic storage via other methods [9,10,12,26]. In most cases, decreases in RBC deformability were observed as storage duration increased; however, none of the RBC units investigated in these studies underwent leukofiltration, a process which has been shown to improve RBC morphology, slow the rate of ATP depletion, and decreases blood viscosity during hypothermic storage [27,28]. Recent studies investigating the deformability of leukoreduced CPD-SAGM blood units have produced contrasting results. Henkelman *et al.* demonstrated that the hypothermic storage of RBCs led to decreases in maximum RBC deformability as measured at 50 Pa using ektacytometry (LORCA) [13].

However, Raat *et al.* found no decreases in the deformability of RBCs stored for 35 days at shear stresses of both 4 and 30 Pa (LORCA). It is possible that the observed discrepancies are related to subtle differences in the storage media, however it may be simply related to the techniques employed to analyze the data. In both studies, only two shear stresses were used to compare RBC rigidity and maximum deformability values [13,29]. This fails to give a complete picture of deformability, as much of the data within the deformability curve is simply discarded. In the present study, the EI_{max} and K_{EI} values obtained via the Eadie-Hofstee linearization technique are derived using elongation index values obtained from seven discrete shear stresses[14].Therefore, the deformability parameters presented in this chapter are more indicative of the kinetics of RBC deformation.

In the current study, statistically significant decreases in RBC maximal deformability are evident following 5 weeks of hypothermic storage. Although it took 5-6 weeks of hypothermic storage for this decrease in EI_{max} to become statistically significant, it is evident from figure 3.4 that a downward trend in maximal deformability began to appear between weeks 3 and 4 of storage, and subsequently dropped throughout the remainder of the storage period. RBC rigidity values, as indicated by the K_{EI} parameter, remained stable throughout storage, however the slight upward trend demonstrated in figure 3.5 suggests that RBC rigidity may also be increasing.

As mentioned in the previous chapter, RBC deformability is primarily a function of cytoplasmic viscosity, RBC membrane/cytoskeleton characteristics,

and the normal surface area to volume ratio of the RBCs [6, 14 30]. Temporal changes in the standard hematological measures performed in this study give clues as to which of these components of deformability are affected during hypothermic storage.

Cytoplasmic viscosity is due primarily to the intracellular hemoglobin concentration [6]. Increases in cytoplasmic viscosity lead to more rigid RBCs and are likely to result in decreases in RBC deformability[6]. If changes in intracellular hemoglobin concentrations are occurring, it should be reflected in the MCH and MCHC values which are shown in tables 3.1. MCH values remained reasonably constant throughout storage and MCHC tended to decreases, especially at later sample points (table 3.1). Theoretically, this drop in MCHC should lead to decreases in cytoplasmic viscosity and therefore, increases in RBC deformability. From the deformability measurements, this is clearly not the case, suggesting that changes in cytoplasmic viscosity are not leading to the deficiencies in RBC deformability reported in this study.

Although the observed decrease in RBC deformability cannot be explained by changes in cytoplasmic viscosity, alterations in the membrane/cytoskeleton complex and surface area to volume ratio are supported by the hematological measurements. Induced damage to the RBC membrane complex may include changes in the viscoelastic properties of the membrane, the membrane lipid composition, the underlying cytoskeleton, or dynamic interactions occurring between the cytoskeletal scaffold and the lipid bilayers [6, 31, 32]. Percent hemolysis is an obvious indicator of RBC membrane health and is shown in figure

3.1. Although percent hemolysis values increased throughout storage duration, these increases were minimal and comparable to values in the literature for CPD SAGM RBCs, suggesting that no critical modifications in RBC membrane integrity are occurring [4,5]. However, the progressive drop in ATP level as indicated in figure 3.2 is likely to have an effect on both the RBC membrane and the normally favourable surface area to volume ratio. Decreases in ATP levels result in the shutdown of transmembrane ion pumps leading to increases in intracellular ion concentrations, changes in osmotic balance, and alteration of the dynamic interactions occurring between the membrane and the underlying cytoskeletal scaffold [33-35]. Increased intracellular ion concentrations induce cell swelling (as indicated by the upward trend in MCV values in table 3.1) as well as the loss of RBC membrane lipid via microvesiculation (induced specifically by increases in intracellular calcium levels due to the shutdown of the CA^{++}ATPase), resulting in the progressive spheroechinocytosis that is associated with the hypothermic storage lesion [32, 36, 37]. The subsequent decrease in surface area to volume ratio due to the synergistic effects of RBC swelling and the loss of lipid via microvesiculation is likely responsible for the decreases in RBC deformability observed in this study.

Further evidence supporting the role of ATP is also presented in figure 3.5, which demonstrates that the eventual drop in EI_{max} values are clearly preceded by decreases in ATP concentration. Given that ATP depletion is likely to play a key role in RBC deformability, RBC rejuvenation was carried out using a PIPA solution to investigate if restoration of RBC intracellular ATP levels has

any effect on the capacity of RBCs to deform. The effect of RBC rejuvenation has been examined extensively in previous studies on both hypothermically stored and cryopreserved RBCs [17-20, 38-40]. Virtually all previous studies suggest that RBC rejuvenation results in the restoration of ATP and 2, 3-DPG levels, as well as the reduction of MCV values. In addition, it has been demonstrated that the observed increase in adhesion of hypothermically stored RBCs to the vascular endothelium, which is thought to be a result of phosphatidyl serine exposure in the outer leaflet of the plasma membrane, can be reversed following rejuvenation treatments [18, 40]. Although numerous studies have investigated the effect of PIPA solutions on the quality of stored RBCs, little research has been done investigating the effect of rejuvenation on RBC deformability[15,16]. In the current study, RBC rejuvenation resulted in expected statistically significant increases in ATP levels (fig 3.7), as well as MCHC values (Table 3.2). This was accompanied by statistically significant decreases in RBC MCV (Table 3.2). Surprisingly, RBC deformability was adversely affected by RBC rejuvenation as demonstrated by the rise in EI_{max} values shown in figure 3.8.

Although a further loss of deformability following rejuvenation seems counter intuitive, previous studies may provide an explanation. It has been shown that the formation of exocytic and endocytic vesicles accompanies the membrane remodelling which occurs during the maturation of reticulocytes [17, 41-43]. This process is thought to occur as a mechanism to adjust the surface area to volume ratio of immature RBCs, resulting in improved deformability and *in vivo* function [17]. A similar membrane remodelling process has been postulated to occur when

ATP levels are restored during rejuvenation, resulting in the loss of membrane lipid via microvesiculation and a subsequent decrease in MCV [17]. Although this process is thought to be beneficial for the maintenance of deformability in reticulocytes, it may be deleterious in the case of mature RBCs. The effect of rejuvenation-induced microvesiculation on RBC deformability would be two fold. Firstly, a loss of lipid is likely to result in a decrease in the surface area to volume ratio of the RBCs, a phenomenon known to impair RBC deformability. Secondly, a loss of lipid will increase the relative concentration of hemoglobin in the cell (as reflected by the increasing MCHC levels), leading to an increase in cytoplasmic viscosity and subsequently, more rigid RBCs. In addition to the effects elicited by the decrease in surface area to volume ratio and increased cytoplasmic viscosity, rejuvenation induced microvesiculation may also further disrupt interactions between the membrane lipid bilayer and the cytoskeleton matrix, preventing the necessary rearrangements required for reversible RBC deformability. Due to these arguments, the observed decrease in deformability following RBC rejuvenation is understandable.

Although it has long been postulated that decreases in RBC deformability during hypothermic storage are due to falling ATP concentrations, the irreversibility of these deficiencies suggest that ATP depletion leads to other downstream mechanisms that result in deformability losses during *ex vivo* storage. The irreversible nature of these changes points to structural modifications that are likely related to the cell membrane, such as a loss of membrane lipid via microvesiculation. As mentioned previously in this discussion, membrane

microvesiculation has the potential to affect the surface area to volume ratio and the gross organization of the membrane/cytoskeleton complex which are both powerful mediators of RBC deformability [6]. Although microvesiculation is thought to occur as a consequence of ATP depletion, it has also been shown to occur prior to ATP depletion in hypothermically stored RBCs [32, 34]. More research is required to fully investigate the mechanism of deformability loss in hypothermically stored RBC; however, due to the multifaceted effect of microvesiculation on RBC deformability, the exploration of preservation strategies focussing on the stabilization of RBC membrane is warranted.

The clinical significance of the findings in the current study as well as the recent studies by Henkelman and Raat are debateable [13, 44]. Despite finding statistically significant decreases in maximum RBCs deformability, Henkelman *et al.* proposed that the decrease in deformability was not likely biologically significant due to the measured values remaining within normal reference ranges [13]. However, numerous studies have demonstrated that the *in vivo* rheology of aged RBCs is impaired, as indicated by decreases in oxygen delivery and increased vascular resistance, as well as increased morbidity and mortality in critical care patients [6, 29]. Due to the complex nature of *in vivo* RBC rheology, a discrepancy between in vitro measured parameters and in vivo function is not surprising. It is highly likely that the adverse changes in *in vivo* rheology observed when transfusing older blood units is not attributed to deformability alone, but rather a combination of deformability, endothelial adhesion, altered RBC aggregation, and nitric oxide scavenging by increased free hemoglobin

encapsulated in microvesicles [45, 46]. It is therefore difficult to deem subtle changes in any one of these factors biologically insignificant, as small changes in any parameter may be magnified by the synergistic interplay occurring *in vivo.* Perhaps the best way to elucidate which rheologic parameter has the greatest effect on transfusion efficacy would be the development of better *in vivo* models of circulation, as the measurement of individual variables *in vitro* may not be sufficient to explain current clinical observations.

3.5 Conclusion

Mounting evidence suggests that the transfusion of hypothermically stored RBC units leads to impaired oxygen delivery and increased vascular resistance *in vivo* [6]. In light of this, methods to effectively assess the hemorheological properties of stored RBCs *in vitro* are needed. By applying the use of ektacytometry and the Eadie-Hofstee linear data transformation investigated in Chapter 1, this chapter has demonstrated that significant decreases in RBC maximum deformability occur during hypothermic storage. Furthermore, this study has shown that the restoration of ATP levels via RBC rejuvenation does not lead to improvements in deformability, indicating that other mechanisms besides reductions in ATP are involved in RBC deformability losses. Due to the fact that the downstream mechanisms are most likely related to the abnormal functioning of the RBC membrane/cytoskeleton complex, it is suggested that preservation strategies focussing on the stabilization of the RBC membrane be investigated.

Table 3.1:RBC indices from receipt to week 7of hypothermic storage (* $p < 0.05$ using nonparametric methods, ǂ denotes $p < 0.05$ using parametric methods)

Storage Duration	MCV (fL)	MCH (pg)	MCHC (g/L)
Week 0	94 ± 1	30.1 ± 0.2	322 ± 3
Week 1	95 ± 1	34.1 ± 0.3 *ǂ	358 ± 3 *ǂ
Week 2	97 ± 1	30.8 ± 0.3	319 ± 1
Week 3	94 ± 1	33.1 ± 0.3 *ǂ	350 ± 4 *ǂ
Week 4	95 ± 1	29.8 ± 0.2	314 ± 2
Week 5	96 ± 1	30.5 ± 0.3	318 ± 2
Week 6	96 ± 1	30.3 ± 0.2	315 ± 2 *
Week 7	98 ± 1	29.1 ± 0.3ǂ	298 ± 3 *ǂ

Table 3.2: RBC indices of 45 day old CPD-SAGM units before and after rejuvenation with PIPA solution (* $p < 0.05$ using nonparametric methods, ǂ denotes $p < 0.05$ using parametric methods)

Indices	Pre-Rejuvenation	Post-Rejuvenation
MCV	97 ± 1	92 ± 1 *ǂ
MCH	31 ± 1	30 ± 1
MCHC	321 ± 3	331 ± 4 *ǂ

Figure 3.1: Percent hemolysis for RBCs stored hypothermically for 7 weeks. All values were below the acceptable limit of < 0.8% in Canada [10]. (* $p < 0.05$ using nonparametric methods, ǂ $p < 0.05$ using parametric methods)

