University of Alberta

The Adhesion of Stored Red Blood Cells to Human Umbilical Vein Endothelial Cells

by

Julien Nunes

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Medical Sciences – Laboratory Medicine and Pathology

Spring 2013
Edmonton, Alberta

Abstract

The field of transfusion medicine is dependent upon the ability to store and preserve the native functionality of red blood cells (RBCs). The subtle membrane changes that the RBC incurs during storage can result in compromised quality and diminished function upon transfusion. A custom adhesion assay was developed which was used to determine the change in red blood cell-endothelial (RBC-EC) interactions in cryopreserved and hypothermic RBCs. This thesis demonstrated an increase in RBC adherence and adherence strength due to the hypothermic storage lesion and cryoinjury. Through these analytical investigations, this thesis has provided a clearer understanding of RBC-EC interactions and have enhanced our understanding of the mechanisms involved in RBC-EC interactions. The resulting benefit of this investigation's findings should result in a better quality product being available for transfusion which will thus benefit the field of biopreservation and transfusion medicine.

Acknowledgements

I wish to express my deepest gratitude to the following individuals who were instrumental in the completion of this thesis:

To Dr. Jelena Holovati (supervisor) and Dr. Jason Acker (co-supervisor) for being such amazing mentors.

To Dr. Rodrigo Onell, Mr. Christopher Ward and Dr Monika Keelan for serving on my examining committee and providing insightful comments and feedback.

To my parents, Marlene and Michael Nunes. thank you for being my biggest supporters.

Dedication

This thesis is dedicated to my parents, Michael and Marlene Nunes and brother, Fabien who gave me unconditional love, support and encouragement during the writing of this thesis and journey as a graduate student. I am especially grateful to my wonderful mother, who instilled faith and the power of prayer in me which gave me the motivation to succeed.

List of Abbreviations

2,3DPG	**2, 3-diphosphoglycerate**
AABB	**American association of blood banks**
ACD	**acid-citrate-dextrose**
APACHE	**acute physiology and chronic health evaluation**
AS	**additive solution**
CPA	**cryoprotective agent**
CPD	**citrate phosphate dextrose**
DEHP	**di-2-ethylhexylphthalate**
DMSO	**dimethylsulfoxide**
EBM-2	**endothelial basal medium -2**
EC	**endothelial cell**
EDTA	**ethylenediaminetetraacetic acid**
EPO	**erythropoietin**
G6PD	**glucose-6-phosphate dehydrogenase**
Hb	**hemoglobin**
hEGF	**human epidermal growth factor**
HES	**hydroxyethyl starch**
hFGF	**human fibroblast growth factor**
HiCN	**cyanmethemaglobin**
HK	**hexokinase**
HMP	**hexose monophosphate**
HSL	**hypothermic storage lesion**

HUVEC	**human umbilical vein endothelial cells**
IgG	**immunoglobulin G**
ISE	**Ion selective electrodes**
LORCA	**laser assisted optical rotational cell analyzer**
LR	**leukoreduction**
Lyso-PC	**lysophosphatidylcholine**
Lyso-PE	**lysophosphatidylethanolamine**
MCH	**mean corpuscular hemoglobin**
MCHC	**mean corpuscular hemoglobin content**
MCV	**mean cell volume**
MODS	**multiple organ dysfunction syndrome**
MRP1	**multidrug resistance protein 1**
NADPH	**nicotinamide adenine dineuclotide phosphate**
NEM	***N*-ethylmaleimide**
PA	**phosphatidic acid**
PAGGSM	**phosphate-adenine-glucose-guanosin-saline-mannitol**
PBS	**phosphate buffered saline**
PC	**phosphatidylcholine**
PE	**phosphatidylethanolamine**
PI	**phosphatidylinositol**
PIP	**phosphatidylinositol-monosphosphate**
PIP2	**phosphatidylinositol-4,5 bisphosphate**
pO2	**oxygen partial pressure**

PS	**phosphatidylserine**
PVC	**polyvinylchloride**
PVP	**polyvinyl pyrrolidone**
R^3-IGF-1	**insulin-like Growth factor-I**
ROS	**reactive oxidative species**
SAGM	**saline-adenine-glucose-mannitol**
SM	**sphingomyelin**
SOFA	**sepsis related organ failure assessment score**
SRAs	**specific research aims**
ssRBC	**sickle cell RBC**
TOTM	**tri-octyl-tri-mellitate**
TRALI	**transfusion related acute lung injury**
VEGF	**vascular endothelial growth factor**
WBC	**white blood cells**

Chapter 1 1

Introduction 1

1.1 Red blood cell bio preservation 2

1.1.1 Synthesis and energetic of red blood cells 3

1.1.2 RBC-EC adhesion in normal physiology 4

1.2 RBC hypothermic storage 8

1.2.1 History of RBC preservative systems 9

1.2.2 RBC hypothermic storage lesions 11

1.3 RBC cryopreservation 15

1.3.1 Clinical cryopreservation 17

1.4 RBC adhesion and deformability 18

1.5 Thesis approaches 21

1.6 Hypothesis and thesis objectives 21

1.7 References 24

Chapter 2 33

Establishment of Quality Assessment for Red Blood Cell-Endothelial Cell Adherence 33

2.1 Introduction 34

2.2 Materials and methods 36

2.2.1 RBC processing 36

2.2.2 Sample preparation 36

2.2.3 HUVEC cell culture 37

2.2.4 Coverslip fibronectin treatment 37

2.2.5 Adhesion assay 38

2.2.5.1 Flow rate determination 40
2.2.5.2 Adherence strength 41
2.2.5.3 Controls 42
2.3 Results 43
2.4 Discussion 44
2.5 Conclusion 50
2.6 References 58
Chapter 3 64
The effects of cryopreservation on erythrocyte adherence and deformability 64
3.1 Introduction 65
3.2 Materials and Methods 68
3.2.1 RBC Processing 68
3.2.2 Cryopreservation and deglycerolization 68
3.2.3 Assays 69
3.2.3.1 Conventional in vitro RBC quality assays 69
3.2.3.2 Non-conventional in vitro RBC quality assays 74
3.2.3.2.1 RBC- EC adhesion assay 74
3.2.3.2.2 Deformability 74
3.2.4 Statistical Analysis 76
3.3 Results 76
3.3.1 Conventional in vitro RBC quality assays 76
3.3.2 Non conventional in vitro RBC quality assays 77
3.4 Discussion 77
3.5 Conclusion 81

3.6 References 88

Chapter 4 93

Red Blood Cell Endothelial Adherence during 42 day Hypothermic Storage 93

4.1 Introduction 94

4.2 Materials and Methods 97

4.2.1 RBC Processing 97

4.2.2 RBC-EC endothelium adhesion 97

4.2.3 Adherence strength 98

4.3 Results 99

4.4 Discussion 100

4.5 Conclusion 103

Chapter 5 109

General Discussion & Future Directions 109

5.1 Review of Thesis Objectives 110

5.2 Study Contributions and Future Directions 111

5.1 References 113

Appendix 114

List of Tables

Table 2.1- Calculated flow rates of corresponding shear stresses for adhesion assay.....40

Table 3.1 – Table of RBC quality measures for pre-freeze and post thaw samples. 83

List of Equations	Equation	Page
Equation 2.1	$\tau_w = 6Q\mu/wh^2$	41
Equation 2.2	$Q = \tau_w wh^2/6\mu$	41
Equation 2.3	$N = N_\infty + \alpha/\tau$	42
Equation 2.4	$N = \alpha \ln\tau + N_\infty$	42
Equation 3.1	Corrected Hb content = (% recovery x [$Hb_{prefreeze}$]) +[Hb_{final}]	71
Equation 3.2	Glucose + ATP → Glucose-6-phosphate + ADP	72
Equation 3.3	Glucose-6-phosphate + NAD^+ → 6-phosphogluconate + $NADH^+ + H^+$	72
Equation 3.4	ATP (µmol/gHb) = 10 x ATP (µmol/dL) / Hct (L/L) x Hb (g/L)	72
Equation 3.5	ATP (µmol/gHb) = 10 x ATP (µmol/dL) / Hct (L/L) x Hb (g/L)	73
Equation 3.6	$c = A_{540}$ x M x F/ε_{540} x l x 1000	74
Equation 3.7	% hemolysis = (100- Spun Hct) x Hb_s) / Hb_T	74

List of Figures

Figure 2.1 Different fields of view of fibronectin treated coverslips after 48 hours of HUVEC confluency using the Leitz microscope at 40× magnification. 51

Figure 2.2- Schematic depiction of the adhesion assay. 52

Figure 2.3 Adhesion apparatus setup; (A) infusion side of the adhesion apparatus; (B) suction side of the adhesion assay; (C) overhead view of the adhesion assay. 53

Figure 2.4 The RBC counting mechanism with untreated RBCs at a hematocrit of 1.5%; arrows indicate the direction of laminar flow (A) RBCs at low flow rates (0.1 Pa); (B) RBCs at medium flow rates (0.25 Pa) (C) RBCs at high flow rates (0.5 Pa)..... 54

Figure 2.5 Adhesion profile for 7 day old (fresh) RBCs adherent to the EC monolayer of HUVEC cells under increasing shear stress τ. 55

Figure 2.6 Adhesion strength profile for day 7 old (fresh) RBCs; that is, the number of RBCs adherent to the EC monolayer of HUVEC cells vs. $1/\tau$. 56

Figure 2.7 Adhesion of treated RBCs to the EC monolayer of HUVEC cells (A) t-BuOOH-treated RBCs (B) vitamin E-treated RBCs (C) ssRBCs. 57

Figure 3.1 – Graph showing the effects of cryopreservation on RBC-EC adherence before and after cryopreservation. Mean number of adherent RBCs at increasing shear stress; control (prefreeze) adherent RBCs versus thawed adherent RBCs..... 84

Figure 3.2 – Graph of the logarithmic transformation of mean number of adherent RBCs versus 1/shear stress (adherence strength) between prefreeze and post thaw RBCs; Significant increase in overall calculated adherent strength observed for pre-freeze and post thawed samples; (Inset) the differences in the RBC adherence strength between the pre-freeze and post-thawed RBC ... 85

Figure 3.3 - Figure showing the Eadie-Hofstee transformation to obtain EI_{max} and K_{EI} values .. 86

Figure 3.4 – Graph showing the comparison of deformability parameters (K_{EI} and EI_{max}) between prefreeze and post thaw RBCs..... 87

Figure 4.1 Graph of number of adherent RBCs at increasing shear stress during 42 day hypothermic storage. Mean number of adherent RBCs at increasing shear stress; comparitive analysis of adherent RBCs days 8, 14, 21, 35, 42; significant differences in mean number of adherent RBCs at 0.05 Pa on days 21, 35 and 42 versus day 8. The y axis represents the mean number of adherent RBCs per field of view at magnification x 60 versus the x axis which represents increasing shear stresses. 104

Figure 4.2 – Graph of adherence coefficient (adherence strength) changes during the 42 day hypothermic storage period. Significant increases in calculated adherence strength observed at day 21 of hypothermic storage..... 105

Chapter 1

Introduction

1.1 Red blood cell bio preservation

Red blood cell (RBC) transfusion from a healthy human to an individual suffering from anemia, leukemia, hemorrhage, or surgical trauma represents an effective therapeutic strategy to increase RBC mass in the diseased person (1-3). RBC biopreservation represents an artificial means of preserving the quality of red blood cells *in vitro* for subsequent transfusion. The low or subzero temperatures involved in red blood cell biopreservation suppress most intracellular biochemical and biomechanical (physical structure) reactions, slowing the natural ageing process and prolonging RBC "shelf life". The safety and efficacy of RBC transfusions depend on the use of suitable biopreservation techniques that maintain RBC quality during *in vitro* storage. Bio preservation techniques such as low temperature are used in blood banks worldwide to maintain RBC quality and stability and achieve maximum RBC survival and functionality.

Blood banking processing and storage procedures in Canada involve the buffy coat removal method (1)whereby whole blood is collected into a blood bag containing an anticoagulant such as citrate phosphate dextrose (CPD) (4). Once collected, the blood is converted into a variety of components including packed red blood cells (4). Centrifugation and filtration to remove white blood cells (WBCs) are performed on the whole blood cell unit which is then extracted to another bag containing a solution with additives that protect the viability of RBCs. Packed RBCs can be used immediately or stored at 1-6^{o} C in a solution containing

nutrient additives for up to 42-49 days (1,5), or cryopreserved using Meryman's high glycerol (40 % w/v) or low glycerol (20 % w/v) methods.

Each year approximately 85 million RBC units are successfully transfused worldwide because of the application of biopreservation techniques to transfusion medicine. Possibly the main biophysicial effect of storage on RBCs is the loss of membrane and hemoglobin through progressive vesiculation and changes in RBC mechanical and rheological (flow) properties. This chapter describes the biophysical and biochemical consequences to RBCs of hypothermic and cryopreservation and discusses the impact of these consequences on RBC survival and performance *in vitro*.

1.1.1 Synthesis and energetic of red blood cells

RBCs are a product of a differentiation process that begins in the bone marrow where hematopoietic stem cells differentiate to nucleate RBCs. After extrusion of nuclei and degradation of the endoplasmic reticulum, reticulocytes emerge in the circulation where they rapidly develop into mature RBCs(6,7). RBCs are biconcave disks 8 μm in diameter and have a 120 day life span inside the human body (6,7). The RBC's carbon dioxide/oxygen exchanger and nitric oxide transporter/signaler (main functions) require a membrane that is flexible and deformable, allowing the RBC to maneuver unhindered, especially through the body's narrow microvasculature. Under instances of oxygen deprivation, the peritubular interstitial cells of the kidney, which are the primary oxygen sensing system of the body, secrete erythropoietin (EPO) to stimulate RBC production (7)

Despite being anucelated, RBCs possess a central core of chaperone proteins and heat shock proteins that maintain and protect the RBC throughout its 120 day lifespan (8). RBCs do not contain organelles and can thus take advantage of diversions or shunts from the glycolytic pathway to generate metabolites that maximize oxygen delivery from several vantage points. Three of these diversions are the hexose monophosphate pathway (HMP) that produces NADPH to prevent oxidative injury, the Rappaport-Luebering pathway that promotes 2,3 DPG syntheses, and the methemoglobin pathway that prevents hemoglobin (Hb) denaturation and oxidative injury (9).

1.1.2 RBC-EC adhesion in normal physiology

Under normal circumstances, RBC adherence to endothelial cells (ECs) lining the blood vessel is insignificant (10). However, during routine blood banking conditions RBC-EC adherence is enhanced (11), creating the possibility for vascular resistance when RBCs are transfused. RBC-EC adherence can be defined as the ability of RBCs to adhere to blood vessel wall ECs in pathological conditions (12). Under normal conditions, red blood cells often flow in single file in capillaries, their adherence is insignificant, and their deformability is sufficient to enable adequate passage through capillaries. They typically assume a nonaxisymmetric shape, with cyclic “tank treading” motion of the membrane around the interior (12). RBC-EC adhesion decreases blood flow and increases the residence time of RBCs in the microcirculation (13)

1.1.3 RBC-EC adhesion in abnormal physiology

Borst *et al* (14) demonstrated experimentally that RBCs that have undergone eryptosis (erythrocyte/RBC suicidal death/apoptosis) have enhanced adhesion to endothelial cells mediated by phosphatidylserine exposure and the endothelial transmembrane CXC chemokine ligand 16 (CXCL16). Phosphatidylserine (PS) exposure on RBCs provide a site for the assembly of coagulant enzymes leading to thrombosis generation and clotting(15). CXCL16 also known as SR-PSOX is a chemokine which in membrane form, acts as a scavenger receptor that binds PS and oxidized low density lipoprotein(16,17). CXCL16 is expressed in human vascular endothelial cells promoting adhesion and firm arrest of leukocytes under flow conditions(14) Adhesion of eryptotic RBCs has been associated with cardiovascular complications such as diabetes or renal failure known to impair the microcirculation (18). Additionally, the adhesion of eryptotic RBCs to phagocytosing cells contributes to their clearance from circulating blood and the predisposition to development of anemia(19).

RBC-EC adhesion is pronounced in many RBC abnormalities including sickle cell disease, malaria and diabetes mellitus. Their unusually strong adherence to the endothelium is mediated by key adhesive molecules expressed on the surface of the abnormal RBC and the endothelial cell when in close proximity (20). Adherence in sickle cell disease is controlled by VLA-4 (integrinα4β1) and Lu/BCAM (Lutheran/Basal cell adhesion molecule) on the sickled RBC with VCAM-1 (vascular cell adhesion molecules 1) and laminin α5 receptors on the endothelial cells (20). The adhesion of malaria infected RBC is mediated by a parasite protein, *plasmodium falciparum* erythrocyte membrane

protein 1 (PfEMP1) Inter Cellular Adhesion Molecule-1(ICAM-1) appears as a ligand for PfEMP1 in addition to CD31, CD36 and PECAM-1. In diabetes mellitus RBC band three protein is glycated and binds to the Receptor for Advanced Glycation End Products (RAGE)(20,21)

1.1.4 Physical structure of the RBC membrane

Similar to most animal membranes, the RBC membrane is composed of 19.5 % (w/w) water, 39.5 % protein, 35.1 % lipid and 5.8 % carbohydrates(22). Of the 35 % lipid portion, 60 % are phospholipids, mainly phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM) and phosphatidylserine (PS). Minor phospholipid membrane components are phosphatidylinositol (PI), PI-monophosphate (PIP), PI-4,5 bisphosphate (PIP2), phosphatidic acid (PA), lysophosphatidylcholine (lyso-PC), and lysophosphatidylethanolamine (lyso-PE). Nonesterified cholesterol represents approximately 30 % of the lipid RBC composition and the remaining 10 % are glycolipids (22).

Glycolipids (sugar bearing lipids) comprise 5 % of the external half of the RBC membrane (23) and are comprised mainly of sphingosines such as glycosphingolipids. Sugar residues present in glycolipids are responsible for numerous functions such as adhesiveness to the extracellular endothelium. They associate in clumps or rafts and support carbohydrate side chains that extend into the aqueous plasma to help form the glycocalyx. The glycocalyx is a layer of carbohydrates whose net negative charge prevents microbial attack and protects the RBC from mechanical damage caused by adhesion to neighbouring RBCs or

the endothelium. Transmembranous (integral) and skeletal (cytoskeletal, peripheral) proteins make up 52 % of the membrane structure by mass. Pasini *et al* documented at least 340 different red cell membrane proteins (6).

Cholesterol which is esterified and largely hydrophobic, resides parallel to the acyl tails of the phospholipids and is equally distributed between the outer and inner layers and evenly dispersed within each layer of the RBC membrane. There is approximately one molecule of cholesterol per phospholipid molecule. Cholesterol's beta hydroxyl group, the only hydrophilic portion of the molecule, is anchored within the polar head groups, while the rest of the molecule is intercalated among and is parallel to the acyl tails. Cholesterol confers tensile strength to the lipid bilayer of the RBC membrane (24).

At physiologic pH, the majority of phospholipid content is electrically neutral, although PS, PA and PI are negatively charged. With the exception of SM and lyso-PC, most of the phospholipids have two fatty acid chains attached to a glycerol backbone. The most common fatty acids in human lipid bilayer membranes are usually observed in the following C=C ratio: (16:0, 18:0, 18:1, 18:2 and 20:4). Phospholipids are asymmetrically distributed across the bilayer, a feature known as 'trans asymmettry'. PS and PE are located almost entirely in the inner monolayer while PC and SM are more common in the outer leaflet. PS exposure results in cell death and apoptosis(22).

Phospholipid distribution is energy dependent, with phospholipid positions relying on a number of membrane-associated enzymes called flippases, floppaes and scramblases (23). When phospholipid distribution is disrupted, such as in

sickle cell anemia and thalassemia, or when RBCs have reached the end of their 120-day life span, PS, the only negatively charged phospholipid, redistributes (flips) to the outer layer of the RBC membrane. Splenic macrophages possess receptors that bind PS allowing the macrophages to destroy senescent RBCs (9). Flippase is responsible for PS and PE translocation from the outer leaflet to the inner leaflet of the cell membrane. Floppase catalyzes the translocation of PC and SM from the inner leaflet to the outer leaflet of the bilayer. The flop mechanism that controls PS externalization and is usually implicated in RBC-EC adherence is Mg^{2+}/ATP dependent. The flop mechanism results from aminophospholipid translocase impairment and occurs very slowly during hypothermic storage (25). Flippase is a member of the Mg^{2+}-dependent, P-glycoprotein ATPase family, whereas floppase is a multidrug resistance protein 1 (MRP1) family member (25). The aminophospholipids located on the inner surface of the RBC membrane are thought to become disrupted during routine blood banking conditions (hypothermic storage) This might induce the thrombogenic cascade that leads to enhanced RBC-EC interaction and the subsequent clearance of RBCs from the circulation by the reticuloendothelial system(26,27).

1.2 RBC hypothermic storage

RBC hypothermic storage involves storage and preservation of red blood cells at below physiological temperatures but above freezing temperatures (usually at 1-6^{o}C) to maintain RBC quality for transfusion purposes. This biopreservation strategy relies on low temperature to suppress intracellular

biochemical reactions, maintain nutrient consumption, and limit waste accrual. During hypothermic storage, however, biochemical processes continue, albeit at a lessened rate. To reduce nutrient consumption and cellular waste accumulation that adversely affect the quality of the storage conditions, red blood cells must be supplemented with specially designed storage solutions that extend the *in vitro* storage time or "shelf life" of RBCs. Specially designed storage solutions contain licensed anticoagulant and salts, sugars, guanosines, and adeninens to maintain the reactions necessary for RBC preservation (28).

1.2.1 History of RBC preservative systems

The maintenance of cellular ATP concentrations by manipulations of the pH and tonicity of preservative solutions (5,29,30) has led to the development of new nutrient storage media(29,30) as well as improvements to current additives. The discovery by Arthus and Pages in 1890 that calcium ions in the plasma of whole blood were responsible for clotting (31), began a search for techniques to extract RBCs and retain red blood cell viability for patient transfusion. In 1914 and later in 1916, Hustin, Rouse and Turner introduced citrate and glucose and anticoagulant and preservative respectively (31), which laid the foundation for modern day blood banking. Citrate and glucose made it possible to separate blood donor and blood recipient in space and time (31). In 1918, Oswald Robertson successfully transfused stored citrated human red blood cells into soldiers on the battlefield during World War 1(32,33). Since that time, anticoagulants and additive solutions have been manipulated to prolong the shelf life of stored RBCs.

A longer RBC storage time can reduce the need for frequent blood donations and increase RBC therapeutic opportunities, especially in remote locations (30).

The ability to lengthen RBC storage life has been achieved using additive solutions (AS-1, AS-2, AS-3, AS-5, SAGM, PAGGSM (5)), which are coupled to anticoagulants such as CPD or acid citrate dextrose (ACD) to enhance preservation of RBCs under hypothermic storage(34). Additive solutions provide additional volume and nutrients for longer storage and better flow of packed RBCs(5). They also reverse some of the adverse reactions due to hypothermic storage lesions from long term storage, resulting in extended RBC shelf-life (42-49 days) (5,35-37). Specially designed plastic storage containers made of leachable plasticizers (DEHP, BTHC) that confer protection to the RBC membrane and minimize hemolysis are common (29,38-40)

Red blood cell concentrates are prepared by the removal of plasma and white blood cells (leukoreduction (LR))(41). Leukoreduction has been clinically successful in maintaining the quality of stored or frozen RBCs since most of the biologically active compounds that cause adverse reaction reactions are removed by the filtration process (42-44). To maintain RBC quality, the final product is usually stored in a slightly hypertonic solution - generally SAGM (sodium, adenine, glucose, mannitol, 376 milliosmoles/liter (mOsm/L) - at $4 \pm 2\ ^{o}C$. There is still controversy however about the length of time blood can be stored before transfusion without compromising the quality of the product and the recipient's health (45).

