Membrane water permeability as a quality predictor for red blood cells during hypothermic storage

by

Abdulrahman Alshalani

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Department of Laboratory Medicine and Pathology
University of Alberta

Abstract

Hypothermic storage of red blood cell is essential in the field of transfusion medicine to fulfill the huge clinical demand for red blood cell products in situations of massive bleeding or red blood cell disorders. The prime goal of hypothermic storage is to maintain the quality and safety of stored red blood cells. The cell membrane is one of the red blood cell main components that needs to be structurally and functionally preserved throughout storage. It is characterized by its semipermeability which allows the movement of water to maintain osmotic equilibrium with external media and allows for cell shrinkage or swelling. The cell membrane, however, experiences progressive damages during storage. Several factors are proposed to influence the storage lesion including the storage length, blood component manufacturing method, and donor characteristics. This thesis will characterize the membrane water permeability properties during storage and will apply water permeability measurements to determine the contributions of the storage length, blood component manufacturing method, and donor-related characteristics to membrane water permeability variation.

Red blood cell units were obtained from volunteer blood donors and categorized according to the duration of storage, manufacturing method, and donor characteristics. Validated techniques were applied to characterize the red cell membrane water permeability and membrane quality properties. The membrane water permeability parameters included the hydraulic conductivity of water permeability, osmotically inactive fraction, and Arrhenius activation energy. The membrane quality measures were deformability, total hemoglobin, supernatant hemoglobin, percent hemolysis, osmotic fragility, hematologic indices of mean corpuscular

volume, the mean corpuscular hemoglobin, and the mean corpuscular hemoglobin concentration, supernatant potassium, and supernatant sodium.

It was found that membrane water permeability measurements were significantly increased as a function of the storage length (at day 21 of storage), which remained elevated for the rest of 42 days of storage. This was accompanied by significant elevations in the osmotically inactive fraction and Arrhenius activation energy. This study, also, found that units processed by a whole blood filtration manufacturing method exhibited significantly higher membrane water permeability from the start of storage until the end of 42 days of storage compared to units manufactured using a red cell filtration. There were no significance differences between groups in dependent to donors' age and sex. These results revealed that both the storage length and manufacturing process had significant contributions to the water permeability and various quality parameters with limited contributions from the blood donors' age and sex.

In conclusion, this thesis has expanded the knowledge of membrane quality during the hypothermic storage by applying a novel method to assess membrane water permeability. According to these findings, it is therefore likely that such connections exist between water permeability increase and membrane damages during storage. This work also tested possible factors affecting the RBCs quality during hypothermic storage. Findings of this thesis proposed that part of the membrane-related storage lesion occur before the first day of storage due to the effect of blood manufacturing process and donor variability. The findings of this thesis highlight the importance of describing membrane water permeability properties during storage which can be a quality predictor of the biophysical and chemical changes that affect the quality of stored red blood cells during hypothermic storage.

Preface

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Role of blood component manufacturing on red blood cell damage and changes in white blood cells”, No. Pro00059754, 2015-12-09. It, also, received research ethics approval from the Canadian Blood Services Ethics Board, Project Name “Effect of gender on hemolysis and rheology during routine storage of red blood cell units”, No. 2013-013, 2015-01-14, and Project Name “Role of blood component manufacturing on red blood cell damage and changes in white blood cells”, No. 2015-032, 2015-09-01.

Dedication

This achievement is dedicated to my parents, Qubilah and Jaser, for unconditional love, prayers, inspiration, and support that they have provided me throughout my life.

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List of Abbreviations

A	cell surface area
ANOVA	analysis of variance
AS-1	adsol additive solution
AS-3	nutricel additive solution
AS-5	optisol additive solution
Atm	standard atmosphere
ATP	adenosine triphosphate
B	osmotically inactive substances
°C	celsius
C_s^i	intracellular concentration of permeate solute
C_s^e	extracellular concentration of permeate solute
C_i^i	intracellular concentration of impermeate solute
C_i^e	extracellular concentration of nonpermeate solute
CBS	Canadian Blood Services
CPD	citrate-phosphate-dextrose anticoagulant
dH2O	distilled water
2,3-DPG	2,3-diphosphoglycerate
E	maximum error of estimate
Ea	Arrhenius activation energy

EI	elongation index
EI_{max}	maximum elongation index
Eq	equation
FDA	Food and Drug Administration
Fig	figure
fL	femtolitre
G	gram
%Hl	percent hemolysis
Hb	hemoglobin
Hb_S	concentration of the supernatant hemoglobin
Hb_T	concentration of the total hemoglobin
Hct	hematocrit
K	kelvin
K	fitting constant
K^+	potassium
kcal	kilocalorie
KCl	potassium chloride
K_{EI}	50% of the maximum elongation
Kg	kilogram
KH2PO4	potassium dihydrogen phosphate
L	litre
LORCA	laser-assisted optical rotational cell analyzer
Lp	water permeability

MCF	mean corpuscular fragility
MCH	mean corpuscular hemoglobin
MCHC	mean cellular hemoglobin concentrating
MCV	mean corpuscular volume
Min	minute
mL	millilitre
µL	microlitre
μm^2	square micrometre
μm^3	cubic micron
mosm	milliosmoles
N	sample size
Na^+	sodium
NADPH	nicotinamide-adenine-dinucleotide-phosphate
NaCl	sodium chloride
Na2HPO4	disodium hydrogen phosphate
netCAD	Canadian Blood Services' Network Center for Applied Development
Nm	nanometre
NO	nitric oxide
Pa	pascal
PBS	phosphate buffered saline
Pg	picogram
PS	phosphatidylserine
p-value	significant level

PVP	polyvinylpyrrolidone
p50	partial pressure of oxygen
QMP	quality monitoring program
R	gas constant
R	Pearson correlation coefficient
R^2	coefficient of determination
RBC	red blood cell
RCF	red cell filtration manufacturing process (top and bottom method)
SAGM	saline-adenine-glucose-mannitol additive solution
SD	standard deviation
SEM	standard error of the mean
SS	shear stress
T	temperature
T	time
V	cell volume
V_0	isotonic cell volume
WB	whole blood donation
WBF	whole blood filtration manufacturing process (top and top method)
$Z_{a/2}$	standard error of the population mean
Π	anisotonic osmolality
π_0	isotonic osmolality
Σ	standard deviation

Chapter 1

Introduction

1.1.Hypothermic storage of red blood cell

Every year, nearly 80 million red blood units are transfused to millions of patients all around the world (1). Red blood cells (RBC) transfusion is used to save lives by increasing the oxygen delivery to tissues and raising the hemoglobin level for patients with severe bleeding or major surgeries. Also, it is a life long treatment for patients with RBC disorders such as severe anemia and hemoglobinopathies (2). The huge clinical need for RBC products has led to development of methodologies for RBCs preservation. Many preservation protocols have been designed to maintain the quality and safety of RBCs. The most common method to preserve RBCs is storage of cells at a condition below the normal physiological temperature and above the freezing point, which is known as hypothermic storage. The hypothermic storage of RBCs is based on the principle that most of biochemical reactions can be suppressed when cooling cells at refrigeration temperatures (1-6˚C). Preserving RBCs at low temperature can inhibit metabolic activities of cells leading to a reduction in the accumulation of cellular metabolic wastes (3).

The first step toward the hypothermic storage was taken in 1916 when Rous and Turner discovered the citrate-glucose solution. They used citrate as an anticoagulant solution and glucose as a preservative nutrient for RBCs. Robertson, during World War I (1917), initiated the first blood bank when he stored blood group O in a bottle containing the citrate-glucose solution for 26 days (1, 3, 4). Blood preservation was improved in the 1950s when plastic blood bags and blood product manufacturing, separation of RBCs from plasma and other blood cells, were

developed (1). The next significant step occurred when the current traditional anticoagulant solution, citrate-phosphate-dextrose (CPD), was introduced in 1957 (5). The quality of the storage further improved when the additive solutions such as Adsol (AS-1), Nutricel (AS-3), Optisol (AS-5), and saline-adenine-glucose-mannitol (SAGM) used with the anticoagulant solution which allows preserving RBCs at 1-6˚C for up to 6 weeks (1, 3). Since then, numerous studies on RBC preservation have focused on the quality of preserved cells in term of their viability and functionality.

During the hypothermic storage, however, RBCs age faster than in the body and undergo some structural and functional changes which are known as the storage lesion (6-8). Several studies have focused on the structural, biochemical, and biomechanical injuries and modifications that occur during storage. These studies have reported a rapid depletion in adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) concentrations which disrupt energy-dependent activities inside cells such as sodium (Na^+) potassium (K^+) pump leading to loss of potassium from RBCs (1, 8). A low ATP level also leads to loss of membrane phospholipid asymmetry and expression of phosphatidylserine which acts as a target for macrophages when exposed on the outer leaflet resulting in the red blood cell destruction (9, 10). It also has been associated with a decrease in the functionality of membrane transporter proteins leading to a change in the intracellular viscosity (11). Another change that has been associated with ATP depletion is an increase in the oxidative damage to hemoglobin and membrane components. Low 2,3-DPG concentration increases hemoglobin affinity for oxygen which may impact the ability of stored RBCs to deliver sufficient oxygen to tissues after transfusion (8, 12). Moreover, RBCs irreversibly lose some hemoglobin molecules and some membrane parts by microvisicleulation (1). Subsequently, the shape of cells changes from biconcave discs to

crenated sphere cells (1). RBCs, as a result, start to lose their ability to deform due to disrupting all deformability-determinant factors (1, 13). There are also other biomechanical injuries that have been reported during hypothermic storage including increased osmotic fragility, RBC aggregability and adhesiveness (12, 14, 15). The progression of the storage lesion continues until RBCs finally lysis (hemolysis) in the storage bag causing release of intracellular hemoglobin and other cell contents into the preservative solution (6, 12, 15). As the RBC is the area of interest for this thesis, an overview of the structure and function of RBC is worth knowing in addition to some factors that may affect the RBCs quality and a brief discussion about quality measurements.

1.1.1. Red blood cell

When looking at the human blood sample, it consists of red blood cells, white blood cells, and platelets suspended in plasma. RBCs, also known as erythrocytes, are the main component of whole blood constituting about 40 to 51 percent of the packed cell volume (16). They have a circular biconcave discoid shape with an average diameter of 8 μm and mean volume of 90 fL. They are unique among all other eukaryotic cells in that they lack a nucleus, mitochondria, and other cytoplasmic organelles (16-18).

RBCs are derived from pluripotent stem cell through a maturation process called erythropoiesis which takes place in the body bone marrow. In this process, the stem cell is normally stimulated by the erythropoietin hormone and some growth factors to proliferate and differentiate along an enucleation maturation process until giving rise to a reticulocyte which lacks nucleus (19). The reticulocyte is then released from bone marrow to the circulation where

RNA is completely lost and the cell matures. The maturation process involves a reduction in cell volume, loss of the nucleus and cell organelles, and an increase in hemoglobin concentration. The mature RBC circulates in blood vessels for 120 days until the cell is removed from the circulation by the reticuloendothelial system. In the normal physiological condition, the production and destruction of RBCs are nearly equivalent and balanced (3, 17, 19).

The ultimate goal of RBCs is to transport oxygen from the lungs to the body tissues followed by carbon dioxide delivery to the lungs. During 120-day of their lifespan, RBCs face several mechanical stresses in the circulation including passing through the microcirculation with a diameter reaching one-third of the normal red blood cell (20). In addition, they experience some biochemical stresses including osmotic and oxidant stresses when traveling through different tissues and organs (21).

To achieve the goal of gas transportation and resist these stresses, red blood cells are dependent on the efficacy of the following three major elements; hemoglobin, metabolism, and cell membrane. Almost all hereditary and acquired defects of RBCs involve abnormalities in one or more of these components (3, 17, 18, 22). A brief review of hemoglobin and metabolism will be discussed before moving to an in-depth overview on the cell membrane.