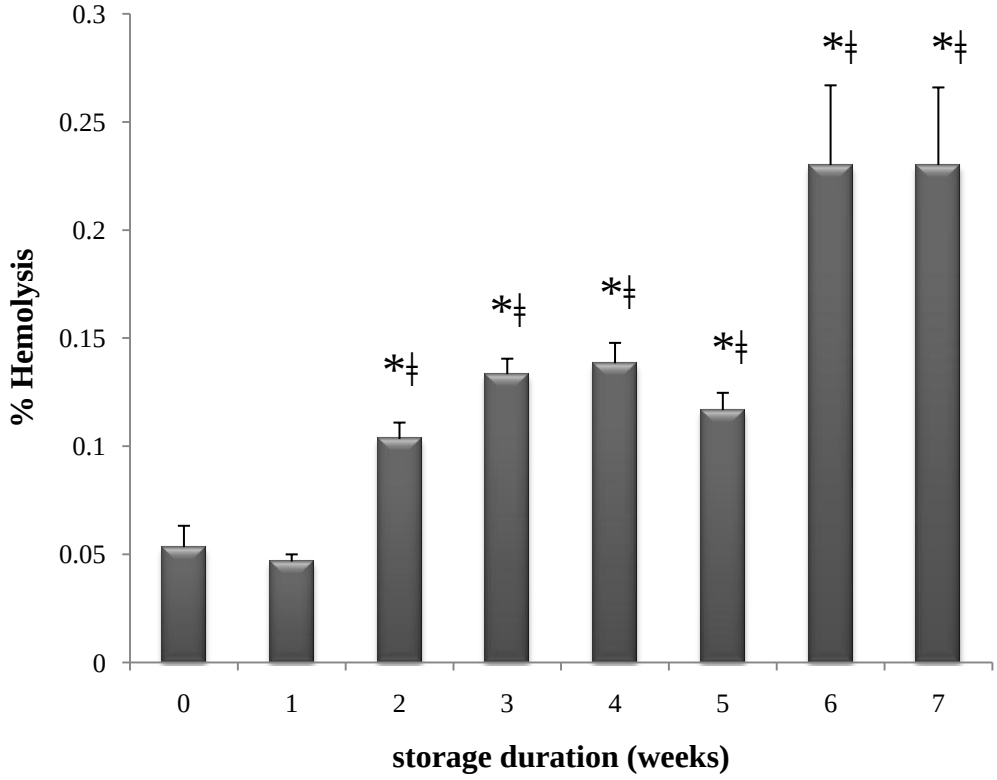


Figure 3.2: ATP levels for RBCs stored hypothermically for 7 weeks. Significant decreases in ATP levels were observed from week five onward. (* $p < 0.05$ using nonparametric methods, ǂ $p < 0.05$ using parametric methods)

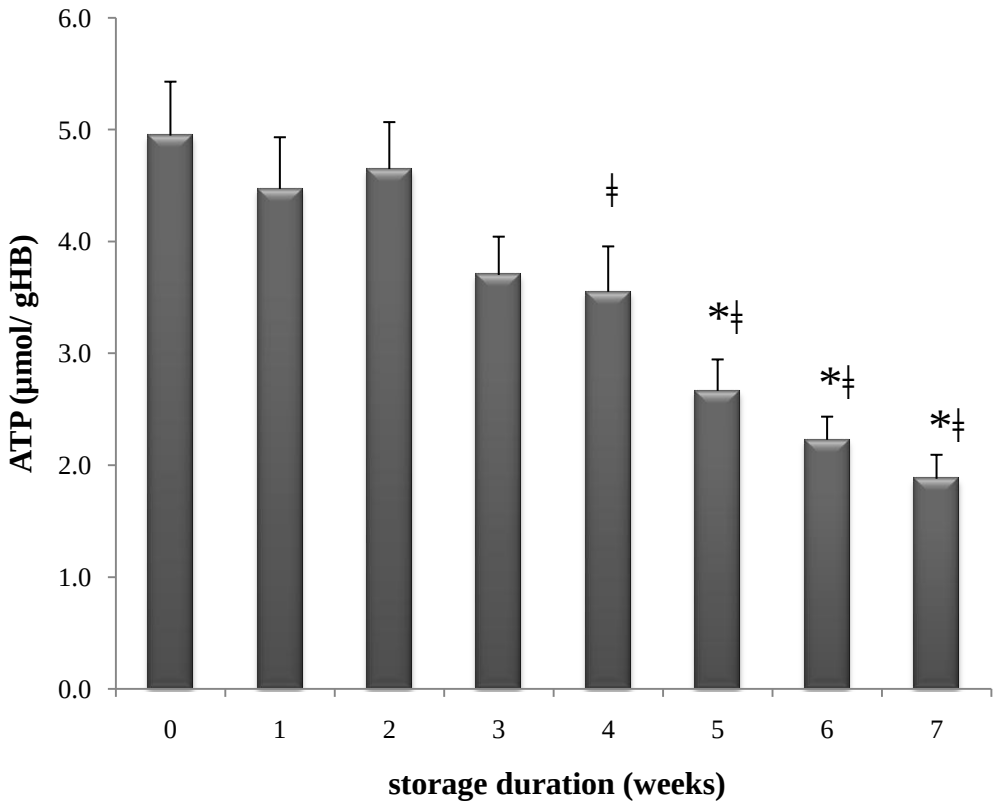


Figure 3.3: Eadie-Hofstee linearization of hypothermically stored RBC deformability curves for weeks 0, 2, 5, 6, and 7 of hypothermic storage at 1-6 ˚C.

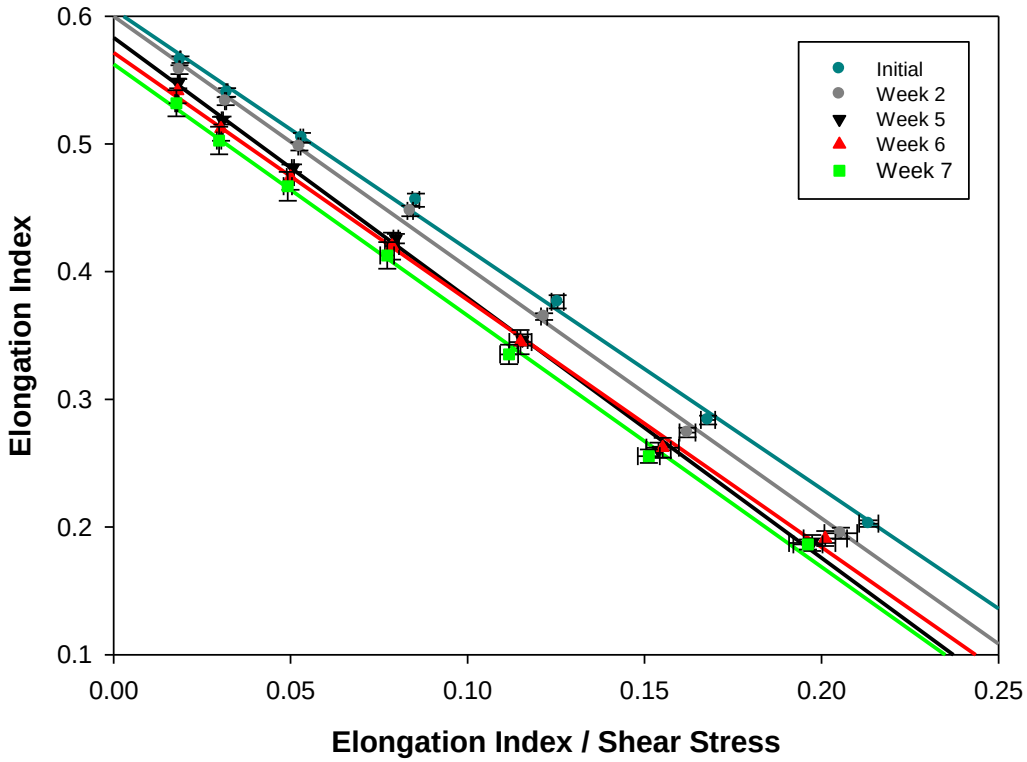


Figure 3.4: EI_{max} values for RBCs stored hypothermically for 7 weeks. Significant decreases in EI_{max} values were observed from week five onward . (* $p < 0.05$ using nonparametric methods, ǂ $p < 0.05$ using parametric methods)

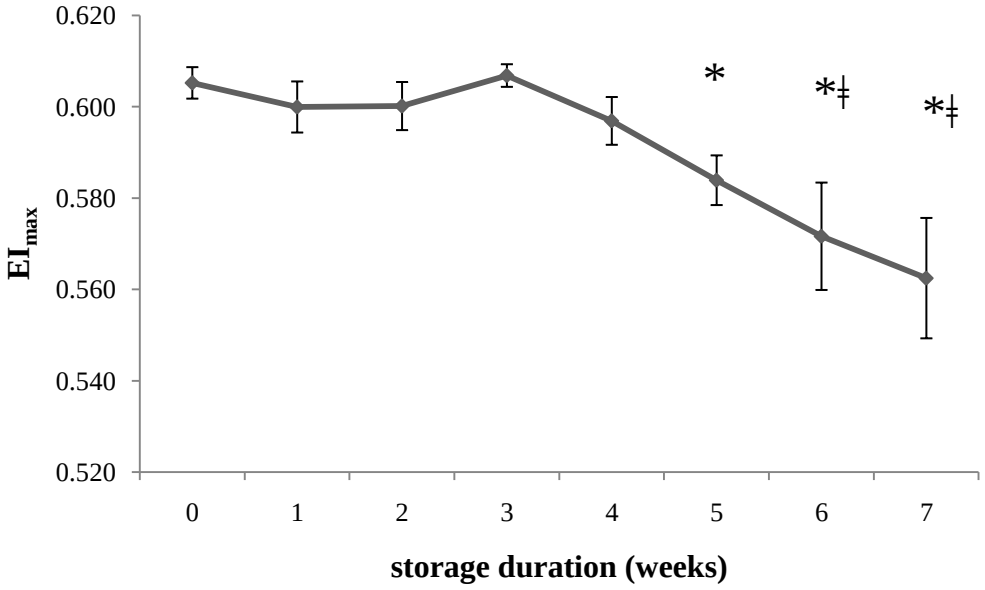


Figure 3.5: EI_{max} and ATP concentrations for RBCs stored hypothermically for 7 weeks.

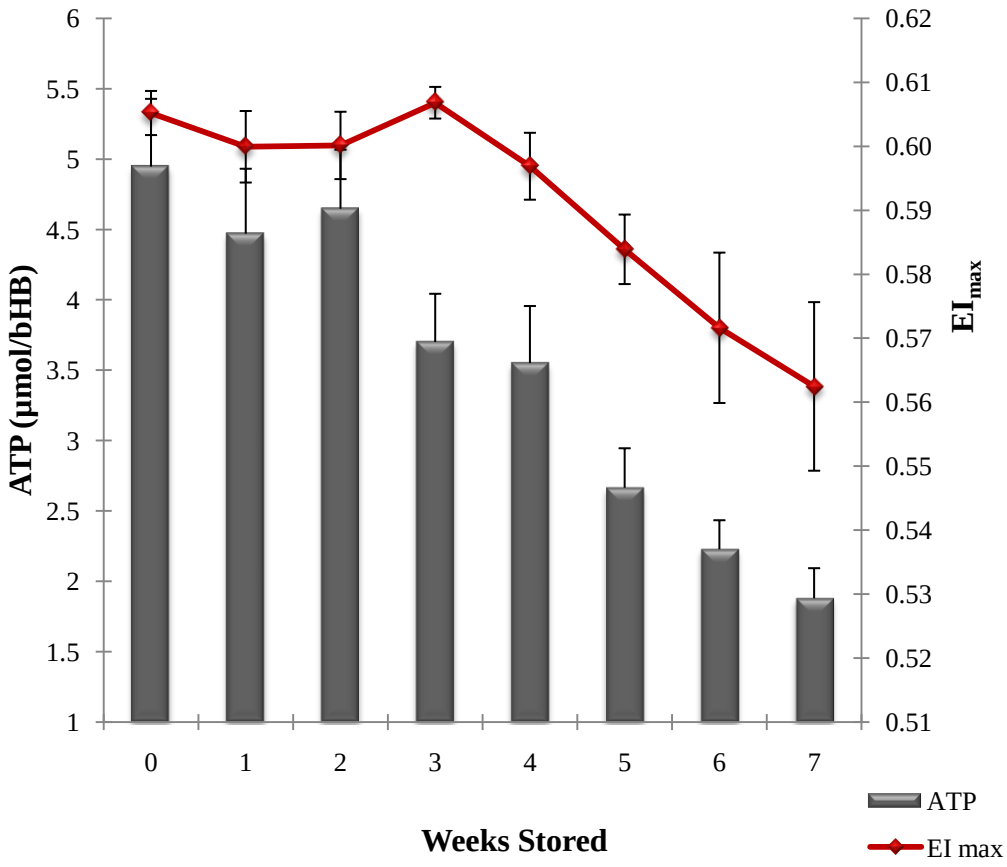


Figure 3.6: K_{EI} values for RBCs stored hypothermically for 7 weeks. No statistically significant changes in K_{EI} values were observed