1.2.2 RBC hypothermic storage lesions

The structural and functional changes that RBCs undergo during hypothermic storage lesion are known collectively as hypothermic storage lesions. RBC storage lesions include the following biochemical and biomechanical changes: morphological alterations, slowed metabolism with a decrease in the concentration of adenosine triphosphate (ATP), acidosis with a decrease in the concentration of 2,3-diphosphoglycerate (2,3 DPG), loss of function of cation pumps and intracellular potassium and accumulation of sodium within the cytoplasm, oxidative damage with changes to the structure of band 3 (integral protein located in the RBC membrane (see section 1.4)(46) and lipid peroxidation, apoptotic changes with the restructuring of membrane phospholipids, loss of parts of the RBC membrane through vesiculation, decreased deformability and increased vascular resistance caused by increased adherence to ECs (47).

Some of these changes occur within the first few hours of hypothermic storage-for example, a decrease in pH or increases in potassium and lactate-while others take days or weeks. These events risk compromising the safety and efficacy of long-stored RBCs because they reduce their capacity to carry and release oxygen. The release of potentially toxic intermediates (for example, free hemoglobin can act as a source of reactive oxygen species) can negatively influence physiological rheology through the increased capacity of the RBCs to adhere to the endothelium or through their enhanced thrombogenic or proinflammatory potential (48-51).

Fluctuating ATP levels during hypothermic storage also appear to inhibit proper membrane skeleton complex formation such as spectrin-actin 4.1, spectrin-spectrin and spectrin-actin 4.1 linkages which all affect RBC shape and, by extension, function (28). The lipid bilayer asymmetry is fundamental for maintaining RBC shape; shape disruption can increase RBC-endothelial cell interaction(28). Calcium influx and changes in pH can contribute to shape loss as the diffusion of intracellular calcium frustrates the Ca^{2+}/Mg^{2+} pump resulting in lowered ATP levels. Decreased RBC survival appears to be caused by a loss of surface area attributable to cholesterol and lipid microvesiculation, processes that are independent of ATP concentration but increase with calcium accumulation or oxidative injury (28). The progressive loss of membrane lipids can prove detrimental to the survival of the RBC, rendering it osmotically fragile and limiting its lifespan in storage or after transfusion.

Another biochemical effect is a clear decrease in the levels of 2,3 DPG (which is consumed within the first week) which translates to an increased affinity of hemoglobin for oxygen and consequently decreases the capacity of RBCs to release oxygen according to local metabolic needs (52). Alongside these reversible changes, various irreversible events occur during the storage process, including the fragmentation and aggregation of proteins and lipids that is achieved by radical species generated by prolonged, continuous oxidative stress (53-55)

Perhaps the most evident changes in red blood cells during the storage period are those that affect its membrane. The cell phenotype varies from a smooth discoid shape to a phenotype characterized by various membrane

protrusions or *specula* (echinocytes) and finally to a spheroid (spheroechinocyte)(56). The reversibility of these changes decreases as the duration of storage increases. Reactive oxidative stress is known to induce diverse damaging processes in tissues and cells, including oxidation of intracellular and surface components of blood cells. During hypothermic storage, different reactive oxidative species (ROS) can induce: oxidation of membrane lipids and alteration of their intra/extracellular distribution (through the exposure of phosphatidylserine at the cell surface), oxidation and cleavage of proteins, degradation of surface proteoglycans, and oxidation of hemoglobin and its adherence to the cell membrane (57,58). Together these changes can alter RBC properties that depend on the composition and physical properties of the RBC membrane (59)

1.2.3 Clinical significance of transfusing stored RBCs

Currently, the criteria for safe and effective RBC transfusions are evaluated by the number of RBCs in the unit, the survival and function of the RBCs, the residual hemolysis and sterility, the residual number and function of WBCs, and the presence of residual biologically active plasma and non-plasma substances. The quality of the donor RBC influences the recipient's clinical outcome.

According to the literature, a liberal transfusion policy with respect to the age of hypothermically stored RBCs negatively affects patients' clinical outcomes(29,60-63) There is also suggestion that stable critically ill children

receiving prestorage leukoreduced RBC units that have undergone prolonged storage time be at risk of developing new or progressive multiple organ dysfunction syndrome (MODS)(63). However, conclusions could not be drawn from that experiment as it described an independent association and not a cause-effect relationship and a randomized clinical trial must be performed to evaluate the true effect. In general, a higher number of severely ill patients, as measured by either APACHE II (Acute Physiology and Chronic Health Evaluation II) or the sepsis-related organ failure assessment (SOFA) score, received RBC transfusions of hypothermic stored units than moderately ill patients (61) Additionally, an epidemiological study provided evidence of an association between blood transfusion and diminished organ function and increased mortality in critically ill patients. A significant association between the number of RBC transfusions and the risk of subsequent infection has been reported in patients following trauma, burns, and a variety of surgical procedures, both elective and emergency (64-67)

Taylor *et al* demonstrated an association between RBC transfusion and nosocomial infection and mortality (67). The potential risks associated with transfusion included hemolytic transfusion reactions, transfusion-related graft-versus host disease, nonhemolytic febrile transfusion reactions, and transmission of disease, allergic reactions, immune suppression and post transfusion infection(68-76). A recent study evaluating clinical outcomes following the institution of a universal prestorage leukoreduction program in Canada noted a reduction in hospital mortality following the introduction of this program(77).Transfusion of stored red blood cells exhibiting signs of storage

lesions has been implicated in adverse effects in patients in intensive care (61,63,78-81), patients undergoing cardiac interventions (82-87) or colorectal surgery (88-90), and patients with multiple trauma(91-93).

Finally, RBCs activate a process of vesiculation to eliminate proteins and lipids that have been altered by oxidative stress. To protect the cell from a further chain reaction of stress and consequent damage to the circulation, the membrane signals the removal of the cell through IgG or complement-mediated phagocytosis by the recipient's Kuppfer cells.(94). These membrane neoantigens, by stimulating the immune system, seem to be related to the onset of proinflammatory events, which are often harmful if not fatal in critically ill patients undergoing transfusion therapy (95,96). Alongside these signals, which are particular to red cell ageing, a series of other markers appear including the exposure of phosphatidylserine (PS) on the external leaflet of the lipid bilayer of the cell membrane. These markers are common in other physiological phenomena associated with programmed cell death or apoptosis.

Many of the problems arising out of the storage lesions lie within the membrane itself and the only way to address this is through a deeper understanding of how RBC ageing during storage impacts its rheological (flow) properties, specifically RBC deformability and adhesion.

1.3 RBC cryopreservation

Cryopreservation is the process of preserving the biological structure and function of a living system by freezing it and storing it at an ultralow temperature

(2). It has been used to preserve RBCs for at least 150 years (97). The concept of cryopreservation is based on the idea that subzero temperatures will suppress all biochemical and biomechanical reactions in living tissues and cells. Although it is an invaluable tool for extended RBC storage, cryopreservation is a labour intensive and expensive bio preservation procedure due to the technically demanding nature of processing and low temperature storage, as well as the limited 24 hour shelf life of deglycerolized units (98).

Since the discovery of glycerol's cryoprotectant properties in the 1950s by Dr. Audrey Smith, freezing has been used to supplement the hypothermic storage system. Cryopreservation has been used for more than 40 years by the military to store RBC before successful reinfusion, as well as for preserving rare RBC phenotypes (99). RBC cryopreservation also allows the quarantine of cells used to immunize donors of Rhesus immune globulin, enables storage of large volumes of autologous blood, and potentially limits the exposure of a given RBC recipient to a single donor.(98).

To meet the demands of modern medicine and surgery, RBCs must be available, safe, effective and cheap. In clinical practice, RBCs can be frozen using either low or high glycerol methods to limit the rate and extent of ice and crystal formation during freezing (100,101). During cryopreservation, structural changes to the RBC membrane might occur that can produce adverse clinical outcomes in transfused patients.

1.3.1 Clinical cryopreservation

During clinical cryopreservation, glycerol slows the rate of ice crystal formation and allows the RBC suspension to freeze as a glass (98). Low concentrations of glycerol (15-20 %) are sufficient to limit rates of crystal growth during rapid cooling with freezing in liquid nitrogen at -197 ^{o}C and storage at -150 ^{o}C. The high glycerol method (40 %) allows the slow growth of ice crystals and is performed under slow cooling conditions.

The low glycerol method is compatible for storage in thin-walled, siliconized aluminium cans, flat steel canisters, or Teflon bags that can withstand rapid freezing and minimize RBC loss due to fluid transfers (98). The high glycerol method is compatible with storage in plastic bags protected by aluminium cassettes or cardboard boxes that can withstand slow freezing and minimize RBC loss in mechanical -80^{o}C freezers, with slow cooling rates of approximately -1^{o}C min^{-1}(98). The high glycerol method is the favored approach in North America where frozen units are shipped on dry ice and processed near the facility of use. The low glycerol method is favored in Europe where units are processed at the storage facility and shipped in liquid form for immediate use. Both method may result in losses up to 10 %(37) and require post thaw washing to reduce the glycerol concentration to less than 1 % weight/volume(102). The controlled addition and removal of glycerol in both methods are necessary to prevent osmotic lysis of the RBCs and to minimize recipient exposure to the toxic chemical cryoprotective agent (CPA).

1.4 RBC adhesion and deformability

Increased changes in biochemical components in the RBC membrane during storage can result in increased RBC-EC interaction. RBC adherence to ECs isolates them from blood impairing the local flow pattern. RBC-EC interactions are generally insignificant in normal conditions but can become greatly enhanced in pathophysiological and oxidative stress states. A similar tendency has also been proposed in stored RBCs, leading to the eventual splenic sequestration and clearance of “old” RBCs (103). Oxidative stress and storage conditions have been associated with the translocation of PS to the outer membrane leaflet. When PS is externalized it can participate in binding to different proteins at the RBC surface resulting in increased RBC adherence to the endothelium.

The band 3 transmembrane protein provides the anion channel of the erythrocyte plasma membrane; it crosses the membrane more than once and has a large amino terminal. The oxidative stress that occurs during hypothermic storage can cause clustering of the band 3 segment exposed on the cytoplasmic side of the membrane and might cause increased RBC adhesion to the endothelium. (104). An increase in sphingomyelin has also been shown to be associated with elevated RBC adhesion to ECs (105). It is important to note that the RBC-EC interaction is influenced by three factor types: RBC related factors, endothelial factors and plasma factors. All these factors act synergistically and changes to any one of these factors during storage can cause serious alteration in RBC-EC adhesion and correspondingly in blood flow(106).

RBC-EC interactions are reportedly enhanced in pathophysiology related to RBC abnormalities such as in sickle cell disease, cerebral malaria, diabetes and thalassemia. Alterations in flow properties of human RBCs measured *in vitro* have been observed and implicated in the pathophysiology of numerous diseases including acute myocardial infarction, unstable angina, sepsis, schizophrenia, obesity, thalassemia and pregnancy induced hypertension(107-110).

RBC deformability refers to the ability of red blood cells to adapt their shape to the dynamically changing flow conditions in order to minimize their resistance to flow. This is particularly important for their passage through capillaries, which have a diameter smaller than that of the red blood cell. Structural changes in RBCs that impair their deformability contribute to the hindrance of blood flow, particularly in low flow states (57,111). RBC deformability has been proposed to be a major determinant of RBC survival, since less deformable cells sequester in the spleen and are destroyed (57). Red blood cells with reduced deformability can impair perfusion and thus reduce oxygen delivery to peripheral tissues; rigid, undeformable RBCs can directly block capillaries.

RBC deformability depends not only on RBC geometry but also on relative cytoplasmic hemoglobin viscosity. The normal mean cell hemoglobin concentration (MCHC) ranges from 290 g/L – 350 g/L. As the hemoglobin concentration rises above 350 g/L, deformability is compromised and RBC shelf life is shortened as the more viscous cells cannot pass through narrow capillaries or splenic pores. As RBCs age, they lose membrane surface area while retaining

hemoglobin. The hemoglobin becomes more and more concentrated and eventually the RBC is unable to pass through the splenic pores and is destroyed by splenic macrophages.

Many studies have linked reduced RBC deformability to circulatory disorders and the anemia observed in diverse pathologies, for example, thalassemia, sickle cell anemia, cerebral malaria, sepsis, diabetes, and disturbed cerebral flow in patients with stroke (112). Loss of membrane elasticity, increased membrane fragility, increased membrane permeability to cations, loss of cell water, and a stiffened membrane cytoskeleton are intrinsic factors that contribute to cellular deformability. Extrinsic factors that contribute to cellular deformability are related to cell surface area, volume, and environment; they include oxygen tension, osmolality, pH, temperature and concentration of plasma proteins. These factors combined with a unique cell geometry (size, shape, excess surface-area to volume ratio) provide the RBC with special mechanical properties and confer remarkable flexibility(113).

Deformability and RBC-EC adherence might actually provide a measure of erythrocyte survival in many conditions. Thus the degrees of reduction in deformability or increases in RBC-EC adherence of the erythrocytes might indicate functional impairment of the cells from a variety of alterations in membrane properties, shape, and internal composition. Conversely, normal deformability and normal EC adherence can provide measures of the functional integrity of the living cell.

Conventional biochemical assays focus only on the biochemical changes that take place as RBCs age during storage; the assays fail to address the changes of function and viability that can occur after cryopreservation and hypothermic storage. Better predictors of RBC quality and function *in vitro* are thus required. The addition of an EC adherence assay to the current conventional assays of RBC quality (including ektacytometry) would provide a more complete picture of cellular function and post transfusion viability of RBCs.

1.5 Thesis approaches

Presently, the criteria for RBC quality during *in vitro* storage is based on a minimal (0.8–1%) haemolysis range and a survival rate that is greater than 75% for post transfused blood cells (112,114). However, current assays do not give a complete picture of the damage incurred as a result of storage that can diminish RBC functional capabilities. More effective determinants of cellular membrane changes that result from bio preservation techniques and *in vitro* storage are necessary. This thesis investigates the RBC membrane alterations that result from cryoinjury and hypothermic storage lesions via two novel methods used to assess biophysical injury—ektacytometry and adhesion assays. Ektacytometry has previously been used to study deformability changes in stored RBCs(113) however for the first time, adhesion changes will be investigated in Canadian RBC products.

1.6 Hypothesis and thesis objectives

This thesis tests the following hypothesis: The adhesion assay is an effective method for detecting adhesion changes in RBCs during cryopreservation and hypothermic storage. The thesis is made up of three experimental studies; three specific research aims (SRAs) are targeted:

SRA1: To develop and establish an adhesion assay to evaluate RBC adhesion properties (*Chapter 2)*

Human umbilical vein endothelial cells (HUVECs) will be cultured to provide the monolayer base for perfusing stored RBCs. This assay will be an ex vivo simulation of a blood vessel, allowing one to monitor the adherence changes of RBCs as a direct consequence of storage in subsequent experiments

SRA2: To determine RBC deformability and adhesion changes in cryopreserved RBCs *(Chapter 3)*

Cryopreservation is usually an intervention strategy used to preserve hypothermic stored RBCs for longer. Eadie Hofstee transformation and adherence strength will be utilized to interpret deformability and adhesion data. A modified Meryman's high glycerol freezing method will be performed on the RBCs and conventional assays (ATP, hematocrit, corrected hemoglobin content, RBC indices, K^+ concentration) will be coupled to nonconventional assays (ektacytometry and adhesion) before and after cryopreservation to better predict viability and functionality of cryopreserved RBCs.

SRA3: To determine RBC adhesion changes in 42 day hypothermic stored RBCs *(Chapter 4)*

The adhesion assay developed and used in Chapters 2 and 3 will be used to measure adherence changes at weekly intervals during the extent of 42 days of hypothermic storage. The change in the number of adherent RBCs and adherence strength comparison with relatively fresh RBC (day 8) and the following days will be performed to determine when exactly during the storage period adherence specifically changes. The results obtained will indicate hypothermic stored RBC's tendency to adhere to the endothelium of the microvasculature in an *in vitro* environment.

1.7 References

(1) Kakaiya R, Aronson CA, Julleis J. Whole blood collection and component processing. AABB technical manual. 16th ed. Bethesda, MD: AABB Press; 2008. p. 201-211.

(2) Scott KL, Lecak J, Acker JP. Biopreservation of red blood cells: Past, present, and future. Transfus. Med. Rev. 2005;19(2):127-142.

(3) Holovati JL, Wong KA, Webster JM, Acker JP. The effects of cryopreservation on red blood cell microvesiculation, phosphatidylserine externalization, and CD47 expression. Transfusion 2008;48:1658-1668.

(4) Clarke G, Blajchman M. Canadian Blood Services Clinical guide to transfusion. 4th ed.; 2007.

(5) Hess JR. An update on solutions for red cell storage. Vox Sang. 2006;91(1):13-19.

(6) Pasini EM, Kirkegaard M, Mortenseen P, Lutz H, Thomas A, Mann M. In depth analysis of the membrane cytosolic proteome of red blood cells Blood 2006(108):791-801.

(7) Rodak B, Fritsma G, Keohane E. Erythrocyte Production and Destruction. In: Kathryn Doig, editor. Hematology: Clinical Principles and Applications. 4th ed.: Elsevier Saunders. p. 86-102.

(8) D'Alessandro a, Righetti P, Zolla L. The red blood cell proteome and interactome: an update. J Prot. Res. 2010;9(1):144-163.

(9) Rodak B, Fritsma G, Keohane E. Energy Metabolism and Membrane Physiology of the Erythrocyte, Part II: Hematopoesis. In: Doig K, Fritsma G, editors. Hematology: Clinical Principles and Applications. 4th ed.: Elsevier Saunders. p. 103-114.

(10) Yedgar S, Kaul DK, Barshtein G. RBC adhesion to vascular endothelial cells: more potent than RBC aggregation in Inducing circulatory disorders. Microcirculation (New York) 2008;15(7):581-583.

(11) Relevy H, Koshkaryev A, Manny N, Yedgar S, Barshtein G. Blood banking-induced alteration of red blood cell flow properties. Transfusion 2008;48(1):136-146.

(12) Secomb TW. Red blood cell mechanics and capillary blood rheology. Cell Biophys. 1991;18(3):231-251.

(13) Hebbel RP. Blockade of adhesion of sickle cells to endothelium by monoclonal antibodies. N Engl J Med 2000;342(25):1910-1912.

(14) Borst O, Abed M, Alesutan I, Towhid TS, Qadri Ms, Foller M, et al. Dynamic adhesion of eryptotic erythrocytes to endothelial cells via CXCL16/SR-PSOX. Am J Physiol - Cell Physiol 2011;302:C644-C651.

(15) Chung S, Bae O, Lim K, Noh J, Lee M, Jung Y, et al. Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procagulant microvesicle generation in human erythrocytees. Arterioscl. Thromb. Vasc. 2007;27:414-421.

(16) Gough P, Garton K, Wille P, Rychlewski M, Dempsey P, Raines E. A distintegrin and metalloproteinase 10-mediated cleavage and shedding regulates the cell surface expression of CXC chemokine ligand 16. J Immunol. 2004;172:3678-3685.

(17) Shimaoka T, Seino K, Kume N, Minami M, Nishine C, Suematsu M, et al. Critical role for CXC chemokine ligand 16 *SSR_PSOX) in Th1 response mediated by NKT cells. J. Immunol. 2007;179:8172-8179.

(18) Bonomini M, Sirolli V, Gizzi,F, Di Stante,S, Grilli A, Felaco M. Enhanced adherence of human uremic erythrocytes to vascular endothelium: Role of phosphatidylserine exposure. Kidney Int. 2002;62(4):1358-1363.

(19) Lang F, Gulbins E, Lerche H, Huber S, Kempe D, Foller M. Eryptosis, a window to systemic disease. Cell. Physiol. Biochem. 2008;22:373-380.

(20) Wautier J, Wautier M. Molecular basis of erythrocyte adhesion to endothelial cells in diseases. Clin. Hemorheol. Micro. 2013;53:11-21.

(21) Grossin N, Wautier M, Picot J, Stern DM, Wautier J. Differential effect of plasma or erythrocyte AGE-ligands of RAGE on expression of transcripts for receptor isoforms. Diabetes Metab. 2009;35:410-417.

(22) Yawata Y editor. Cell membrane: The red blood cell as a model. : Wiley; 2003.

(23) Alberts B, Johnson A, Lewis J editors. Membrane structure. Molecular biology of the cell 5th ed.: Garland Science; 2007.

(24) Cooper R. Influence of the increased membrane cholesterol in membrane fluidity and cell function in human red blood cells. J. Supramol. Struc. 1978(8):413-430.

(25) De Oliveira S, Saldanha C. An overview about erythrocyte membrane. Clin. Hemorheol. Micro. 2010;44:63-74.

(26) Tanaka Y, Schroit A. Insertion of fluorescent phosphatidylserine into the plasma-membrane of red-blood-cells - recognition by autologous macrophages. J. Biol. Chem. 1983;258(18):1335-1343.

(27) Hebbel RP, Yamada O, Moldow CF, Jacob HS, White JG, Eaton JW. Abnormal adherence of sickle erythrocytes to cultured vascular endothelium: possible mechanism for microvascular occlusion in sickle cell disease. J Clin Invest. 1980;65(1):154-160.

(28) Hogman CF. Preparation and preservation of red cells. Vox Sang. 2011;74(S2):177-187.

(29) ChinYee I, Arya N, dAlmeida MS. The red cell storage lesion and its implication for transfusion. Transfus. Sci. 1997;18(3):447-458.

(30) Hess JR, Greenwalt TG. Storage of red blood cells: New approaches. Transfus. Med. Rev. 2002;16(4):283-295.

(31) Mollison PL. The introduction of citrate as an anticoagulant for transfusion and of glucose as a red cell preservative. Br. J. Haematol. 2000;108:13-18.

(32) Maizels M, Paterson J. Survival of stored blood after transfusion. Lancet 1940;2:417-420.

(33) Robertson O. Transfusion with preserved red blood cells. Br. Med. J. 1918;1(2999):691-695.

(34) Acker J. Liposomes in Transfusion Medicine: An approach for reducing the RBC HSL. 2008.

(35) D'Alessandro A, Liumbruno G, Grazzini G, Zolla L. Red blood cell storage: The story so far. Blood Transfus. 2010;8(2):82-88.

(36) Zehnder L, Schulzki T, Goede JS, Hayes J, Reinhart WH. Erythrocyte storage in hypertonic (SAGM) or isotonic (PAGGSM) conservation medium: influence on cell properties. Vox Sang. 2008;95(4):280-287.

(37) Hogman CF. Liquid-stored red blood cells for transfusion - A status report. Vox Sang. 1999;76(2):67-77.

(38) AuBuchon J, Davey R, Estep T, Miripol J. Effect of the plasticizer di (2-ethylhexyl)phthalate on survival of stored red cells. Transfusion. 1984;24:422.

(39) Myhre B, Johnson D, Marcus C, Demaniew S, Carmen R, Nelson E. Survival of red cells stored for 21 and 35 days in a non di (2-ethylhexyl) phthalate plastic container. Vox Sang. 1987;53:199-202.

(40) Hogman C, Ericson A, Repucci A. Storage of saline-adenine-glucose-mannitol-suspended red cells in a new plastic container: polyvinylchloride plasticizer with butyryl-n-trihexylcitrate Transfusion 1991;31:26-29.

(41) Valeri C. Status report on the quality of liquid and frozen red blood cells. Vox Sang. 2002;83:193-196.

(42) Grimshaw K, Sahler J, Spinelli SL, Phipps RP, Blumberg N. New frontiers in transfusion biology: identification and significance of mediators of morbidity and mortality in stored red blood cells. Transfusion 2011;51(4):874-880.

(43) Hess JR, Sparrow RL, van der Meer PF, Acker JP, Cardigan RA, Devine DV, et al. Red blood cell hemolysis during blood bank storage: using national quality management data to answer basic scientific questions. Transfusion 2009;49(12):2599-2603.