Hemoglobin (Hb) is a complex protein consisting of one pair of α- globin chains and another pair of non-α- globin chains associated with four heme groups. The most abundant hemoglobin in the adult human is Hb A with a small quantity of two other hemoglobin types, Hb A2 and Hb F, (23). Hemoglobin molecules constitute approximately 95% of the RBC dry weight. Each hemoglobin molecule can carry four oxygen molecules (3). Association and releasing of oxygen is regulated by 2,3-DPG which binds to the hemoglobin molecule to help release oxygen to tissues. Hemoglobin has more affinity to bind oxygen in the absence of 2,3-

DPG, but the oxygen affinity decreases when the concentration of 2,3-DPG increases inside the cell (17). In addition to gas transportation, hemoglobin also plays a role in maintaining the blood flow rate inside blood vessels by binding the nitric oxide (NO) which is produced from epithelial cells. Binding of NO molecules to hemoglobin causes vasoconstriction and increases the blood flow in arteries. In veins, hemoglobin releases NO causing vasodilation and slows down blood flow (24).

Since the RBC lacks a nucleus and other cell organelles, it depends on specific metabolic pathways to do all the followings; protect the cell from oxidative stress, maintain the osmotic stability, control the process of uptake and release of oxygen by the hemoglobin, and keep the membrane integrity (23). The main source of energy in RBC is through the biochemical degradation of glucose via the Embeden-Mayerhof metabolic pathway in which one glucose molecule is anaerobically metabolized to form two lactate molecules with the generation of two molecules each of ATP and nicotinamide-adenine-dinucleotide-phosphate (NADPH). The ATP is considered the only source of energy used to fill the requirement of all energy-dependent activities in the RBC such as sodium- potassium pump, transporter proteins, and phosphorylation of membrane which maintain the surface level to the internal volume of the cell. The NADPH is used to protect hemoglobin and the cell membrane from oxidative damage. This metabolic pathway is also linked to the production of 2,3-DPG (17, 23). The availability of functional hemoglobin and a source of energy in normal quantity is very critical for RBC to achieve the function of oxygen delivery to tissues.

The red cell membrane is composed of about 40% lipid, 52% protein, and the remaining is carbohydrate (25, 26). The lipid bilayer is composed of cholesterol and phospholipids which are present in equivalent amounts. Unlike phospholipids, cholesterol seems to be equally

distributed between the inner and outer leaflets of the lipid bilayer. The majority of choline phospholipids (phosphatidylcholine and sphingomyelin) are located in the outer monolayer of the lipid bilayer while most of phosphatidylethanolamine and phosphatidylserine (PS) is located in the inner monolayer facing the cytoplasm (9, 11, 25-27). Maintenance of asymmetric distribution of internal and external phospholipids is crucial especially for PS which acts as a signal for macrophages when exposed on the outer leaflet leading to the red blood cell destruction and removal from the circulation by the reticuloendothelial system (9, 10).

The red cell membrane proteins can be divided into two general groups; integral and skeletal proteins (9-11, 25-28). The integral proteins are bound to the cell membrane by forming a strong bond to the hydrophobic portion of the lipid bilayer. They act as anchors for linking the lipid bilayer to the underlying skeleton membrane. This attachment is very important to maintain the shape, cohesion, and flexibility of the RBC in the circulation. Band 3 represents about 20% of the integral protein, and it has the skeleton-linking function through interaction with ankyrin and protein 4.1 (25, 26, 29). The glycophorins are also a major family of integral protein. They have a cytoplasmic end that connects the membrane skeleton to the lipid bilayer (25) and extracellular end that determines the antigenic properties of the cell including blood group antigens (29). In addition, some of the integral proteins work as transporters and channels such as aquaporin (water transporter), UT-B (urea transporter), and band 3 which acts as a channel for respiratory gasses in the blood (29). Underlying the lipid bilayer is a filamentous network of proteins called membrane skeleton. This network is primarily composed of spectrin, ankyrin, actin, and protein 4.1 (27), and they have the function of regulating the membrane stability and cellular discoid shape (11, 26). In fact, extraction of the skeletal proteins leads to membrane fragmentation and vesiculation (25, 26).

The primary function of the cell membrane of most eukaryotic cells is to form a barrier between the intracellular components and the extracellular environment. The cell membrane also maintains the cellular integrity by holding the cell together forming a particular shape. The cell membrane also has surface proteins called protein markers that allow cells to identify and communicate with each other. These proteins often work as a receptor for specific biochemicals allowing the cell to undergo particular signaling such as immune proteins (11, 29).

The cell membrane is considered as the prime factor for the RBC to undergo deformation. Deformability allows the normal biconcave erythrocytes with diameter 8 μm to undergo reversible shape changes to flow through the microcirculation with diameters 3-5 μm (20). However, it is widely known that the reduction of erythrocyte deformability can shorten the erythrocyte survival in the circulation (10, 30-33). This is possible because erythrocytes have to resist some external forces during their 120 days of the lifetime. The RBC deformability is regulated by three main factors; the cell surface area to volume ratio, the cytoplasmic viscosity, and the membrane elasticity (9, 26). The RBC membrane area is regulated by stable skeletal proteins and the firm cohesion between membrane components to prevent vesiculation and breakup (33). The integral transporter proteins regulate the cell volume and cytoplasmic viscosity by maintaining the water and cation contents of the cell. The strong cohesion and stability between lipid bilayer and membrane proteins play a role in the maintenance of the normal biconcave shape of the red blood cell (11).

The red cell membrane also plays a critical role in maintaining the cell discoid shape by maintaining the intracellular viscosity and contents through a process called selective permeability. This is regulated primarily by the anion (band 3) and water (aquaporin 1) transporter proteins by maintaining the cellular Na^+/K^+ and water contents (21, 34, 35). When

the extracellular osmolality exceeds the intracellular osmolality, water leaves the cell causing cell shrinkage. In contrast, cell swelling occurs as water enters the cell when intracellular osmolality exceeds the extracellular osmolality. Normally, RBCs are suspended in isotonic extracellular plasma fluid (≈ 290 mOsmol/kg). However, RBCs may flow through some organs that have a high or low osmolality medium such as the renal medulla. Thus, RBCs use some osmoregulatory mechanisms including Na^+/K^+ pump and water transport to maintain the cell volume stability after osmotic swelling or shrinkage. These mechanisms are essential for optimum RBCs function, structure, and survival (21, 34, 35). As red blood cells age and are at the end of their average 120 days of lifespan, they lose some of their intracellular contents followed by water loss to obtain the osmotic equilibrium (21). This normal mechanism has a direct impact on the instability of the cell shape and volume which leads to an inability to pass through the spleen followed by splenic sequestration and destruction of these impaired RBCs (36).

Numerous disorders have been associated with the previously mentioned RBCs components; hemoglobin, metabolic pathway, and cell membrane. These include thalassemia, sickle cell disease and drug-induced methemoglobinemia which are all linked to hemoglobinopathies (22). Defects associated with abnormalities in metabolic activities, such as glucose-6-phosphate dehydrogenase deficiency, cause the RBC to be more susceptible to oxidant stress which leads to a decrease in RBC survival (23). There are also some pathological alterations in the red cell membrane including primary red cell membrane defects, such as hereditary spherocytosis, and secondary induced red cell membrane disorders, such as malaria and sickle cell disease. These have been connected with disruption in the RBC deformability and osmoregulatory mechanisms (34, 36-40). Hereditary spherocytosis caused by a defect of one or

more of the transporter proteins (Band 3) and skeletal proteins (spectrin) which lead to a loss of membrane surface area and change in the cell discoid shape. This disorder has been associated with a reduction in the anion transport activity which reduces the cell deformability and survival and eventually causing hemolytic anemia (37, 39). Colton-null patients are characterized by deficiencies in RBC water transporter protein (aquaporin 1) with a significant reduction in the membrane water permeability (40, 41). At a highly anisotonic medium such as in renal medulla where the environment is extremely hypertonic and the osmolality may reach 1400 mosmole/kg (21), Colton-null RBCs are unable to regain water rapidly causing an increase in the cell viscosity (40, 41). Invasion of RBCs with malaria parasites have also been associated with changes in cell Na^+/K^+ and water concentrations (38). As the parasite grows inside RBC, it starts to control the cellular transporter proteins to fill the nutrient demands. The cell becomes more permeable to molecules such as sugars, vitamins, and inorganic ions resulting in increased the cell rigidity and volume (38). The polymerization of hemoglobin in sickle cell disease is associated with loss of cellular osmotically active substances followed by water loss causing cell shrinkage, dehydration, and rigidity (21, 34, 36).

1.1.2. Factors affect RBCs quality

The hypothermic storage protocols licensed by regulation agencies including the US Food and Drug Administration (FDA) allow for the storage of RBCs at refrigeration temperatures (1-6˚C) for up to 6 weeks as long as hemolysis in the bag not exceed 1% and 75% of transfused RBCs survive in the circulation after 24 hours of transfusion (8, 42). However, these rules do not assure the safety of transfusion as some studies have associated the red blood

cell transfusion to serious post-transfusion clinical complications. Numerous studies have linked the storage length to the storage lesion in which the quality of stored RBCs decrease as the storage duration proceed (43-46). In fact, some clinical studies have also examined the storage period as the main risk factor for post-transfusion adverse outcomes (14, 15, 42, 47). They warned the correlation between transfusing old stored RBCs and serious clinical consequences especially with clinically ill patients such patients who undergo cardiac surgery or suffer from multiple trauma. These adverse outcomes include increased length of stay in hospital, organ failures, infection, immunological effects, and mortality (8, 14, 15, 42, 47). Hypotheses have been generated to explain the association between transfusing old stored RBCs with post-transfusion clinical harms. Hod et al. have suggested this could be due to the accumulation of non-transferrin bound iron which is derived from the free hemoglobin in RBCs bag and from the acceptable in vivo hemolysis of less than 25% of transfused RBCs (47, 48). The complications also have been linked to some other storage injuries such as the formation of microvesicles which may impact the immune system (7) and deformability loss which induce RBCs destruction by the reticuloendothelial system (49). However, other studies have shown that transfusing fresh stored RBCs is not any different than old stored RBCs as long as RBCs are viable (49, 50). Lacroix et al. found in their recent study of more than 2400 patient cases who received blood transfusion that there were no clinical differences between fresh or old RBCs transfusions (50). Another large cohort study for 9669 patients who received 46,868 RBCs indicates that there is no evidence for an association between the storage length and clinical mortality (51).

Clearly, there are other factors, besides the storage length, affecting RBCs quality that need to be considered including the blood manufacturing process and donor characteristics (52, 53). The blood manufacturing process is a series of methods of collecting whole blood from

donors, separating blood into different components, and preserving these different components in different medium for transfusion. Most of blood donation centers separate blood manually in which the whole blood is collected in a bag containing CPD anticoagulant. The whole blood is then processed by one of two methods: red blood filtration method (RCF, top and bottom system), which also known as buffy coat (BC) method, or whole blood filtration method (WBF, bottom and bottom system). These two methods used to separated whole blood donations into three components; packed RBCs, platelets (in RCF method only), and plasma (54). Apheresis is also an advanced method used to collect one or more specific components directly from a donor (55, 56).

During separation, RBCs experience several mechanical stresses including centrifugation and filtration. Acker et al. and Hansen et al. have monitored the quality of stored RBCs derived from different component processing. They found that quality measures including hematocrit and hemolysis are different between methods indicating that manufacturing can affect cell characteristics (54, 57). Also, the amount of residual leukocytes and platelets after buffy coat removal and leukoreduction have shown to be a direct impact on storage quality, and it has been proposed to have an immunological effect after transfusion (49). Moreover, the type of bag plasticizer, anticoagulant, and the additive solution used during manufacturing may also have an impact on the final product (58) in which RBCs move from the normal physiological medium to a medium of different pH, temperature, and osmolality (56, 59, 60). It has shown that the acidic CPD affects the metabolic activities of RBCs leading to reduce the ATP production during storage (61). The CPD is also a hypertonic solution with an osmolality of ≈500 mOsmol/kg. This may cause an osmotic shock followed by lysis for the first portion of RBCs mixed with the anticoagulant solution during manual collection (56, 60). In addition, Radwanski et al. has found

that the time of preparation and exposing the RBCs to the room temperature have effects on the quality of storage. They found that the 2,3-DPG concentration decreased in overnight room temperature compared to apheresis collection (58).