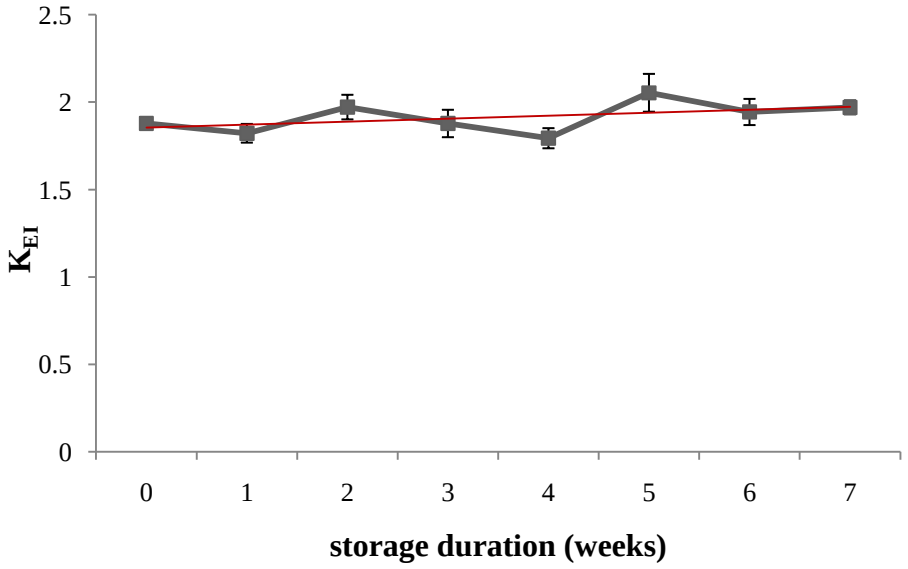


Figure 3.7 – ATP concentration of 45 day old CPD-SAGM RBC units before and after rejuvenation with PIPA solution. Significant increases in ATP concentration compared to non-rejuvenated RBCs were observed. (* $p < 0.05$ using nonparametric methods, ǂ $p < 0.05$ using parametric methods)

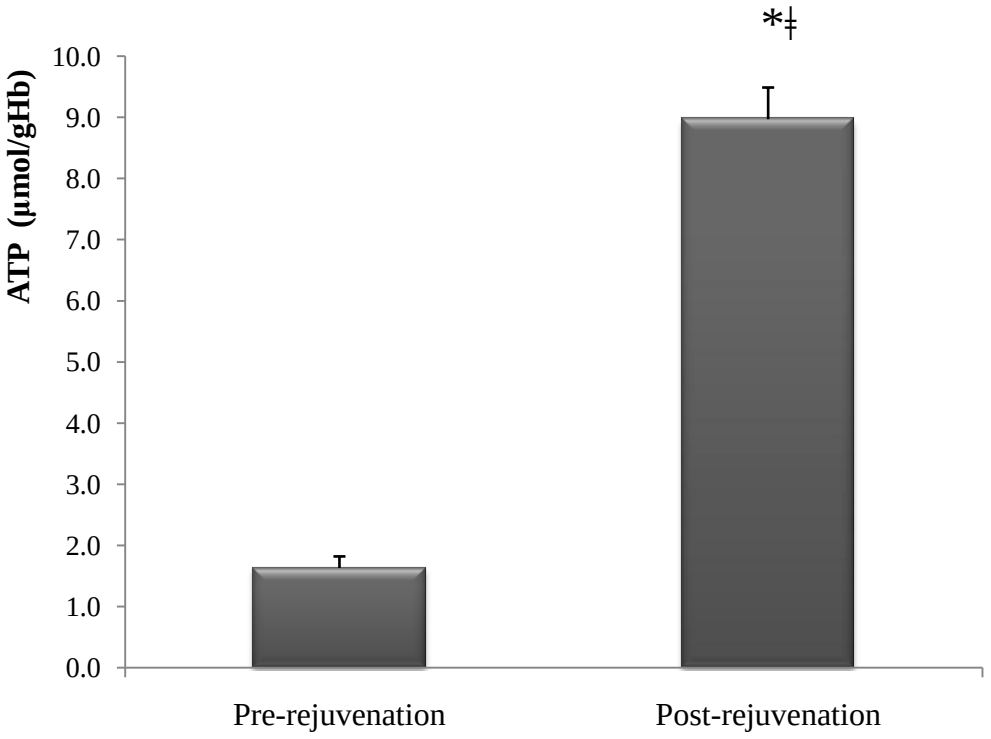
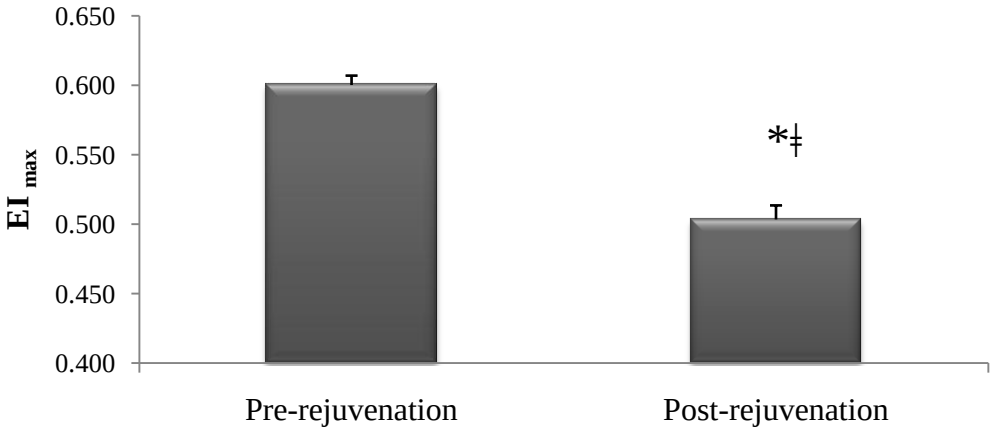
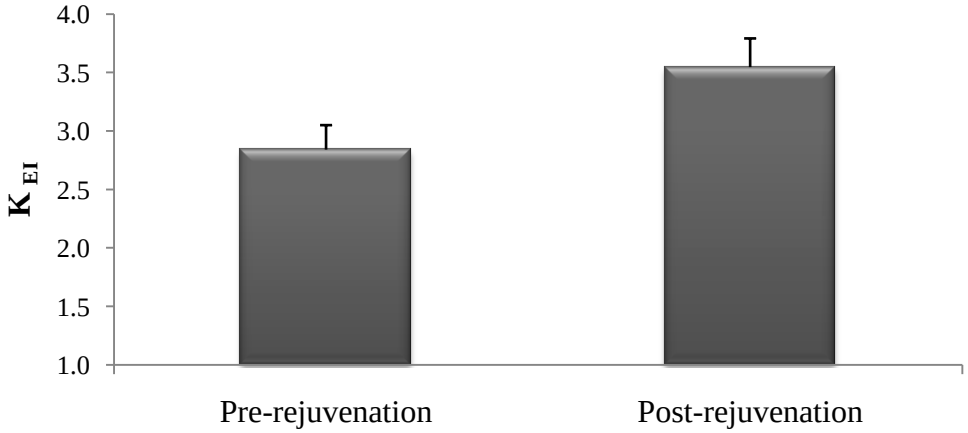


Figure 3.8 – Deformability of 45 day old CPD-SAGM RBC units before and after rejuvenation with PIPA solution (within 1 hour). Rejuvenation resulted in significant decreases in EI_{max} **A.** EI_{max} values **B.** K_{EI} values . (* $p < 0.05$ using nonparametric methods, ǂ $p < 0.05$ using parametric methods)

A.



B.



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Chapter 4

The effect of liposome treatment on the deformability of hypothermically stored red blood cells

4.1 Introduction

With the advent of modern preservative solutions and processing techniques, the shelf life of hypothermically stored RBCs destined for transfusion has been extended to 42 days in Canada [1,2]. Although preservative solutions have addressed some components of the RBC 'storage lesion' and have led to the improved overall quality of hypothermically stored RBCs, biochemical and biomechanical changes are still evident[1]. In addition, the transfusion of older RBC units has been associated with adverse clinical outcomes and increased morbidity and mortality in critically ill patients [3,4]. Despite the incremental advances that have occurred in the last three decades regarding our understanding of the RBC storage lesion and the development of new storage solutions, studies investigating more novel approaches to preserve RBC functionality during hypothermic storage are certainly warranted.

As mentioned in the previous chapters, many of the effects of hypothermic storage are manifested in the membrane of the cell. A distortion in the normal asymmetric distribution of lipids in the membrane is often observed, specifically the translocation of phosphatidyl serine from the inner to outer leaflet of the plasma membrane, which may lead to the premature removal of RBCs from the circulation once transfused[1]. In addition, microvesicles are shed from RBC membranes during storage, resulting in morphology changes as well as decreases in the surface area to volume ratio and the eventual build up of biologically active lipids and free hemoglobin in the storage supernatant [5]. The negatively charged

microvesicles are also known to have pro-inflammatory and pro-thrombotic activity, neither of which being desirable for transfusion [6]. The third chapter of this thesis demonstrated that routine hypothermic storage of RBCs also leads to significant decreases in maximum deformability prior to the current expiry date of 42 days in Canada. These changes in deformability were shown to be independent of ATP level decreases, suggesting that decreases in RBC deformability are related to deficits in normal membrane function. Although decreases in hypothermically stored RBC deformability have been reported previously, the clinical significance of this finding is still unknown; however, it has been associated with adverse clinical outcomes [4]. In light of all these findings, it is apparent that new preservation strategies focussing on the membrane component of the hypothermic storage lesion are necessary.

The use of liposomes may be a possible method to modify the plasma membrane of hypothermically stored RBCs. Liposomes can be defined as microscopic vesicles composed of an intact lipid membrane surrounding an aqueous core. Liposomes were originally used in research contexts as synthetic models for the study of the structural properties of membranes as well as membrane fluidity [7]. The synthetic nature of liposomes allows the researcher to prepare vesicles with specific membrane lipid and aqueous core compositions which has led to the use of liposomes in multiple disciplines. Fusion of liposomes with biological membranes allows for the transfer of intraliposomal components into the cytoplasm of target cells [8,9]. This gives the advantage of being able to transfer substances that are non-lipophilic across target membranes and has led to

the effective use of liposomes in the pharmaceutics industry as carriers of bioactive compounds for the treatment of infectious diseases, cancers, and arthritis [10-12]. In addition to disease therapies, liposomes have also been used in the biopreservation field. Numerous studies have utilized liposomes to study how sugars stabilize membranes during lyophilisation [13-15]. In addition, liposomes have been used as membrane models in cryobiology to study the protective mechanisms of permeating and non-permeating cryoprotectants, as well as being utilized as membrane protectants themselves for the cryopreservation of bovine and equine sperm [16-20]. More recently, Holovati *et al.* investigated the effect of trehalose loaded liposomes on the RBC membrane following cryopreservation [21]. The results of the study demonstrated that the treatment of RBCs with liposomes prior to cryopreservation resulted in increased membrane integrity and post thaw recovery. Interestingly, trehalose containing liposomes and saline containing liposomes both elicited the protective effects, suggesting that lipid delivery to the RBC membrane, rather than intracellular trehalose delivery, resulted in cryoprotection [21].Additionally, studies investigating RBC freeze drying have also demonstrated that the inclusion of liposomes in the lyophilisation buffer preserves RBC membrane integrity and reduces RBC hemolysis [22].

Although it has been established that the treatment of RBCs with liposomes results in improved membrane quality following cryopreservation and lyophilisation, there have been no studies investigating the effect of liposomes on the quality of hypothermically stored RBCs. In the current study, we investigated

if the membrane quality and cellular deformability of RBCs could be modified with liposome treatment prior to routine hypothermic storage. The effects of different liposome compositions were also investigated. Freshly isolated RBCs were treated with liposomes of varying lipid compositions (different lipid acyl chain lengths and degrees of saturation) and stored hypothermically for up to 42 days. Weekly assessments of RBC quality were performed including percent hemolysis, MCV, MCH, MCHC, RBC morphology, and RBC deformability analysis. The results of this study will be instrumental in determining if liposomal modification of RBC membranes is of any utility for the successful preservation of RBC membrane quality at hypothermic temperatures.

4.2 Materials and Methods

4.2.1 Liposome Preparation

Liposomes were synthesized in the same manner as Holovati *et al.*[7], with slight modifications. Dry 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was dissolved in chloroform (Alfa Aesar, Ward Hill, MA) and vigorously mixed with 1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and 1, 2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS) to a final molar ratio of 8:1:1 and a final total lipid concentration of approximately 25 mM. 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE), and 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (DMPS), as well as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-

glycero-3-phospho-L-serine (DOPS) were also prepared in an identical manner resulting in 8:1:1 molar ratios and final lipid concentrations of approximately 25 mM. All lipids stocks were purchased from Avanti Polar Lipids (Alabaster, AL) and with the exception of dry DPPC, all utilized lipids were obtained pre-dissolved in chloroform or chloroform: methanol mixtures. Multilamellar vesicles (MLVs) were produced by evaporating the chloroform: methanol solvents from the various lipid solutions under dry nitrogen stream in a fume hood. The product is a semi-dry lipid cake coating the inside surface of a glass 100 mL volumetric flask. To ensure the total evaporation of any residual solvent, lipid solution flasks were lyophilized for approximately 12 hours (VirTis advantage lyophilizer, Warminster, PA) and stored at -80°C until ready for hydration.