(44) Moenninghoff J, Moog R. Investigation of a new in-line leukocyte reduction filter for packed red blood cells. Transfus. Apher. Sci. 2012;46(3):253-256.

(45) Zimrin AB, Hess JR. Current issues relating to the transfusion of stored red blood cells. Vox Sang. 2009;96(2):93-103.

(46) Karon B, Hoyer J, Stubbs J, Thomas D. Changes in band 3 oligomeric state precede cell membrane phospholipid loss during blood bank storage of red blood cells. Transfusion 2009(49):1435-1442.

(47) Bosman GJCGM, Werre JM, Willekens FLA, Novotny VMJ. Erythrocyte ageing in vivo and in vitro: structural aspects and implications for transfusion. Transfus. Med. 2008;18(6):335-347.

(48) Anniss A, Glenister K, Killian, JJ, Sparrow,RL. Proteomic analysis of supernatants of stored red blood cell products. Transfusion 2005(49):2136-2143.

(49) Koshkaryev A, Zelig O, Manny N, Yedgar S, Barshtein G. Rejuvenation treatment of stored red blood cells reverses storage-induced adhesion to vascular endothelial cells. Transfusion (Malden) 2009;49(10):2136-2143.

(50) Sweeney J, Kouttab N, Kurtis J. Stored red blood cell supernatant facilitates thrombin generation. Transfusion 2009;49(8):1569-1579.

(51) McFaul S, Corley J, Mester C, Nath J. Packed blood stored in AS-5 become proinflammatory during storage. Transfusion 2009(49):1451-1460.

(52) Beutler E, Wood L. In vivo regeneration of red cell 2,3 DPG after transfusion of stored blood. J. Lab. Clin. Med. 1969'74(2):300-304.

(53) Sharifi S, Dzik W, Sadrzadeh S. Human plasma and tirilazad mesylate protect stored human erythrocytes against the oxidative damage of g-irradiation. Transfus. Med. 2000(10):125-130.

(54) Wolfe L. Injuries to the red cell membrane during conventional blood preservation. Sem. Hematol. 1989;26(4):307-312.

(55) Racek J, Herynkova R, Holecek V. Influence of antioxidants on the quality of stored blood. Vox Sang. 1997(72):16-19.

(56) Liumbruno G, Calten D, Petropulacos K. The Chikununga epidemic in Italy and its repercussion on the blood system. Blood Transfus. 2008;6:199-210.

(57) Mohandas N, Chasis JA. Red-blood-cell deformability, membrane material properties and shape - regulation by transmembrane, skeletal and cytosolic proteins and lipids. Sem. Hematol. 1993;30(3):171-192.

(58) Junker M, Creutz CE. Ca2+-dependent binding of endonexin (annexin-IV) to membranes - analysis of the effects of membrane lipid-composition and development of a predictive model for the binding interaction. Biochemistry 1994;33:8930-8940.

(59) Chang G, Absolom D, Strong A, Stubley G, Zing W. Physical and hydrodynamic factors affecting erythrocyte adhesion to polymer surfaces. J. Biomed. Mat. Res. 1988;22:13-29.

(60) Marik PE, Sibbald WJ. Effect of stored-blood transfusion on oxygen delivery in patients with sepsis. JAMA 1993;269(23):3024-3029.

(61) Martin C, Sibbald W, Hebert P, Schweitzer I. Age of transfused red blood cells is associated with ICU length of stay. Clin. Invest. Med. 1994;17:124.

(62) Purdy FR, Tweeddale MG, Merrick PM. Association of mortality with age of blood transfused in septic ICU patients. Can. J. Anaesth. 1997;44(12):1256-1261.

(63) Gauvin F, Spinella PC, Lacroix J, Choker G, Ducruet T, Karam O, et al. Association between length of storage of transfused red blood cells and multiple organ dysfunction syndrome in pediatric intensive care patients. Transfusion 2010;50(9):1902-1913.

(64) Landers D, Hill G, Wong K, Fox I. Blood transfusion-induced immunomodulation. Anesth. Analg. 1996;82:187-204.

(65) Mickler T, Longnecker D. The immunosuppressive aspects of blood transfusion. J. Intensiv. Care Med. 1992;7:176-188.

(66) Blajchman M. Immunomodulation and blood transfusion. Am. J. Ther.. 2002;9(5):389-395.

(67) Taylor R, Manganaro L, OBrien J, Trottier S, Parkar N, Veremakus C. Impact of allogeneic packed red blood cell transfusion on nosocomial infection rates in the critically ill patient. Crit. Care Med. 2002;30:2249-2254.

(68) Dodd R. The risk of transfusion-transmitted infections. N. Engl. J. Med. 1992;192(322):419-421.

(69) Schrenber G, Busch M, Kleinman S, Korelitz J. The risk of transfusion transmitted viral infections. N. Engl. J. Med. 1996;344:1685-1690.

(70) Holland P. Viral infections and the blood supply. N. Engl. J. Med. 1996;334:1734-1735.

(71) Valeri C editor. Physiology of blood transfusion. : Little Brown & Co.In: Barie PS, Shires GT Surgical Intensive Care; 1993.

(72) Valeri C. Transfusion medicine and surgical practice. Bull. Am. Coll. Surg. 1993;78:20-24.

(73) Triulzi D, Vanek J, Ryan DH, Blumberg N. A clinical and immunological study of blood transfusion and postoperative bacterial infection in spinal surgery. Transfusion 1992;32:517-524.

(74) Vamvakas E, Carven, JH, HIbberd, PL. Blood transfusion and infection after colorectal cancer surgery. Transfusion 1996;36:1000-1008.

(75) Jeter E, Spivey M. Noninfectious complications of blood transfusion. Hematol/Oncol. Clin. N. Am. 1995;9:187-204.

(76) Klein H. New insights into the management of anemia in the surgical patient. Am J Med 1996;101(suppl 2A):125-155.

(77) Hebert PC, Fergusson D, Blajchman MA, Wells G, Kinetic A, Goyle D. Clinical outcomes following institution of the Canadian Universal leukoreduction program for red blood cell transfusions. JAMA 2003;289:1941-1949.

(78) Fernandez CJ, Akamine N, De Marco F. Red blood cell transfusion does not increase oxygen consumption in critically ill septic patients. Crit. Care 2001;5(6):362-367.

(79) Hebert P, Chin-Yee I, Fergusson D. A pilot trial evaluating the clinical effects of prolonged storage of red cells. Anesth. Analg. 2005;100:1433-1438.

(80) Taylor R, O' Brien, J Trottier, SJ. Red blood cell transfusions and nosocomial infections in critically ill patients. Crit. Care Med. 2006;34:2302-2308.

(81) Sakr Y, Chierego M, Piagnerelli M. The microvascular response to red blood cell transfusions in patients with severe sepsis. Crit. Care Med. 2007;35:1639-1644.

(82) Vamvakas EC, Carven JH. Length of storage of transfused red cells and postoperative morbidity in patients undergoing coronary artery bypass graft surgery. Transfusion 2000;40(1):101-109.

(83) Leal-Noval S, Jara-Lopez I, garcia-Garmendia J. Influence of erythrocye concentrate storage time on postsurgical morbidity in cardiac surgery patients. Anesthesiology 2003;98:815-822.

(84) Van de Watering L, Lorinser J, Versteegh M. Effects of storage time of red blood cell transfusions on the prognosis of coronary artery bypass graft patients. Transfusion 2006;46:1712-1718.

(85) Basran S, Frumento R, Cohen Aea. The association between duration of storage of transfused red blood cells and morbidity and mortality after reoperative cardiac surgery. 2006 Anesth. Analg.;103:15-20.

(86) Koch CG, Li L, Sessler DI, Figueroa P, Hoeltge GA, Mihaljevic T, et al. Duration of red-cell storage and complications after cardiac surgery. N .Engl. J. Med. 2008;358(12):1229-1239.

(87) Yap C, Lau L, Krishnaswamy Mea. Age of transfused red cells and early outcomes after cardiac surgery. Ann. Thor. Surg. 2008;86:554-559.

(88) Edna T, Bjerkeset T. Association between transfusion of stored blood and infective bacterial complications after resection for colorectal cancer. Eur. J. Surg. 1998;164:449-456.

(89) Mynster T, Nielsen H. The impact of storage time of transfused blood on postoperative infectious complications in rectal cancer surgery. Scand. J. Gastroenterol. 2000;35:212-217.

(90) Mynster T, Nielsen H. Storage time of transfused blood and disease recurrence after colorectal cancer surgery. Dis. Colon Rectum 2001;44:955-964.

(91) Offner P, Moore E, Biffl Wea. Increased rate of infection associated with transfusion of old blood after severe injury. Arch. Surg. 2002;137:711-716.

(92) Keller M, Jean R, Lamorte Eea. Effects of age of transfused blood on length of stay in trama patients: a preliminary report J Trauma 2002;53:1023-1025.

(93) Murrell Z, Haukoos, JS, Putman, B, Klein S. The effect of older blood on mortality, need for ICU care and the length of ICU stay after major trauma. Am. Surg. 2005;71:781-785.

(94) Willekens F, Werre J, Groenen-Dopp Y. Erythrocyte vesiculation: a self protective mechanism. 2008 Br. J Haematol.;141:549-556.

(95) Tinmouth A, Chin-Yee I. The clinical consequences of the red cell storage lesion. Transfus. Med. Rev. 2001;15(2):91-107.

(96) Lelubre C, Piagnarelli M, Vincent J. Association between duration of storage of transfused red blood cells and morbidity and mortality in adult patients: myth or reality? Transfusion 2009;49:1384-1394.

(97) Rowe A. Cryopreservation of red blood cells. Vox Sang. 1994;67:201-206.

(98) Hess J. Red cell freezing and its impact on the supply chain. Transfus. Med. 2004;14:1-8.

(99) Valeri C, Ragoo G, Pivacek L, Cassiday G, Srey R, Hansson-Wicher M, et al. An experiment with glycerol frozen red blood cells stored at -80C for up to 37 years Vox Sang. ;79:168-174.

(100) Pert J, Moore R, and Schork P. Low temperature preservation of human erythrocytes. Bibl. Hematol. 1965;23:674-682.

(101) Meryman H. Red cell freezing by the American National Red Cross. Am. J. Med. 1975;41:265-282.

(102) Valeri C. Blood banking and the Use of Frozen Blood Products. Chemical Rubber Company, Boca Raton, FL 1976.

(103) Ami R, Barshetin G, Zelster D, Goldberg Y, Shapira I, Roth A, et al. Parameters of red blood cell aggregation as correlates of the inflammatory state. Am. J. Physiol. Heart Circ. Physiol. 2001;280:H1982-H1988.

(104) Telen M. Red blood cell surface adhesion molecules: their possible roles in normal human physiology and disease. Sem. Hematol. 2000;37(2):130-142.

(105) Sarr N, Sall N, Toure M, Diatta A, Seck I. Phospholipid composition and content of the erythrocyte membrane in carriers of sickle cell trait. Dakar Med. 1998;43(1):5-8.

(106) Schlager A, Zamir G, Barshtein G, Yedgar S, Arbell D. Plasma factor in red blood cells adhesion to endothelial cells: humans and rats. Cell Biochem Biophys. 2010;58(3):157-161.

(107) Ben-Ami R, Barshetin G, Mardi T, Deutch V, Elkayam O, Yedgar S, et al. A synergistic effect of albumin and fibrinogen on immunoglobulin-induced red blood cell aggregation. Am. J. Physiol. Heart Circ. Physiol. 2003;285:H2663-H2669.

(108) Barshtein G, Ponizovsky A, Nechamkin Y, Ritsner M, Yedgar S, Bergelson L. Aggregability of red blood cells of schizophrenia patients with negative syndrome is selectively enhanced. Schizophr. Bull. 2004;30:913-922.

(109) Chen S, Eldor A, Barhstein G, Zhang S, Goldfarb A, Rachmilewitz E, et al. Enhanced aggregability of red blood cells of beta-thalassemia major patients. Am J Physiol. 1996;270(6 Pt 2):H1951-H1956.

(110) Hovav T, Goldfarb A, Artmann G, Yedgar S, Barshtein G. Enhanced adherence of beta thalassaemic erythrocytes to endothelial cells. Br. J. Haematol. 1999;106:178-181.

(111) Barshtein G, Ben-Ami R, Yedgar S. Role of red blood cell flow behavior in hemodynamics and hemostasis. Expert Rev. Cardiovasc. Ther. 2007;5(4):743-752.

(112) Palis T. Ontogeny of erythropoeisis. Curr. Opin. Hematol. 2008;15:155-161.

(113) Stadnick H, Onell R, Acker JP, Holovati JL. Eadie-Hofstee analysis of red blood cell deformability. Clin. Hemorheol. Micro. 2011;47:229-239.

(114) Hogman CF, Meryman H. Storage parameters affecting red blood cell survival and function after transfusion. Transfus. Med. Rev. 1999;13(4).

Chapter 2

Establishment of Quality Assessment for Red Blood Cell-Endothelial Cell Adherence

2.1 Introduction

At the relatively low shear stress of normal physiological conditions, all RBCs are detached from ECs of the blood vessel wall. RBC-EC adhesion occurs in post capillary venules at physiological shear stresses of less than 0.1 Pa(1) . RBC-EC adhesion can become greatly enhanced in pathophysiological and oxidative stress states. Oxidative stress represents an imbalance between enhanced generation of reactive oxygen species (ROS) and a low cellular content of antioxidants such as reduced glutathione (GSH), the major intracellular scavenger of ROS (2). A similar tendency to adhere to ECs has also been proposed in stored RBCs, leading to eventual splenic sequestration and clearance (3). Various states (oxidative stress and especially RBC storage) have been associated with the translocation of PS to the outer membrane leaflet, and this is considered to be a major determinant of RBC adherence to the endothelium (4).

RBC-EC interaction involves RBC related factors, endothelial factors, and plasma factors, all acting synergistically. Changes to any one of these factors during storage can cause serious alteration in RBC-EC adhesion and correspondingly in post transfusion blood flow. Plasma factors and polysaccharides such as fibrinogen, thrombospondin, and dextran have also been implicated in increased RBC-EC adherence (5-8). During hypothermic storage, membrane proteins such as band 3 become altered (9) and can activate vascular ECs causing inflammation and the expression of adhesion molecules (10-16). Several studies have reported the expression of a large number of RBC adhesion

molecules, such as CD44, CD47, CD58, LW/ICAM-4, RAGE, and Lu (9,17-33). Other RBC adhesion molecules include LW/ICAM-4, BCAM/LU, and sialyl moieties (34). Many adhesion proteins belong to the immunoglobulin superfamily (IgSF) of proteins (17) and play a crucial role in cell-cell interactions. These adhesion molecules are differentially expressed at distinct stages of the RBC lifespan (25). They have also been implicated in a large range of biological functions such as erythropoiesis (differentiation, maturation, enucleation, and release of RBCs), self-recognition, red cell turnover, and cellular aging (25,30).

Enhanced RBC-EC adhesion has been implicated in the pathophysiology of numerous RBC pathophysiology, including sickle cell disease, cerebral malaria, diabetes, thalassemia, acute myocardial infarction, unstable angina, sepsis, schizophrenia, and pregnancy-induced hypertension (35,36,37). The resultant impairment of normal RBC flow properties can lead to decreased post transfusion RBC survival, adversely affecting patient outcomes. Previously, multiple organ failure, nosocomial infection, and increased mortality and morbidity have been linked to the transfusion of aged RBCs that might have impaired flow properties (38-42). Increased RBC-EC adherence can contribute to decreased tissue oxygenation and subsequent blockage of the microvasculature resulting in tissue infarction and necrosis (43). A wide array of methods and devices, each with advantages and disadvantages, exist to assess RBC rheological properties (see section 2.4). They include: the micropipette assay, the concentric cylinder or Couette viscometer, the cone and plate viscometer, the static (gravity sedimentation) assay, the human umbilical vein model, the *ex vivo* rat mesenteric

model, microfluidic devices, and parallel plate flow chambers (flow adhesion assays) (44,45,45,46).

In this chapter we wish to introduce a custom parallel plate flow adhesion chamber to examine RBC-EC interactions. This device enables the measurement of subtle lesions of membrane damage that might occur during RBC storage. Rheological parameters might be better indicators of blood viability and function than the conventional biochemical assays. The developed assay will be used to examine adhesion changes in hypothermic-stored and cryopreserved RBCs in subsequent chapters (3 and 4).

2.2 Materials and methods

2.2.1 RBC processing

Leukoreduced, CPD-SAGM, packed RBC units (n = 5) from buffy coat produced whole blood was obtained from netCAD (Network Centre for Applied Development, Vancouver, Canada). Ethics approval was obtained from both the Canadian Blood Services and the University of Alberta Research Ethics Board prior to study commencement

2.2.2 Sample preparation

RBCs (2 mL) were aliquoted from each unit and washed three times at 1000 × g (RCF) for 5 minutes at 4 °C in 1× PBS (Phosphate buffered saline, Cellgro, Mediatech Inc. Manassas, VA). Following the final wash, the supernatant was aspirated and the RBCs were stored on ice until needed for adhesion experiments.

Prior to conducting the assay, the RBC sample was removed from the ice and resuspended to 1.5% hematocrit in 3 ml of PBS/1% HSA in labeled 50 ml conical tubes.

2.2.3 HUVEC cell culture

HUVEC cells (Lonza, Cat # C2517A) were cultured in EBM-2 Bulletkit media (Lonza, Walkersville, MD, USA Cat # CC-4176) composed of 10 mL (2%) fetal bovine serum (FBS), 0.5 mL human epidermal growth factor (hEGF), 0.2 mL hydrocortisone, 0.5 mL vascular endothelial growth factor (VEGF), 0.2 mL human fibroblast growth factor B hFGF-B (with heparin), 0.5 mL insulin-like growth factor-I (R^3-IGF-1), 0.5 mL ascorbic acid, and 0.5 mL heparin. Cell culture media was changed according to the seeding density (24 hours after passaging and subsequently every 48 hours until sub culturing). Cells were cultured in 75 cm^2 tissue culture treated Corning flasks at 37 ^oC and 5% CO_2 and passaged with 0.25% trypsin-EDTA (Lonza, Walkersville, MD, USA) when the cell density reached approximately 80% of the culture surface, which was determined by bright field microscopy. Either banked or fresh HUVEC (Cat # CC-2517) were grown and used until passage eight for adhesion assay experiments. An HUVEC bank was created by suspending subcultured HUVECs to a final concentration of 8 x 10^5-1 x 10^6 cells/mL in 10 % DMSO in liquid nitrogen.

2.2.4 Coverslip fibronectin treatment

According to calculations for optimal cell growth performed by Kluthe (47), 10 µg/cm^2 was optimal for endothelial cell (EC) growth on a 12 mm^2 coverslip. The fibronectin density appeared proportional to the surface area of the coverslip. 1.13 cm^2 (12 mm^2) required 10 µg/ml of fibronectin. Therefore the optimal fibronectin application on a 15 mm^2 coverslip (1.77 cm^2) required 15.66 µg/ml of fibronectin. A 1 mg/ml stock of bovine fibronectin (Sigma-Aldrich, Canada) stock solution in 15 mL conical tube was used to prepare 15.66 µg/ml in 1× PBS (Ca^{2+}/Mg^{2+} free) (Cellgro, Mediatech Inc, Manassas, VA 20109). Sterile (autoclaved) coverslips were placed in wells of 12 multiwell plates using flame-sterilized tweezers and 500 µl of 15.66 µg/mL fibronectin was placed in each well; plates were incubated for 40 minutes at room temperature, and wells were washed twice with 1× PBS (Ca^{2+}/Mg^{2+} free). After the final wash, immediately, 1 mL of EBM-2 (endothelial basal media, Lonza, Walkersville, MD, USA) nutrient media containing all the appropriate growth supplements for healthy HUVEC proliferation was added to each well followed by HUVEC suspensions at a density of 2×10^5 cells/ml. HUVECs on coverslips were confluent typically 48 hours after initial seeding.

2.2.5 Adhesion assay

Adhesion analysis of RBCs to cultured HUVECs was performed using the adhesion assay apparatus (Warner Instruments, Connecticut, USA) shown in Figure 2.2. Figure 2.2 illustrates the setup of the apparatus. The adhesion assay apparatus consisted of two syringe pumps, an infusion pump (Econoflo Syringe Pump, Harvard Apparatus) and an extraction pump (Syringe Pump Fusion200 model, Chemyx Inc., Stafford, Texas) placed on either side of a flow chamber

(RC-20 small volume closed bath imaging chamber, Harvard Apparatus, Canada) on the microscope stage. A dual in-line heater/cooler device (Warner Instruments, CT, USA) was connected to a water reservoir that maintained the system at physiological temperature (37 °C). A video camera (Sony NEX-5, Sony Canada) was connected to capture adherent RBCs. A computer monitor was connected to the system to upload videos of adherent RBCs. HUVECs were grown to confluence (Figure 2.1) in 75 cm^2 flasks then transferred to fibronectin treated coverslips (Thermo Scientific, Rochester, NY) which were used to form the base of the flow chamber (RC-20, Harvard Apparatus, Canada). The assembled flow chamber was mounted on a microscope (Nikon Eclipse TE-2000 U Microscope, Nikon, Canada) on the microscopic stage. An RBC suspension at 1.5% hematocrit in 1× PBS (Ca^{2+}/Mg^{2+} free), 1% human serum albumin (HSA) was placed on the HUVEC monolayer at zero flow rate for 20 minutes at 37 °C. PBS was then perfused across the suspension in stepwise increases of shear stress (representative of physiological shear stress) from 0.1 Pa to 0.5 Pa in 30 second increments as shown in Table 2.1.

Table 2.1- Calculated flow rates of corresponding shear stresses for adhesion assay

Stress (Pa)	0.10	0.15	0.20	0.25	0.30	0.40	0.50
Pump flow rate (ml/min)	0.60	0.90	1.20	1.50	1.80	2.40	3.00

Intervals for the 20 minute incubation, 10 minute RBC perfusion, buffer wash, and stepwise increases of shear stress were timed with preset stop watches.

2.2.5.1 Flow rate determination

RBC flow rates (Q) were determined with equation 2.1 (48) that describes the wall shear stress τ_w of a Newtonian fluid with constant viscosity in a parallel plate system. Equation 2.1 shows that the shear stress is zero at the centre line between the parallel plates and increases linearly to a maximum on the plate surface:

$\tau_{w =} 6Q\mu/wh^2$, **(2.1)**

where

Q = the volumetric flow rate (cm^3/s),

μ = is the viscosity of the medium (0.1 dyne s/cm^2), (48)

w = is the chamber width, and

h = is the chamber height. (48-50).

The volumetric flow rate Q was determined from equation 2.1 and the corresponding shear stress values and entered into the infusion syringe pumps.

The volumetric RBC flow rate Q was determined with equation 2.2:

$Q = \tau_w wh^2/6\mu$. **(2.2)**

With syringes and flow chamber in place and all temperature control units on, experiments were begun by pressing RUN and START buttons on the infusion and withdrawal (push and pull) pumps, respectively. RBCs adherent to HUVECs were recorded for playback, counted manually using a camera (Nex-3, Sony, Canada), and the number of RBCs was plotted as a function of increasing shear stress. Adherent RBCs were counted within one field of view at 60× magnification.

2.2.5.2 Adherence strength

A logarithmic relationship was derived from the initial plot of mean number of adherent RBC to HUVEC cells versus the reciprocal shear stress according to Relevy *et al.* (51) and equation 2.3, to determine the adherence strength.

$N = N_{\infty} + \alpha/\tau$ (49) **(2.3)**

where

N = the number of adherent RBCs at a specific shear stress (τ),

α = the adherence coefficient expressing the strength of intercellular interactions,

$1/\tau$ = the reciprocal shear stress (τ), and

N_{∞} = the number of adherent RBCs extrapolated at infinite shear stress.