Donor characteristics also have an impact on RBCs quality. Blood is collected from different populations in term of age, sex, frequency of donation, origin, and ABO/Rh blood group. A better understanding of the effects of theses differences would help understand the source of the storage lesion and minimize clinical outcomes. The effects of donor age on RBCs properties is obvious. As RBCs age at the end of their 120 days lifespan, the bone marrow generates new RBCs to replace the older cells. However, after the age of 50, the erythropoiesis process decreases which impacts the ratio of old to new RBCs causing increase concentration of the older cells (62, 63). It is also has been revealed that males have a high level of older RBCs compared to premenopausal females. Kameneva et al. in his study of 97 blood samples collected from young men and women of less than 32 years of age has found that men have a higher percentage of older RBCs. Male blood has different rheological properties with lower deformability and higher aggregability and fragility compared to female blood (62). In addition, stored blood from female donors revealed less hemolysis (53) and lower mechanical fragility index (64) which indicate that female RBCs are less fragile, more intact, and less likely to be subjected to phagocytosis after transfusion. A logical explanation of these differences between young men and women blood is that because of the monthly blood loss of menstruation which allows releasing of new RBCs to the circulation and increases the new to old RBCs ratio (62-64). Thus, it is crucial to characterize the impact of manufacturing process and donors factor to improve storage quality.

1.1.3. In-vitro RBC quality measures

Studies on stored RBCs quality aim to define the desirable standards of products where risks are minimized, and benefits are maximized. As studies on the risks of blood transmitted diseases have decreased in the past few decades, there have been increasingly rapid advances in the field of the RBC in vitro quality measures during the six weeks of storage (52, 65). Several attempts have focused their quality measures on the regulatory standards of 24 hours' recovery after transfusion and keeping hemolysis less than 0.8% (12). The Canadian regulatory standards state that hematocrit must not exceed 0.8 L/L, and hemoglobin must be at least 35 g/unit. However, other parameters are routinely monitored such as predonation hemoglobin level and volume of RBC product (65).

Canadian Blood Services (CBS) created a quality monitoring program (QMP) in 2005 which includes a panel of in vitro testing of structural and functional properties of stored RBCs collected from different CBS sites. QMP involves assessment of the quality of RBCs at the beginning (3-7 days) and the end (40-43 days) of storage. The quality measures have been expanded in this program to cover the structural characteristics of RBCs during storage including morphological alterations, RBCs indices, hemolysis, deformability, and extracellular potassium concentration. Oxygen delivering capacity of RBCs has also been measured through the hemoglobin oxygen affinity of partial pressure of oxygen (p50), 2,3-DPG, and ATP concentration (54).

The red cell membrane must be considered when studying the quality of stored RBCs. RBCs with impaired cell membranes are unable to resist the post-transfusion mechanical and osmotic stresses and lead to shortening of cell survival and an increase in the incidence of cells removal from the circulation (36). Therefore, maintenance of the cell membrane integrity is

extremely critical to improve storage quality. With all of these in mind, however, limited reports in the literature have focused on studying the cell membrane properties. The quality of the red cell membrane can be determined by combining various in vitro quality measures. The membrane integrity can be assessed by testing hemolysis, potassium leakage, and microvisiculation which represent the breakdown of the cell membrane. RBC deformability can also be measured to determine the ability of cell membrane to undergo reversible shape changes when subjected to various shear stresses. The ability of RBCs to resist osmotic stresses can be estimated through osmotic fragility testing. The mean corpuscular volume (MCV) and the mean cellular hemoglobin concentrating (MCHC) can also help to define cellular rigidity by defining the internal molecules to volume ratio. By combining these parameters along with water permeability testing, which will be discussed in detail in the next section, a better understanding of cell membrane quality during storage can be derived

1.2. Water Permeability as a membrane quality predictor

The red cell membrane, like other eukaryotic cells, allows the movement of water as it is the essential molecule of the life. The red cell membrane has a transmembrane protein channel known as aquaporin 1, previously called CHIP 28. The aquaporin 1 allow RBC to function as an osmometer enabling the movement of water across the membrane to maintain the osmotic equilibrium with the extracellular medium (23, 37). Thus, water moves through the cell membrane aquaporin from the medium of lower osmolality to the medium of higher osmolality to maintain the osmotic equilibrium causing cell shrinkage or swelling. When the RBC is placed in a hypertonic medium, water moves from inside the cell, in which the concentration is lower, to the extracellular medium until the difference between internal and external osmolality is

equalized (osmotic equilibrium). As the hypertonic solution shrinks the cell, a hypotonic solution leads to cell swelling and increases the cell volume. The structure and the composition of the RBC membrane controls the rate of movement of water between the cell and the extracellular environment (66-70).

Water permeability has been considered one of the major determinants of cellular quality during the cryopreservation of RBCs at freezing temperatures. It has been used widely to define an optimal protocol for cryopreservation (71, 72). However, there are limited reports in the literature that investigate the water permeability properties of RBCs during hypothermic storage. The cell membrane of stored RBCs experience various injuries including breakdown and loss of membrane parts which may have an impact on the ability of cells to overcome the post-transfusion osmotic stresses. Therefore, osmotic behavior of stored RBCs may be a predictive indicator for the quality of the cell membrane and how it may response to storage and post-transfusion osmotic stresses.

1.2.1. Osmotic parameters

Three main osmotic parameters govern the movement of water through the cell membrane. These are the water permeability, the osmotically inactive fraction, and the Arrhenius activation energy. The water permeability (Lp) is the rate at which water crosses the cell membrane (69). Most methods measure the water permeability by measuring the rate of cell volume change when placing RBCs into an anisotonic solution (66, 70, 73). Briefly, when placing the RBCs in an anisotonic solution, water moves in and out of the cell depend on the concentration of the solution until it reaches an equilibrium volume (69). RBCs contain substances such as hemoglobin that have no role in the osmotic exchange of water. These

substances are known as osmotically inactive substances (b). Therefore, the volume of these substances must be subtracted from the total cell volume to get the osmotically active volume of water. The Arrhenius activation energy (Ea) is used to show the effect of temperature on the water permeability (69, 74).

A number of equations have been used to determine the permeability characteristics of the cell. Jacobs and Stewart (1932) developed a mathematical equation to describe the rate of volume change in isotonic solutions. The rate of volume change depends on the difference between intracellular and extracellular concentrations and the cell membrane surface area (75). That is:

$$\frac{dV}{dt} = L\mathrm{p}ART\left[\left(C_s^i - C_s^e\right) + \left(C_i^i - C_i^e\right)\right] \qquad \text{Eq. 1-1}$$

Where V is the cell volume (μm³), t is the time (min), Lp is the membrane water permeability (μm³/min/atm), A is the cell surface area (μm²), R is the gas constant (μm³atm/mole/K), T is the absolute temperature (K), C_s^i is the intracellular concentration of permeate solute (moles), C_s^e is the extracellular concentration of permeate solute (moles), C_i^i is the intracellular concentration of nonpermeate solute (moles), and C_i^e is the extracellular concentration of nonpermeate solute (moles) (76, 77). It is assumed in Eq. 1-1 that the cell membrane thickness and surface area remain constant throughout the experiment. Other assumptions are that the concentration of experimental solutions also remain constant during the experiment, and both of the experimental solutions and intracellular fluids are homogenous in composition (75).

The osmotically inactive volume (b) can be estimated by using Boyle-van't Hoff equation through plotting equilibrium cell volume as a function of osmolality after placing cells into various anisotonic solutions. The following equation can give the volume of the inactive fraction:

$$\frac{V}{V_0} = (1 - b)\frac{\pi_0}{\pi} + b \qquad \text{Eq. 1-2}$$

Where V is the equilibrium cell volume (μm³), V_0 is the isotonic cell volume (μm³), b is the cell volume at infinite osmolality or also known as the osmotically inactive volume, π_0 is the isotonic osmolality (osmoles/kg), and π is the anisotonic osmolality (osmoles/kg) (71, 72, 78). It is assumed in Eq. 1-2 that the Boyle-van't Hoff relation is true in which the osmotically active volume is constant during the entire course of experiment (78).

To determine the Arrhenius activation energy (Ea), measurement of of Lp at different temperatures is needed. It can be determined from the slope of plotting the logarithm of the Lp versus the inverse temperature, 1/T:

$$Lp = k * exp\left(\frac{-Ea}{RT}\right) \qquad \text{Eq. 1-3}$$

Where Lp is the membrane water permeability (μm³/min/atm), k is the fitting constant, Ea is the activation energy for Lp (kcal/mol), R is the gas constant (μm³atm/mole/K), and T is absolute temperature (K) (76, 79).

1.2.2. Techniques for measuring water permeability of RBCs

Several methods have been developed to measure the membrane permeability of RBCs. The stopped-flow analyzer is considered as the most common instrument used to measure rapid

volume changes induced by osmotic changes. There are three major methods for using the stop-flow to measure the membrane water permeability. The intensity of light scattering method has been used to monitor the osmotic changes in cell volume, and is based on the relationship between the light that is scattered from cells on the cell volume change. This approach was applied to measure water permeability in a variety of small cells including RBCs and membrane vesicles (68, 80-83). Another method is the fluorescence quenching method that measures the RBCs volume by using an intracellular fluorescent volume indicator such as carboxyfluorescein diacetate. The cell volume changes can be determined directly from the intensity of fluorescence of the fluorescent indicator which is trapped within the cell (73, 80, 81, 83). However, these techniques have been associated with some limitations such as factors that affect the intensity of the light scattering and the fluorescence intensity including mixing artifacts, the value of cell refraction index (73, 81, 83), and the use of a fluorescent dye (73, 83).

A new method was introduced in 2014 to rapidly measure the cell response to anisotonic solutions by measuring the intrinsic fluorescence of intracellular hemoglobin (73). It has been demonstrated that the globin chains of RBC hemoglobin exhibit autofluorescent properties due to the presence of tryptophan. Hemoglobin molecules, as a result, emit light with a maximum intensity at 310-330 nm when excited by light at 280 nm (84, 85). Zhurova et al. scanned the fluorescence spectrum, as a part of this technique optimization, of adult RBCs after being excited at 280 nm using stopped-flow analyzer and concluded that the maximum emission was at 314 nm (73). The principle of the new method is based on measuring the quenching of hemoglobin autofluorescence that results from changes in cell volume. The rate of change of cell volume with time when RBCs are place in anisotonic solutions can be measured directly from the intensity of intracellular hemoglobin fluorescence and used to calculate the water permeability

characteristics of the cell. More specifically, fluorescence intensity increases when mixing of RBCs with a hypotonic solution to induce cell swelling. However, cells will shrink when placed into a hypertonic solution leading to a reduction in autofluorescence due to the quenching of the fluorescence (73). This method was applied to determine the osmotic parameters of cord and adult RBCs (76). It will also be used, for this project, to monitor the cell membrane quality of stored RBCs at different time points during storage.

1.3.Hypothesis

If the storage length, blood manufacturing process, and donors' age and sex affect membrane remodeling during hypothermic storage, then membrane water permeability of red blood cells can be used to evaluate red blood cell membrane quality.

1.4.Objectives and Approach

During hypothermic storage, the red cell membrane is considered one of the critical elements in the cellular response as it very sensitive to the extracellular medium when RBCs move from a normal physiological medium to a medium of different osmolality. It regulates the cellular volume and intracellular contents by maintaining the movement of water and solutes between cytoplasm and the external environment. A better understanding of cell membrane osmotic properties is essential in predicting a good outcome from hypothermic storage. However, limited reports in the literature have studied the cell membrane osmotic behavior during storage. This novel idea will be examined in this thesis as we will assess the osmotic parameters of the red cell membrane during hypothermic storage. With that we can evaluate red

cell membrane integrity and how the cell membrane would behave to different osmotic stresses during storage by determining the elasticity and rigidity of the cell membrane as cell swells and shrinks.

The specific objectives of this thesis are the following:

1. Characterize the RBC water permeability, osmotically inactive fraction, and Arrhenius activation energy as a function of storage time.