All dry lipid cakes were hydrated with filter sterilized HEPES-NaCl buffer (pH 7.4, 274 mOsm which was preheated to 65°C which is above the gel-liquid crystal transition temperature (Tm) of the utilized lipids. The hydration process consists of alternating cycles of vigorous vortexing and heating for 3 minutes each a total of 5 times. It is favourable to maintain the temperature of the lipid suspensions above the highest lipid Tm throughout this process. Following hydration, the MLV lipid suspensions were subjected to repeated freeze/thaw/vortex cycles. All suspensions were frozen in liquid nitrogen, thawed in a 65°C water bath, and vortexed for 3 minutes for a total of 5 cycles. This freeze/thaw protocol results in improved homogeneity of the lipid suspensions and an increased buffer entrapment within the MLV intralamellar spaces [23].

The MLV suspensions were then subjected to an extrusion protocol to obtain a homogenous population of large unilamellar vesicles (LUVs) in a manner similar to Holovati *et al.*[7] . Approximately 10 mL of the MLV suspension was introduced into the inlet valve of an extruder (Lipex Extruder, Northern Lipids, Vancouver, BC) and forced under nitrogen pressure (200 psi) through two polycarbonate filters (Nucleopore Polycarbonate, Whatman, Newton, MA) with a pore size of 200 nm. The extruder was kept at 65-70 °C throughout all extrusion steps, allowing the lipid suspensions to be pushed through the polycarbonate filters at a steady rate. Ten cycles of extrusion were performed for each liposome composition prepared. Prepared liposome solutions were stored under nitrogen gas to minimize any lipid peroxidation and maintained at 4°C until further use for a maximum of five weeks. For the remainder of this chapter, all liposome preparations will be referred to by the predominant lipid in each respective preparation (DPPC, DOPC, and DMPC).

4.2.2 Liposome Characterization

The prepared liposomes were characterized for both size and phosphate content.

4.2.2.1 Total Phosphate Content

Phosphoplipids consist of a polar head group linked to a glycerol-fatty acid chain backbone via a phosphorus molecule. By determining the phosphorus content of the prepared liposome suspensions, the total lipid concentration can be

calculated. This information is essential when determining liposome dose for RBC incubation. We determined total phosphate content spectrophotometrically via the Fiske – Subbarow colorimetric assay [24]. An aliquot of the liposome suspensions (25 µL) is treated with 0.7 mL of perchloric acid (Sigma-Aldrich, St. Louis, MO), resulting in the release of phosphorus from the phospholipid molecules and subsequent oxidation to inorganic phosphate when heated to 180 °C (1.5 hours) [149] .A 7 mL solution of ammonium molybdate solution (11 mM ammonium molybdate in a 1 M sulphuric acid solution, Sigma-Aldrich, St. Louis, MO) was then added to the assay tubes, converting the inorganic phosphate to phospho-molybdic acid[25]. Upon the addition of 0.9 mL Fiske-Subbarow reagent (1.4 M sodium bisulfite, 40 mM sodium sulphite, 10mM 4-Amino-3-hydroxy-1-naphthalenesulfonic acid, Sigma-Aldrich, St. Louis, MO) and subsequent boiling (20 min), a blue coloured phospho-molybdic acid complex is formed. The absorbance of this complex was measured at 815 nm on a spectrophotometer (Spectramax Plus, Molecular Devices, Sunnyvale, CA) and the phosphate concentration was calculated by comparing sample absorbencies to that of a phosphate standard curve (0, 25, 50,100,200,300 µL of NaH_2PO_4, Sigma-Aldrich, St. Louis, MO). The total lipid concentration of the liposome suspensions was calculated by the following equation:

$$total\ lipid\ concentration(mM) = \frac{C_{PO_4}}{V_S \times \%PO_4} \times 100 \qquad (4.1)$$

Where:

C_{PO_4} = phosphate content (nmol) from phosphate standard curve

V_S = volume of sample solution in μL

$\%PO_4$ = percent of phosphate in the sample

All doses for RBC incubations were determined via the total lipid concentration in each liposomes formulation sample.

4.2.2.2 Liposome Size

The mean diameter of the synthesized liposomes was determined using a Malvern 3000 HS Zetasizer (Malvern Instruments, Worcestershire, UK). This instrument operates on the principle of dynamic light scattering which measures the Brownian motion of particles in suspension and relates this to their size. Brownian motion can be defined as the random movement of particles in solution due to the constant bombardment of these particles by solvent molecules. Smaller particles move faster than larger particles, and the relationship between the size of a particle and its speed has been previously described by the Stokes-Einstein equation[26]. A small volume of liposome suspension (10 μL) was diluted in 1 mL HEPES-NaCl buffer and loaded into the instrument cuvette. A laser is then shone through the suspension resulting in a scattered light signal of a characteristic intensity which is constantly fluctuating due to Brownian motion of the sample. All scattered light was detected at an angle of 90˚. The change in signal intensity over time is then analyzed by the instrument software and a mean diameter size distribution is calculated and expressed as the Z-average size (nm). A polydispersity value (PDV) is also calculated which indicates the degree of size

variability in the analyzed sample. Polydispersity values of less than 0.1 indicate a sample with acceptably uniform size [27]. Polystyrene and silica sizing beads (200 and 400 nm) were used as controls for liposome size measurements (Bangs Laboratories Inc., Fishers, IN). †

4.2.3 RBC Collection

Fresh RBCs (30 mL) were collected from healthy volunteers (n=6) into 7 mL vacutainer tubes containing K_3-EDTA anticoagulant (BD, Franklin Lakes, NJ) using standard phlebotomy procedures. Following collection, whole blood was centrifuged at 1500 x g for 10 minutes at 4 ˚C (Eppendorf Centrifuge 5810R, Westbury, NY) and the supernatant and buffy coat were removed by vacuum aspiration. The RBC pellets were then washed with HEPES-NaCl buffer 3 times and all samples from each individual donor were pooled into a single tube. Following pooling, the RBCs were counted on a hematology analyzer (Beckman Coulter AcT, New York, NY) and the hematocrit was determined via microhematocrit centrifugation (Chapter 2, section 2.2.1). RBC dilutions for liposome experiments were based on hematocrit values. All RBCs were utilized for liposome experiments within 4 hours of collection.

4.2.4 Liposome Incubation with RBCs

† All size measurements of control sizing beads and liposome formulations were performed by Dr. Mitali Banerjee.

Each liposome formulation investigated was incubated with RBCs (12 mL, Hct ≈ 5 %) on a nutating mixer (VWR, West Chester, PA) for 1 hour at 37 ˚C. HEPES-NaCl buffer was used to dilute RBC samples and final concentrations of lipid were 2 mM for each respective liposome preparation. In addition, a small amount of SAGM was added to provide some nourishment for the RBCs. The ratio of SAGM to RBCs was similar to that of pRBCs units when hematocrit and sample volume were taken into account (275 μL of SAGM was added to 12 mL of RBCs with a hematocrit of 5%). RBC samples were subsequently stored hypothermically at 1 to 6 ˚C for up to 42 days and quality assessments were performed at one week intervals throughout hypothermic storage duration.

4.2.5 RBC Quality Assessment

4.2.5.1 Conventional RBC Quality Analysis

Conventional RBC quality was assessed by percent hemolysis, MCV, MCH, MCHC and RBC morphology. Percent hemolysis and RBC indices values were determined using the cyanmethhemoglobin method and a hematology cell analyzer (Beckman Coulter AcT, New York, NY) respectively. Both methods have been previously described in Chapter 3, section 3.2.2.

RBC morphology was assessed via light microscopy at 100x magnification following RBC staining with a Hema 3 staining kit (Fisher Diagnostics, Middleton, VA). RBCs are assigned a morphological score depending on the stage of transformation from biconcave disc to spherocyte

during progressive spheroechinocytosis [28]. Each cell type is assigned a factor and multiplied by the percentage of cells at this stage in a given sample. The 6 cell types and associated scores are as follows: smooth disc (1.0), crenated disc (0.8), crenated discoid (0.6), crenated spheroid (0.4), crenated sphere (0.2) and smooth sphere (0.0). 100 RBCs are scored and a morphology index is calculated by adding the products of each cell type. A score close to 100 would be indicative of RBCs with normal smooth disc morphology while lower scores indicate the extent of spheroechinocytosis.

4.2.5.2 RBC Deformability Assessment

RBC deformability was assessed as in Chapters 2 and 3 with subsequent Eadie-Hofstee Analysis (Chapter 2, Section 2.2.3). In summary, RBCs were suspended in PVP and analyzed with the LORRCA at shear stresses between 0.95 and 30 Pa. Following Eadie-Hofstee linearization, EI_{max} and K_{EI} values for liposome treated RBCS were calculated after each week of storage. RBC samples diluted and stored in HEPES-NaCl buffer and SAGM without liposome treatment served as controls. All EI_{max} and K_{EI} values are expressed as means ± SEM.

4.2.6 Statistical Analysis

The data were analyzed using commercial statistical software (SPSS Version 12.0, Lead Technologies, Charlotte, NC.) as well as Mynova (developed b y Dr. Stephen PJ Brooks, 1992). Nonparametric (Mann-Whitney U test) as well as parametric (One-way ANOVA with SNK post-hoc) statistical analysis was

performed to examine the differences in RBC *in vitro* parameters on a week to week basis. Unless specified, significant differences are expressed relative to age matched control RBC samples that are stored in HEPES-NaCl buffer and SAGM under the same conditions but without liposome incubation. Probabilities less than 0.05 were considered significant.

4.3 Results

The phosphate standard curves used to calculate the lipid content of the liposome preparations is depicted in figure 4.1 A and B. In batch one the concentration of DPPC, DOPC and DMPC liposomes was 18.37 ± 0.09, 24.18 ± 0.41, and 22.92 ± 0.11 mM respectively (Fig 4.1 A). The concentration of DPPC, DOPC, and DMPC liposomes in batch two was 26.80 ± 1.01, 27.94 ± 0.11, and 25.59 ± 1.58 mM respectively (Fig 4.1 B). In each case, the phosphate content of liposome preparations was measured in duplicate.

The average diameter of the liposome preparations (batch 1) are shown in table 4.1. The size distributions of all liposome preparations and polystyrene sizing beads were unimodal.

The percent hemolysis of RBCs increased in control and liposome treated samples as storage duration increased (Fig 4.2). RBCs treated with DMPC liposomes showed the most marked increase in percent hemolysis reaching a maximum of 53 ± 7 % after 42 days of hypothermic storage (Fig 4.2). At all time points, statistically significant increases in percent hemolysis were evident in DMPC treated RBCs. Although percent hemolysis in DOPC treated samples

remained similar to control values for the first 14 days of hypothermic storage, statistically significant increases were observed from day 21 of storage onward using nonparmetric methods ($p = 0.03$) and day 35 using parametric methods ($p < 0.05$)(Fig 4.2). By day 42, the percent hemolysis in DOPC treated samples was comparable to DMPC samples (49 ± 7 %). In contrast to DMPC and DOPC treated samples, DPPC treated sample showed percent hemolysis values similar to that of control RBCs (Fig 4.2). Although there were no statistically significant decreases observed, percent hemolysis in DPPC treated RBCs tended to be slightly lower than untreated control RBCs from day 21 of hypothermic storage onward. DPPC treated RBCs exhibited significantly less hemolysis compared to DMPC treated samples at all time points and significantly less hemolysis compared to DOPC treated samples from day 21 onward (Fig 4.2). By 42 days of storage, hemolysis in DPPC treated samples reached 12 ± 1 % compared to 16 ± 1 % in control samples.

MCV tended to increase during storage duration in both untreated control and liposome treated samples (Fig 4.3). At all sample points, DMPC treated RBCs had higher MCV values compared to untreated controls and DPPC/DOPC treated RBCs. This increase was statistically significant at all time points using parametric methods ($p < 0.05$) and 14 days of hypothermic storage using nonparametric methods ($p = 0.04$). There was no statistically significant difference in MCV observed between DPPC and DOPC treated samples or between DPPC / DOPC samples and untreated controls (Fig 4.3). MCH values of DMPC treated RBCs also tended to be higher than control and DPPC treated

RBCs throughout storage, however this was only statistically significant at 21 days of storage (nonparametric $p = 0.02$) (Fig 4.4). By the last two weeks of storage, DOPC MCH values approached that of DMPC treated RBCs, however DPPC treated and control RBCs remained very similar throughout storage and within standard reference ranges (Fig 4.4) ([9]. MCHC values tended to decrease in all samples for the first 35 days of storage followed by a subsequent rise in values on day 42, specifically in DOPC and DMPC treated cells (Fig 4.5). Due to the high variability in the samples, significant differences between liposomes treated and control samples were not observed.