The line of best fit was used to derive the coefficient of adherence (strength of RBC-EC interaction). The gradient from newly derived (modified) equation 2.4, from the logarithmic line of best fit gave the adherence strength:

N = $\alpha \ln\tau + N_{\infty}$ **,** **(2.4)**

where

N = the number of adherent RBCs at a specific shear stress (τ),

α = the adherence coefficient expressing the strength of intercellular interactions,

$\ln\tau$ = shear stress, (τ) expressed as a natural logarithm, and

N_∞ = the number of adherent RBCs extrapolated at infinite shear stress.

Figures 2.5 and 2.6 represent data obtained when a plot of mean number of adherent RBCs versus increase in shear stress τ and the reciprocal of shear stress ($1/\tau_w$) are transformed to the line of best fit to determine adherence strength.

2.2.5.3 Controls

7 days old RBCs were used as the negative controls since they are relatively not adherent to the EC monolayer of HUVEC cells (51). *In vitro* membrane damage was induced with oxidants (tert-butyl-hydro peroxide, t-BuOOH (Sigma-Aldrich) and NEM N-ethylmaleimide (Sigma Aldrich) (the gold standard for inducing oxidative injury (52)) and the antioxidant vitamin E (α-tocopherol (53)). Further assessment of RBC-EC interactions were evaluated using sickle-shaped RBCs (ssRBCs) which are known to be exceptionally adherent to EC monolayers (53,54).

NEM and vitamin E (α-tocopherol) treatments of RBCs were performed according to Stewart *et al.* (55) and Koshkaryev *et al.* (53), respectively. Briefly, RBCs were adjusted to 30% hematocrit and incubated for 30 minutes at room temperature with TBS (Tris-buffered saline) containing 10 mmol/L NEM. RBCs were washed twice in 1× TBS without NEM then diluted to 16% hematocrit in TBS containing 1.1 mmol/L $CaCl_2$ for 3 minutes at 37 °C. Calcium ionophore was added to a final concentration of 4 µmol/L and the RBCs were washed three times in TBS. For vitamin E treatment, an RBC sample was isolated from plasma by centrifugation for 10 min at 1000 × g, washed three times in Ca^{2+}/Mg^{2+} free PBS, and suspended to 40% hematocrit in PBS supplemented with 1 mmol/L

$CaCl_2$. The RBC suspension was then incubated with vitamin E at 37 °C for 60 min at a final concentration of 200 µM vitamin E.

t-BuOOH treatment was performed at an RBC hematocrit of 15% according to Stadnick *et al.* (56). Briefly, after initial processing, RBC samples were treated with t-BuOOH at a final concentration of 2 mM t-BuOOH and incubated for 15 minutes at 37 °C on a rotating shaker. Samples were then washed three times with 1.5 ml PBS at 1000 g for 5 minutes and the RBC pellet was resuspended in PBS buffer to a final hematocrit of 1.5% in preparation for the adhesion assay analysis. Video recordings and images of treated RBCs adherent to HUVEC cells were obtained to establish positive controls for the method.

ssRBCs were obtained with informed consent from a patient at the Edmonton Sickle Cell Clinic. Images of ssRBCs adherent to HUVEC cells per field of view at 0.1 Pa were obtained. Sickled RBCs were selected as they are known to be adherent to the EC monolayer in parallel plate flow chambers under flow conditions mimicking those in post capillary venules. (57),(58)

2.3 Results

Figure 2.1 depicts images of HUVECs taken from different coverslips after 48 hours to assess HUVEC proliferation before performing the RBC adherence assay. All HUVECs appeared to be confluent and healthy.

Figures 2.2 and 2.3 show the adhesion apparatus setup. Figure 2.2 is an illustration of the complete system; Figure 2.3 shows a side view of infusion, a side view of extraction/pull, and an overhead view of the complete setup. RBCs were perfused from the infusion side of the pump into the flow chamber where

they were incubated for 20 minutes in static conditions. The RBC suspension was then flushed out of the chamber via the extraction syringe and RBCs adherent to HUVEC cells inside the chamber were counted.

Figure 2.4 shows how the RBCs were counted at varying degrees of shear stress. The arrow below the images indicates the direction of RBC flow. The highest number of adherent RBCs were seen at the lowest shear stress (0.1 Pa) (Figure 2.4A) followed by a decrease in RBCs adherence as a medial shear stress range of 0.25 Pa (Figure 2.4B) was reached. Some RBCs that were previously adherent had been detached. At the highest shear stress (0.5 Pa) (Figure 2.4C), most or all of the RBCs were detached from the EC monolayer.

Figures 2.5 and 2.6 represent the information obtained after performing the adhesion assay. Figure 2.5 is a plot of the number of RBCs adherent to the EC monolayer of HUVEC cells versus increasing shear stress τ for a 7 day old (fresh) RBC sample. The adherence strength for 7 days old RBCs was determined to be 6.8 $dyne^{-1}$. Figure 2.6 shows a plot of the number of RBCs adherent to the EC monolayer of HUVEC cells versus $1/\tau$ for a 7 days old RBC sample.

Figure 2.7 shows that at a consistent shear stress of 0.1 Pa, 55 ssRBCs were adherent, 27 t-BuOOH-treated RBCs were adherent, 22 vitamin E-treated RBCs were adherent, and 14 fresh RBCs were adherent to the EC monolayer of HUVEC cells; NEM-treated RBCs were completely hemolyzed.

2.4 Discussion

The adhesion assay developed was successful in differentiating between RBCs that were adherent from RBCs that were not adherent or simply

aggregating to HUVEC cells. RBCs that were immobile or had deformed cytoskeletons and membranes but were attached to HUVEC cells and resisted stepwise increases of shear stress were determined to be adherent. The assay took approximately an hour per sample to perform which may be more time consuming and less efficient than other types of adhesion assay (11,50,59),however, with further validation and research the assay time can be shortened. The assay detected adherence to HUVEC cells of oxidized/modified RBCs. Oxidizing agents were demonstrated by Koshkaryev *et al.* to significantly induce PS externalization (53,54) and hence increase RBC-EC interactions. ssRBCs are also known to be adherent (58,60,61) and results confirmed that 55 RBCs were adherent to HUVEC cells at 0.1 Pa.

Other methodologies to demonstrate adhesion utilize automatic RBC counting software such as Image Pro Plus (49,51,62) whereas we used a manual approach. Hematocrits of 1–1.5% allowed for adequate visualization of adherent RBC behaviour during and after the wash procedure. A higher hematocrit might extend the time necessary to clear the field of view and could make counting difficult. Washing was done to remove plasma and prevent nonspecific binding of RBCs to the EC monolayer. This might have removed older or younger populations of RBCs, however, washing was essential to visualize adherent RBC behaviour.

In Barshtein *et al.* (50) a transducer and a formula to determine chamber pressure was used to analyse RBC adhesion whereas we opted to use the momentum balance equation for a fluid assumed to have a Newtonian nature

(section 2.2.5.1). Transducers might have been more sensitive instruments to use to the pressure changes encountered in the flow chamber. Although there were inherent limitations that existed with our method of analysis through the use of the equations 2.1 and 2.2, R^2 values obtained for the graphical determination of adherence strength were 0.85 and greater

Barshstein *et al.* (49,51) counted the number of adherent RBCs within 10 random fields of view. We counted adherent RBCs within one field as it proved difficult to keep track of multiple fields of view while attempting to maintain the *in vitro* environment at physiological conditions. Moving the stage without a reference point would have made accurate counting difficult and sources of error might be introduced.

HUVECs up to 14 passages old were used by other investigators such as Sparrow *et al.* (59) whereas we stopped at passage eight to reduce the biological variability that would occur as the monolayers aged and were continuously passaged, possibly changing the cell's adhesion properties and the integrity of the assay. The specificity of the assay was a nonissue as all units were leukoreduced and adherent RBCs were easily discernible from the HUVEC monolayer background as illustrated in Figures 2.4 and 2.7, respectively.

It is important to compare our RBC adhesion assay to other methodologies that have a similar goal. The micropipette assay allows for the determination of adherent strength using a micropipette and suction to add and remove a single RBC from an EC monolayer. Adherent strength is quantified by reaspiration at increasing pressures. Measuring the amount of cell membranes pulled into a

micropipette allows a direct assessment of membrane properties, however, this method has limited ability to study the effects of cell viscosity and reduced surface to volume ratio, and is not well suited to model RBC physiological adhesion to endothelial cells. Moreover, this method is not applicable in a clinical laboratory as it is a very laborious method where only a single RBC can be examined at a time.

The cone and plate viscometer consists of a cone that rotates and a plate that is held stationary and can accommodate several coverslips (44). RBC suspensions are placed between the rotating cone and stationary plate where endothelialized coverslips can be inserted. Shear stress can be calculated using a formula for a Newtonian fluid which is different from the formula used for a parallel plate flow chamber (44).

The cone-and-plate device has a small operating fluid volume and the sample is subject to significant evaporation. As a result of the small medium volume and lack of gas exchange/pH control, the local increase of compounds released from RBCs or ECs in this system is not equal to a normal physiological response and can lead to misleading conclusions concerning proper RBC-EC interactions in *in vivo* settings (44).

Microfluidic devices for investigating sickle cell vaso-occlusion on endothelial cells are recent innovations that are now in use. An *in vitro* microfluidic platform features micro channels the size of post-capillary venules (30 μm) with human endothelial cells cultured within and completely lining the entire inner surface of those micro channels. (63,64). The “microvasculature-on-a-

chip" enables visualization of blood cell-endothelial cell interactions during vaso-occlusion under a controlled hemodynamic environment and provides a platform to study the effect of vaso-occlusion on endothelial cells. It represents a robust but expensive means of determining vaso-occlusive properties (45,46).

In static assays RBCs are incubated with the HUVECs under no-flow conditions at 37 °C for 20-30 minutes then non adherent RBCs washed off leaving the adherent RBCs behind to be counted. The adhesion of RBCs to the extracellular matrix of many cell types (epithelial cells and fibroblasts) can be assessed with this method (65). The assays are relatively simple to perform and provide a valuable assessment of the adhesiveness of cells of a defined extracellular matrix substrate (e.g., fibronectin), however they simulate adhesion that occurs in blood under shear stress poorly (63).

The human umbilical vein model represents an environment closer to *in vivo* conditions than the conditions used in static assays. RBC suspensions are pumped through cannulated umbilical veins followed by cell-free medium washing and the adherent RBCs are counted. However, the flow type and local wall shear stress cannot be controlled as the flow channel geometry is only approximately known. The umbilical vein however can withstand shear stresses at the arterial level (44).

The *ex vivo* rat mesenteric vascular model assay performs in the isolated, acutely denervated, and artificially perfused rat mesocecum vasculature. More importantly, the flow path is characterised by an *in vivo* architecture and shear forces normally encountered by RBCs due to blood flow (44). The assay utilizes

intravital fluorescence microscopy and fluorescein isothiocyanate (FITC) labelling of RBCs to determine RBC-EC adhesion (10). It appears to be a robust *in vivo* means of determining RBC-EC adhesion.

Finally, parallel plate flow chambers incorporate coverslips with endothelial cells cultured and coated on one side and red blood cells injected and extracted by syringe pumps on either side of the chamber. A microscope and a video camera capture images of adherent RBCs under a controlled environment that mimics the environment in post capillary venules (65,66). Parallel plate flow chambers are appropriate for analysis of blood cell adhesion to endothelial cells, to each other, or extracellular matrix proteins. They are suited to simulating blood flow in an *in vitro* environment while applying shear stresses that cannot be characterized under static conditions (63,64). Assuming Newtonian fluid behavior, the wall shear stress τ_w applied on the cell monolayer can be calculated using equation 2.1 as previously described in Section 2.2.5.1 (62). The calculation of shear stress τ_w (equation 2.1) and the value for adherence strength $1/\tau_w$ provide a mathematical means of determining adherence strength. Adherence strength in other studies (8,56) was determined as the shear stress required to detach 50% of adherent RBCs.

Parallel plate chambers are robust and inexpensive alternatives to the aforementioned systems for investigating RBC-EC adhesion. They are designed to simulate the fluid mechanical environment encountered by cells in the vasculature. In establishing this assay, we ensured that experimental conditions

closely mimicked the physiological temperature (37 °C) by using syringe and flow chamber temperature regulators (heaters).

Future work will determine the reproducibility and accuracy of the adhesion assay described in this thesis. Comparative assessments can be made to the work of Sparrow *et al.* (59) at weekly intervals (days 7, 14, 21, 28, 35 and 42 of hypothermic storage). By determining the y-intercept of a plot of the number of adherent RBCs/shear stress (this method) versus number of adherent RBCs/shear stress (Sparrow *et al* method*.)* the standard error in the system can be estimated. The appropriate hematocrit for the adhesion assay can be determined by plotting the number of adherent RBCs versus hematocrit %. A control/run chart might be used to assess the effectiveness of this approach. Assays should be performed by different technologists on different days in different units to establish the variability of results that might be obtained beyond a research setting.

2.5 Conclusion

An assay for determining the *in vitro* RBC membrane quality of stored blood products has been developed utilizing ssRBCs as positive controls and 7 day old RBCs as negative controls. The calculated fibronectin concentration used to pre-treat the coverslips before HUVEC culturing were optimal for adequate HUVEC confluency within 48 hours. Excellent differentiation between EC adherent and EC no adherent RBCs was achieved with the adhesion assay developed here. The developed assay will be used to determine the adherent properties of stored RBCs in chapters 3 and 4.

Figure 2.1- Different fields of view of fibronectin treated coverslips after 48 hours of HUVEC confluency using the Leitz microscope at 40× magnification.

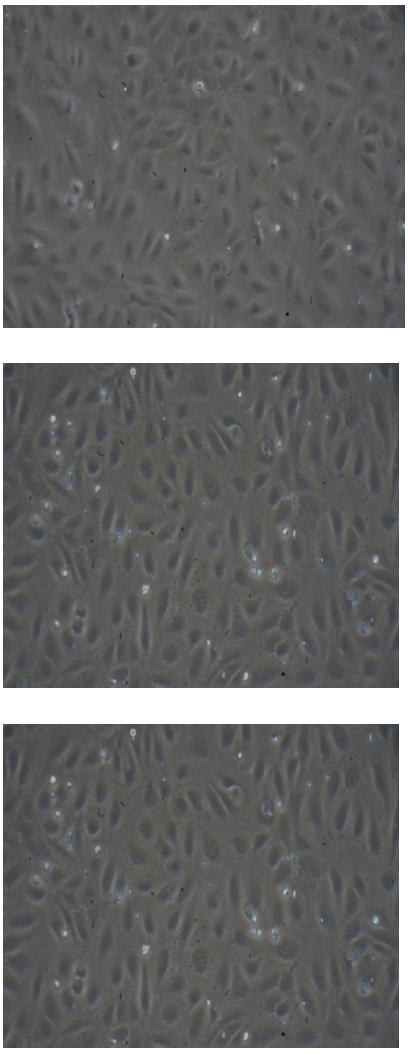
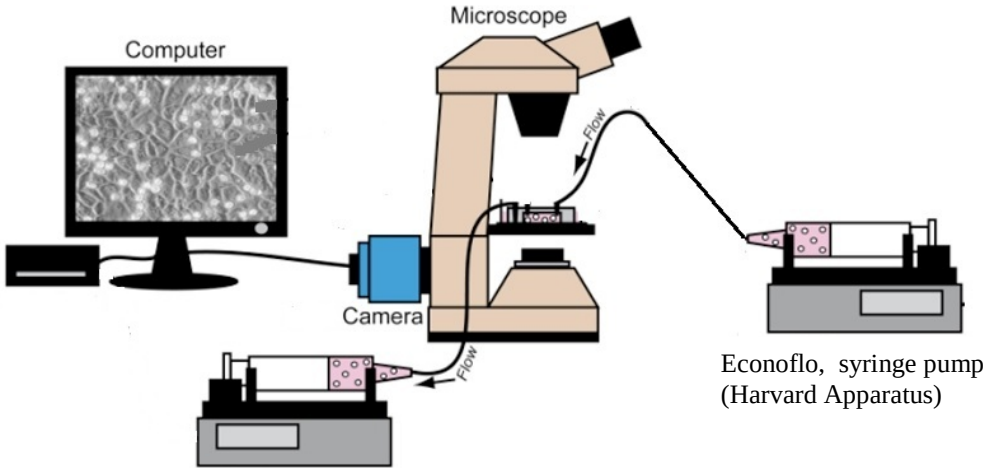


Figure 2.2- Schematic depiction of the adhesion assay.

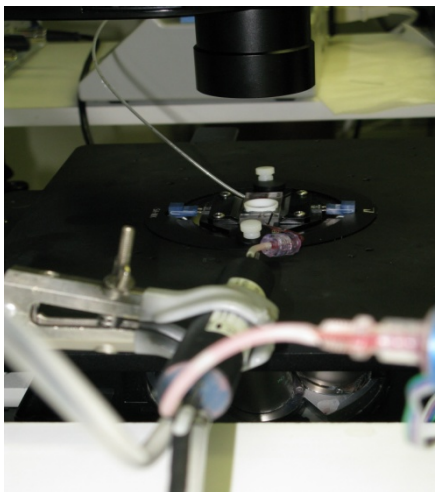


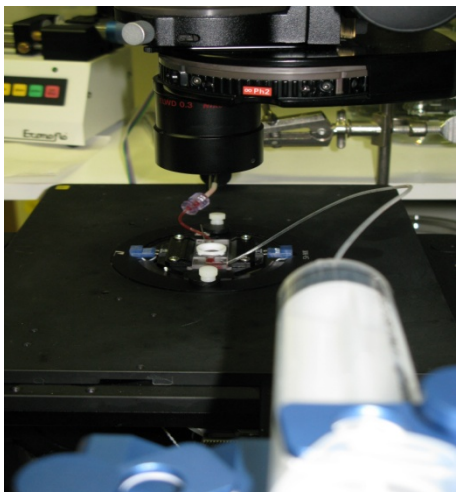
Econoflo, syringe pump (Harvard Apparatus)

Syringe pump Fusion200 model (Chemyx Inc., Stafford, Texas)

Figure 2.3- Adhesion apparatus setup; (A) infusion side of the adhesion apparatus; (B) suction side of the adhesion assay; (C) overhead view of the adhesion assay.

(A) (B)





(C)

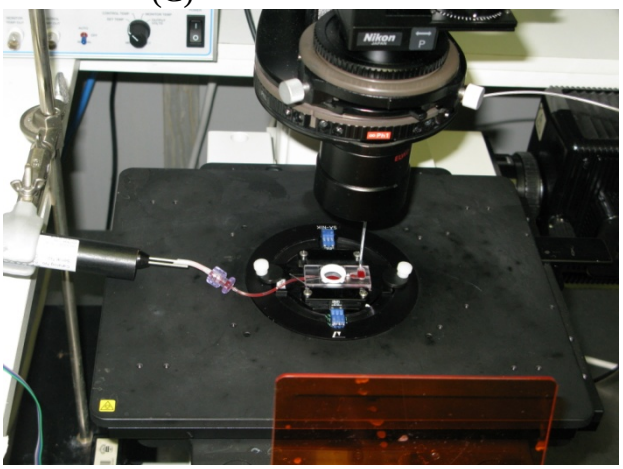


Figure 2.4- The RBC counting mechanism with untreated RBCs at a hematocrit of 1.5%; arrows indicate the direction of laminar flow (A) RBCs at low shear stress (0.1 Pa); (B) RBCs at medium shear stress (0.25 Pa) (C) RBCs at high shear stress (0.5 Pa).

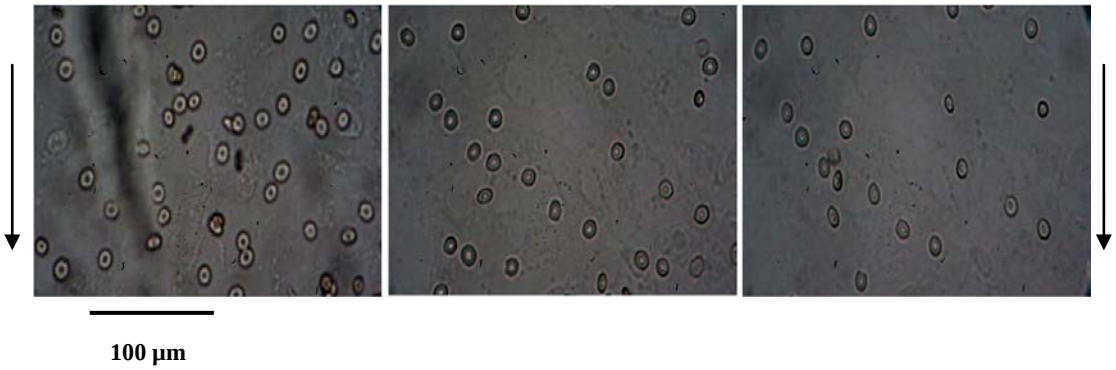


Figure 2.5- Adhesion profile for 7 day old (fresh) RBCs adherent to the EC monolayer of HUVEC cells under increasing shear stress **τ**.

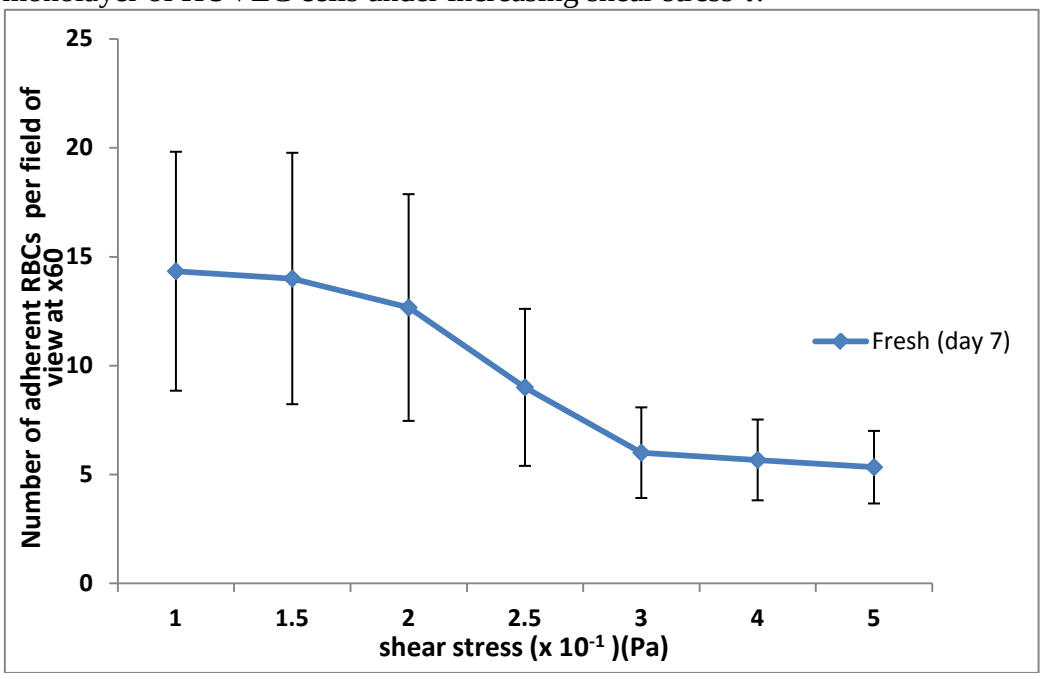


Figure 2.6- Adhesion strength profile for day 7 old (fresh) RBCs; that is, the number of RBCs adherent to the EC monolayer of HUVEC cells vs. 1/**τ**.