   It is known that the quality of the cell membrane is affected during the hypothermic storage which is revealed as an increase in hemolysis, microvisiculation, and decrease in deformability (86). However, the effects of storage on the water permeability and other osmotic parameters are unknown. In Chapter two, differences in water permeability will be investigated at various time points during storage. The two other osmotic parameters that regulate the movement of water through the cell membrane, osmotically inactive fraction and Arrhenius activation energy, will also be assessed along with water permeability. These osmotic parameters will be measured using a stopped-flow analyzer (73).

2. Determine the effect of blood component manufacturing method as a key factor for water permeability changes of hypothermically stored RBCs

   Recent evidence suggests that the blood manufacturing methods used to separate RBCs from whole blood donations have an impact on the quality of stored RBCs (54, 58). Chapter three attempts to show the influence of blood component manufacturing methods on the membrane water permeability and on other membrane quality parameters. Whole blood donations used for this study will be processed by the two blood

manufacturing methods that are commonly used by CBS, red cell filtration (RCF) and whole blood filtration (WBF). Another knowledge can be obtained from this study is, also, the relationship between the storage length and manufacturing methods and if one factor is enhanced by the other.

3. Define and understand the relationship between donor-specific factors (age and sex) with water permeability changes during storage.

In chapter four, water permeability differences in response to the donors' age and sex will be investigated. This chapter aims to determine the extent to which the donor-associated factors could affect the membrane quality and whether that enhanced by the previous factors of the storage length and manufacturing method.

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Chapter 2[1]

Membrane water permeability, osmotically inactive fraction, and Arrhenius activation energy of red blood cells during six weeks of hypothermic storage

2.1.Introduction

Red blood cells (RBCs) experience various structural, biochemical, and functional changes during hypothermic storage which are commonly referred to in the literature as the storage lesion (1, 2). The red cell membrane is one of the most critical components that is affected during storage in which stored cells irreversibly lose some membrane constituents and hemoglobin molecules by a process called mcrovisicleulation (3). This has been connected with altering the RBCs shape, volume, and hemoglobin concentration (4). Other membrane changes have been reported including deformability alterations and an increase in osmotic fragility, membrane aggregability, and adhesiveness (5). Membrane injuries during storage are proposed to be a risk factor in some post-transfusion outcomes including of proinflammatory consequences, oxidative damage, an increased incident of cell destruction, and impaired oxygen delivery (6-8).

The red cell membrane regulates the osmotic water transport between the cell and surrounding environment to maintain the equilibrium volume through the movement of water from a medium of lower osmolality to a medium of higher osmolality causing cell shrinkage or swelling (9, 10). RBCs are usually soaked in isotonic extracellular plasma fluid where the osmolality is nearly 290 mOsmol/kg. However, they may flow through some organs that have different osmolality medium such as renal medulla where the osmolality is extremely hypertonic

[1]This chapter has been submitted for publication.

and may reach to 1400 mOsmol/kg (9). RBCs with impaired membrane water transporter, such the case in Colton-null patients, are unable to resist osmotic stresses in the circulation leading to shortening cell survival and increase the incidence of cells removal from the circulation (11).

Monitoring of the cell membrane osmotic properties at different storage time points is extremely crucial in order to assess the membrane quality during storage as the hypothermic storage protocol was designed to preserve the quality of cell membrane and other RBC main elements (12). In addition to all of the overwhelming reports on the membrane changes during the storage, membrane water movement testing can provide more estimation about the cell membrane response to osmotic stresses. Several osmotic parameters are used to characterize water transport across the cell membrane including the water permeability, osmotically inactive fraction, and Arrhenius activation energy. The water permeability (Lp) describes the rate of movement of water through the cell membrane which is proportional to the differences between intracellular and extracellular concentrations (13). The osmotically inactive fraction (b) is the volume of the cell that is not involved in the osmotic response (14). The Arrhenius activation energy (Ea) shows the dependence of water permeability on temperature (15). A stopped- flow analyzer has been used to measure the membrane water transport through methods of osmotically induced volume change (16-19).

Assessing the osmotic parameters has been used widely to determine membrane structural and functional properties of a variety of cells. A number of reviews have discussed in detail assumptions, theories, and applications of osmotic principle (13, 14, 20, 21). However, limited reports in the literature have studied the cell membrane osmotic parameters during hypothermic storage. The majority of attempts to characterize a cell's osmotic parameters have centred on assessing the quality of cells during cryopreservation (16, 22-24). It has never been

investigated to show the functionality of RBCs during hypothermic storage. This study comes with the idea that monitoring storage membrane osmotic changes may be a storage predictor of the biophysical and chemical changes that affect the quality of RBCs.

In this study, the osmotic properties of the red cell membrane have been determined at sequent testing points during storage by using a newly established method that measures the kinetics of osmotically induced volume using the intrinsic fluorescence of intracellular hemoglobin (16). The main aim of this study is to investigate the RBCs membrane water permeability and the related osmotic parameters including osmotically inactive fraction and Arrhenius activation energy throughout storage. This study also attempts to explore the impact of storage length on the membrane water permeability as well as on the membrane quality parameters.

2.2.Materials and methods

2.2.1. Blood collection & processing

Nine RBC units were collected from age and gender matched male donors over 50 years of age. Units were obtained from the Canadian Blood Services' Network Center for Applied Development (netCAD) in Vancouver. Whole blood in citrate phosphate dextrose (CPD) anticoagulant was collected and the RBCs were separated from plasma and buffy coat using the red cell filtration (top and bottom) method. RBC units were leukoreduced and mixed with and saline adenine glucose mannitol (SAGM) preservative solution (25). All units were stored at 1 to 6ºC for six weeks and sampled at days 3, 14, 21, 28,3 5, and 42 for the osmotic and quality parameters. Additionally, nine other units were derived from the same donor group and manufacturing process. These units were stored in the same conditions and tested for supernatant

potassium (K^+) and sodium (Na^+). Ethics approval was obtained from the Canadian blood services Ethics Board and the research Ethics Board of the University of Alberta.

2.2.2. RBC osmotic parameters

Osmotic measurements were based on the osmotically induced volume changes which was performed by mixing RBCs with experimental solutions prepared from concentrated phosphate buffered saline (10x PBS). To prepare one litter of 10x PBS, 80 g of sodium chloride (NaCl, Sigma-Aldrich, Lot SLBK2622v), 2 g of potassium chloride (KCl, Sigma-Aldrich, Lot SLBJ0462v), 14.20 g of disodium hydrogen phosphate (Na2HPO4, Sigma-Aldrich, Lot SLBJ0462v), and 2.45 g of potassium dihydrogen phosphate (KH2PO4, Sigma-Aldrich, Lot 14312LO) were dissolved in 800 mL distilled water (dH2O). Volume was then adjusted to 1L by adding more dH2O. This solution was filtered and stored at room temperature. Experimental solutions were then prepared by diluting 10x PBS concentrated solution with a proper amount of dH2O to achieve 0.5x, 1x, 5x, and 8x PBS solutions. The osmolality of the experimental solutions was measured in triplicate using a freezing-point depression osmometer (Precision Systems Inc., Natick, Massachusetts). Briefly, 100 µL of the experimental solution was loaded into the osmometer. The instrument started to cool the solution below its freezing point until ice crystallization is induced by mechanical agitation. The instrument, then, started to heat the solution up to the freezing point which is then converted to osmolality as mOsmol/kg. At each testing point, 20 mL of RBC suspension was prepared for each sample by mixing 400 µL RBCs with 20 mL of 1x PBS solution.

Monitoring the cell volume change when placing RBCs into an anisotonic solution was determined by tracking the change in the intrinsic hemoglobin fluorescence intensity. A stopped-

flow spectrometry SX18 MV (Applied Photophysics, Leatherhead, UK) was used to measure kinetic changes of RBC intrinsic fluorescence by rapidly mixing the same volume of the RBC suspension and experimental solutions of approximately 120 μL. The instrumental sittings and conversion of fluorescence to RBC volume were described in details by Zhurova et al. (16), and it has been used to determine osmotic parameters of RBC (15). Briefly, the stopped-flow spectrophotometer was programmed to record one thousand data points of RBC fluorescence intensity during 10 seconds of reaction. The excitation wavelength and the emission detection were set at 280 nm and 314 nm, respectively. To assure accurate osmolality during each experimental run, the stopped-flow circuit was flushed with the same solution to be tested before each experimental run. Fluorescence of each experimental solution was measured to allow baseline subtraction of the buffer fluorescence from each fluorescent point of RBCs upon mixing with the same experimental solution. Fluorescence of RBCs when mixing with each experimental solution was then measured. Excel software was used to convert fluorescence data to volume as a function of time using correlation equation between fluorescence and volume. Some changes to this technique were applied to remove unwanted scattered excitation light as described in Appendix 1.

To determine Lp, RBCs suspended in 1x PBS were mixed with 0.5x PBS to reach a final concentration of 0.75x PBS with a final osmolality of 217 ± 0.5 mOsmol/kg. As a control, the change in intrinsic fluorescence of RBCs were determined for an isotonic concentration (1x PBS) with the final osmolality of 289 ± 2 mOsmol/kg. The Excel solver was then used to examine the goodness of fit of data to equation 2-1 (15, 20, 26, 27):

$$\frac{dV}{dt} = L\mathrm{p}ART\left[\left(C_s^i - C_s^e\right) + \left(C_i^i - C_i^e\right)\right] \qquad \text{Eq. 2-1}$$

Where V is the cell volume (μm³), t is the time (min), Lp is the membrane water permeability (μm³/min/atm), A is the cell surface area (μm²), R is the gas constant (μm³atm/mole/K), T is the absolute temperature (K), C_s^i is the intracellular concentration of permeate solute (moles), C_s^e is the extracellular concentration of permeate solute (moles), C_i^i is the intracellular concentration of nonpermeate solute (moles), and C_i^e is the extracellular concentration of nonpermeate solute (moles).

The osmotically inactive volume (b) was determined in this experiment to specify the fraction of substances that have no role in the osmotic exchange of water. The b was determined from the intercept of the Boyle-van't Hoff plot of relative cell volume ($\frac{V}{V_0}$) with inverse osmolality ($\frac{\pi_0}{\pi}$) in which V is the equilibrium cell volume, V_0 is the isotonic cell volume, π_0 is the isotonic osmolality, and π is the anisotonic osmolality (14, 28). In this study, RBC suspensions were mixed with equal amount of 0.5x, 1x, 5x, and 8x PBS to achieve the final osmolality of 217 ± 0.5, 289 ± 2, 844 ± 1, and 1272 ± 1 mOsmol/kg, respectively. The mean volume of the last 3 seconds of the 10 s collected on the stopped-flow analyzer was used to calculate the equilibrium volume (V).

The Arrhenius activation energy (Ea) was determined in this study to show the effect of temperature on the water permeability. The value of Ea can be determined from the slope of plotting the logarithm of the Lp versus the inverse temperature, 1/T (15, 22). To determine the Ea, experimental values of Lp for RBCs with a solution of final osmolality of 217 ± 0.5 mOsmol/kg were also characterized at three different temperatures; 4,20 and 37 ± 0.1 ºC.

2.2.3. RBC quality parameters

RBC deformability was performed to measure the ability of RBCs to undergo reversible shape changes when subjected to shear stress. This was assessed by using the principle of ektacytometry in which RBC suspension was subjected to shear stress resulting in cells elongating by different degrees using a laser-assisted optical rotational cell analyzer (LORCA, Mechatronics, the Netherlands). Briefly, a 10 µL of RBC was diluted in 1 ml of polyvinylpyrrolidone (PVP) and assessed at 37 ºC (± 0.1). A laser beam is scattered by the RBC suspension that are stressed at different levels of shear stress ranging from 0.95 to 30 Pa which then displayed as a deformability curve based on the diffraction of cell shape. Data was then transformed to the Eadie-Hofstee plot to determine two RBC deformation parameters. EI_{max} which represents the predicted maximum elongation index at an infinite shear stress and that can be obtained from the y-intercept of the plot. K_{EI} is the shear stress needed to obtain 50% of the maximum elongation which can be determined from the slope (29, 30).