Due to the low hematocrit and presence of liposomes in the storage buffer, easily readable blood smears were difficult to obtain. Due to this, RBC morphology scores were only computed for the first set of liposome incubation experiments ($n = 3$). Statistical analysis was not performed due to the small sample size. RBC morphology scores tended to decrease over storage duration for both untreated controls and liposome treated samples (Figure 4.6 and 4.7). DPPC treated RBCs tended to have higher scores than untreated controls and DOPC/DMPC treated RBCs from day seven onward (Fig 4.6). RBC ghosts present in DOPC and DMPC samples due to the high percent hemolysis values were scored as zeros, however in some cases, ghosts were not always visible (Figures 4.7). Due to this, morphology index values for DOPC and DMPC treated samples are likely inflated.

RBC deformability deteriorated throughout storage duration for both untreated controls and liposome treated samples (Fig 4.8 and 4.9). A downward

trend in EI_{max} was observed in all liposome treated samples as well as untreated controls (Fig 4.8). Statistically significant decreases in EI_{max} became evident in DMPC treated RBCs after 14 days of hypothermic storage ($p < 0.001$ using both statistical methods) and 21 days of storage in DOPC treated RBCs ($p < 0.05$ using both statistical methods). The decreases in EI_{max} observed in DOPC/DMPC treated samples remained statistically significant for the duration of storage (Fig 4.8). In contrast to DOPC/DMPC treated RBCs, DPPC treatment did not result in any significant differences compared to untreated control RBCs (Fig 4.8). Two separate trends are visible in the K_{EI} values illustrated in figure 4.9. Following 7 days of hypothermic storage, an increase in K_{EI} was evident in all liposome treated and untreated control samples. Subsequent to this, K_{EI} values in DPPC treated RBCs and untreated controls increased through duration (Fig 4.9). In contrast, K_{EI} values of DMPC and DOPC treated RBCS dropped following 7 and 14 days of hypothermic storage respectively (Fig 4.9). K_{EI} values of these two samples continued to decrease until day 28 where the K_{EI} values begin to increase for the last two weeks of hypothermic storage. Despite the visible trends, no statistically significant difference was detected in K_{EI} values between liposome treated RBCs and untreated controls due to the large variation in calculated K_{EI} values.

4.4 Discussion

Although liposomes have been previously used as membrane stabilizers in freeze-dried and cryopreserved RBCs, this is the first study investigating the

effect of liposomes on RBCs stored hypothermically [21,22]. Liposome treatment of RBCs prior to hypothermic storage resulted in contrasting effects on RBC quality depending on liposome composition. DOPC and DMPC liposome treatment resulted in more drastic changes to RBC quality compared to DPPC liposomes, including greater percent hemolysis values and decreases in maximum deformability. In contrast, RBCs treated with DPPC liposomes showed slightly less hemolysis than untreated controls (not statistically significant) and retained deformation parameters that were similar in magnitude to that of untreated control RBCs after 42 days of hypothermic storage.

Previous studies have demonstrated that the type of interaction occurring between liposomes and target cell membranes is dependent upon a combination factors including the liposome lipid composition, properties of the target cell membrane, as well as liposome incubation conditions [25, 30-32]. Three basic types of liposome interaction with target cells have been described: adsorption to the cell membrane (which may or may not be accompanied by lipid/cholesterol transfer between the liposome and the target membrane), fusion with the cell membrane resulting in the delivery of intraliposomal contents, or endocytosis of the liposome by the target cell [32-34]. Due to the fact that mature RBCs are incapable of endocytosis, this mode of interaction is highly unlikely and will not be considered further [29]. It is known that membrane fluidity during liposome incubation greatly affects the strength of liposome interaction, with stronger interaction types (fusion vs. adsorption vs. no interaction) being more probable when incubation temperatures are above the T_m of both the liposomes and the

target cell membrane [30]. Therefore, liposome composition is arguably the dominant factor determining which type of interaction occurs as the liposomal lipid composition determines the T_m of a given liposome formulation. Due to these facts, it is logical to expect liposomes which are in the fluid phase during incubation to elicit stronger effects on target cells than liposomes which are in the gel phase. In the current study, RBC incubation took place at 37 ˚C for all liposome compositions investigated. The T_m of the predominant lipid species in DPPC, DMPC, and DOPC liposome formulations was 41, 23, and -20 ˚C respectively. The more pronounced effects of DOPC and DMPC liposomes on RBCs in the current study are therefore understandable, as both liposome formulations have T_m values that are below the incubation temperature currently employed and are therefore more likely to strongly interact with RBCs. In contrast, the minimal effects elicited by DPPC liposomes seem logical due to the fact these liposomes are most certainly in the gel phase during RBC incubation, likely resulting in minimal interaction with RBCs. Although the assays performed in this study do not permit the unequivocal determination of the exact type of liposome RBC interaction occurring, the T_m values of DOPC and DMPC liposomes suggest that at the incubation conditions investigated in this study, liposome adsorption and/or fusion are likely to occur.

Due to the fact that the RBCs in this study are diluted and stored suboptimally in HEPES-NaCl buffer, it is not surprising that hemolysis values as a whole are much higher than one would typically observe [35]. Typically, the hemolysis values of RBC units stored with additive solutions in blood banks are

less than 0.2 to 0.4 % following 42 days of hypothermic storage [35]. From a hemolysis standpoint, the advantage of storing RBCs under suboptimal conditions is that this allowed us to more easily detect any changes that liposome treatment had on RBC membrane quality. Although it is likely that lipid was transferred to the RBCs by interaction with DOPC and DMPC liposomes, it is clear that this interaction did not stabilize the membrane as indicated by the extremely high percent hemolysis values in figure 4.2. It has been previously reported that the depletion of RBC membrane cholesterol with the use of cyclodextrins induces RBC hemolysis, likely by removal of associated membrane components [36,37]. In addition, it has been shown that adsorption or hemifusion of liposomes often results in the depletion of membrane cholesterol in target cells, and that this process is relatively fast [38,39]. Stoll *et al.* recently demonstrated that following interaction with RBCs and subsequent isolation via Ficoll density gradient separation, cholesterol was detectable in the isolated liposome fraction [40]. It is therefore logical to deduce that treatment with DMPC and DOPC liposomes in the current study resulted in an extraction of RBC membrane cholesterol and the subsequent disruption of membrane components, leading to eventual membrane failure. The apparent protective effect of DPPC liposomes, although not statistically significant, is more difficult to explain. The study by Stoll *et al*. demonstrated that DPPC liposomes interact very little with RBCs, therefore membrane protection via lipid transfer is unlikely [40]. However, since the liposomes in this study were not removed from the storage buffer following incubation, it is possible that DPPC liposomes may have had a coating effect on

the RBC membranes, resulting in some sort of membrane protection. More research is required to establish if DPPC liposomes are indeed protective against hemolysis during hypothermic storage.

Minimal effects on RBC MCV were observed in this study, as shown in figure 4.3. The gradual increase in MCV observed in all samples is a normal response as decreasing ATP level during storage eventually lead to the shutdown of membrane ion pumps (Na^+/K^+ ATPase and Ca^{++} ATPase) which control the electrochemical gradient across the RBC membrane [41]. This results in the characteristic influx of water and cell swelling following storage for extended periods of time. The significantly higher MCV levels in DMPC treated RBCs may be related to the higher levels of hemolysis observed, as the membrane health of these samples was clearly compromised. The increased MCH values observed in DMPC treated cells throughout storage and DOPC treated cells in the final two weeks of storage are likely inflated due to high hemolysis values. The MCH is calculated in a hematology analyzer by dividing the sample hemoglobin content by the RBC count [29]. However, the total hemoglobin measurement is indicative of all the hemoglobin in the sample, not just the hemoglobin contained within the RBCs. Therefore, we would expect MCH values to be elevated in samples where hemolysis is elevated. Elevated MCHC in DMPC and DOPC treated RBCs at various time points are also probably related to the high hemolysis in the samples. MCHC is calculated by dividing the hemoglobin in a sample by the hematocrit [29]. This hematocrit value is calculated by multiplying the MCV by the RBC count, therefore in samples with a high degree of hemolysis, this value will be

diminished, thus resulting in inflated MCHC values. Due to these arguments, the use of RBC indices to help describe the effect of liposome treatment on RBCs is of limited value.

In addition to having slightly less hemolysis compared to untreated controls, DPPC treated RBCs also had greater morphology index scores for much of the storage duration (Fig 4.4). However, this observation was not confirmed statistically, as only 3 donors were scored for RBC morphology due to difficulties in preparing quality blood smears from samples with such low hematocrits. As previously stated in the results section, there is likely inflation in the morphology scores of DOPC and DMPC treated samples, as the degree of hemolysis present often resulted in the lack of observable RBCs available for morphology analysis. In DOPC treated samples (Fig 4.5C), RBC ghosts are clearly evident, thus visually confirming the high hemolysis values measured. The apparent improvement in morphology scores for DPPC treated cells is once again surprising considering this liposome composition has been shown to interact the least with RBCs [40]. The progressive change from biconcave disc to echinocyte to spherocyte that is observed in hypothermically stored RBCs is due mainly to the loss of membrane in the form of microvesicles [5]. This is thought to occur due to falling ATP levels resulting in a perturbation of the dynamic interactions between the membrane and underlying cytoskeleton. The low hematocrits used in this study prevented us from calculating ATP values throughout storage with any confidence; however, the drop in ATP normally associated with hypothermic storage is demonstrated in chapter 3. As mentioned previously, a coating effect of

DPPC liposomes on the RBCs without any direct interaction or lipid transfer may inhibit portions of the membrane from blebbing into microvesicles, thus limiting the development of echinocytic spicules. This would in turn lead to higher morphology scores. However, further studies measuring the degree of microvesiculation after DPPC liposome treatment are need. In addition, it would be beneficial to visualize the DPPC treated RBCs via electron microscopy to confirm if DPPC vesicles are indeed coating the RBCs throughout the storage period.

Although the effect of liposomes on numerous RBC quality indicators has been previously investigated for cryopreserved RBCs, the deformability of liposome treated RBCs has not yet been investigated [21]. Chapter 2 of this thesis demonstrated that *in vitro* modifications to the RBC membrane and cytoskeleton do in fact lead to detectable changes in RBC deformability; therefore, we would expect that membrane modifications induced by liposome interaction would be manifested by alterations in measured deformability parameters as well [42]. The results of this study demonstrate that the treatment and subsequent storage of RBCs with both DOPC and DMPC liposomes results in significant decreases in EI_{max} values, while DPPC treatment results in nearly no EI_{max} changes compared to control RBCs (Fig 4.8). Considering that DOPC and DMPC liposome preparations are likely to result in both lipid transfer to RBCs and cholesterol extraction, these findings are not unexpected. Changes in EI_{max} are said to reflect alterations in the ultrastructure of the membrane or cytoskeleton matrix, rather than just minor changes in RBC membrane rigidity [43,44]. Although lipid

delivery to the RBC membrane could be manifested by gross changes in EI_{max} values, it is once again more likely that cholesterol extraction from the membrane is occurring resulting in a disruption of membrane/cytoskeleton components. Numerous studies have investigated the effect of modifying the cholesterol content in RBC membranes [36-38,45]. Ohtani *et al.* demonstrated that the treatment of RBCs with cyclodextrins and subsequent extraction of cholesterol from the RBC membrane resulted in a discoid to spherocyte morphology transformation [37].Due to the decreases in the surface area to volume ratio associated with this morphology change, a decrease in maximal deformability is highly probable. In addition, chapter two of this thesis demonstrated that the extraction of cholesterol from RBCs with MβCD resulted in dose dependant decreases in RBC EI_{max} values[44]. Furthermore, the EI_{max} values obtained in the current chapter following treatment with DMPC liposomes were comparable to those obtained in chapter 2 following RBC treatment with 10 mM MβCD[44]. The idea that cholesterol depletion is leading to the observed changes in RBC deformability is also supported by the K_{EI} values in figure 4.9. If cholesterol depletion is occurring during liposome incubation and subsequent storage, we would expect an increase in membrane fluidity as the phospholipids in the membrane will be less tightly packed [46]. This was shown to be the case when treating RBCs with higher concentrations of MβCD in chapter two[44]. This increase in membrane fluidity is likely to result in less rigid membranes, which should be reflected in the K_{EI} values. DPPC treated RBCs retained K_{EI} values similar to that of untreated controls throughout storage duration, which is

consistent with findings suggesting that DPPC liposome interact weakly with RBCs (Fig 4.9)[40]. K_{EI} values for DMPC and DOPC treated RBCs exhibit an interesting, albeit non-statistically significant, decrease in K_{EI} values compared to that of both DPPC treated and control RBCs for much of the storage duration. The extraction of cholesterol from RBC membranes is a logical explanation for this phenomenon.