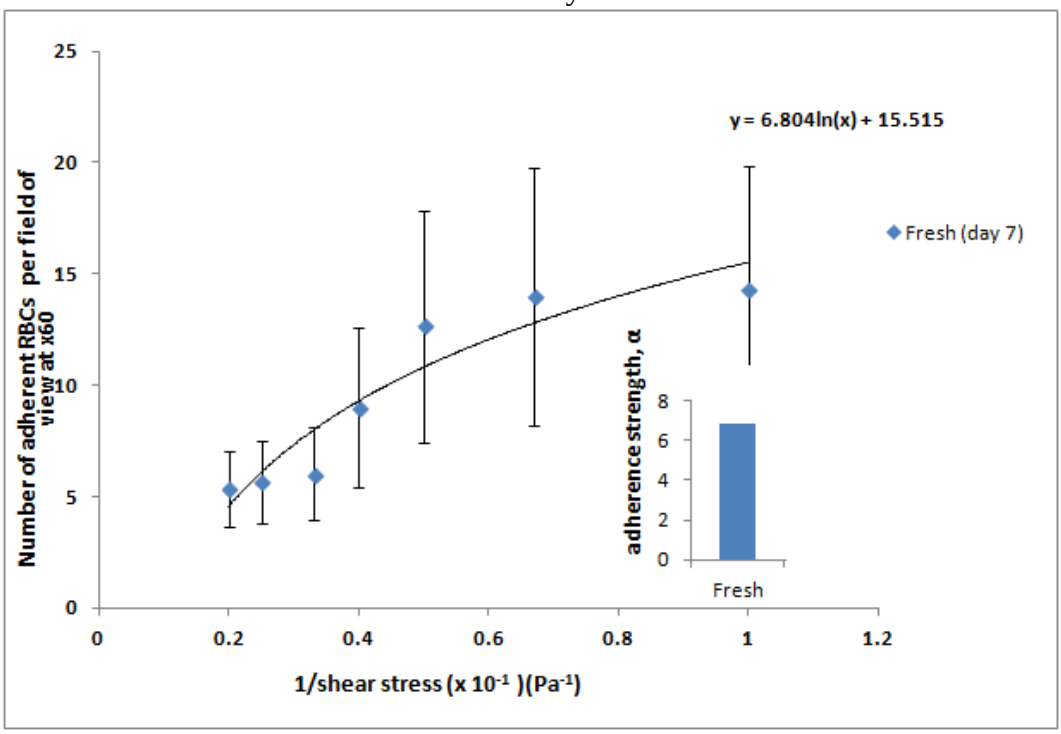
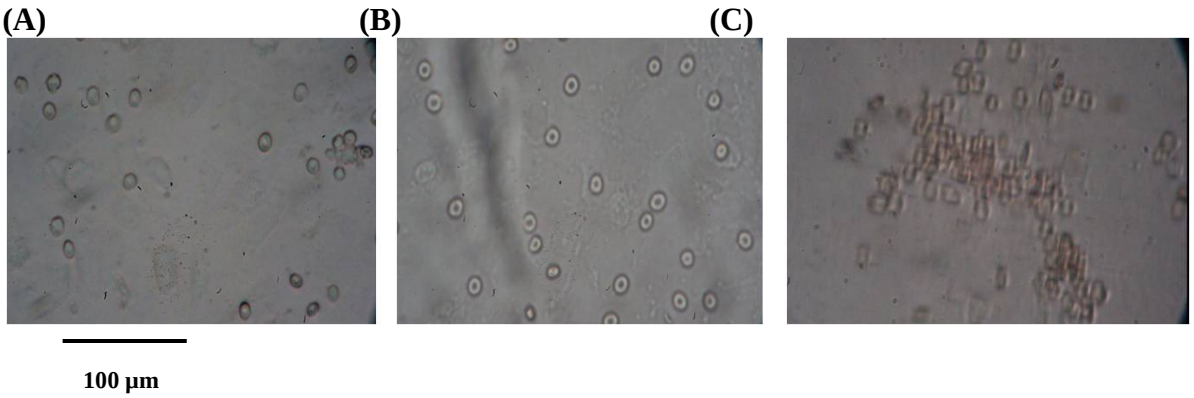


Figure 2.7-Adhesion of treated RBCs to the EC monolayer of HUVEC cells (A) t-BuOOH-treated RBCs (B) vitamin E-treated RBCs (C) ssRBCs.



2.6 References

(1) Lipowsky HH. Microvascular rheology and hemodynamics. Microcirculation 2005;12(1):5-15.

(2) Hedley DW, Chow S. Evaluation of methods for measuring cellular glutathione content using flow cytometry. Cytometry 1994;15:349-358.

(3) Yedgar S, Kaul DK, Barshtein G. RBC adhesion to vascular endothelial cells: more potent than RBC aggregation in inducing circulatory disorders. Microcirculation (New York) 2008 2008;15(7):581-583.

(4) Barshtein G, Ben-Ami R, Yedgar S. Role of red blood cell flow behavior in hemodynamics and hemostasis. Expert Rev. Cardiovasc. Ther. 2007;5(4):743-752.

(5) Hebbel RP, Moldow CF, Steinberg MH. Modulation of erythrocyte-endothelial interactions and the vasocclusive severity of sickling disorders Blood 1981;58(5):947-952.

(6) Brittain HA, Eckman JR, Swerlick RA, Howard RJ, Wick TM. Thrombospondin from activated platelets promotes sickle erythrocyte adherence to human microvascular endothelium under physiologic flow: a potential role for platelet activation in sickle cell vaso-occlusion Blood 1993;81(8):2137-2143.

(7) Wautier JL, Pintigny D, Wautier MP, Paton RC, Galacteros F, Passa P, et al. Fibrinogen, a modulator of erythrocyte adhesion to vascular endothelium. J. Lab. Clin. Med. 1983;101(6):911-920.

(8) Yang Y, Eng HL, Neu B. Erythrocyte-Endothelium Adhesion Can Be Induced by Dextran. Langmuir 2010;26:2680-2683.

(9) Telen M. Red blood cell surface adhesion molecules: their possible roles in normal human physiology and disease. Sem. Hematol. 2000;37(2):130-142.

(10) Chin-Yee IH, Gray-Statchuk L, Milkovich S, Ellis CG. Transfusion of stored red blood cells adhere in the rat microvasculature. Transfusion 2009;49(11):2304-2310.

(11) Anniss AM, Sparrow RL. Variable adhesion of different red blood cell products to activated vascular endothelium under flow conditions. Am. J. Hematol. 2007;82(6):439-445.

(12) Luk CS, Gray-Statchuk LA, Cepinkas G, Chin-Yee IH. WBC reduction reduces storage-associated RBC adhesion to human vascular endothelial cells under conditions of continuous flow in vitro. Transfusion 2003;43(2):151-156.

(13) Brown MD, Wick TM, Eckman JR. Activation of vascular endothelial cell adhesion molecule expression by sickle blood cells. Fetal Pediatr. Pathol. 2001;20(1):47-72.

(14) Madamanchi NR, Hakim ZS, Runge MS. Oxidative stress in atherogenesis and arterial thrombosis: the disconnect between cellular studies and clinical outcomes. J. Thromb. Haemost. 2005;3(2):254-267.

(15) Rattan V, Shen Y, Sultana C, Kumar D, Kalra VK. Diabetic RBC-induced oxidant stress leads to trans endothelial migration of monocyte-like HL-60 cells. Am. J. Physiol. 1997;273(2 Pt 1):E369-E375.

(16) Sultana C, Shen Y, Rattan V, Johnson C, Kalra VK. Interaction of sickle erythrocytes with endothelial cells in the presence of endothelial cell conditioned medium induces oxidant stress leading to trans endothelial migration of monocytes Blood 1998;92(10):3924-3935.

(17) Brittain J, Hans J, Ataga K, Orringer E, Paris L. Mechanism of CD47 induced alpha beta integrin activation and adhesion in sickle reticulocytes. J. Biol. Chem. 2004;279(41):42393-42402.

(18) Brittain J, Mlinar K, Anderson C, Orringer E, Paris L. Integrin associated proteins is an adhesion receptor on sickle red blood cells for immobilized thrombospondin. Blood 2001;97(7):2159-2164.

(19) Brown E, Frazier W. Integrin associated protein (CD47) and its ligands. Trends Cell Biol. 2001;11(3):130-135.

(20) Dahl K, Westhoff C, Discher D. Fractional attachment of CD47 (IAP) to the erythrocyte cytoskeleton and visual colocolization with Rh protein complexes. Blood 2003;101(3):1194-1199.

(21) Garratty G, Telen M, Petz L. Red cell antigens as functional molecules and obstacles to transfusion. Hematology 2002:445-462.

(22) Goel M, Diamond S. Adhesion of normal erythrocytes aat depresses venous shear rates to activated neutrophils activated platelets and fibrin polymerized from plasma. Blood 2002;100(10):3797-3803.

(23) Hermand P, Gane P, Huet M, Jallus V, Kaplans C, Sonneborn H, et al. Red cell ICAM-4 is a novel ligand for platelets activated alpha IIb beta 3 integrin. J. Biol. Chem. 2002;278(7):4892-4898.

(24) Hillery C, Du M, Montgomery R, Scott J. Increased adhesion of erythrocytes to components of the extracellular matrix: isolation and characterization of a red

blood cell lipid that binds thrombospondin and laminin. Blood 1996;87(11):4879-4886.

(25) Ihanus E, Uotila A, Toivanen M, Varis M, Gahmberg C. Red cell ICAM4 is a ligand for the monocyte/macrophage integrin CD11c/CD18:characterization of the binding sites on ICAM-4. Blood 2007;109(2):802-810.

(26) Mankelow T, Sping F, Parsons S, Brady R, Mohandas N, Chasis J, et al. Identification of critical amino acid residues on the erythroid intercellular adhesion molecule-4 (ICAM-4) mediating adhesion to alpha v integrin. Blood 2004;103(4):1503-1508.

(27) Matsui N, Borsig L, Rosen M, Yaghmai A, Varki and SH Embury. P-selectin mediates the adhesion of sickle erythrocytes to the endothelium. Blood 2001 98;6:1955-1962.

(28) Pasini EM, Kirkegaard M, Mortenseen P, Lutz H, Thomas A, Mann M. In depth analysis of the membrane cytosolic proteome of red blood cells Blood 2006(108):791-801.

(29) L. Sargento C, Saldanha C, Monteiro C, Perdigao and J Martins-Silva. Evidence of prolonged disturbances in the haemostatic, hemorheologic and inflammatory profiles in trans mural myocardial infarction survivors. Thromb. Haemost. 2003;89(5):892-903.

(30) Telen M. Erythrocyte adhesion receptors: blood group antigens and related molecules. Transfus. Med. Rev. 2005;19(1):32-44.

(31) Wautier J, Wautier M. Erythrocytes and platelet adhesion to endothelium are mediated by specialized molecules. Clin. Hemorheol. Micro. 2004;30(3,4):181-184.

(32) Wautier M, Khodabandehlou C, Le Devehat and Wautier, L. Modultion of RAGE expression influences the adhesion of red blood cells from diabetic patient. Clin. Hemorheol. Micro. 2006;35(3):379-386.

(33) Zen Q, Cottman M, Truskey G, Fraser R, Telen M. Critical factors in basal cell adhesion molecule/ Lutheran-mediated adhesion to laminin. J. Biol. Chem. 1999;274(2):728-734.

(34) De Oliveira S, Saldanha C. An overview about erythrocyte membrane Clin. Hemorheol. Micro. 2010;44:63-74.

(35) Montes R, Eckman J, Hsu L, Wick T. Sickle erythrocyte adherence to endothelium at low shear: role of shear stress in propagation of vaso-occlusion. Am. J. Hematol. 2002;70:216-227.

(36) Yedgar S, Hovav T, Barshtein G. Red blood cell intercellular interactions in oxidative stress states. Clin. Hemorheol. Micro. 1999;21(3-4):189-93

(37) Yedgar S, Koshkaryev A, Barshtein G. The red blood cell in vascular occlusion. Pathophysiol. Haemost. Throm. 2002;32(5-6):263-268.

(38) Purdy FR, Tweeddale MG, Merrick PM. Association of mortality with age of blood transfused in septic ICU patients. Can. J. Anaesth. 1997;44(12):1256-1261.

(39) Basran S, Frumento R, Cohen Aea. The association between duration of storage of transfused red blood cells and morbidity and mortality after reoperative cardiac surgery. Anesth. Analg. 2006;103:15-20.

(40) Gauvin F, Spinella PC, Lacroix J, Choker G, Ducruet T, Karam O, et al. Association between length of storage of transfused red blood cells and multiple organ dysfunction syndrome in pediatric intensive care patients. Transfusion 2010;50(9):1902-1913.

(41) Vamvakas e, Carven J. Transfusion and postoperative pneumonia in coronary bypass graft surgery:effect of the length of storage of transfused red cells. Transfusion 1999;39:701-710.

(42) Moore FA, Moore EE, Sauaia A. Blood Transfusion: An Independent Risk Factor for Postinjury Multiple Organ Failure. Arch. Surg. 1997;132(6):620-625.

(43) Barshtein G, Manny N, Yedgar S. Circulatory Risk in the Transfusion of Red Blood Cells With Impaired Flow Properties Induced by Storage. Transfusion Medicine Reviews 2011;25:24-35.

(44) Shiu Y, Mcintyre L. In vitro studies of erythrocyte-vascular endothelium interactions. Ann. Biomed. Eng. 2003;31:1299-1313.

(45) Tsai M, Kita A, Leach J, Rounsevell R, Huang JN, Moake J, et al. In vitro modeling of the microvascular occlusion and thrombosis that occur in hematologic diseases using microfluidic technology. J. Clin. Invest. 2012;122(1):408-418.

(46) Rosenbluth MJ, Lam WA, Fletcher DA. Analyzing cell mechanics in hematologic diseases with microfluidic biophysical flow cytometry. Lab. Chip 2008;8(7):1062-1070.

(47) Cell adhesion and viability in HUVEC monolayers after low temperature exposure - ProQuest Dissertations & Theses (PQDT) - ProQuest Available at: http://search.proquest.com/pqdtft/docview/304787664/fulltextPDF/13955397DAB571211E8/1?accountid=14474. Accessed 9/22/2012, 2012.

(48) Bird, R.B., Stewart, W.E., and Lightfoot, E.N. Transport Phenomena. New York: Wiley; 1960. p. 47.

(49) Koshkaryev A, Zelig O, Manny N, Yedgar S, Barshtein G. Rejuvenation treatment of stored red blood cells reverses storage-induced adhesion to vascular endothelial cells. Transfusion (Malden) 2009;49(10):2136-2143.

(50) Barshtein G, Relevy H, Koshkaryev A, Sayag L, Manny N, Yedgar S. Blood banking-induced alteration of red blood cell flow-affecting properties. Bio rheology 2005;42(1-2):40.

(51) Relevy H, Koshkaryev A, Manny N, Yedgar S, Barshtein G. Blood banking-induced alteration of red blood cell flow properties. Transfusion 2008;48(1):136-146.

(52) Closse C, Dachary-Prigent J, Boisseau MR. Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. Br. J. Haematol. 1999;107(2):300-302.

(53) Koshkaryev A, Barshtein G, Yedgar S. Vitamin E Induces Phosphatidylserine Externalization and Red Cell Adhesion to Endothelial Cells. Cell Biochem. Biophys. 2010;56(2-3):109-114.

(54) Kaul DK, Koshkaryev A, Artmann G, Barshtein G, Yedgar S. Additive effect of red blood cell rigidity and adherence to endothelial cells in inducing vascular resistance. Am. J. Physiol Heart Circ. Physiol. 2008;295(4):H1788-H1793.

(55) Stewart A, Urbaniak S, Turner M, Bessos H. The application of a new quantitative assay for the monitoring of integrin-associated protein CD47 on red blood cells during storage and comparison with the expression of CD47 and phosphatidylserine with flow cytometry. Transfusion 2005;45(9):1496-1503.

(56) Stadnick H, Onell R, Acker JP, Holovati JL. Eadie-Hofstee analysis of red blood cell deformability. Clin. Hemorheol. Micro. 2011;47:229-239.

(57) Barabino GA, McIntire LV, Eskin SG, Sears DA, Udden M. Endothelial cell interactions with sickle cell, sickle trait, mechanically injured, and normal erythrocytes under controlled flow Blood 1987;70(1):152-157.

(58) Barabino G, Platt M, Kaul DK. Sickle Cell Biomechanics. Ann. Rev.Biomed. Eng. 2010;12:346-367.

(59) Anniss AM, Sparrow RL. Storage duration and white blood cell content of red blood cell (RBC) products increases adhesion of stored RBCs to endothelium under flow conditions. Transfusion 2006;46(9):1561-1567.

(60) Barabino GA, McIntire LV, Eskin SG, Sears DA, Udden M. Rheological studies of erythrocyte-endothelial cell interactions in sickle cell disease. Prog. Clin. Biol. Res. 1987;240:113-127.

(61) Barabino G, Mcintire L, Eskin S, Sears D, Udden M. Endothelial-cell interactions with sickle-cell, sickle trait, mechanically injured, and normal erythrocytes under controlled flow. Blood 1987;70(1):152-157.

(62) Koshkaryev A, Manny N, Zeleg O, Yedgar S, Barshtein G. "Rejuvenation" treatment repairs cold storage-induced damage of red blood cell hemodynamic properties. Biorheology 2008 2008;45(1-2):150.

(63) Kucik DF, Wu C. Cell migration; Cell-Adhesion Assays 2004;294:43-54.

(64) Lawrence MB. Threshold levels of fluid shear promote leukocyte adhesion through selections (CD62L,P,E). J. Cell Biol. 1997;136(3):717-727.

Chapter 3

The effects of cryopreservation on erythrocyte adherence and deformability

[1]

[1] A version of this chapter has been presented at Extreme Cryo and has been submitted for publication in Biopreservation and Biobanking. It is currently in press. Conventional assay data were collected and analyzed by Jayme Tchir, Tracey Turner and Adele Hansen. Nonconventional assays(ektacytometry and adhesion) were performed by Julien Nunes

3.1 Introduction

Red blood cell (RBC) cryopreservation is based upon the idea that storage of cells at sub-zero temperatures halts all biochemical and biomechanical reactions to extend cellular *ex vivo* shelf life. This bio preservation technique has been around for at least 150 years and led to the development of a clinical method for RBC cryopreservation in 40 percent (wt/vol) glycerol at -80^{o}C for up to 10 years(1,2). Cryopreservation is a valuable tool for the long term storage of rare blood groups from donors and for military deployment (1,3-5). It is also beneficial in emergency and clinical settings where the demand exceeds the supply of RBCs (1,3-6).RBCs are safer in some circumstances for transfusion in limiting allogeneic transmission of diseases because of the numerous washings performed during the deglycerolization process(5,7)

A lot is known about the *in vitro* quality of cryopreserved RBCs as a result of conventional biochemical assays such as % recovery, percent hemolysis, extracellular K^{+} levels, ATP concentrations, MCV, MCHC and MCH that detects the final stages of the storage lesion(7-9), but little is known about early subtle membrane damage. Previously, the effects of cryopreservation on red blood cell membrane quality parameters such as microvesiculation, phosphatidylserine externalization and CD47 expression (9) were investigated. Results of that study demonstrated an increase in the PS expression in prefreeze samples. PS expression and externalization is correlated to increased RBC-EC interactions (16, 29). The need for more accurate predictors of *in vitro* RBC membrane quality for determining post-transfused RBC performance and its impact on clinical outcomes is needed. The current post storage quality parameters previously

highlighted in Section 1.5 of the introductory chapter stipulates that a minimal hemolysis of < 0.8-1% and at least a 75% survival rate of RBCs in the circulatory system within 24 hours of transfusion are necessary criteria for basing RBC viability (1). However these are not adequately insightful measures of subtle RBC membrane lesions, as they are not proper indicators of *in vivo* cellular function and quality (1).

RBC adhesion to vascular endothelium has been correlated to a significant increase in various pathological diseases such as malaria, stroke, sickle cell disease, beta thalassemia and diabetes mellitus (10-12). Altered RBC rheological properties have been clinically linked to many circulatory disorders (41, 42) as well as independently associated with many adverse transfusion outcomes (43-47). Subtle membrane changes arising from clinical RBC cryopreservation may induce further alterations to deformability and adhesive properties that can effect micro vascular occlusion upon subsequent transfusion.

In diseased states, studies have indicated that altered RBC membrane properties may play an important role in enhancing RBC endothelial cell (EC) interactions. Perhaps more indicative of RBC function post transfusion, is the health of the RBC membrane and its hemorheological parameters, specifically adhesion and deformability. Similarly to pathological states, RBCs for transfusion are known to have membrane shape and deformability changes as a consequence of refrigerated storage (12). It still remains to be determined whether similar changes within the membrane and cytoskeleton can occur as a result of RBC

cryopreservation. Perturbations to the RBC structure can occur which can be injurious to its membrane compromising deformability and adhesion properties.

The formation of extracellular ice crystals as well as exposure to hypertonic and isotonic solutions during the freezing and thawing process creates osmotic forces that cause cellular shrinkage and expansion which can potentially disrupt RBC cell membranes (5,13). *In vitro* disruptions in the cell membrane as a result of mechanical stress imparted by subsequent RBC volume changes during cryopreservation and deglycerolization process can lead to alterations to the peripheral and integral proteins that form the cytoskeletal framework of the RBC membrane as well as in the asymmetric distribution of the phospholipid bilayer cell membrane. Asymmetric redistribution in the phospholipid composition of the red blood cell has been associated with the *in vivo* mechanisms of erythrocyte senescence signalling and clearance through erythrophagocytosis by macrophages(9,14,15). Erythrophagocytosis involves PS externalization which has been shown to be a major participant in RBC endothelial cell interaction and blood coagulation (16,17). In addition to influencing RBC-endothelium interactions, impairment to the cytoskeletal structure as a direct consequence of cellular resizing during cryopreservation and deglycerolization might also potentially affect RBC deformability, causing an increase in cellular rigidity. The potential to impart alterations to rheological properties has been recently evaluated in frozen RBCs by Henkelman, S *et al.* (18) in which deformability was determined to be enhanced after cryopreservation. Changes to RBC adherence properties in cryopreserved RBCs still remain unaddressed.

This chapter aims to assess the subtle membrane changes that may result from the clinical RBC cryopreservation process as it relates to changes in flow properties, specifically adherence and deformability of frozen RBCs. In this study we present the data of both RBC deformability and adherence changes as a direct consequence of freezing and deglycerolization in relation to the post thaw quality of the RBC membrane utilizing ecktacytometric analysis (19) and an adhesion assay (introduced in Chapter 2), together with other conventionally used *in vitro* determinants of RBC quality.

3.2 Materials and Methods

3.2.1 RBC Processing

Leukoreduced, CPD-SAGM, packed RBC units (n = 7)from whole blood buffy coat production method that were matched for ABO groups using the pool and split study design in which one arm was studied, were obtained from the Canadian Blood Services National Inventory and netCAD (Network Centre for Applied Development, Vancouver, Canada). Pre-freeze *in vitro* RBC quality assessments included conventional assays described in detail in section 3.2.3.1 such as % recovery, hematocrit (20), % hemolysis (21), RBC indices(22) (mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin MCH), and haemoglobin content, ATP concentration(23-27), extracellular K+ concentration, % recovery). Non-conventional (adherence assay previously developed in chapter 2 of this thesis and deformability (19)) assays of biomechanical change were also performed.

3.2.2 Cryopreservation and deglycerolization

RBCs that were hypothermically stored at 1-6^{o}C for 14 days were cryopreserved and stored at < -80^{o} C for at least 24 hours according to a modified Meryman's high glycerol method (2). Briefly, glycerol solution (Glycerolyte 57, Baxter Healthcare Corp., Deerfield, IL) was added drop wise to the RBC unit to a final concentration of approximately 40 % (wt/vol.) and then placed in a metal container for freezing at a freezing rate of 1^{o} C per minute using a thermostat (1,2). The RBC units were quickly thawed for 10-15 minutes at 36 – 38^{o}C in a circulating water-bath and deglycerolized by washing with 150 mL of 12 % NaCl, 2 L of 1.6% NaCl, and 2 L of 0.2 % dextrose-0.9 % NaCl solutions (Baxter Healthcare Corp.) by serial centrifugation in a cell washer (COBE 2991, Model 1, IBM, Princeton, NJ). Post thaw assessments were performed 20-24 hours after the deglycerolization process and hypothermic storage (1-6^{o}C).