Total hemoglobin, supernatant hemoglobin, and percent hemolysis were performed in this study to monitor the release of free hemoglobin from RBCs. A Drabkin's spectrophotometric method was used to determine total and supernatant hemolysis (31). In this method, RBCs and supernatants were diluted with Drabkin's reagent. The resultant solution was then measured spectrophotometrically (SpectraMax 384 Plus, Molecular Devices Corp., Sunnyvale, CA) at wavelength of 540 nm which directly related to the concentration of hemoglobin in the sample (4, 32). The percent hemolysis was calculated by dividing the supernatant hemoglobin to the total hemoglobin as shown in equation 2-2 (33). The hematocrit was used to determine the volume of the supernatant in the sample.

$$\%\mathrm{Hl} = \frac{(100 - \mathrm{Hct}) \times \mathrm{Hb_S}}{\mathrm{Hb_T}} * 100 \qquad \text{Eq. 2-2}$$

Where %Hl is the percent hemolysis (%), Hct is the hematocrit (%), $\mathrm{Hb_S}$ is concentration of the supernatant hemoglobin (g/L), and the $\mathrm{Hb_T}$ is concentration of the total hemoglobin (g/L).

The ability of RBCs to resist osmotic stresses was assessed by determining osmotic fragility which is the concentration of saline solution where 50% hemolysis occurs. The point of 50% hemolysis is known as mean corpuscular fragility (MCF). This was done by diluting RBCs 1:100 in a series of decreasing concentration from the normal physiological saline ranging from 0.9 g/L to 0.0 g/L in dH2O. Percent hemolysis for each suspension was then determined using spectrophotometry at wavelength of 540 nm. 50% hemolysis was determined by plotting the percent hemolysis with the concentration of solutions (4, 34).

The hematologic indices including the mean corpuscular volume (MCV, fL), the mean corpuscular hemoglobin (MCH, pg), and the mean corpuscular hemoglobin concentration (MCHC, g/L) were also assessed in this study by using automated cell counter (Beckman Coulter ACT 8, Fullerton, CA).

2.2.4. Supernatant potassium (K^+) and supernatant sodium (Na^+)

Nine additional units were tested as a part of the quality monitoring program (QMP) for red blood cell components performed in Canadian Blood Services Edmonton center. The fresh (day 7) and the expiry (day 42) measurements of supernatant K^+ and supernatant Na^+ were obtained for the additional units. Sample were spun (2200x g, 10 min, 4 °C), and an aliquot of

supernatant was sent out to a local hospital for Na+ and K+ qualification by ion selective electrochemistry on a chemistry analyzer (DXC800, Beckman Coulter) (34).

2.2.5. Statistical Analysis

The sample size of 9 units was estimated from a statistical equation based on a specific margin of error and confidence level for the previous results of Lp (15). That is

$$n = \left(\frac{z_{a/2} \cdot \sigma}{E}\right)^2 \quad \text{Eq. 2-3}$$

Where n is the sample size, $Z_{a/2}$ is the standard error of the population mean (constant number depend on the confidence level), σ is the standard deviation, and E is the maximum error of estimate. Data analysis was conducted using SPSS statistical software (Version 23, IBM, NY). One-way analysis of variance (ANOVA) was used to identify the significance of storage length on all parameters. If there is any significant result, further statistical testing of Tuckey post hoc was performed to identify the contribution of each testing point. The Pearson correlation was used to determine the relationship of water permeability with quality parameters of RBC. A p-value of less than 0.05 was considered significant for both analyses. Samples were measured in triplicate for all osmotic parameters, and in duplicate for all quality parameters except for osmotic fragility which was only tested once at each time points. Mean, standard deviation (SD), standard error of the mean (SEM) were reported for all parameters.

2.3.Results

2.3.1. Osmotic parameters

There were significant elevations in the water permeability of RBCs during 42 days of storage ($p < 0.0001$). Water permeability measurements at day 3, 14, 21, 28, 35 and 42 are shown in Figure 2.1. The water permeability was 9.9 ± 1.9 μm³/min/atm at the beginning of storage (day 3) which significantly increased at day 21 to 29.3 ± 8.3 μm³/min/atm ($p < 0.0001$) and that significant elevation remained for the rest of storage where Lp reached 27.05 ± 5.7 μm³/min/atm at the end of storage (day 42). One sample was selected as a representative for the nine samples to show the kinetic of volume changes as a function of time after mixing of RBCs suspension with 0.5x PBS (final osmolality 217 ± 0.5 mOsmol/kg) at three different storage periods (Figure 2.2). Analysis of the kinetic of volume change throughout storage time shows that RBCs reach equilibrium volume within approximately 0.02 minute (1.2 second) on day 21 and 42, while it needs more than 0.04 minute (2.4 second) to reach the same equilibrium volume on day 3.

The Boyle-van't-Hoff plot for osmotically inactive fraction of RBCs is shown in Figure 2.3 for days 3, 21, and 42 of storage upon mixing RBC suspensions of 9 units with 1x, 0.5x, 5x, and 8x PBS gave a final osmolality of 289 ± 2, 217 ± 0.5, 844 ± 1 and 1272 ± 1 mOsmol/kg, respectively. The osmotically inactive fraction of fresh testing of day 3 (b_1) was 0.47 ± 0.2 which significantly increased to 0.75 ± 0.1 at the end testing (b_3) ($p < 0.001$). The average equilibrium volume when mixing RBCs with solutions of final osmolality of 289, 217, 844 and 1272 mOsmol/kg were 92.8 ± 1.2 μm³, 111.3 ± 4.2 μm³, 64.5 ± 3.2 μm³, and 55.0 ± 3.1 μm³ at the fresh testing (day 3) which shifted to be 92.9 ± 1.3 μm³, 103.2 ± 3.6 μm³, 76.9 ± 3.1 μm³, and 77.1 ± 2.1 μm³ at day 42 of testing, respectively. The Arrhenius activation energy (Figure 2.4)

was 3.52 ± 1.4 kcal/mol at the beginning of the storage which significantly increased ($p < 0.005$) on day 42 of storage to be 4.89 ± 0.2 kcal/mol.

2.3.2. Impact of storage on in-vitro quality parameters

Deformability assessments at day 3, 21, and 42 of storage are presented in Figure 2.5 on the Eadie-Hofstee plot. EI_{max} decreased significantly within 21 day of storage ($p < 0.05$) which continue to decrease until the end of storage ($p < 0.001$). However, K_{EI} was not affected during the storage. Table 2.1 displays the absolutes values of quality parameters at day 3, 21, and 42 of storage. Significant changes were seen in the supernatant hemoglobin and hemolysis but not on the total hemoglobin concentration. At expiry, the concentration of the supernatant hemoglobin and the percent hemolysis were significantly increased ($p < 0.001$). The mean corpuscular fragility did not change during storage ($p = 0.47$). The hematologic indices were also affected by storage. The MCV was elevated but not significantly ($p = 0.19$) in which RBCs average volume increased from 94.2 ± 3.7 fL at the fresh testing to 97.4 ± 4.1 fL at the end of storage. The MCH did not also significantly change during storage ($p = 0.57$). The MCHC significantly decreased throughout the storage. The significant reduction was first observed on day 21 which continued until the end of storage ($p < 0.0001$).

Data on the supernatant K^+ and supernatant Na^+ is shown in Table 2.2 The supernatant K^+ level increased significantly throughout storage ($p < 0.001$) in contrast to the supernatant Na^+ which significantly decreased with storage ($p < 0.001$).

Correlations of the osmotic parameters to the quality parameter show that water permeability has a significant correlation ($p < 0.001$) with changes in hemolysis ($r = 0.473$),

MCV (r = 0.499), supernatant K^+ (r = 0.895), and supernatant Na^+ (r = - 0.897) as shown in Figure 2.6. Correlation analysis of Lp, also, indicated that there were also significant relationships ($p < 0.01$) with deformability (r = 0.364), the concentration of supernatant hemoglobin (r = 0.448), MCF (r = 0.359), and MCHC (r = 0.512). MCH was not significantly correlated to the Lp.

2.4.Discussion

This study examined the effect of storage length on osmotic properties and routine quality parameters of RBCs. The water permeability, the osmotically inactive fraction, and Arrhenius activation energy were significantly increased within 21 days of storage, which were previously unreported. The value of Lp at the day 21 and day 42 testing had elevated to a degree of almost three times more than the value of fresh testing at day 3. Also, the expiry values of *b* and Ea had raised with 159.5% and 138.9%, respectively, from the fresh testing. The EI_{max}, percent hemolysis, MCHC, supernatant K^+, and supernatant Na^+ were also significantly affected by the storage length. The remaining quality parameters, including K_{EI} , MCV, MCH, and the osmotic fragility, were also affected but to lesser degree.

A possible reason for the significant increase of water permeability during storage is that RBCs lose their semipermeability and become leakier as the storage proceeds. This is in line with other reported storage membrane injuries. It has been shown that some parts of membrane are progressively lost during storage of RBCs which is associated with hemolysis elevation, morphological alteration, and K^+/Na^+ disruption (1, 3, 4). Correlation analysis in this study has indicated that there is significate relationship between Lp and other quality measures including hemolysis, MCV, supernatant K^+/Na^+, MCHC, and supernatant hemoglobin. This could be

explained by that storage lesion is associated with changes in membrane properties in which membrane become permeable to substances that are not normally able to cross the cell membrane followed by allowing hemoglobin and some other internal contents to exit the cell. As a result, Lp is increased because cells eventually lose their intact functional membrane. Findings of this paper corroborate the ideas of Lucke and McCutcheon who reviewed consequences of water permeability increase in various cells (13). They indicated that injured cells become more permeable to water before they lose their contents, which may explain why RBCs, in the current study, had a 178% change in water permeability at day 14 of storage compared to the fresh testing. The findings of this research provide insights for that the water permeability increase during storage is more likely to be due to membrane damages as part of quality loss during storage.

This study showed that the value of Lp varied depending on the storage length. Explaining the consistency of the current results of Lp with the literature need to be applied with caution because most of the previous studies report the RBC water permeability at only one-time point. For example, Zhurova et al. used the same intrinsic fluorescence experiment to compare water permeability of RBCs as a function of various osmolalities in which all RBC samples were examined within 15 days of collection (15). They found that when mixing RBCs with a hypotonic solution with the osmolality of 214 mOsmol/kg, the water permeability was 9.51 ± 0.35 μm^3/min/atm which is in agreement with those obtained by the current study at the fresh testing on day 3. Moreover, Agre et al. tested red cell osmotic water permeability within two days of collection at 37°C by monitoring the light scattering with a stopped-flow analyzer (35). They reported that the Lp of samples obtained from adult participants was 0.037 ± 0.007 cm/s.

The unit of cm/s can be converted to μm³/min/atm using the following equation according to Zhurova et al. (15):

$$\mathrm{Lp} = \frac{P_f}{T * 7.5926 * 10^{-6}} \qquad \text{Eq. 2-4}$$

Where the Lp is water permeability with the unit of μm³/min/atm, P_f is the water permeability with the unit of cm/s, and T is the temperature (k). By applying the preceding equation (Eq. 2-4), the osmotic water permeability of Agre et al.'s study is equivalent to 15.71 ± 2.97 μm³/min/atm which is approximately 160% higher than that of reported for the day 3 of storage in this study.

The osmotically inactive fraction also showed a significant increase during storage. The initial value of osmotically inactive fraction at day 3 (b_1= 0.47 ± 0.2) is in agreement with reported values in the literature which are *b* = 0.47 ± 0.02 (36), 0.41 ± 0.01 (37), and 0.51 ± 0.02 (38). As the storage proceeded, however, cells are unable to shrink to the same degree as in fresh testing (Figure 2.3). There are two possible explanations for the significant increase in *b* during storage. It could be that cells gain more volume which does not participate in the osmotic activity and that is consistent with an increase in MCV change during storage (Table 2.1). Second, the ability of red blood cells to response to the osmotic pressure by undergoing osmotically-induce volume regulation is highly dependent on the elasticity of the cell membrane which is mainly regulated by the membrane skeleton (39, 40). It has been reported that storage induces a decrease in RBCs elasticity and rheological properties (41, 42). As that is the case, the ability of membrane skeleton to rearrange their position to undergo volume change is limited as the storage proceed. This appears with decrease elasticity and increase the rigidity and therefore the inactive fraction. However, the one way ANOVA of the rigidity indicator (K_{EI}) showed no statistically

significant results throughout the storage. Future studies on the reasons for significant inactive volume increased during storage and relationship of that with cell rigidity are therefore recommended.