Despite the apparent negative effects of DOPC and DMPC liposome preparations on the membrane quality of hypothermically stored RBCs, the results of this study are still encouraging. If the depletion of membrane cholesterol is indeed the main factor contributing to RBC hemolysis and deformability deficiencies, it is likely this effect can be modified by adjusting the composition of the liposomes[31]. Stoll *et al.* demonstrated that inclusion of cholesterol into DLPC liposomes which were subsequently incubated with RBCs resulted in little depletion of RBC cholesterol [40]. Therefore, if we can identify an appropriate ratio of cholesterol to lipid in prepared liposomes, it would be theoretically possible deliver lipid to the RBC membrane without depleting RBC cholesterol to levels that result in membrane failure. By this rationale, it would then be possible to enhance RBC membrane fluidity via the delivery of lipids that are in the fluid phase at low temperatures. If the membrane fluidity of RBCs can be enhanced via liposome treatment without causing destabilization of the membrane, it may then be possible to decrease the membrane rigidity of hypothermically stored RBCs resulting in the maintenance of RBC deformability during storage. More research is required to optimize the liposome composition that will elicit this effect.

4.5 Conclusion

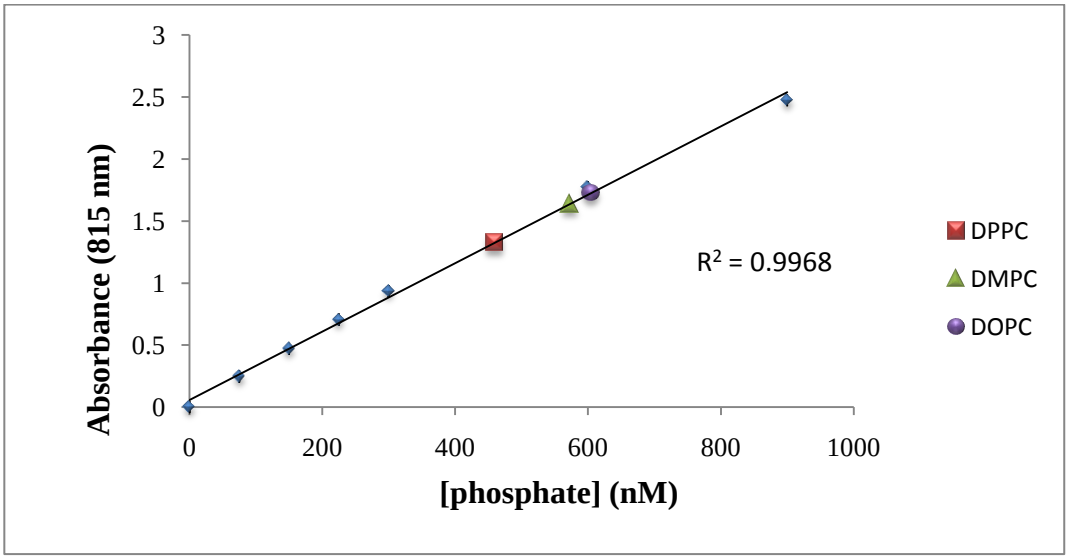
The ability to systematically modify the membranes of RBCs could lead to future improvements in the quality of blood destined for transfusion. The results of this study demonstrate that liposomes have the capacity to alter the membrane characteristics and specifically, the deformability of hypothermically stored RBCs differentially depending on the liposome lipid composition. DOPC and DMPC liposome treatment resulted in the greatest effect upon RBC membranes, while DPPC liposomes showed minimal interaction. We have also suggested that the destabilizing effects on the RBC membrane exerted by DOPC and DMPC are likely related to the transfer of cholesterol to adhered liposomes rather than via lipid transfer. The apparent protective effects elicited by DPPC liposome treatment require further investigation as no obvious membrane stabilization mechanism is evident. With future investigations into the optimal liposome lipid compositions for membrane stabilization of stored RBCs, the development of effective strategies to combat the membrane component of the RBC hypothermic storage lesion may be achievable.

Table 4.1: Sizes of polystyrene beads and batch 1 of prepared liposome formulations. All size distributions were unimodal.

Sample	Z-average size (nm)	Polydispersity value (PDV)
200 nm sizing beads	198	0.04
400 nm sizing beads	386	0.08
DPPC liposomes	153	0.04
DOPC liposomes	148	0.08
DMPC liposomes	152	0.06

Figure 4.1: Standard phosphate curves used to calculate the total lipid content of the extruded liposome formulations. Figures A and B are liposome batches 1 and 2 respectively

A.



B.

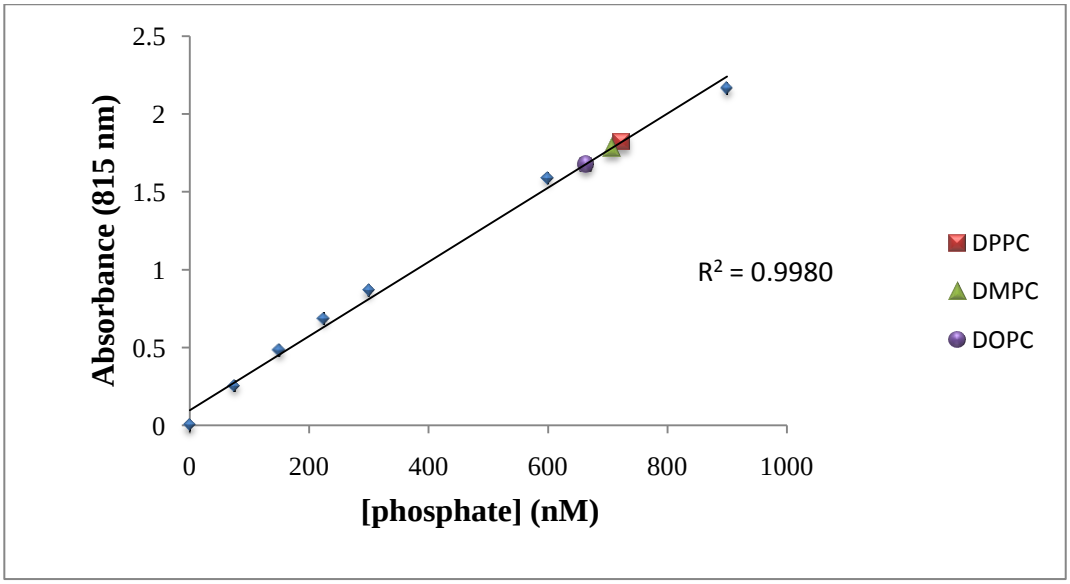


Figure 4.2: Percent hemolysis of liposome treated RBCs (DPPC, DOPC, and DMPC formulations) following hypothermic storage for up to 7 weeks . (* $p < 0.05$ using nonparametric methods, ǂ $p < 0.05$ using parametric methods)

.

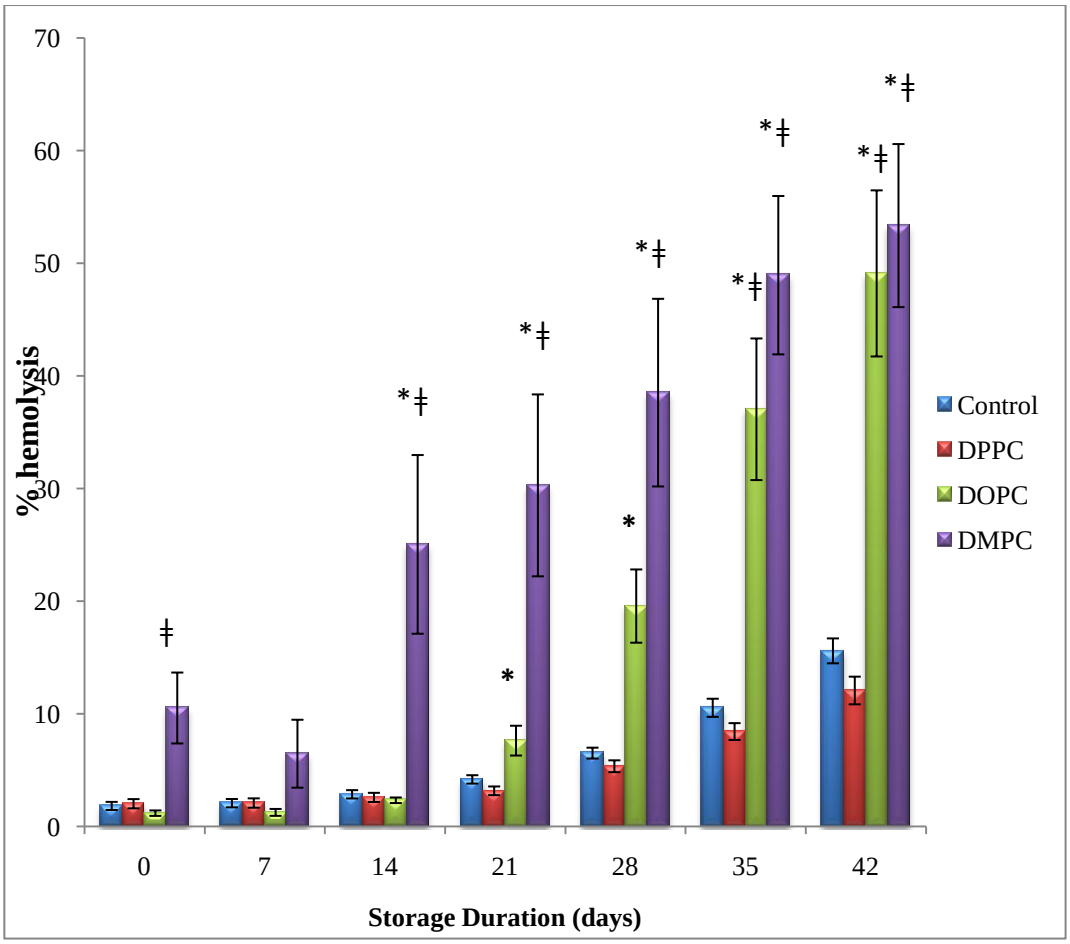


Figure 4.3: MCV of RBCs following liposome treatment and subsequent hypothermic storage for up to 7 weeks . (* $p < 0.05$ using nonparametric methods, ǂ $p < 0.05$ using parametric methods)

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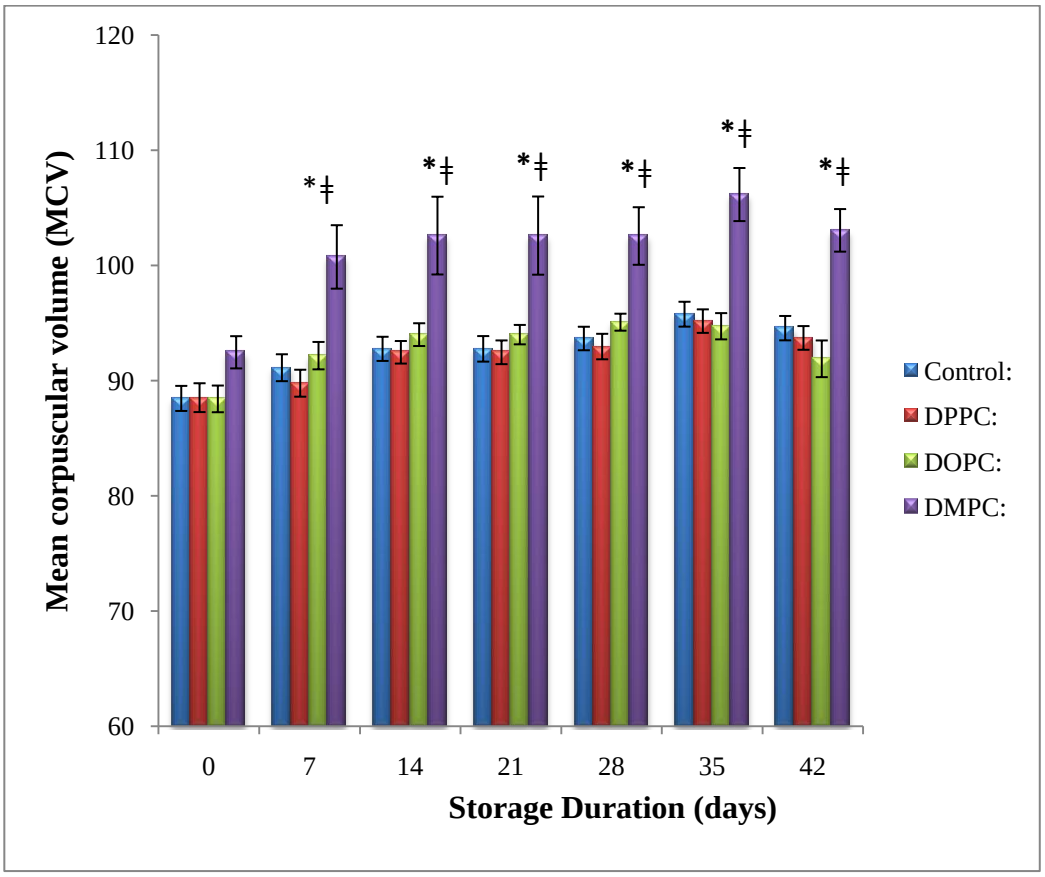