3.2.3 Assays

3.2.3.1 Conventional in vitro RBC quality assays

Conventional biochemical RBC quality assays were performed for pre-cryopreserved and post-deglycerolized samples. Briefly, the spun hematocrit (L/L) was calculated via centrifugation of the deglycerolized samples drawn up into capillary tubes from a ratio analysis of the separation of red blood cells versus the plasma. The volume of packed red cells in a given volume of whole blood is measured.

The corrected haemoglobin content was calculated by multiplying the percent recovery by the haemoglobin concentration in the sample prior to

freezing. This estimate is then added to the hemoglobin content measured in the final product.

Corrected hemoglobin content = (% recovery x [$Hb_{prefreeze}$]) +[Hb_{final}] **(3.1)**

RBC indices (mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) was determined from a Coulter automated cell counter (Coulter AcT, Beckman Coulter, New York, NY).

An electric field is applied to the aperture of the instrument once the electrode is submersed in the sample solution and the impedance between the 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 and is expressed as the average volume of cells crossing the aperture given in femtoliters (fL). 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.

RBC ATP concentrations (μmol/g Hgb) were measured in de-proteinized extracts by an enzymatic method, using a commercially available kit (Rolf

Greiner Biochemical, Flacht, Germany(9)). ATP concentrations were calculated from an enzymatic reaction whereby the amount of NADH produced from the breakdown of glucose is directly proportional to the concentration of the ATP in the original sample measured spectrophotometrically at 340 nm.

The reaction for the above assay is as follows:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glucose-6-phosphate} + \text{ADP} \qquad \textbf{(3.2)}$$

$$\text{Glucose-6-phosphate} + NAD^{+} \xrightarrow{\text{G6PD}} \text{6-phosphogluconate} + NADH + H^{+} \qquad \textbf{(3.3)}$$

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:

$$\text{ATP}\ (\mu\text{mol/dL}) = \Delta A \times V \times F \times 100 / \varepsilon_{340} \times v \times d \qquad \textbf{(3.4)}$$

Where:

ΔA = (Absorbance of the sample solution at 540 nm) – (absorbance of blank at 340 nm)

V = total volume of the sample

F = dilution factor of sample preparation

ε_{540} = extinction coefficient of NADH

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 (µmol/gHb) = 10 x ATP (µmol/dL) / Hct (L/L) x Hb (g/L) **(3.5)**

Extracellular K^+ concentrations were determined by immersing ion selective electrodes (ISE) (Beckman Coulter Unicel Synchron DXC 800 LX Clinical Systems, Beckman, Coulter, Fullerton, CA) which measures the membrane potential of ions in a diluted blood sample solution. RBC samples were centrifuged, supernatants extracted and sent to the University of Alberta Hospital Clinical Chemistry Laboratory for quantitative determination of potassium. University of Alberta Hospital Clinical Chemistry Laboratory routinely analyzes high volume of biological fluid samples for potassium concentration. The recorded membrane potential is proportional to the activity of the ion of interest which is then multiplied by the dilution factor to obtain the total ion concentration.

Percent hemolysis was determined spectrophotometrically via the cyanmethemoglobin formation (HiCN) of supernatant hemoglobin binding with Drabkin's reagent. The amount of light absorbed at 540 nm is proportional to the

supernatant hemoglobin concentration. The resultant hemoglobin concentration can be obtained from the following equation:

$$c = A_{540} \times M \times F / \varepsilon_{540} \times \ell \times 1000 \quad \textbf{(3.6)}$$

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 Hi CN at 540 nm (11.0 $cm^{-1}. mM^{-1}$)

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:

$$\% \text{ hemolysis} = (100 - \text{Spun Hct}) \times Hb_s) / Hb_T \quad \textbf{(3.7)}$$

Hct = hematocrit (%)

Hb_s = supernatant hemoglobin concentration (g/L)

Hb_T = total hemoglobin concentration (g/L)

The hematocrit 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) by independently verifying the assay is working.

3.2.3.2 Non-conventional in vitro RBC quality assays

3.2.3.2.1 RBC- EC adhesion assay

The assay was performed in accordance with chapter 2, Sections 2.2.4 and 2.2.5 of this thesis. Fresh RBCs were used as negative control and ssRBC were the positive controls. To reiterate, aliquots (1ml) were extracted from each unit and washed three times with 1X calcium and magnesium (Ca^{2+}/Mg^{2+}) free Phosphate Buffered Saline (PBS) (1000 g for 5 minutes at 4^{o}C). pRBC suspensions (1.5% hematocrit in 1X Ca^{2+}/Mg^{2+} free PBS containing 1% human serum albumin (HSA), VWR Canada) were incubated for 20 minutes at 37^{o}C under static conditions over a confluent human umbilical vein endothelial cell (HUVEC, Lonza, Cat # CC-2517) monolayer. HUVECs were grown to confluence in 75 cm^2 flasks then transferred to fibronectin treated sterilized coverslips (Thermo Scientific, Rochester, NY) which were used to form the base of the flow chamber (RC-20, Harvard Apparatus, Canada). The assembled flow chamber was mounted upon a microscope (Nikon Eclipse TE-2000 U Microscope, Nikon, Canada) using a stage adaptor and flow chamber platform for RBC-endothelial cell (EC) interaction. The number of adherent RBCs versus increases of shear stress followed by the number of adherent RBCs versus the reciprocal of the shear stress were plotted to determine adherence strength as previously described in 2.2.5.1 and 2.2.5.2.

3.2.3.2.2 Deformability

Deformability of pre-freeze and post-thaw samples was determined by ektacytometric analysis (19). RBCs were suspended in high molecular weight polymer polyvinylpyrrolidone (PVP MW~30,000) and subjected to shear stresses on a Laser Assisted Optical Rotational Cell Analyzer (LORCA, Mechatronics, the Netherlands) ranging from 0.95 to 30 Pa at a temperature of 37ºC. An internal laser beam was directed through the RBC suspension, resulting in a characteristic diffraction pattern projected onto a small screen. By rotation of the outer cylinder of the LORCA, shear stress was imparted upon the RBCs, causing their major axis to elongate. As the shear stress imparted upon the RBCs increases, the diffraction pattern detected on the projection screen transitions from circular to elliptical. An elongation index (EI) can be calculated from these diffraction patterns by the computer. As the diffraction pattern becomes more elliptical, the major elongation axis increases while the minor axis decreases. Higher elongation indexes at higher shear stresses are obtained. The resultant deformability curves were linearized using an Eadie-Hofstee transformation similar to Stadnick, H *et al* (19). The transformation requires plotting the measured elongation index vs. elongation index/respective shear stress to obtain the maximum elongation index (EI_{max}) and K_{EI}. By transforming ektacytometric RBC deformability curves, significant insight into RBC deformability changes which would otherwise be difficult to obtain by analyzing deformability plots at single shear stresses is obtained.The y-intercept of the plot corresponds to the theoretical EI_{max} while the slope represents the $-K_{EI.}$ EI_{max} can be defined as the maximum elongation index predicted at an infinite shear stress while K_{EI} is the shear stress required to

achieve half of EI_{max} (19) Essentially, K_{EI} values are a measure of RBC rigidity while EI_{max} values are an overall indicator of RBC deformability An Eadie-Hofstee transformation is illustrated in figure 3.3.

3.2.4 Statistical Analysis

Non-parametric Wilcoxon tests were performed for n = 7 samples using SPSS software version 14.0 (Lead Technologies, Charlotte, NC). Results are expressed as mean $\pm$ SEM unless otherwise specified. Comparisons were made between pre-freeze and 24 hour post-thawed/deglycerolized samples for deformability, adherence and conventional *in vitro* biochemical RBC quality parameters. Probabilities less than 0.05 were considered significant.

3.3 Results

The effects of clinical RBC cryopreservation on RBC *in vitro* quality were examined using both conventional and non-conventional parameters of RBC membrane quality.

3.3.1 Conventional in vitro RBC quality assays

The results are summarized in Table 3.1 and RBC post thaw percent recovery was determined to be 91.19 $\pm$ 1.55 %. There was a statistically significant decrease ($p = 0.017$) in hematocrit with pre-freeze versus post-thawed samples. RBC indices revealed a statistically significant increase ($p = 0.012$) in MCV as a result of cryopreservation and/or deglycerolization. MCHC values statistically decreased ($p = 0.026$) after deglycerolization. MCH values of remained statistically unchanged ($p = 0.325$) after thawing. The post-

deglycerolization extracellular K^+ after thawing was significantly reduced ($p = 0.001$).

3.3.2 Non conventional in vitro RBC quality assays

Figure 3.1 denotes the effects of cryopreservation on the mean number of RBCs interacting with the endothelial cell (EC) monolayer at increasing shear stress by comparing control pre-freeze adherent number of RBCs versus thawed adherent number of RBCs. The y axis represents the number of adherent RBCs per field of view at x 60 magnification versus increases of shear stresses on the x axis. Non-parametric tests revealed no statistically significant differences in mean number of adherent RBCs for the pre-freeze samples and the post thaw samples. Figure 3.2 is the logarithmic transformation of the mean number of adherent RBCs versus 1/shear stress. The coefficient of the gradient from the calculated transformation is known as the adherence coefficient and it is representative of the adherence strength as defined previously in Chapter 2 of this thesis. Significant increases in the overall adherence strength for post thawed RBCs were observed (Figure 3.2, inset $p = 0.005$). Figure 3.4 is a comparison of the deformability parameters (K_{EI}) and EI_{max} between pre-frozen and post thawed RBCs. The elongation index versus the shear stress was also represented internally by the inset figure. There were no significant changes for EI_{max} or K_{EI} for pre-freeze versus post thaw RBCs (Figure 3.4, $p = 0.735$ and $p = 0.310$ respectively).

3.4 Discussion

RBCs under hypothermic storage in the blood banks are subjected to many *in vitro* stresses, generated by component production, storage conditions, white blood cell remnants and RBC hemolysis which can result in increased *in vitro*

stresses. These *in vitro* stresses may play a role in subtle cellular damage incurred by the RBC membrane during cryopreservation. Furthermore, during cryopreservation, membrane restructuring properties may also disrupt the phospholipid bilayer because of osmotic pressures on RBC membrane leading to reorientation of PS towards the outer leaflet of the membrane, resulting in increased RBC-EC adherence. Additionally, extracellular ice formation resulting from slow cooling during the modified Meryman's method may further cause membrane damage that can lead to increased RBC-EC interactions after cryopreservation. Red blood cell deformability and endothelial cell adherence are important rheological determinants of the microcirculatory flow. Throughout the years, the rheological properties of liquid stored RBCs have been extensively investigated (31-40), however the influence of RBC cryopreservation on RBC adherence still remains to be determined.

Our results indicate that conventional determinants of *in vitro* RBC quality and membrane integrity are in agreement with other published data examining quality of cryopreserved RBCs (50). Despite the additional risk of RBC membrane injuries due to the cryopreservation/deglycerolization process, hemolytic values (0.46 $\pm$ 0.02) % remained well within CSA standard ranges of < 0.8 % together with percent recovery (91.0 $\pm$ 2.0) %, (Table 3.1). ATP levels were higher (3.82 $\pm$ 0.14) µg/Hglb than established values (2.7 µg/Hglb) which has been shown to be adequate indicators of post transfused RBC survival (50). This is to be expected as ATP loss is not significant until week 4-5 of hypothermic storage (51) and further decreases might have been halted by the

cryopreservation process at 14 days of hypothermic storage before cryopreservation. Moreover, re-suspension in saline dextrose solution for 24 hours probably provided extra nutrients to maintain ATP levels post cryopreservation.

The deglycerolization process might have made the RBC membrane permeable to cations and hence induced cell swelling which correlates with the increased MCV values obtained. Although RBC swelling was observed reflected by an increase in the MCV and a decreased MCHC, neither of these factors appeared to affect overall cellular deformability in agreement with similarly published results (18) which showed no correlation between increased MCV, reduced MCHC and changes to cellular deformability. The saline washes to adjust the hematocrit might have been responsible for the lowered MCHC values observed at post thaw. It is known that resuspension in autologous plasma upon transfusion might reverse the swelling upon transfusion (40). However, the clinical implications in patients who may have massive transfusion where a substantial volume of additive solution is transfused at the same time warrant further study as an added risk might be present for effecting adverse responses in multiple transfused patients. The lowered hematocrit observed might be due to resuspension (agglomeration) and washes in increasing osmolalities of saline solutuon which concurrently reduces its intracellular viscosity. The subsequent deglycerolization and resuspension in saline/dextrose solution was sufficient to significantly reduce the supernatant K^+ concentration post thaw ($p = 0.001$).

Non-conventional determinants of *in vitro* RBC quality revealed deformability in cryopreserved RBCs remained unchanged. This study is in

agreement with similar studies by Henkelman,S *et al* which demonstrated that deglycerolization does not impact RBC deformability (18) as there were no change to either K_{EI} and EI_{max} values. Usually, elevation of RBC/EC adhesion is observed at the second week of storage (37) which could be concluded to be halted at the beginning of freezing lasting up until post thaw. This correlated well with the referenced ATP values (50) as ATP is considered to be connected to membrane alterations and survival of RBCs. There was a change in the adherence strength without any effect on deformability values which may be due to the deglycerolization process (18) that might have reoriented PS from the inner to the outer membrane leaflet promoting vascular adhesion. RBCs remaining adherent were shown to adhere stronger to the endothelium after cryopreservation. This may have adverse clinical impact on the patient as shear stresses encountered in pathophysiological states are lower and therefore not strong enough to detach adherent RBCs, exacerbating an already serious condition. Alternatively, the RBCs observed to be adhering strongly ($p=0.005$) could be due to a small subpopulations of older RBCs (53) in the greater pooled pRBC sample as mixed RBC units with different storage times were used could have contributed to the increased adherence observed.

Previous findings (9) implicated PS externalization as the prominent factor which might be associated with the biomechanical damages resulting from the two week hypothermic storage, cryopreservation and 24 hour deglycerolization process. Currently, RBC units are stored for a maximum of 14 days prior to cryopreservation in Canada. It may be necessary to limit the pre-freeze storage

length prior to the cryopreservation process as they may adversely affect patient transfusion outcomes(18,32,46,) and hence current regulations regarding the pre-freeze storage length needs to be addressed.

Military personnel being the primary candidates for multiple transfusions of cryopreserved RBCs are at a greater disadvantage of receiving RBC units that have altered rheological properties due to pre-freeze hypothermic storage and cryopreservation. Altered rheological properties are linked to microvascular occlusion and circulatory disorders (18,29,30,37,59,60). Further assessments to corroborate these results and its clinical implications in an *in vivo* model are warranted. Limitations of this study included the small number of units used.

3.5 Conclusion

All conventional *in vitro* measurements of analysis remained within CSA and/or AABB regulatory standards with statistically significant deviations noted only in MCV and MCHC scores. This study reports no significant change in mean number of adherent RBC due to cryopreservation. However RBCs remaining adherent to EC were showing increasing strength of attachment which might have clinical consequences. Hence cryopreservation serves as a suitable alternative in mitigating the effects of the hypothermic storage lesion with no change to RBC deformability or significant increase in the mean number of adherent RBCs. Pre-freeze stored RBCs should not exceed 14 days as a tendency to adhere stronger to the EC monolayer was found. Further assessments of subtle membrane damages are required to ascertain the consequences of prior liquid storage and cryopreservation on RBC membrane integrity, specifically adherence strength in

an *in vivo* model. Since current regulations allow for storage of hypothermic-stored blood up to 42 days before freezing the proceeding chapter will determine the effects of hypothermic storage on RBC-EC adherence during 42 day hypothermic storage.

Table 3.1- Table of RBC quality measures for pre-freeze and post thaw samples. Values are expressed as mean ± SEM

RBC Quality Assay	Pre-freeze	Post thaw expired	Regulatory Standard	
% recovery	100	91.0 ± 2.0	≥80% in 100% of units tested	
Corrected haemoglobin content (g/unit)		49.2 + 1.45	≥ 35g/unit in at least 90% of units tested	
% hemolysis		0.46 ± 0.02	< 0.8 % (CSA standard)	
Hematocrit(L/L)	0.65 + 0.01	0.62 + 0.01	> 0.50 L/L and < 0.70 L/L in 90% of units tested	
MCV(fL)	99.76 ± 0.63	105.1 ± 0.71	80-100 fL	
MCH(pg)	30.03 ± 0.14	30.25 ± 0.15	27-31 pg	
MCHC (g/L)	300.88 ± 1.27	287.75 ± 1.57	290 g/L-350 g/L	
ATP (µgmol/Hglb)		3.82 ± 0.14	> 2.7	
% hemolysis		0.46 ± 0.02	< 0.8 % (CSA standard)	
			5 day HS	**42 day expired**
K^+ concentration		2.81 ± 0.09	12 ± 0.49	45.73 ± 0.41

.

Figure 3.1- Graph showing the effects of cryopreservation on RBC-EC adherence before and after cryopreservation. Mean number of adherent RBCs at increasing shear stress; control (prefreeze) adherent RBCs versus thawed adherent RBCs

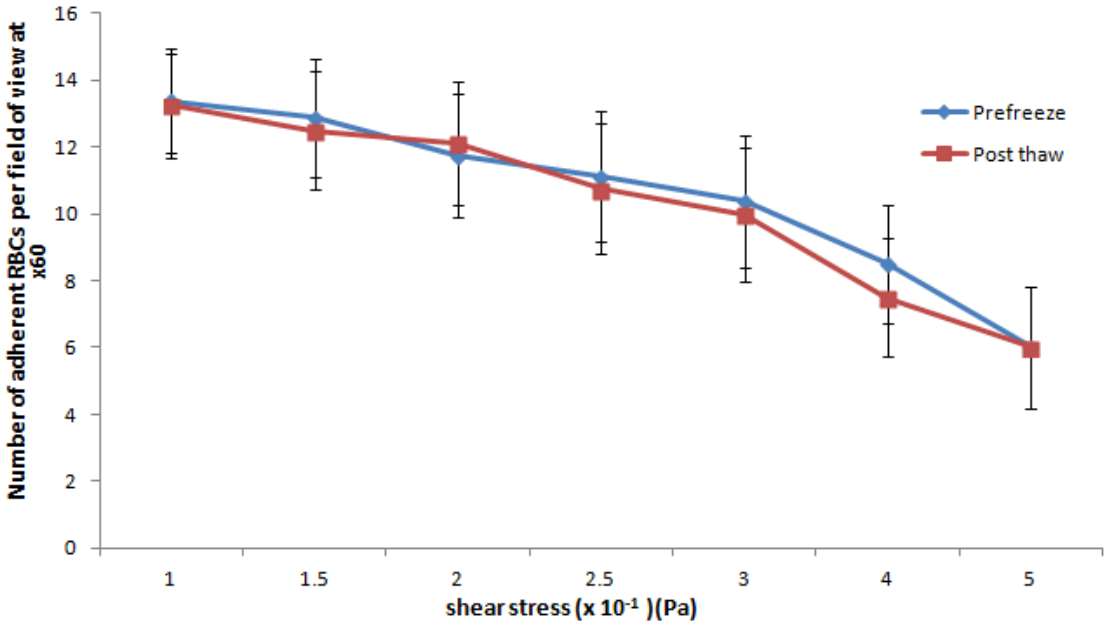


Figure 3.2- Graph of the logarithmic transformation of mean number of adherent RBCs versus 1/shear stress (adherence strength) between prefreeze and post thaw RBCs; Significant increase in overall calculated adherent strength observed for pre-freeze and post thawed samples; (Inset) the differences in the RBC adherence strength between the pre-freeze and post-thawed RBC

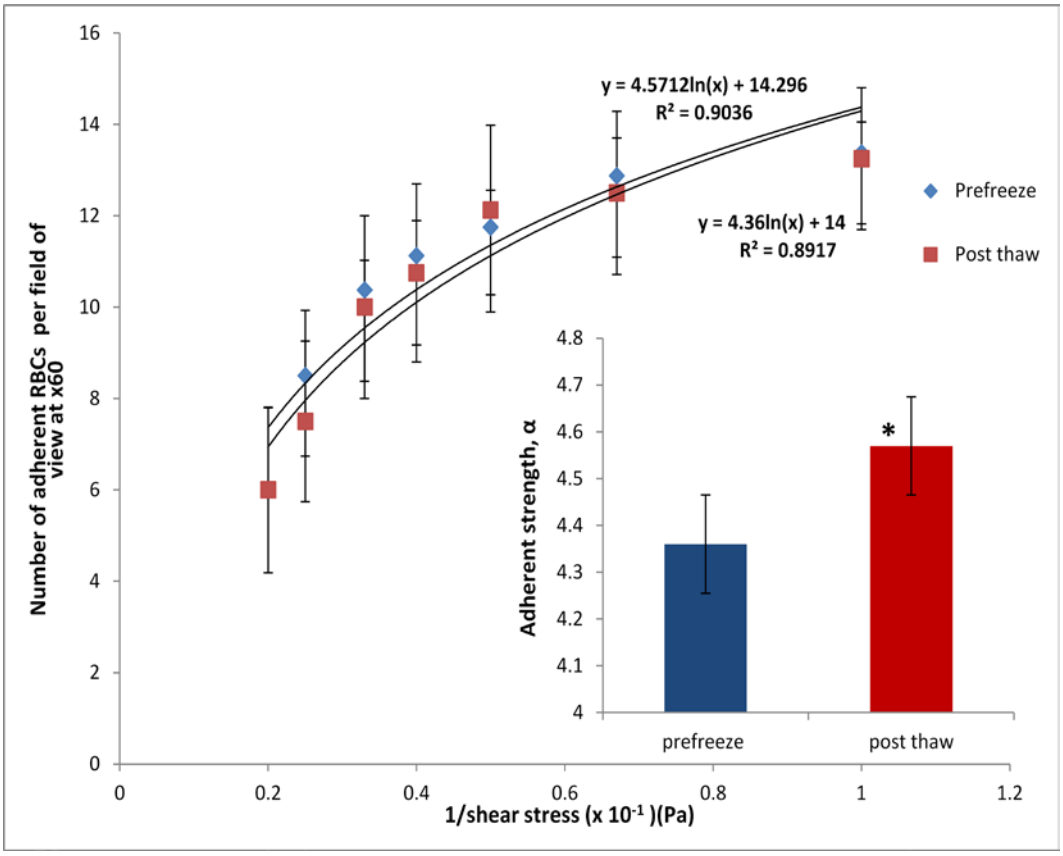


Figure 3.3- Figure showing the Eadie-Hofstee transformation to obtain EI_{max} and K_{EI} values

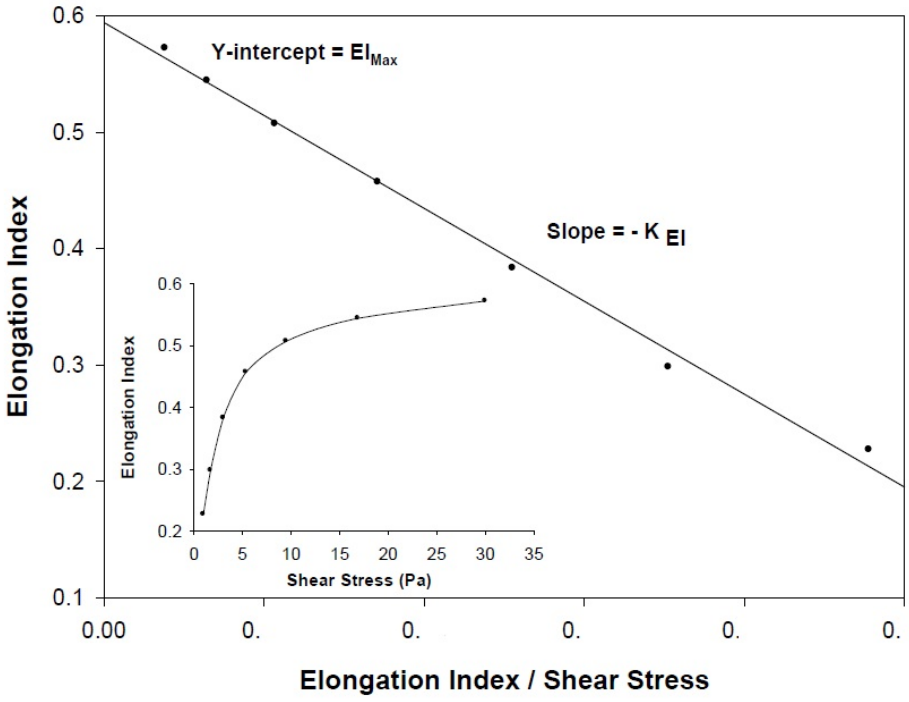
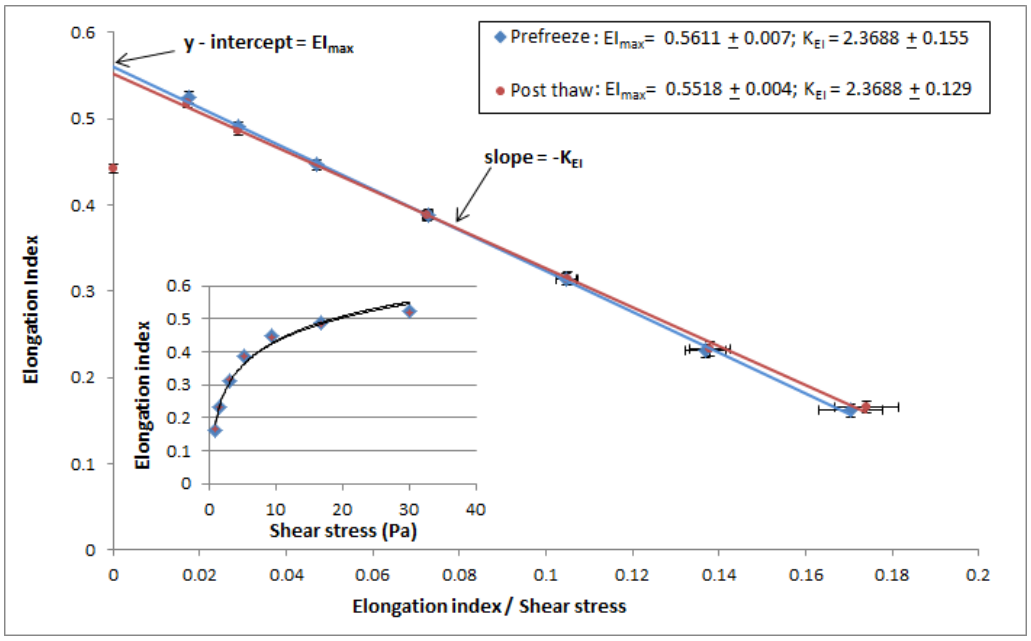


Figure 3.4- Graph showing the comparison of deformability parameters (K_{EI} and EI_{max}) between prefreeze and post thaw RBCs.