The third osmotic parameter, Arrhenius activation energy, is also significantly increased during storage. In general, therefore, it seems that the water permeability of cells become more dependent on temperature as the storage proceed. It has been suggested that the Ea is related to formation and breaking of hydrogen bonds as water passes through membrane layers (43). If that is the case, the high Ea at the end of storage may be related to the rearrangement of membrane layers during storage. In addition, these results corroborate the ideas of Elmoazzen et al. who suggested that the value of Ea is related to the structure of cell membrane. That is, cells with membrane water transporter proteins seem to have lower Ea compared with cells without membrane water transport (44). As the membrane water permeability increases at the end of storage, it is therefore likely that such connections exist between the storage length and loss of membrane aquaporins which eventually influences the membrane semipermeability.

According to these data, we can infer that water permeability can be used as a predicted quality measures of red cell membrane during hypothermic storage. There is a large volume of published studies describing the cell membrane quality of stored RBCs as the maintenance of red cell membrane is essential for preserving cell unique shape, rheology, and osmotic properties. Henkelman et al. have assessed the rheological properties of the red cell membrane in term of aggregability and deformability, and they used that to show the effect of leukoreduction on the functionality of red cell membrane (45). Microvesicle counting using flow cytometry has also been used as a quality predictor of cell membrane quality during storage (4, 46). Usry et al. have monitored the morphological properties of cells and connected that with cells viability after cells

being rejuvenated (47). There are some other parameters have been used to assess the quality of cell membrane during storage including osmotic fragility and hematologic indices (4, 32, 34).

Overall, findings of the current research strengthen the idea that the cell membrane of stored RBCs significantly changes with storage time. This study has identified the membrane water permeability, osmotically inactive fraction, and Arrhenius activation energy differences as a function of storage length. Findings of the current research have raised important questions about the nature of red cell membrane during hypothermic storage and when damage to RBC begins to occur during storage. More research investigating the effect of storage length on the osmotic properties RBCs especially the water permeability is required to confirm the degree of accuracy of this study findings. Further research could also use the membrane water permeability as an indicator of other factors that may affect the storage quality such as storage processing factors including blood manufacturing method and additive solution, as well as, donor-associated characteristics including age and sex.

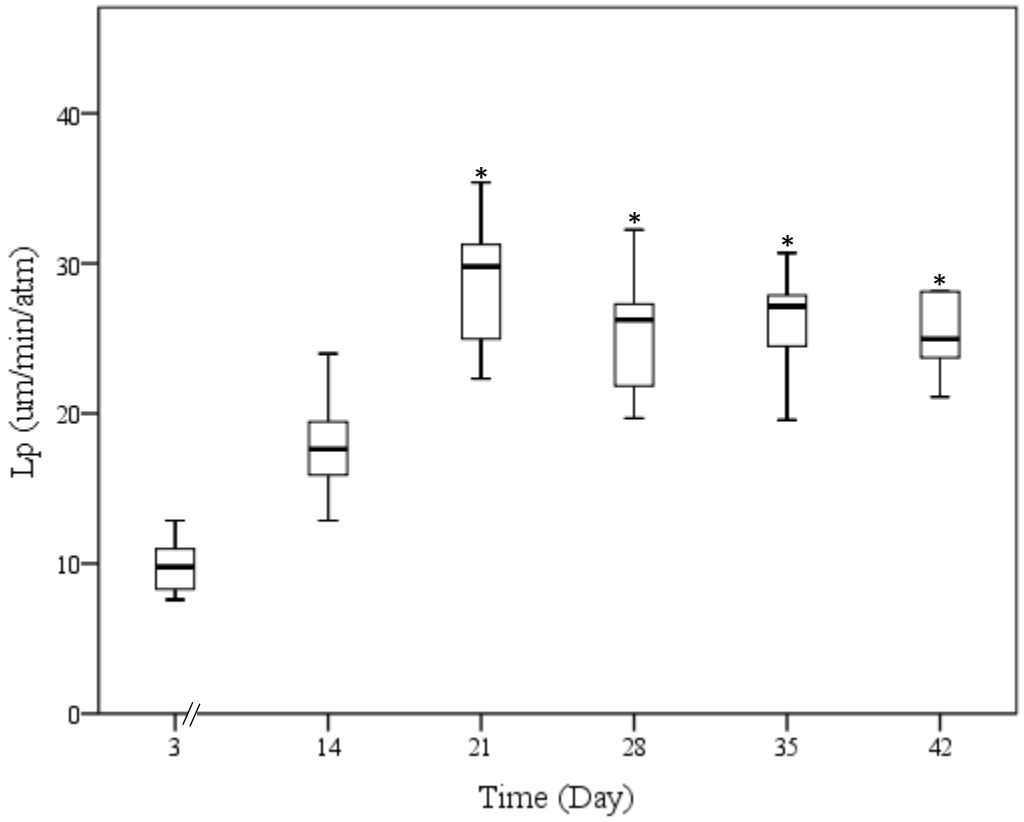

Figure 2.1 Water permeability of RBCs for days 3, 14, 21, 28, 35, and 42 of storage. Water permeability was measured over 42 days of storage by using stop flow analyzer. Box at each testing time point represents the first and third quartiles of the data, the band inside the box is the median, and the top and bottom of the whiskers represent the full range of the minimum and maximum limit of all of the data (n = 9).

* Significant difference when compared with fresh testing (day 3) with p value < 0.05.

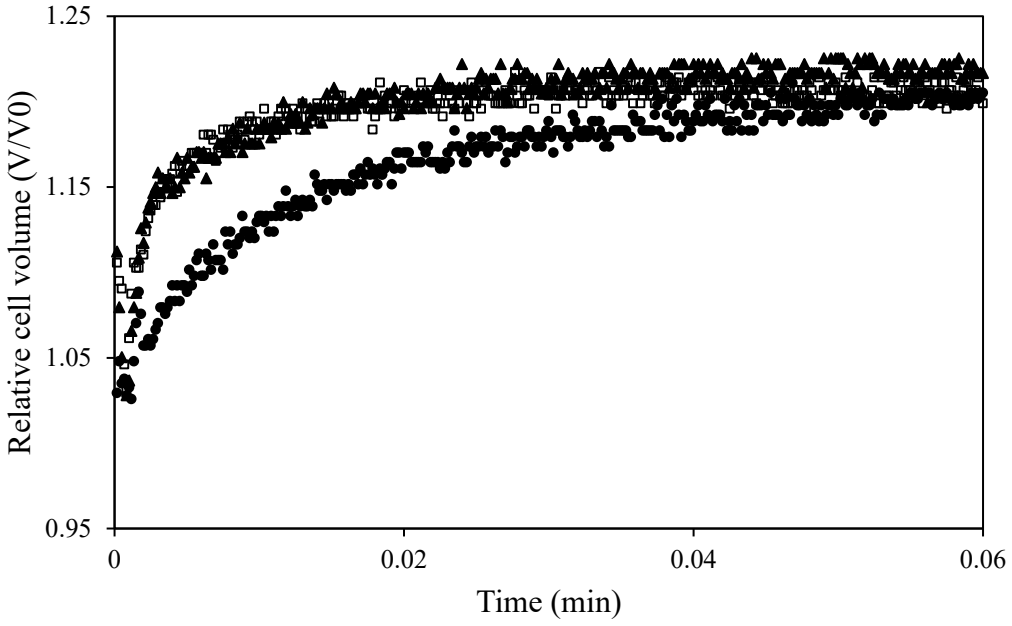

Figure 2.2 The kinetic of RBCs volume changes as a function of storage time for one representative sample. Dots representing kinetics of RBC volume at day 3 , 21 and 42 after mixing RBCs suspension with 0.5x PBS. (●) Day 3; (▫) Day 21; (▲) Day 42

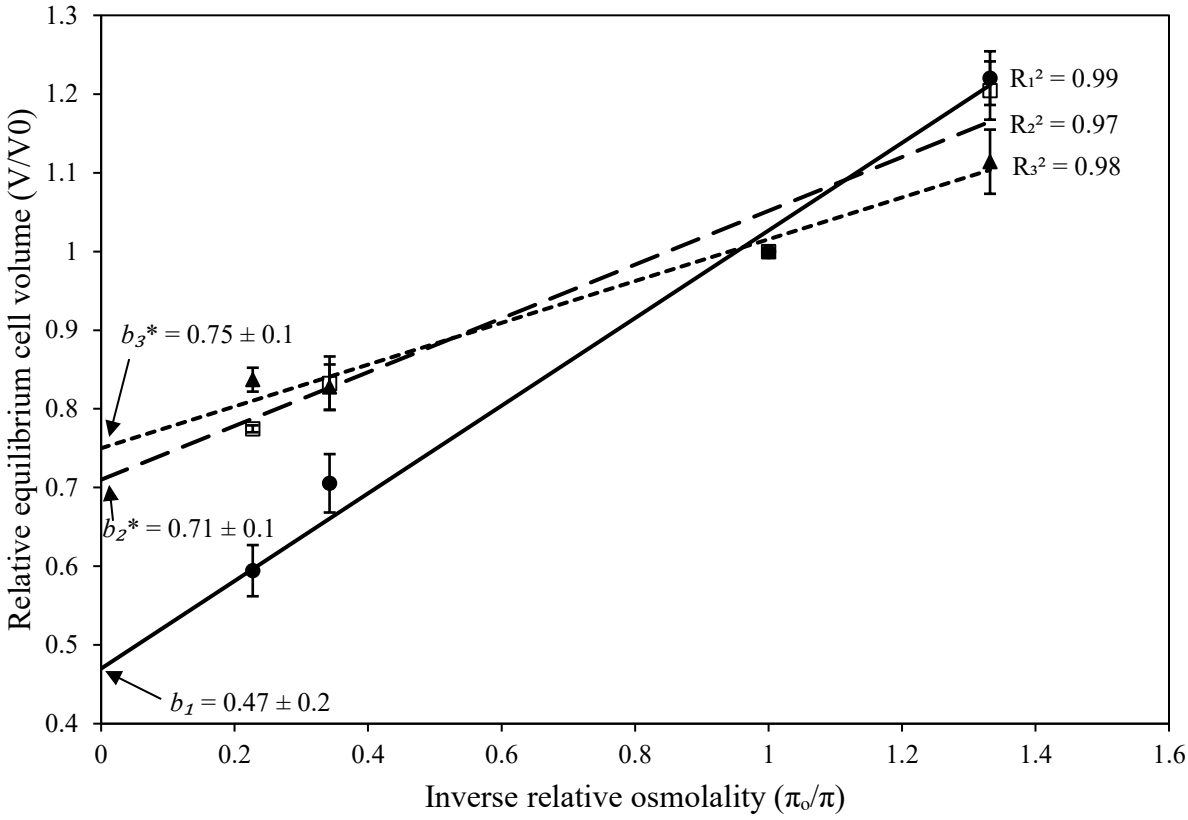

Figure 2.3 Boyle-van't-Hoff plot for RBCs during storage. Osmotically inactive fraction for day 3 (b_1), day 21 (b_2) and day 42 (b_3) were calculated from y-intercept of the linear regression fit for units at each testing time point. Reported *b* are mean ± SD (n = 9). Markers are the relative equilibrium cell volumes (mean ± SEM) for 9 RBC units in different experimental solutions (0.75x, 1x, 3x, and 4.5x PBS). (●) Day 3; (▫) Day 21; (▲) Day 42.

* Significant mean difference when compared with fresh testing (day 3) with p value < 0.05.