Figure 4.4: MCH of RBCs following liposome treatment and subsequent hypothermic storage for up to 7 weeks . (* $p < 0.05$ using nonparametric methods, ǂ $p < 0.05$ using parametric methods)

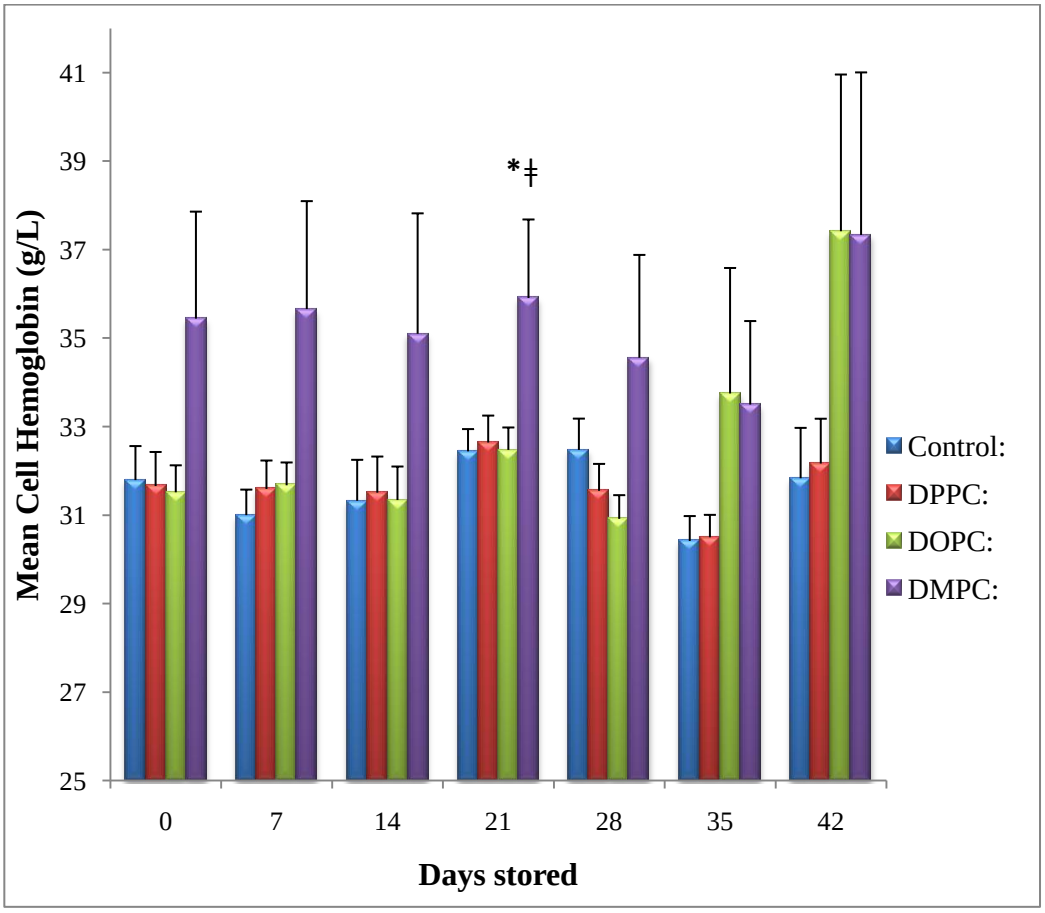


Figure 4.5: MCHC of RBCs following liposome treatment and subsequent hypothermic storage for up to 7 weeks. (* $p < 0.05$ using nonparametric methods, ǂ $p < 0.05$ using parametric methods)

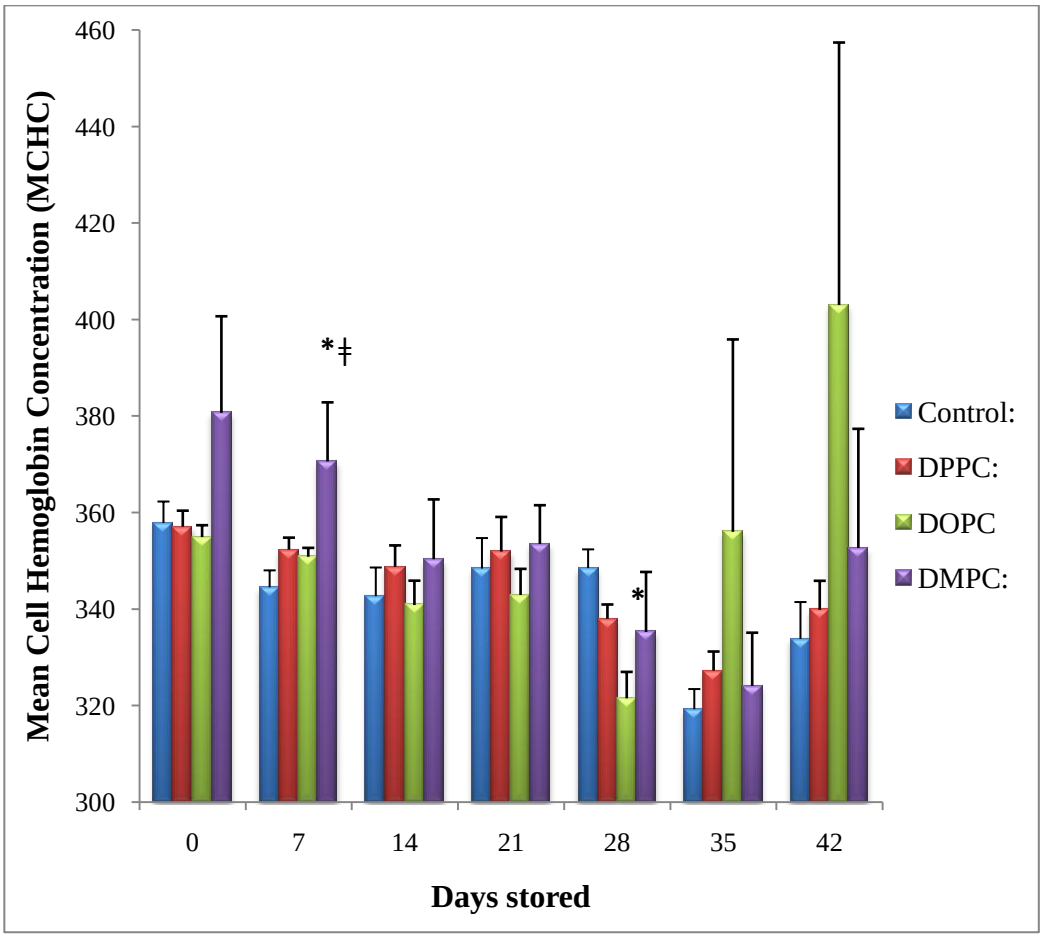


Figure 4.6: Morphology Index of RBCs treated with liposomes and stored hypothermically for up to 7 weeks (n=3).

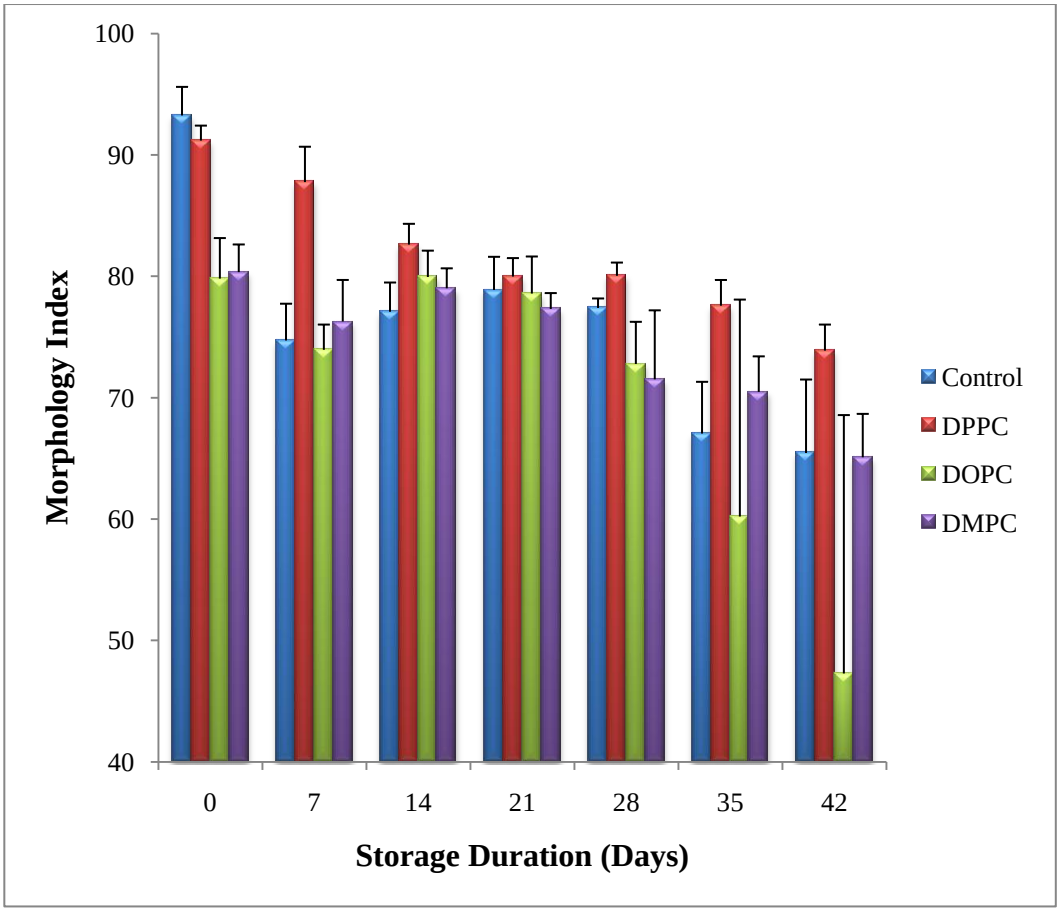
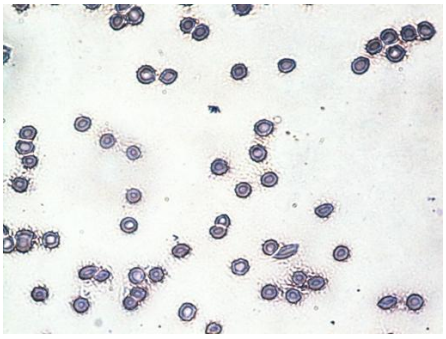
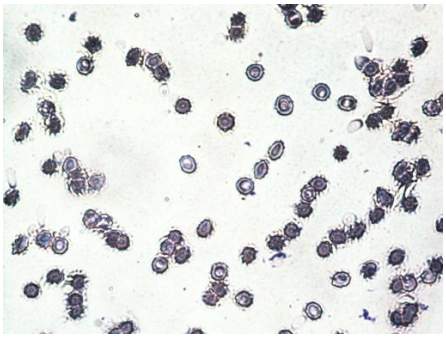


Figure 4.7 RBC morphology of RBC treated with liposomes after 42 days of storage. (A: Control, B: DPPC treated, C: DOPC treated)

A.



B.



C.