3.6 References

(1) Kakaiya R, Aronson CA, Julleis J. Whole blood collection and component processing. AABB technical manual. 16th ed. Bethesda, MD: AABB Press; 2008. p. 201-211.

(2) Meryman, H, Hornblow, M. Method for Freezing and Washing Red Blood-Cells using a High Glycerol Concentration. Transfusion 1972;12(3):145-&.

(3) Rowe AW. Cryopreservation of red blood cells. Vox Sang. 1994(67):201-206.

(4) Valeri CR, MacGregor H, Ragno G, Healey N, Fonger J, Khuri SF. Effects of centrifugal and roller pumps on survival of autologous red cells in cardiopulmonary bypass surgery. Perfusion-Uk 2006;21(5):291-296.

(5) Hess JR. Red cell freezing and its impact on the supply chain. Transfus. Med. 2004;14:1-8.

(6) Lelkens CCM, Noorman F, Koning JG, Truijens-de Lange R, Stekkinger PS, Bakker JC, et al. Stability after thawing of RBCs frozen with the high- and low-glycerol method. Transfusion 2003;43:157-164.

(7) Valeri C. Status report on the quality of liquid and frozen red blood cells. Vox Sang. 2002;83:193-196.

(8) Lee S, Ducoff H. The effects of ionizing radiation on avian erythrocytes. Rad. Res. 1994 137(1):104-110.

(9) Holovati JL, Wong KA, Webster JM, Acker JP. The effects of cryopreservation on red blood cell microvesiculation, phosphatidylserine externalization, and CD47 expression. Transfusion 2008;48:1658-1668.

(10) Yedgar S, Koshkaryev A, Barshtein G. The red blood cell in vascular occlusion. Pathophysiol. Haemost. Throm. 2002;32:263-268.

(11) Kaul DK, Finnegan E B, G.A. Sickle red cell-endothelium interactions. Microcirculation (New York, N.Y.: 1994) 2009. 16(1): 97–111.

(12) Sparrow RL. Red blood cell storage and transfusion-related immunomodulation. Blood Transfus. 2010;8(Suppl. 3):s26-s30.

(13) Pegg DE, Diaper MP. On the mechanism of injury to slowly frozen erythrocytes. Biophys. J. 1988 9;54(3):471-488.

(14) Schroit AJ, Madsen JW, Tanaka Y. In-vivo recognition and clearance of red blood cells containing phosphatidylserine in their plasma membranes. J.Biol. Chem. 1985;260(8):5131-5138.

(15) Tanaka Y, Schroit A. Insertion of fluorescent phosphatidylserine into the plasma-membrane of red-blood-cells - recognition by autologous macrophages. J. Biol. Chem. 1983;258(18):1335-1343.

(16) Koshkaryev A, Zelig O, Manny N, Yedgar S, Barshtein G. Rejuvenation treatment of stored red blood cells reverses storage-induced adhesion to vascular endothelial cells. Transfusion (Malden) 2009;49(10):2136-2143.

(17) Chung S, Bae O, Lim K, Noh J, Lee M, Jung Y, et al. Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant micro vesicle generation in human erythrocytes. Arterioscl. Thromb Vasc Biol. 2007;27(2):414-421.

(18) Henkelman S, Lagerberg JWM, Graaff R, Rakhorst G, van Oeveren W. The effects of cryopreservation on red blood cell rheological properties. Transfusion 2010;50(11):2393-2401.

(19) Stadnick H, Onell R, Acker JP, Holovati JL. Eadie-Hofstee analysis of red blood cell deformability. Clin. Hemorheol.Micro. 2011;47(3):229-239.

(20) Harmening DM . Guide to the preparation, use and quality assurance of blood components. Clinical Hematology and Fundamentals of Hemostasis. 4th ed. Philadelphia: F.A. Davis Company: Council of Europe Publishing; 2008.

(21) Haematology ICfSi. Recommendations for reference method for haemoglobinometry in human blood and specifications for international haemoglobin cyanide standard. J. Clin. Pathol. 1996;49:271-274.

(22) Turgeon ML. Theory and Procedures. Clinical Hematology. 4th ed.: Lippincott Williams and Wilkins; 2005. p. 441.

(23) Heaton WAL. The quality of red-blood-cells. Immunol. Invest. 1995;24(1-2):371-390.

(24) Dern, R.J., Brewer, GJ, Wiorkowski, J.J. Studies on the preservation of human blood. II The relationship of erythrocyte adenosine triphosphate levels and other in vitro measures to red cell storage ability. J. Lab. Clin. Med. 1967;69:968-978.

(25) Meryman,H.T., Hornblower M, Syring, RL. Prolonged storage of red cells at 4C. Transfusion 1986;26:500-505.

(26) Pittmeyer, I.C., Nydegger, U.E. Influence of the cryoprotective agents glycerol and hydroxyethyl starch on red blood cell ATP and 2,3 DPG levels. Vox Sang. 1992;62:141-145.

(27) Brecher, ME., Zylstra-Halling, VW, Pineda, AA. Rejuvenation of erythrocytes preserved with AS-1 and AS-3. Am. J. Clin. Pathol. 1991;91(6):767-769.

(28) Bird, R.B., Stewart, W.E., and Lightfoot, E.N. Transport Phenomena. New York: Wiley; 1960. p. 47.

(29) Barshtein G, Relevy H, Koshkaryev A, Sayag L, Manny N, Yedgar S. Blood banking-induced alteration of red blood cell flow-affecting properties. Biorheology 2005;42(1-2):40.

(30) Barshtein G, Manny N, Yedgar S. Circulatory risk in the transfusion of red blood cells with impaired flow properties induced by storage. Transfus. Med. Rev. 2011;25(1):24-35.

(31) Bennett-Guerrero E, Veldman TH, Doctor A, Telen MJ, Ortel TL, Reid TS, et al. Evolution of adverse changes in stored RBCs. PNAS 2007;104:17063-17068.

(32) Berezina TL, Zaets SB, Morgan C, Spillert CR, Kamiyama M, Spolarics Z, et al. Influence of storage on red blood cell rheological properties. J. Surg. Res. 2002;102(1):6-12.

(33) Hovav TF, Yedgar SF, Manny NF, Barshtein G. Alteration of red cell aggregability and shape during blood storage. Transfusion JID - 1999 39(3):277-281.

(34) Izzo P, Manicone A, Spagnuolo A, Lauta VM, Di Pasquale A, Di Monte D. Erythrocytes stored in CPD SAG-mannitol: Evaluation of their deformability. Clin. Hemorheol. Micro. 1999;21(3-4):335-339.

(35) Kirkpatrick UJ, Adams RA, Lardi A, McCollum CN. Rheological properties and function of blood cells in stored bank blood and salvaged blood. Br. J. Haematol. 1998;101(2):364-368.

(36) Nagaprasad V, Singh M. Sequential analysis of the influence of blood storage on aggregation, deformability and shape parameters of erythrocytes. Clin. Hemorheol. Micro. 1998;18(4):273-284.

(37) Relevy H, Koshkaryev A, Manny N, Yedgar S, Barshtein G. Blood banking-induced alteration of red blood cell flow properties. Transfusion 2008;48(1):136-146.

(38) Mansouri Taleghani B, Grossmann R, Waltenberger G, Geise W, Langer R, Henrich HA, et al. Storage-induced rheological and biochemical changes in erythrocyte concentrates with added solution and possible correlations. Beitr Infusionsther Transfus. Med. 1996;33:141-144.

(39) Taleghani BM, Langer R, Grossmann R, Opitz A, Halbsguth U, Buchheister A, et al. Improvement of the biochemical and rheological quality of whole blood and red cell concentrates - Measured data and their significance. Anesthesiology Intensivmedizin Notfallmedizin Schmerztherapie 2001;35:S11-S19.

(40) Zehnder L, Schulzki T, Goede JS, Hayes J, Reinhart WH. Erythrocyte storage in hypertonic (SAGM) or isotonic (PAGGSM) conservation medium: influence on cell properties. Vox Sang. 2008;95(4):280-287.

(41) Barshtein G, Ben-Ami R, Yedgar S. Role of red blood cell flow behavior in hemodynamics and hemostasis. Expert Rev. Cardiovasc. Ther. 2007;5(4):743-752.

(42) Ramot Y, Koshkaryev A, Goldfarb A, Yedgar S, Barshtein G. Phenyl hydrazine as a partial model for beta-thalassemia red blood cell hemodynamic properties. Br. J. Haematol. 2008;140(6):692-700.

(43) Hayes MA, Timmins AC, Yau EHS, Palazzo M, Hinds CJ, Watson D. Elevation of systemic oxygen delivery in the treatment of critically ill patients. N. Engl. J. Med. 1994;330(24):1717-1722.

(44) Gong MN, Thompson BT, Williams P, Pothier L, Boyce PD, Christiani DC. Clinical predictors of and mortality in acute respiratory distress syndrome: Potential role of red cell transfusion. Crit. Care Med. 2005;33(6):1191-1198.

(45) Zallen G, Offner PJ, Moore EE, Blackwell J, Ciesla DJ, Gabriel J, et al. Age of transfused blood is an independent risk factor for post injury multiple organ failure. Am. J. Surg. 1999;178(6):570-572.

(46) Koch CG, Li L, Sessler DI, Figueroa P, Hoeltge GA, Mihaljevic T, et al. Duration of red-cell storage and complications after cardiac surgery. N. Engl. J. Med. 2008;358(12):1229-1239.

(47) Marik PE, Sibbald WJ. Effect of stored-blood transfusion on oxygen delivery in patients with sepsis. JAMA 1993;269(23):3024-3029.

(48) Marossy A, Švorc P, Kron I, Grešová S. Hemorheology and circulation. Clin. Hemorheol. Micro. 2009;42(4):239-258.

(49) Alt E, Banyai S, Banyai M, Koppensteiner R. Blood rheology in deep venous thrombosis - Relation to persistent and transient risk factors. Thromb. Res. 2002;107(3-4):101-107.

(50) de Korte D FAU - Verhoeven, A.J., Verhoeven AJ. Quality determinants of erythrocyte destined for transfusion. Cell. Mol. Biol (Noisy-le-Grand, France) 2004;50(2):187-195

(51) Kor DJ, Van Buskirk CM, Gajic O. Red blood cell storage lesion. Bosn. J. Basic Med. Sci. 2009;9(Suppl.):S21-S26.

(52) Farges EF, Grebe RF, Baumann M. Viscoelastic and biochemical properties of erythrocytes during storage with SAG-M at +4 degrees C. Clin. Hemorheol. Micro. 2002;27(1):1-11 .

(53) Kuypers FA, Lewis RA, Hua M, Schott MA, Discher D, Ernst JD, et al. Detection of altered membrane phospholipid asymmetry in subpopulations of human red blood cells using fluorescently labeled annexin V. Blood 1996;87(3):1179-1187.

(54) Vandromme MJ, McGwin G, Weinberg JA. Blood transfusion in the critically ill: does storage age matter? Scand. J. Trauma Resus. Emerg. Med. 2009;17(1):35.

(55) Leal-Noval SR, Muñoz-Gómez M, Arellano-Orden V, Marín-Caballos A, Amaya-Villar R, Marín A, et al. Impact of age of transfused blood on cerebral oxygenation in male patients with severe traumatic brain injury. Crit Care Med. 2008;36(4):1290-1296.

(56) Walsh TS. Is stored blood good enough for critically ill patients? Crit. Care Med. 2005;33(1):238-239.

(57) Edgren G, Kamper-Jorgensen M, Eloranta S, Rostgaard K, Custer B, Ullum H, et al. Duration of red blood cell storage and survival of transfused patients (CME). Transfusion 2010;50(6):1185-1195.

(58) Zubair AC. Clinical impact of blood storage lesions. Am. J.Hematol. 2010;85(2):117-122.

(59) Koshkaryev A, Manny N, Zelig O, Yedgar S, Barshtein G. Key role for phosphatidylserine externalization in storage-induced adhesion of red blood cells to vascular endothelial cells. Biorheology 2008;45(1-2):61-61.

(60) Yedgar S, Kaul DK, Barshtein G. Red blood cell adhesion to vessel wall endothelial cells: A potent catalyst of circulatory disorders. Biorheology 2008;45(1-2):29-29.

Chapter 4

Red Blood Cell Endothelial Adherence during 42 day Hypothermic Storage

4.1 Introduction

Worldwide, approximately 85 million RBC units are transfused (1) as a therapeutic strategy in patients suffering from hematological disorders (see Chapter 1). Interest in RBC-EC adhesion is increasing (2-5) as it is thought to catalyse vascular blockage in the microcirculatory system and is correlated with adverse clinical outcomes (3,6).

It has been determined that the RBC capacity to adhere to ECs is elevated in "aged" RBCs due to PS externalization on the RBC outer membrane leaflet which facilitates splenic sequestration and clearance of RBCs (4,7). In transfused patients, RBC-EC interactions are pronounced in low flow regions such as capillaries and post capillary venules where physiological shear stresses have been estimated to be 0.1 Pa (8). Evidence of increased vascular resistance during extended cold storage of RBCs suggests that perturbations or alterations occur within the RBC membrane. Hence RBCs must be evaluated before transfusions to ensure that hypothermic storage lesions are not present.

Deterioration during storage of red blood cell quality is marked by a steady decline in biochemical and biomechanical properties of the RBCs. These alterations are collectively termed hypothermic storage lesions (HSLs). HSLs are represented by a marked decrease in ATP, 2,3 DPG, morphological changes, changes in intracellular pH, decreases in levels of surface sialic acid, membrane dehydration, microvesiculation (loss of cellular hemoglobin and membrane lipids and proteins through vesicles), and increased impairment of RBC hemorheological properties (9). Despite attempts to maintain these parameters in normal physiological ranges with the use of anticoagulants and additive solutions

under specially adapted conditions (storage at 1–6 ^{o}C, anoxic conditions), rheological properties continue to change under storage conditions; increased adherence of RBCs to the microvasculature is one of the most notable changes (10). RBC-EC interaction has been reported to be susceptible to modification under typical blood banking conditions (4,7,11,12), with increased microvasculature adherence due to hypothermic storage.

In other techniques for assessment of RBC-EC adhesion, investigators varied the RBC incubation time of RBC suspension over the HUVEC monolayer (7,11,12). Transducers were also used to determine blood pressure (13) whereas the momentum balance equation assuming blood to be a Newtonian fluid was used in this thesis to determine adherence and adherence strength. In other similar studies measuring adhesion in stored blood products (12,13), adhesion was evaluated for buffy coat-poor, nonleukoreduced, and leukoreduced RBCs, and all displayed a tendency to adhere to ECs after some time in storage(12,13). The tendency to adhere was quantitatively (flow cytometry) connected to an increase in PS concentration in micro particles shed by the stored RBCs (12,13).

Usually PS is located at the membrane inner leaflet but becomes reoriented to the outer leaflet in hemoglobinopathies and oxidative stress states. PS exposure results in binding to different plasma proteins such as fibrinogen, thrombospondin and dextrans at the RBC surface correlated to increased RBC adhesion to EC (14). Possible contributing factors to increased RBC-EC adhesion during hypothermic storage has been the reorientation of phospholipid from the inner RBC leaflet to the outer leaflet, loss of sphingomyelin and sialic acid

portions of the RBC membrane, and lipid and protein peroxidation loss due to the oxidative stress associated with hypothermic storage (15). Oxidative stress is associated with the iron overload caused by hemolysis (16) and imposes thrombogenic and immunomodulatory effects on the endothelial cells of the vascular system. In diseased states such as thalassemia and sickle cell anemia which are characterized by low flow rates, enhanced RBC-EC interaction has resulted in micro vessel occlusion in capillaries and post capillary venules, leading to reduced perfusion ischemia and necrosis (14,17,18,19). Reduced delivery of oxygen to tissues and increased risk of micro capillary obstruction in critically ill patients with sepsis (20) are also adverse effects of transfusing RBCs with impaired flow properties because these patients generally require multiple blood transfusions.

Increased RBC-EC adhesion has been implicated in the pathophysiology of circulatory disorders including thalassemia, sickle cell anemia and malaria (16). Several studies have shown that the transfusion of RBCs stored for more than three weeks decreased microcirculatory oxygen delivery, contributing to tissue hypoxia and increased morbidity and mortality (21,22). Furthermore, storage duration of transfused RBCs is reported to be an independent risk factor for development of post injury multiple organ failure, post operative pneumonia and increased mortality.

The subtle membrane injuries that are occurring within the RBC membrane needs to be addressed in order to ensure a superior product is available for patient transfusion. Chapter 3 indicated a significant increase in adherence

strength from day 14 (prefreeze) of hypothermic storage before cryopreservation therefore it was important to investigate the added impact of extended storage on RBC adherent properties. Hence this chapter aims to determine the effect of 42 days of hypothermic storage on RBC-EC interactions utilizing the adhesion methodology developed in Chapter 2 of this thesis. RBC units are generally transfused up until unit expiry (day 42), which coincided with the experimental time frame.

4.2 Materials and Methods

4.2.1 RBC Processing

Leuko-reduced, CPD-SAGM, packed RBC units (n = 6) using buffy coat production and whole blood filtration were obtained from the Canadian Blood Services National Inventory and netCAD (Network Centre for Applied Development, Vancouver, Canada). All RBC units were hypothermically stored at 1 – 6 ^{o}C and sampled aseptically (using sterile technique) at day 2 post collection (day 8) then at weekly intervals (14, 21, 35, 42) until unit expiry (day 42).

4.2.2 RBC-EC endothelium adhesion

RBC-EC adhesion and strength were assessed utilizing the developed adhesion assay as previously described in chapter 2 (Sections 2.2.4 and 2.2.5). Briefly, aliquots (1mL) were extracted from each unit and washed three times with 1X calcium and magnesium (Ca^{2+}/Mg^{2+}) free Phosphate Buffered Saline (PBS) at 1000 x g for 5 minutes at 4 ^{o}C. pRBC suspensions (1% hematocrit in 1X Ca^{2+}/Mg^{2+} free PBS containing 1% human serum albumin (HSA), VWR Canada) were perfused at 37^{o}C for 5 minutes at a sustained shear stress of 0.025 Pa over a

confluent human umbilical vein endothelial cell (HUVEC, Lonza) monolayer that formed the base of a parallel plate flow chamber. HUVECs were grown to confluence in 75cm^2 flasks then transferred to fibronectin treated coverslips (Thermo Scientific, Rochester, NY) which were used to form the base of the flow chamber (RC-20, Harvard Apparatus, Canada). The assembled flow chamber was mounted upon a microscope (Nikon Eclipse TE-2000 U Microscope, Nikon, Canada) for RBC-endothelial cell (EC) interaction. PBS was then perfused across in stepwise increases of shear stress from 0.01 Pa to 0.2 Pa in 30 second increments. Shear stress, τ_w, was calculated using the equation (23) below for a Newtonian fluid with constant viscosity in a parallel plate system as stipulated in chapter 2. Positive controls were ssRBCs and negative controls were fresh RBCs. Adherent RBCs were recorded for playback using a camera (Nex-3, Sony, Canada) and counted manually with Grid Cell Counter freeware-software (http://www.dnabaser.com/index.html). A plot of adherent RBCs versus increases of shear stress was performed. Adherent RBCs were counted within ten fields of view at x 60 magnification.

4.2.3 Adherence strength

The change in mean adherent RBCs over increasing shear stress conditions was transformed onto a linear relationship to determine the adherence strength. This was determined by plotting the mean number of adherent RBCs versus the reciprocal shear stress. The linear equation (4.1) was used to derive the overall adherence coefficient or strength of RBC-EC interaction which was determined as the equation gradient,

$\mathbf{N = N_{\infty} + \alpha/\tau}$ (7) **(4.1)**

Where,

N = number of adherent RBCs at a specific shear stress (τ)

α = the adherence coefficient expressing the strength of intercellular interactions

$1/\boldsymbol{\tau}$ = the reciprocal shear stress

N_{∞} = number of adherent RBCs at extrapolated shear stress as it approaches infinity.

4.2.4 Statistical analysis

Non-parametric Kruskal Wallis and Mann-Whitney U tests were performed for n = 6 samples using SPSS software version 14.0 (Lead Technologies, Charlotte, NC). Comparisons for mean number of adherent RBC and adherent strength of hypothermic-stored RBC units were made at the different time intervals (day 8, 14, 21, 35 and 42). Probabilities less than 0.05 were considered significant.

4.3 Results

The effects of hypothermic storage on RBC *in vitro* quality were examined using the adhesion assay developed previously in chapter 2. Nonparametric analysis was used to examine statistical differences in RBC adherence parameters following storage.