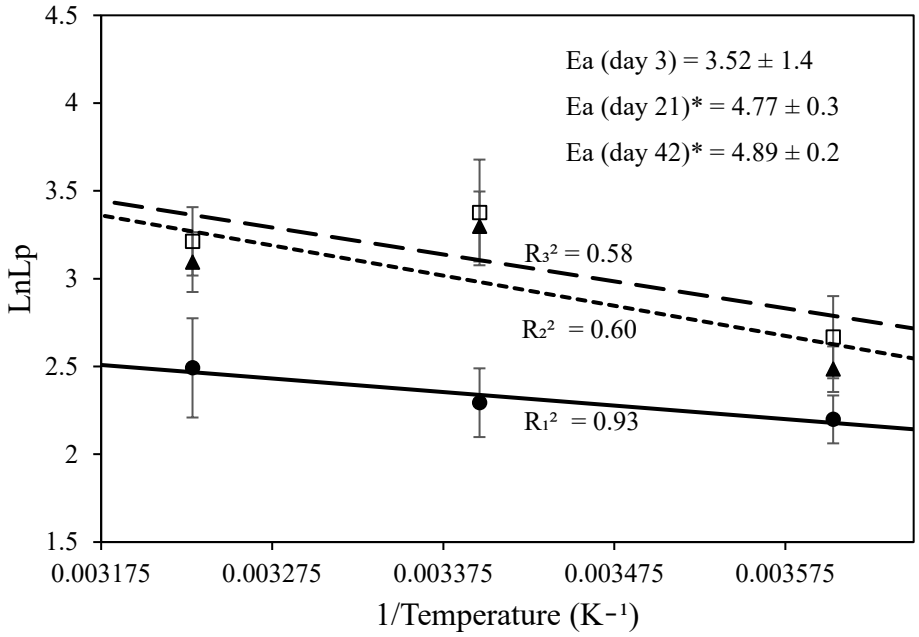

Figure 2.4 Arrhenius plot for RBCs during storage. Activation energy for day 3, day 21, and day 42 were determined from the slope of the linear regression fit for units at each testing date. Reported Ea are mean ± SD (n = 9). Markers are the natural logarithm for Lp (mean ± SD) for 9 RBC units in different temperature (4, 20, 37ºC ± 0.1). (●) Day 3; (▫) Day 21; (▲) Day 42.

* Significant mean difference when compared with fresh testing (day 3) with p value < 0.05.

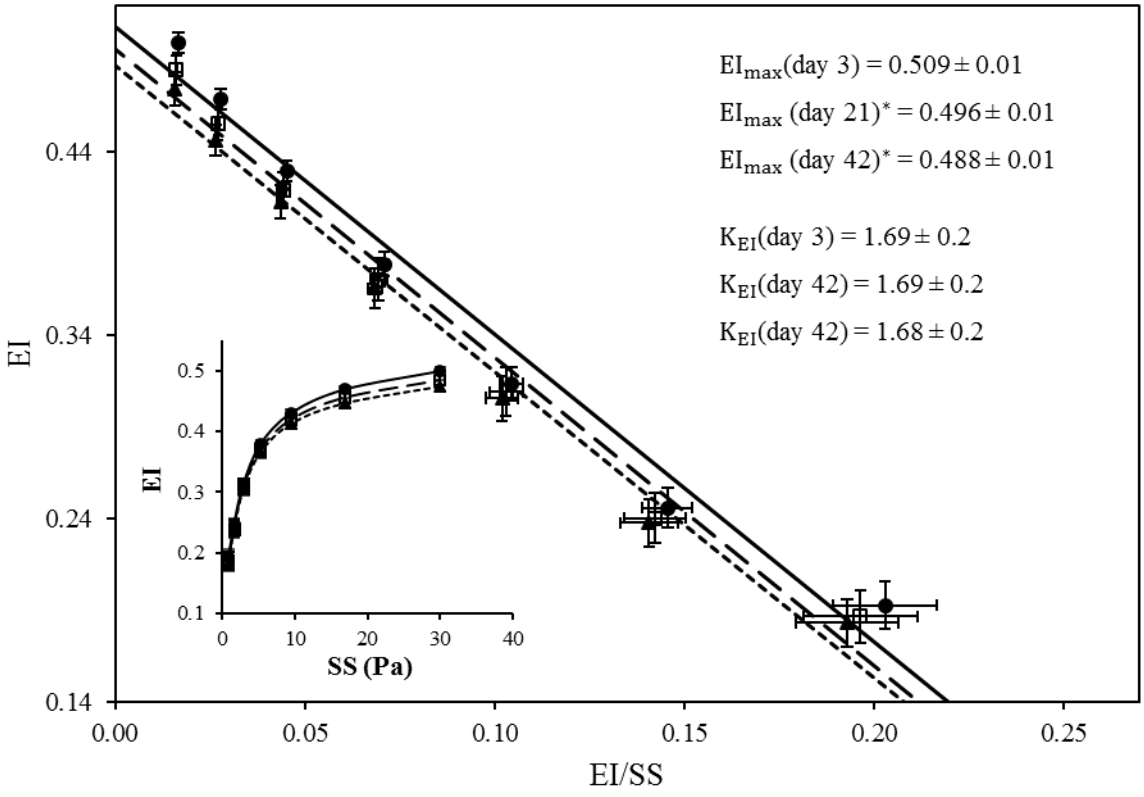

Figure 2.5 Eadie-Hofstee plot of RBCs deformability during storage. Deformability curves were obtained for 9 RBC units at 3 sequence testing time points. Reported EI_{max} and K_{EI} are mean ± SD. (●) Day 3; (▫) Day 21; (▲) Day 42.

* Significant mean difference when compared with fresh testing (day 3) with p value < 0.05.

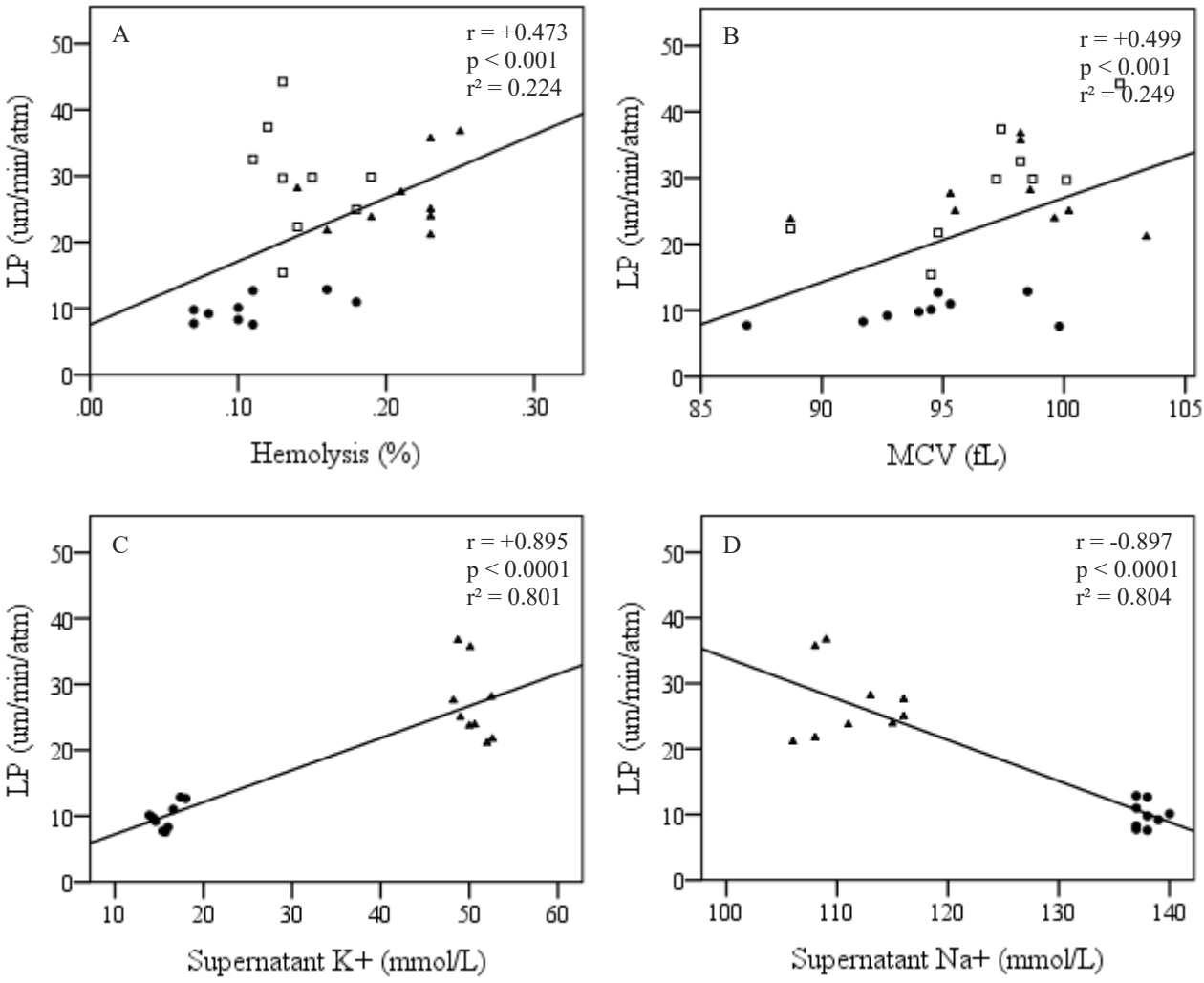

Figure 2.6 Correlation between the water permeability and hemolysis (A), MCV (B) at 3 testing time points of fresh testing (day 3), mid-time testing (day 21), and expiry testing (day 42). Correlation between the water permeability at the fresh and expiry time points with supernatant K^+ (C), and Supernatant Na^+ (D). The values of Pearson correlation coefficient (r) and the significance level (P) are shown in each figure in which the sign of r represents the direction and its absolute value indicate the strength of the relationship between Lp and other quality parameter. (●) fresh testing of day 3 or day 7; (▫) mid-time testing of day 21; (▲) expiry testing of day 42.

Table 2.1 Quality parameters during 42 days of storage.

Quality Parameters	Length of storage		
	Day 3	Day 21	Day 42
Total hemoglobin, g/L	189.1 ± 9.3	199.4 ± 8.9	191.2 ± 8.6
Supernatant Hemoglobin, g/L	0.507 ± 0.2	0.724 ± 0.1	1.107 ± 0.4 *
Hemolysis, %	0.11 ± 0.04	0.14 ± 0.03	0.23 ± 0.07 *
MCF, g/L	4.93 ± 0.2	5.00 ± 0.2	5.06 ± 0.2
MCV, fL	94.2 ± 3.7	96.9 ± 3.8	97.4± 4.1
MCH, pg	30.86 ± 1.5	30.60 ± 1.4	30.15 ± 1.4
MCHC, g/L	327.4 ± 5.0	315.3 ± 3.4 *	309.4 ± 2.6 *

Data are reported as mean ± SD (n = 9).

* Significant mean difference when compared with fresh testing (day 3) with p value < 0.05.

Table 2.2 Supernatant potassium (K^+) and supernatant sodium (Na^+).

Quality Parameters	**Length of storage**	
	Day 7	Day 42
Supernatant K^+, mmol/L	15.7 $\pm$ 1.4	50.4 $\pm$ 1.6 *
Supernatant Na^+, mmol/L	137.9 $\pm$ 1.1	111.3 $\pm$ 3.8 *

Data obtained from the Quality Monitoring Program. Units were tested for days 7 and 42 (n = 9). Data are reported as mean ± SD (n = 9).

* Significant mean difference when compared with fresh testing of day 7 with p value < 0.05.

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Chapter 3[1]

Effect of blood manufacturing on water permeability and membrane quality parameters during hypothermic storage of red blood cells

3.1.Introduction

Currently, red blood cell (RBC) units stored at hypothermic temperature (1-6°C) for up to six weeks are licenced for clinical use as long as 75% of transfused RBCs remain in the circulation for 24 hours after transfusion and the degree of hemolysis in the bag is less than 1% (1, 2). These regulations have been placed to ensure that the quality of products is maintained during collection, processing, and storage and to minimize the possibility of adverse harms to patients. However, studies in the literature have reported that stored RBCs lose some of the membrane parts, hemoglobin, adenosine triphosphate (ATP), and 2,3-diphosphoglycerate (2,3-DPG). These structural and biochemical alterations have been associated with cell rigidity, morphological changes, and eventually, degradation in all deformability dependent factors which are collectively known as the storage lesion (1, 3, 4). Several studies and reviews have focused on linking the storage length to the storage lesion and the loss of quality and efficacy of stored RBCs (5-8). Various clinical cohort studies have also considered that the risk of post-transfusion adverse outcomes, including immunomodulatory effects, increased length of stay in hospital, and organ failures, increases with transfusing prolonged stored RBCs especially when they have been transfused to critically ill patients (9, 10).