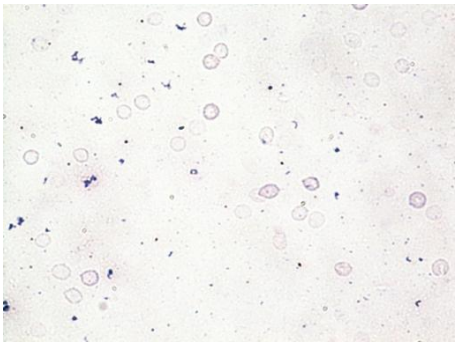


Figure 4.8: EI_{max} of RBCs following liposome treatment and subsequent hypothermic storage for up to 7 weeks . (* $p < 0.05$ using nonparametric methods, ǂ $p < 0.05$ using parametric methods)

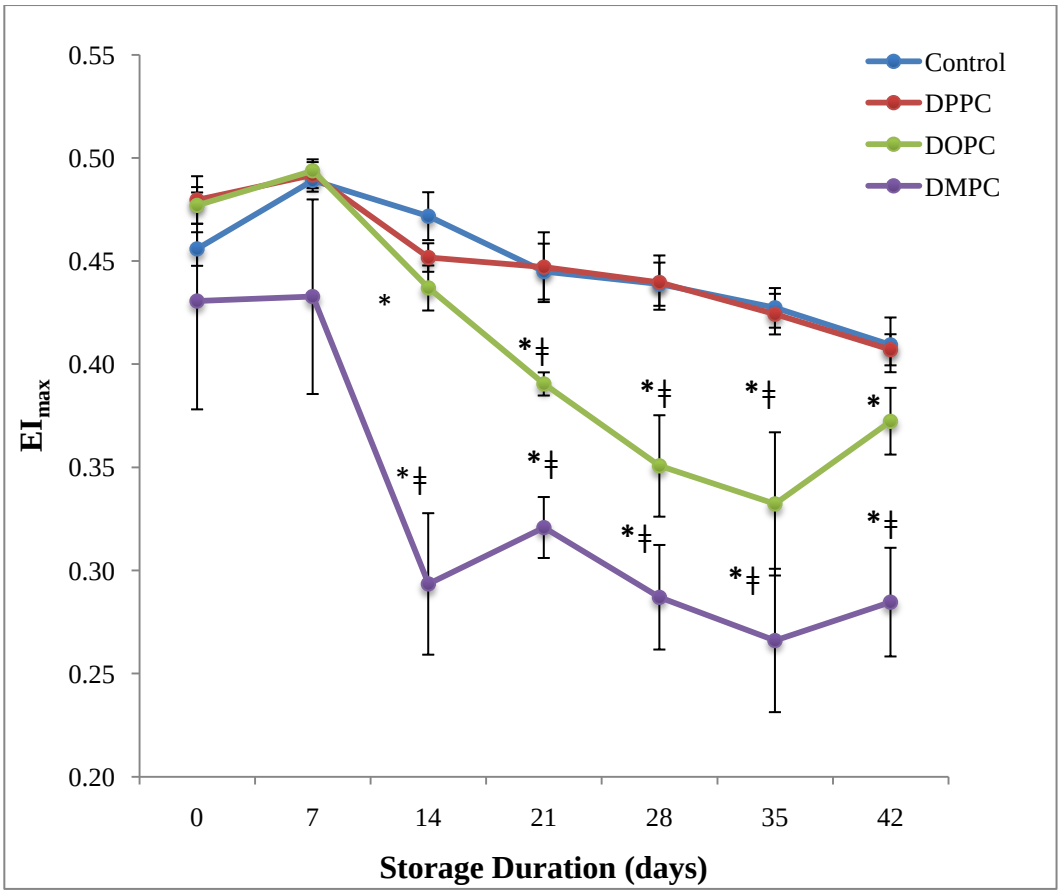
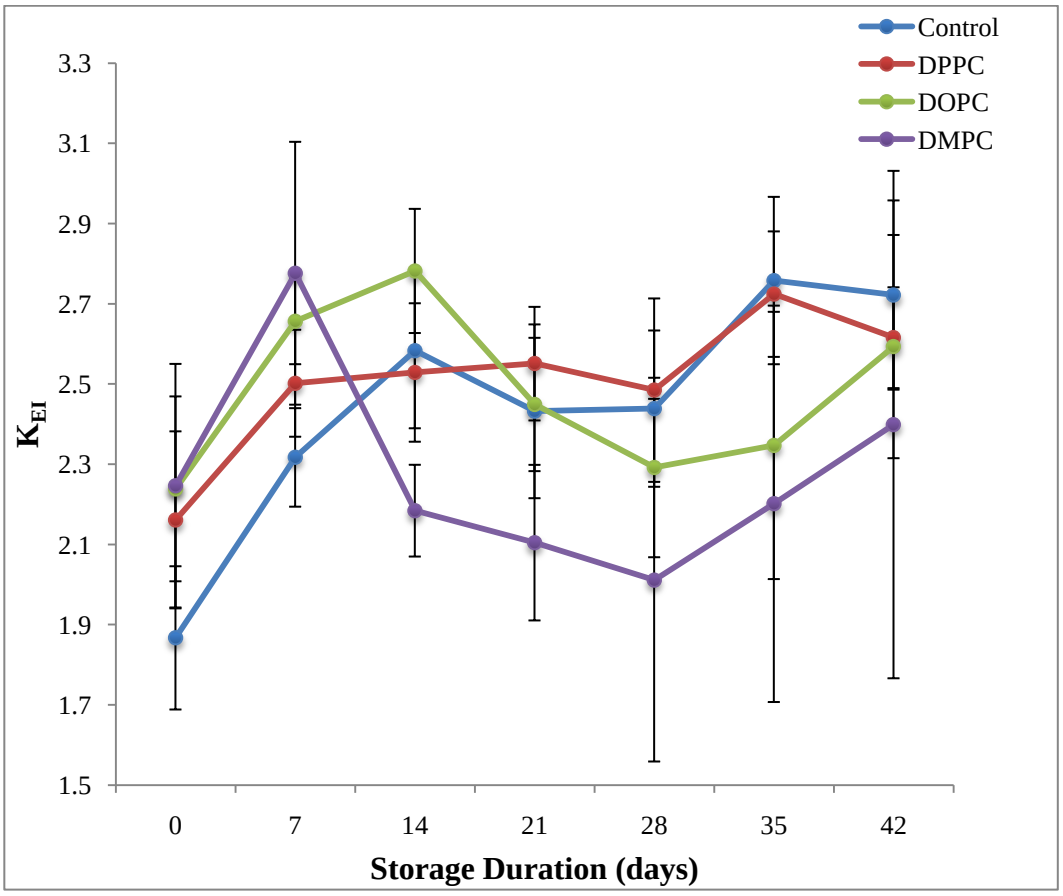


Figure 4.9: K_{EI} of RBCs following liposome treatment and subsequent hypothermic storage for up to 7 weeks.



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Chapter 5

General Discussion and Conclusions

5.1 Review of Thesis Objectives

The current practice of transfusion medicine is heavily dependent on the ability to preserve the structure and function of RBCs at hypothermic temperatures for extended periods of time. This ability has been greatly enhanced in the last few decades with the advent of modern RBC processing techniques and preservative solutions; however, significant changes in the biochemical and biomechanical properties of hypothermically stored RBCs are still evident. Collectively, these changes are known as the hypothermic storage lesion [1-6]. Due to accumulating evidence suggesting that the hypothermically stored RBCs have impaired *in vivo* rheology, more attention has been given to the biomechanical components of the storage lesion and the effects these changes have on rheologic properties such as RBC deformability [7-10]. Despite this increased awareness, no standard method to interpret RBC deformability data has been developed and studies investigating the effect of hypothermic storage on RBC deformability have resulted in contrasting results [11, 12]. In light of this, the objective of this thesis has been to improve the current methods of RBC deformability assessment and analysis to better elucidate if impairments in RBC deformability are indeed evident during hypothermic storage. In addition, a secondary goal of this thesis was to investigate the use of liposomes as possible RBC membrane modification tools, in the hope of better preserving RBC rheology during hypothermic storage. The results of three experimental studies have been presented in this thesis to test the following hypotheses: Ektacytometry with Eadie-Hofstee data analysis is an effective method to assess RBC

deformability during hypothermic storage; RBC deformability during hypothermic storage can be modified by liposome treatment.

The first experimental objective in this thesis was to determine if *in vitro* induced changes in RBC deformability could be detected via ektacytometry (Chapter 2). Numerous methods to interpret RBC deformability data have been explored previously; however recent studies have employed linearization techniques to simplify the comparison of otherwise complex RBC deformability curves [11, 13-20]. In Chapter 2, Eadie-Hofstee analysis was introduced as an alternative linearization method to determine the RBC deformation parameters EI_{max} and K_{EI}, which are indicative of RBC maximal deformability and RBC rigidity respectively [21]. The results of this study, which were recently published in Clinical Hemorheology and Microcirculation, demonstrated that the treatment of RBCs with oxidizing agents known to affect the membrane and cytoskeleton led to changes in RBC deformability as detected via ektacytometry with subsequent Eadie-Hofstee data linearization. The extraction of membrane cholesterol with cyclodextrin was also shown to induce changes in RBC deformability. In addition to simply detecting changes in deformability, the obtained EI_{max} and K_{EI} values reflected the type of chemical treatment, indicating that the analysis technique is also capable of dissecting changes in RBC deformability depending on the type of damage induced. Furthermore, pathologic RBC samples known to have defects in RBC cytoskeletal proteins (hereditary spherocytosis) and abnormal hemoglobins (α-thalassemia) were also shown to have altered deformability profiles. This study demonstrated that the use of

ektacytometry with Eadie-Hofstee data analysis is a suitable method for the detection and resolution of changes in RBC deformability. It was the development of this technique that served as a foundation for the deformability measurements performed on hypothermically stored RBCs in the subsequent chapters of this thesis.

After establishing a technique to examine changes in RBC deformability, the aim of Chapter 3 of this thesis was to apply the deformability analysis method established in the previous chapter to investigate the effect of routine hypothermic storage on the deformability of hypothermically stored RBCs. In addition, the relationship between deformability and intracellular ATP concentration was investigated to determine if ATP depletion alone is responsible for RBC deformability deficiencies arising during hypothermic storage. Decreases in RBC ATP concentrations were observed during hypothermic storage followed by subsequent decreases in RBC maximum deformability. However, it was also shown that the restoration of ATP levels with PIPA solution did not lead to improvements in RBC deformability, but rather led to further deformability impairment. The results of this study demonstrate that reductions in RBC deformability during hypothermic storage are preceded by ATP depletion; however, unlike many biochemical aspects of the hypothermic storage lesion, the restoration of ATP levels by rejuvenation does not reverse deformability losses incurred during storage. The irreversible nature of the deformability deficiencies suggested that other downstream mechanisms beside a simple reduction in ATP levels contribute to the impaired deformability of hypothermically stored RBCs.

Because of this, the investigation of new methods to improve the preservation of RBC deformability during hypothermic storage was recommended. It was also recommended that more research investigating the effect of PIPA rejuvenation on RBC deformability be performed to determine if rejuvenated RBCs have impaired rheology.

Considering that the RBC deformability impairments demonstrated in the previous chapter are likely due to lipid loss from the RBC membrane, specific preservation strategies that focus on the RBC membrane are worth investigating. Previously, liposomes have been utilized to modify cell membranes in biopreservation contexts [22-26].The specific aim of Chapter 4 of this thesis was to investigate the use of liposomes as a possible tool to modify the deformability of hypothermically stored RBCs. Three different compositions of liposomes with different acyl chain lengths and degrees of saturation were synthesized, incubated, and stored with fresh drawn RBCs. RBC deformability was determined via the ektacytometric technique established in Chapter 2 and subsequently employed in Chapter 3. In addition to RBC deformability, conventional measures of RBC quality including percent hemolysis, RBC indices, and RBC morphology were also performed. The results of this study demonstrate that liposome treatment of RBCs leads to detectable changes in both maximal RBC deformability and RBC rigidity. Perhaps the most significant finding was the effect that liposome composition had on RBC quality. Although DPPC liposome treatment resulted in minimal effects on RBC deformability, treatment with DOPC or DMPC liposomes destabilized the RBC membrane leading to significant decreases in

RBC maximal deformability and increases in percent hemolysis. These changes were attributed to the extraction of RBC membrane cholesterol during liposome interaction as described by Stoll *et al.* [27]. A mild protective effect of DPPC liposomes was also observed in terms of percent hemolysis and RBC morphology; however, further research was proposed to explain this occurrence. Despite the apparent negative effects of DOPC and DMPC liposomes, the results of this study provided convincing evidence that RBC deformability can be modified by liposome treatment. Further research was proposed to determine the optimal liposome composition for the successful stabilization of the RBC membrane and possible maintenance of RBC deformability during hypothermic storage.

5.2 Contributions to Science and Future Directions

This thesis has made several valuable contributions to the fields of biopreservation and transfusion medicine. The development of an effective technique to monitor the deformability of RBCs during hypothermic storage is a significant contribution as RBC quality measures that are reflective of the *in vivo* functioning of preserved RBCs are currently lacking. In light of the clinical implications of transfusing RBCs with impaired deformability, the potential exists for the use of this deformability analysis technique in blood bank quality monitoring protocols to ensure RBC products delivered to patients are of the highest standard possible. Furthermore, the use of this technique has demonstrated that hypothermically stored RBCs have a decreased capacity to deform and that

RBC rejuvenation has compounding effects on the already present deformability impairments. Further research into the effect of rejuvenation on the RBC membrane and its relationship to deformability and RBC rheology are certainly warranted. In addition to the development and application of an analytical technique, this thesis has demonstrated that RBC deformability can be modified by liposome incubation. This creates a unique opportunity for the development of a new biopreservation strategy to combat the membrane component of the RBC hypothermic storage lesion. Future studies investigating the optimal liposome composition for the stabilization of the RBC membrane and preservation of RBC deformability during storage are strongly recommended. These studies will facilitate a better understanding of the effects of hypothermic storage on the organization of the RBC membrane and with refinement, may lead to future improvements in the rheologic properties of transfused blood.

Although technical advancements with respect to RBC processing and additive solutions have improved both the allowable duration of storage and quality of hypothermically stored blood, further advancements in RBC biopreservation are still required. To fully address the clinical data suggesting that transfused RBCs lead to rheological complications *in vivo,* a firm understanding of the biomechanical components of the RBC storage lesion is needed. This thesis has provided a methodology to help understand the mechanisms of deformability loss in RBCs, as well as a membrane modification technique to be further explored in the future as a possible RBC biopreservation method. It is my sincere hope that the ideas and techniques generated in this thesis lead to future

investigations that improve our knowledge of RBC rheological properties as well as the quality and safety of RBCs destined for transfusion.

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