Figure 4.1 represents the effects of hypothermic storage on RBC adherence over the course of the 42 day RBC storage period. Statistical analysis yielded differences when comparing mean number of adherent RBCs at 0.05 Pa

on days 21, 35 and 42 versus day 8 ($p < 0.05$). Kruskal Wallis analysis revealed significant differences in the mean number of adherent RBCs from day 8 to day 42 across all shear stresses ($p<0.05$). A significant increase in the number of adherent RBCs were also observed for days 35 and 42 only ($P<0.05$) at 0.1 Pa in figure 4.1

Figure 4.2 is the adherence coefficient, a measure of the adhesive strength of the RBCs measured at distinct time periods of hypothermic storage (days 8, 14, 21, 35 and 42). It was derived from the linear transformation of the mean number of adherent RBCs per field of view at x 60 magnification versus the reciprocal shear stress according to equation 4.1. Negligible adherence strength were seen for Day 8 and 14 however significant increases in calculated adherence strength were observed onward from day 21 until 42 (unit expiry) of hypothermic storage.

4.4 Discussion

In this study, we demonstrated that there were significant changes observed in RBC adherent strengths and the number of adherent RBCs over the hypothermic storage period. As the RBCs are subjected to shear forces close to physiological conditions (0.1 – 0.5 Pa), an attachment profile is generated that determines how many RBCs remain adherent to the EC monolayer. An adherence number curve versus increases of shear stress indicates this whereas an adherent strength profile shows just how strongly the RBCs are adherent to the monolayer over an increasing shear period. A relationship exists when the number of adherent RBCs are plotted versus the inverse of the shear stresses which are determined to be the overall adherence coefficient or strength of RBC attachment.

Our results are in agreement with data obtained by Sparrow, R *et al* (12) which have shown significant increases in the number of adherent RBCs and adherence strength at day 21 of hypothermic storage at 0.05 Pa ($p < 0.05$). Koshkareyv, A *et al* already showed that elevation of RBC-EC interaction is already observed at the second week of storage (13) which correlated well with our results of increased RBC-EC interaction already occurring after 14 days of hypothermic storage. Barshtein, G *et al* (16,17)as well as Bonomini, M *et al* (24)have reported that PS translocation to RBC surface can be induced by elevation of intracellular Ca^{2+} elevation concentration which in turn may be exerted by reactive oxidative species (ROS), a by-product of RBC storage, known to activate specific and nonspecific ion channels in RBCs(16,17,25). Additionally a study conducted by Holovati, J *et al* (25) showed an increase in PS expression in the microparticles of RBCs stored older than two weeks suggesting a link exists between membrane damage, PS externalization and adherence after two weeks of hypothermic storage.

Translocation of PS to the outer membrane leaflet is considered to be a major mediator of RBC adherence to the endothelium (14,17,24). PS at RBC surface may activate platelets and accelerate thrombin-forming processes and has been proposed as a mechanism for thrombosis in sickle cell disease, beta-thalassemia, and hereditary hydrocytosis. Adherence of oxidized RBCs, such as sickle cells and even hypothermic stored RBCs to ECs activates ECs to express VCAM-1, E-selectin, and ICAM-1 which are well-known adhesion molecules (26-30).

Alternatively, a reasonable explanation for the increase in adherence that was noticed at week three could be related to the loss of sialic acid, increase in sphingomyelin or the loss of membrane lipids (13,31) under prolonged storage; increased loss of vital membrane lipids and proteins as micro vesicles and PS externalization from the intact RBC membrane during hypothermic storage is known to occur (4,7) especially from the second week of hypothermic storage. Altered RBC membrane properties may play an important role in enhancing RBC-EC interactions. Conversely, the increased intercellular interactions observed from day 21 might be attributed to the presence of biologically active substances caused by white blood cell (WBC) remnants in the stored units. However universal leukoreduction is practised in Canada (32,33) but residual cells (< 500 x 10^6/L) may be present (32,33) contributing to that spike in adherence on day 21 that was observed as some may have escaped the filtration system. Quality monitoring program (QMP) phosphatidylserine (PS) results however indicate a significant decrease in PS suggesting that RBC-EC interactions may be mediated by factors beyond PS externalisation.

The major limitation of this chapter was the use of a smaller shear stress range of 0.01 Pa to 0.2 Pa and small sample size. This was unavoidable at the time as excessive leaking was encountered using higher pressures. In the microcirculation pressures are typically approximately less than 0.1 Pa therefore if cells are adherent at shear stresses far higher than normal then there is an added risk of microvascular occlusion where the shear stresses needed to detach adherent RBCs are much lower. Patients receiving multiple transfusions although

less liberally practised might be more susceptible to developing the side effects associated with transfusion of rheologically impaired blood. A larger sample size would impart greater statistical power to the results obtained.

Although the data presented in this chapter are preliminary and based upon an assay that requires further validation as previously explained in chapter 2, the information obtained still correlates well with the current literature for examining RBC-EC adherence (4,12). Overall there was a significant increase in the number of adherent RBCs and strength of adhesion during the hypothermic storage period.

4.5 Conclusion

Changes in RBC rheological and biomechanical properties, specifically adherence, can act independently and/or synergistically in inducing circulatory disorders. Although RBCs are routinely stored for a maximum of 42 days, in Canada and the United States, there is the risk that patients transfused with blood older than three weeks might be susceptible to the adverse clinical effects associated with altered RBC membrane properties These patient groups are exclusively those that require large RBC transfusions to immediately increase RBC mass. The data from this study demonstrates RBC-EC adherence is indeed an aspect of the hypothermic storage lesion that is prominent from 21 days of hypothermic storage.

Figure 4.1- Graph of number of adherent RBCs at increasing shear stress during 42 day hypothermic storage. Mean number of adherent RBCs at increasing shear stress; comparative analysis of adherent RBCs days 8, 14, 21, 35, 42; significant differences in mean number of adherent RBCs at 0.05 Pa on days 21, 35 and 42 versus day 8. The y axis represents the mean number of adherent RBCs per field of view at magnification x 60 versus the x axis which represents increasing shear stresses**.**

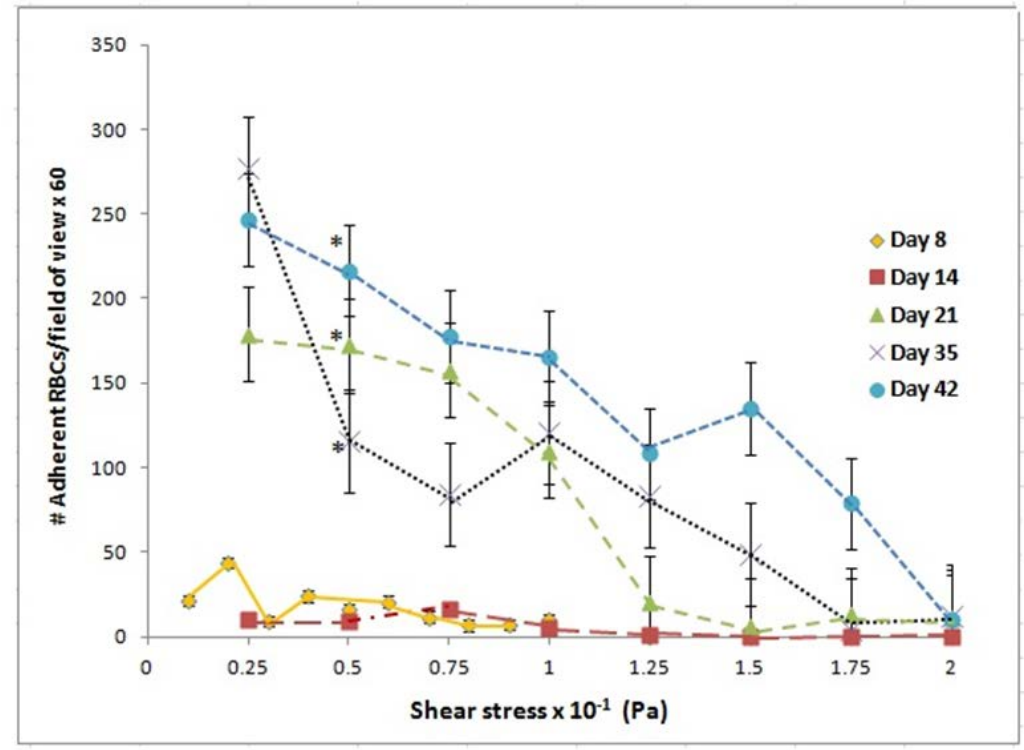
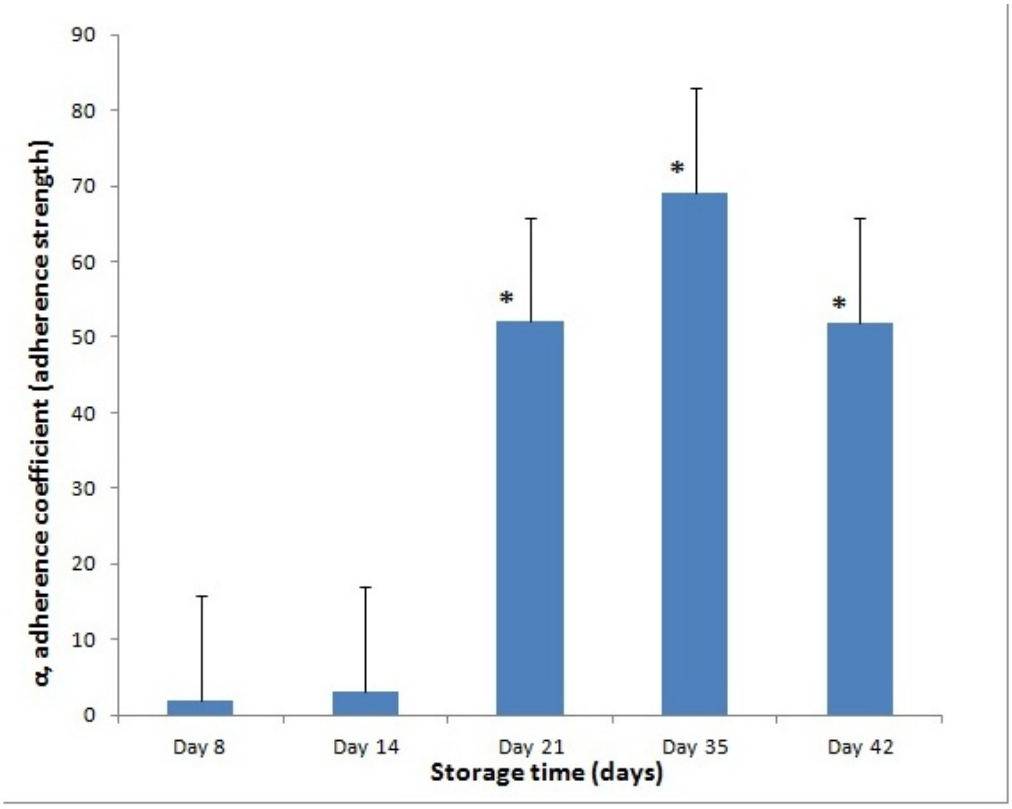


Figure 4.2- Graph of adherence coefficient (adherence strength) changes during the 42 day hypothermic storage period. Significant increases in calculated adherence strength observed at day 21 of hypothermic storage



4.6 References

(1) Acker J. Tissue Engineering II. In: Lee K, Kaplan D, editors. Biopreservation of cells and Engineered Tissues: Berlin:Springer-Verlang; 2005. p. 157-187.

(2) Henkelman S, Dijkstra-Tiekstra M, de Wildt-Eggen J, Graaff R, Rakhorst G, van Oeveren W. Is red blood cell rheology preserved during routine blood bank storage? Transfusion 2010 04;50(4):941-948.

(3) Barshtein G, Manny N, Yedgar S. Circulatory risk in the transfusion of red blood cells with impaired flow properties induced by storage. Transfus. Med. Rev. 2011;25(1):24-35.

(4) Barshtein G, Relevy H, Koshkaryev A, Sayag L, Manny N, Yedgar S. Blood banking-induced alteration of red blood cell flow-affecting properties. Biorheology 2005;42(1-2):40.

(5) Chin-Yee IH, Gray-Statchuk L, Milkovich S, Ellis CG. Transfusion of stored red blood cells adhere in the rat microvasculature. Transfusion 2009;49(11):2304-2310.

(6) Kaul DK, Koshkaryev A, Artmann G, Barshtein G, Yedgar S. Additive effect of red blood cell rigidity and adherence to endothelial cells in inducing vascular resistance. Am. J.Physiol. Heart Circ. Physiol. 2008;295(4):H1788-H1793.

(7) Koshkaryev A, Zelig O, Manny N, Yedgar S, Barshtein G. Rejuvenation treatment of stored red blood cells reverses storage-induced adhesion to vascular endothelial cells. Transfusion (Malden) 2009;49(10):2136-2143.

(8) Lipowsky HH. Microvascular rheology and hemodynamics. Microcirculation 2005;12(1):5-15.

(9) Kor DJ, Van Buskirk CM, Gajic O. Red blood cell storage lesion. Bosn. J.Basic Med. Sci. 2009;9(Suppl.):S21-S26.

(10) D'Alessandro A, Liumbruno G, Grazzini G, Zolla L. Red blood cell storage: The story so far. Blood Transfus. 2010;8(2):82-88.

(11) Anniss AM, Sparrow RL. Variable adhesion of different red blood cell products to activated vascular endothelium under flow conditions. Am. J. Hematol. 2007;82(6):439-445.

(12) Anniss AM, Sparrow RL. Storage duration and white blood cell content of red blood cell (RBC) products increases adhesion of stored RBCs to endothelium under flow conditions. Transfusion 2006;46(9):1561-1567.

(13) Relevy H, Koshkaryev A, Manny N, Yedgar S, Barshtein G. Blood banking-induced alteration of red blood cell flow properties. Transfusion 2008;48(1):136-146.

(14) Yedgar S, Koshkaryev A, Barshtein G. The red blood cell in vascular occlusion. Pathophysiol. Haemost.Thromb. 2002;32(5-6):263-268.

(15) Bosman GJCGM, Werre JM, Willekens FLA, Novotny VMJ. Erythrocyte ageing in vivo and in vitro: structural aspects and implications for transfusion. Transfus. Med. 2008;18(6):335-347.

(16) Ramot Y, Koshkaryev A, Goldfarb A, Yedgar S, Barshtein G. Phenylhydrazine as a partial model for beta-thalassaemia red blood cell hemodynamic properties. Br. J. Haematol. 2008;140(6):692-700.

(17) Yedgar S, Hovav T, Barshtein G. Red blood cell intercellular interactions in oxidative stress states. J. Vasc. Res. 2000;37(Suppl. 1):56.

(18) Bishop J, Nance P, Popel, AS, Intaglietta, M, Johnson P. Effect of erythrocyte aggregation on velocity profiles in venules. Am. J. Physiol Heart Circ. Physiol. 2001;280:H222-H236.

(19) Tomita M, Tanashashi N, Takeda H, Schiszler I, Osada T, Unekawa M, et al. Capillo-venous flow in the brain: significance of intravascular RBC aggregation for venous flow regulation. Clin. Hemorheol. Micro. 2006;34:51-57.

(20) Marik PE, Sibbald WJ. Effect of stored-blood transfusion on oxygen delivery in patients with sepsis. JAMA 1993;269(23):3024-3029.

(21) Hayes MA, Timmins AC, Yau EHS, Palazzo M, Hinds CJ, Watson D. Elevation of systemic oxygen delivery in the treatment of critically ill patients. N. Engl. J. Med. 1994;330(24):1717-1722.

(22) Zallen G, Offner P, Moore Eea. Age of transfused blood is an independent risk factor for postinjury multuple organ failure. Am. J. Surg. 1999;178:570-572.

(23) Bird, R.B., Stewart, W.E., and Lightfoot, E.N. Transport Phenomena. New York: Wiley; 1960. p. 47.

(24) Bonomini M, Sirolli V, Gizzi,F, Di Stante,S, Grilli A, Felaco M. Enhanced adherence of human uremic erythrocytes to vascular endothelium: Role of phosphatidylserine exposure. Kidney Int. 2002;62(4):1358-1363.

(25) Holovati JL, Wong KA, Webster JM, Acker JP. The effects of cryopreservation on red blood cell microvesiculation, phosphatidylserine externalization, and CD47 expression. Transfusion 2008;48:1658-1668.

(26) Gallagher PG, Chang SH, Rettig MP, Neely JE, Hillery CA, Smith BD, et al. Altered erythrocyte endothelial adherence and membrane phospholipid asymmetry in hereditary hydrocytosis Blood 2003;101(11):4625-4627.

(27) Andrews D, Low P. Role of red blood cells in thrombosis : Current Opinion in Hematology. Curr. Opin. Hematol. 1999;6:76-82.

(28) Brown MD, Wick TM, Eckman JR. Activation of vascular endothelial cell adhesion molecule expression by sickle blood cells. Fetal Pediatr. Pathol. 2001;20(1):47-72.

(29) Stuart M, Setty B. Acute chest syndrome of sickle cell disease. Curr. Opin. Hematol. 2001;8:111-122.

(30) Rattan V, Shen Y, Sultana C, Kumar D, Kalra VK. Diabetic RBC-induced oxidant stress leads to transendothelial migration of monocyte-like HL-60 cells Am. J. Physiol. 1997;273(2 Pt 1):E369-E375.

(31) Barshtein G, Ben-Ami R, Yedgar S. Role of red blood cell flow behavior in hemodynamics and hemostasis. Expert Rev. Cardiovasc. Ther. 2007;5(4):743-752.

(32) Kakaiya R, Aronson CA, Julleis J. Whole blood collection and component processing. AABB technical manual. 16th ed. Bethesda, MD: AABB Press; 2008. p. 201-211.

(33) Amin M, Fergusson D, Wilson K, Tinmouth A, Aziz A, Coyle D, et al. The societal unit cost of allogenic red blood cells and red blood cell transfusion in Canada. Transfusion 2004;44(10):1479-1486.

Chapter 5

General Discussion & Future Directions

5.1 Review of Thesis Objectives

The work reported in this thesis comprises (1) the development of an assay to evaluate RBC adherence to the endothelial cell (EC) monolayer (Chapter 2), (2) determination of the effects of RBC cryopreservation on RBC-EC adherence and RBC deformability (Chapter 3), and (3) an estimation of the effect on RBC adherence of 42 days of hypothermic RBC storage (Chapter 4).

The adhesion assay developed in chapter 2 attempted to detect differences in RBC-EC adhesion between RBCs treated with t-BuOOH, NEM, and vitamin E, and untreated RBCs, and compared adhesion strengths of normal RBCs with ssRBCs. We demonstrated that ssRBCs were more strongly adherent to ECs than t-BuOOH-treated RBCs, vitamin E-treated RBCs, and normal RBCs (NEM-treated RBCs were haemolysed); therefore, ssRBCs were used as a positive control for RBC-EC adhesion analysis.

The adhesion assay showed significant differences between RBCs stored under hypothermic and freezing conditions (cryopreservation). Previous research identified subtle membrane lesions that conventional RBC quality assays fail to address when RBCs were stored at temperatures below 0 °C (1,2). Utilizing the RBC-EC adhesion measurement technique established in Chapter 2, we demonstrated that there was an overall increase in the adhesion strength of adherent RBCs after cryopreservation. The clinical implications of these results warrant investigation in an *in vivo* model to more accurately determine the consequences to RBC structure during cryopreservation. The inability of

transfused RBCs to flow freely, especially in low flow regions, can limit their oxygen carrying capability and cause microvascular occlusion and clearance from the circulatory system by splenic macrophages (3). Premature removal of RBCs reduces the long term benefits of blood transfusion.

The work reported in chapter 3 demonstrated that RBC adherence to ECs can begin at day 14 of hypothermic storage under typical blood banking conditions (1–6 ^{o}C for 42 days in CPD-SAGM). RBCs adherent to the EC monolayer increased in number and exhibited increased adherence strength after 21 days of storage. The results of this study agree with adhesion experiments performed by Anniss and Sparrow (1) that showed increases in adherence from 21 and 28 days of storage; however, our studies differed from those of Koshkaryev and Relevy *et al.* (4,5) that showed an association of RBC-EC adherence and PS externalization. Further investigation might establish whether increases in RBC-EC adherent number and adherence strength resulted from subtle membrane lesions associated with PS externalization or perturbations to the membrane during hypothermic storage and cryopreservation. Intravital video microscopy of the flow of fluorescently labelled stored RBCs through an animal's microvasculature, an *in vivo* model used by Chin Yee *et al.* (6), could elucidate the clinical implications of our findings.

5.2 Study Contributions and Future Directions

This thesis contributes to the field of RBC bio preservation and transfusion medicine. During storage, RBCs can undergo membrane changes affecting their rheological properties in patients after blood transfusion. An assay to determine

RBC adherence to the endothelial cell monolayer has been developed. The assay is an *ex vivo* simulation of blood flow in the human microcirculation allowing changes in the EC adherence of blood products before and after storage to be visualized. This assay evaluated the effects on *in vitro* RBC quality of hypothermic storage lesions and cryoinjury. RBCs with impaired hemorheological properties (flow) due to membrane alterations will be less effective in delivering tissue oxygenated RBCs, resulting in tissue ischemia and infarction (tissue death). The mechanisms associated with increased RBC adherence to ECs must be elucidated to ensure that blood products intended for patient transfusion are of good quality. With more time and further research, the assay developed in the current study has the potential to be implemented in routine RBC quality monitoring processes. I truly believe that after further research, development, and validation, this assay can lead to improvement in patient blood transfusion outcomes in Canada.

5.1 References

(1) Anniss AM, Sparrow RL. Storage duration and white blood cell content of red blood cell (RBC) products increases adhesion of stored RBCs to endothelium under flow conditions. Transfusion 2006;46(9):1561-1567.

(2) Koshkaryev A, Zelig O, Manny N, Yedgar S, Barshtein G. Rejuvenation treatment of stored red blood cells reverses storage-induced adhesion to vascular endothelial cells. Transfusion (Malden) 2009;49(10):2136-2143.

(3) Kuypers FA, de Jong K. The role of phosphatidylserine in recognition and removal of erythrocytes. Cell Mol Biol (Noisy-le-grand) 2004;50(2):147-158.

(4) Relevy H, Koshkaryev A, Manny N, Yedgar S, Barshtein G. Blood banking-induced alteration of red blood cell flow properties. Transfusion 2008;48(1):136-146.

(5) Koshkaryev A, Manny N, Zelig O, Yedgar S, Barshtein G. Key role for phosphatidylserine externalization in storage-induced adhesion of red blood cells to vascular endothelial cells. Biorheology 2008;45(1-2):61.

(6) Chin-Yee IH, Gray-Statchuk L, Milkovich S, Ellis CG. Transfusion of stored red blood cells adhere in the rat microvasculature. Transfusion 2009;49(11):2304-2310.

Appendix

Econoflo Syringe Pump Operation
Push Pump

The main power switch located on the rear panel of the syringe pump was turned on and the display indicated POWER FAIL (this is normal as the pump indicates on the display if the power was disrupted since last use). The power adaptor was connected to the TC-124 temperature controller located below the syringe pump shelf and the syringe heater inserted around the front of the 10cc syringe before loading the syringe onto it. The syringe pusher was released to load the syringe by pressing the bronze button located on the side of the pusher. This was achieved by holding the bronze button "in" and sliding the pusher to the left. The spring loaded syringe retainer was raised out of the way to allow the loaded syringe to be placed in the 'V' shaped holder. Once placed there it was released to hold the syringe in place and the syringe was checked to make sure that it rested comfortably in front of the syringe holder and the appropriate flow rates were selected. Connections were subsequently made between syringe, in line heater device and chamber.

Chemyx Fusion200
Suction Pump

A 60 cc syringe was installed in a similar fashion to the push pump secured by the withdraw holder. The power switch at the back of the pump was turned on by pressing START/STOP to begin or stop suction. The suction rates (3 ml/mn) and syringe diameters have already been preset. Connections were made

between the syringe and chamber and once experiments were ready to begin with syringes and flow chamber in place and all temperature control units on, RUN/STOP and START buttons on the infusion and withdrawal pumps were pressed to begin flow.