[1] This chapter is in preparation to be submitted for publication.

A considerable literature increases, recently, with the argument of that along with the storage length, the blood component manufacturing also has an effect on the stored RBCs quality (11-13). Two manufacturing processes that are commonly used by Canadian Blood Services (CBS) to separate RBCs from whole blood (WB) collection include the red cell filtration method (RCF, top and bottom) and the whole blood filtration method (WBF, top and top) (14). The main differences between these two methods are summarized in four factors including the duration and temperature of holding before processing, the speed and length of centrifugation, component derived from whole blood after centrifugation, and the step of leukoreduction (13, 14). Recent studies have described the significant impact of the manufacturing process on the quality measures (13, 15, 16). However, the quality of the functional characteristics of RBC cell membrane as a function of manufacturing has not fully explored.

Monitoring of the membrane water permeability (Lp) as a function of storage length has shown significant results in the study of chapter two of this thesis. It has been measured widely in the literature to describe the cell membrane structure and integrity of various cells (17-19). Lp represents the rate at which water moves across the cell membrane when subjected to anisotonic conditions (19). Thus, the cell membrane regulates the osmotic properties of RBCs especially when flowing through a medium of different osmolality by maintaining the movement of water and solutes between cytoplasm and the extracellular environment until reaching the osmotic equilibrium volume (20). During blood manufacturing, RBCs experience various osmotic stresses starting from moving cells from normal physiological medium of the body circulation to a bag with a hypertonic anticoagulant solution with an approximate osmolality of 450 mOsmol/kg (21). These stresses, along with several mechanical stresses including centrifugation and filtration, may affect the membrane water permeability of RBCs.

In this study, membrane water permeability along with membrane quality measures were examined in this study as a function of RBC manufacturing methods. An objective of this research was to assess the influence of two CBS' blood processing methods on the membrane water permeability as well as membrane related parameters. Other objectives were to investigate whether the effect of blood processing methods is enhanced by storage length, and to test the correlation between the Lp and membrane quality measure for RBC units papered by RCF and WBF.

3.2.Materials and methods[1]

3.2.1. Blood collection and processing

Fifty-one WB units from healthy blood volunteers were collected and processed at three CBS production sites; British Columbia & Yukon (19 units), Calgary (13 units), and Dartmouth (19 units). WB, with a target collection volume of 480 mL, were mixed with 70 mL of citrate-phosphate-dextrose (CPD) anticoagulant (13) and either processed by red cell filtration (RCF, n_1 = 27) or whole blood filtration (WBF, n_2 = 24) (Figure 3.1). Briefly, RCF units were processed using the top-and-bottom system in which WB units were stored for up to 20 hours at room temparature and then centrifuged at 3493x g for 11 min. Saline-adenine-glucose-mannitol (SAGM) additive solution was added to RBC units after component separation, followed by leukoreduced filtration. In WBF (top-and-top system), WB units were held in the refrigerator for up to 72 hours before leukoreduction and then centrifuged at 4552x g for 6 min. SAGM was added to extracted RBCs (13-15). RBC units were packed in a temperature maintained box (1-10

[1] This study was a part of research collaboration called the Quality Monitoring Program (QMP), led by Dr. Jason Acker and Anita Howell at Canadian Blood Services. The experimental design and data analysis referred to in this chapter were developed by me.

°C) and shipped to CBS Edmonton site (Acker's lab). All units were stored at 1-6°C on receipt. Testing points were set at three timings including fresh testing (day 5 ± 2), middle testing (day 21), and expiry testing (day 42). At each testing point, units were gently mixed, and 4 to 6 mL were drawn from the bag and used for in vitro measurements[1]. This research protocol was reviewed and approved by the Research Ethics board of the University of Alberta and the Canadian Blood Services Research Ethics Board.

3.2.2. In vitro measurements

The experimental methods for water permeability, deformability, percent hemolysis, osmotic fragility, supernatant potassium (K^+) and supernatant sodium (Na^+), and RBCs indices are described in detail in the methods section of chapter 2. A brief description of these measurements will follow.

3.2.2.1. Water permeability (Lp)[2]

Water permeability (Lp) was measured at each of three testing points using an intrinsic hemoglobin fluorescence intensity method. Briefly, RBCs suspensions were prepared for each unit by diluting 400 µL RBCs in 20 mL 1x PBS solution with a final osmolality of 285 ± 1 mOsmol/kg. An SX18 MV stopped-flow analyzer (Applied Photophysics, Leatherhead, UK) was used to obtain one thousand data points of RBCs intrinsic hemoglobin fluorescence intensity during 10 seconds reaction period after mixing equal amount of RBC suspension with 0.5x PBS

[1] Sampling was performed by Anita Howell and Angela Hill.
[2] I performed the water permeability measurements.

(147.5 ± 1 mosm/kg) to reach a final concentration of 0.75x PBS (final osmolality 217 ± 1 mosm/kg). Fluorescence points were converted to volume as a function of time. Lp was then calculated using an Excel fitting software (22, 23).

3.2.2.2.Quality measures

Deformability[1] of RBCs was determined at fresh and expiry testing points by ektacytometry using a Laser-assisted Optical Rotational Cell Analyzer (LORCA, Mechatronics, the Netherlands). Outcome data was used to determine the maximum elongation index (EI_{max}) and the 50% of the maximum elongation (K_{EI}) (24). At the three testing points, the percent (%) hemolysis[2] was determined using Drabkin's method. The %hemolysis was determined from the ratio of supernatant hemoglobin concentrations to the total hemoglobin concentrations with correction for the hematocrit (13). The mean corpuscular fragility (MCF)[3] was also used to assess the ability of RBCs to resist osmotic stresses of buffered salt solutions of decreasing concentrations. The concentration of salt required to cause 50% hemolysis in a sample represents the MCF (25). A spectraMax 384 Plus spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) was used in both %hemolysis and MCF to measure the absorbance of hemoglobin at 540 nm which is directly related to the amount of hemoglobin in the solution. The RBCs indices[4] including the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH), and the mean corpuscular hemoglobin concentration (MCHC) were also assessed at all three testing

[1] Deformability was performed by April Xu and Angela Hill.
[2] Hemolysis fresh and expiry measurements were performed by Tracey Turner. The middle hemolysis testing was done by me.
[3] I performed MCF measurements.
[4] RBCs indices measurements were performed by April Xu and Angela Hill.

points using automated cell counter (Beckman Coulter ACT 8, Fullerton, CA). Supernatant potassium (K^+) and supernatant sodium (Na^+)[1] were also obtained for fresh and expiry samples.

3.2.3. Statistical analysis[2]

Results are reported as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed to show differences between the two manufacturing processes. Storage length effect was assessed using two way ANOVA. Tukey post hoc was used to identify significance among pairwise comparisons. In the case of significant differences, mixed between-within subjects analysis of variance (mixed ANOVA) was then performed to assess the adjusted main effects and interaction effect between manufacturing process and storage length of whether there were significant effects in results of RCF and/or WBF manufacturing process, and whether these results were affected by the interaction with the storage length. The type of manufacturing process (RCF vs. WBF) was considered as the between-subjects independent variable, and the testing points time (fresh vs. middle vs. expiry) was the within-subjects independent variable. Pearson correlation was performed to determine the strength of the relationship between water permeability and membrane quality measures in both processing methods. A p value of less than 0.05 was considered significant. Statistical analysis was conducted using SPSS statistical software (Version 23, IBM, NY).

[1] Sample were spun (2200x g, 10 min, 4°C), and an aliquot of supernatant was prepared by Anita Howell and April Xu.
[2] I run the data analysis.

3.3.Results

3.3.1. Water permeability (Lp)

RCF units had a significantly lower Lp compared to RBC units prepared by WBF at all three testing points (Figure 3.2). At fresh testing, the Lp of RCF units was 15.26 ± 4.8 μm^3/min/atm, which was significantly different ($p < 0.01$) than the 28.96 ± 4.7 μm^3/min/atm value obtained from WBF units. The significant differences between units of RCF and WBF were also observed on the middle and expiry testings where Lp was 24.27 ± 7.0 μm^3/min/atm and 27.91 ± 6.0 μm^3/min/atm, respectively, for units prepared by RCF, and 30.78 ± 5.4 μm^3/min/atm and 35.20 ± 5.5 μm^3/min/atm for WBF units, respectively. In addition, investigating the storage length effect revealed that the Lp of RCF units was significantly increased on day 21 and 42 compared to the fresh testing ($p < 0.05$). In contrast, the Lp of WBF was not significantly affected on day 21 and 42. The mixed ANOVA analysis indicated that there was a significantly large interaction effect between the manufacturing process and storage length ($p < 0.01$) (Table 3.1). That means the effect of the manufacturing process on water permeability is dependent on the storage length. Therefore, to describe the influence of unit processing, a related storage time is needed to be specified. The main effects of each independent factor (manufacturing process and storage length) were also large and significant ($p < 0.01$). However, the storage length alone had a more significant main effect than the manufacturing method alone.

3.3.2. In vitro quality measures

Deformability measurements of RCF and WBF for fresh and expiry testing points are shown in Eadie-Hofstee plot (Figure 3.3). There were no significant differences between manufacturing methods at each testing points for EI_{max} and K_{EI}. The EI_{max} of units prepared by

RCF had significantly decreased at the end of storage compared to the fresh testing ($p < 0.05$). However, the EI_{max} of WBF units and the K_{EI} of both RCF and WBF units were not significantly affected throughout the storage. The interaction effect between manufacturing process and storage length and the main effects of RCF and WBF processing did not significantly affect the EI_{max} (Table 3.1). However, the main effect of the storage length on the EI_{max} was significantly large ($p < 0.01$).

Units processed by RCF method had significantly lower hemolysis at day 21 and day 42 testing points compared to WBF ($p < 0.05$) (Figure 3.4-A). However, there were no significant differences between units of the two processing methods at the fresh testing point. RCF units also had significantly increased hemolysis at day 42 testing compared to the fresh testing of the same units ($p < 0.05$). In contrast, hemolysis in WBF units was significantly increased, earlier than RCF, at the day 21 testing remained significantly elevated until the end of storage ($p < 0.05$). The mixed ANOVA analysis showed that there was a significantly large interaction effect from the storage length and the manufacturing process ($p < 0.01$) (Table 3.1). In addition, the main effect of each of storage duration and manufacturing methods were also large and significant ($p < 0.01$).

The osmotic fragility and RBC indices parameters (Table 3.2) had shown no significant results in both of storage length and manufacturing process, except for MCHC (Figure 3.3-B). MCHC measurements throughout the storage revealed that there was no significant difference between RCF units and WBF units at all of three testing points. There was, however, significant elevation at the middle and expiry testing points compared to the fresh testing of units prepared by RCF manufacturing method ($p < 0.05$). WBF units were not significantly affected by the storage length. The storage length and the manufacturing process interaction effect and the

manufacturing process main effect on the MCHC were not significant (Table 3.1). The main effect of the storage length was significant and large ($p < 0.01$).

The fresh and expiry measurements of supernatant potassium (K^+) and supernatant sodium (Na^+) are shown in Figure 3.3-C and D. Units manufactured using RCF had significantly higher supernatant K^+ and significantly lowered supernatant Na^+ at each testing points compared to WBF units ($p < 0.05$). Supernatant K^+ was also significantly increased as a function of storage length in both of RCF and WBF units ($p < 0.05$). Supernatant K^+ mixed ANOVA indicated no significant interaction effect with the large significant main effect of each of manufacturing method and storage length ($p < 0.01$) (Table 3.2). Supernatant Na^+ was significantly decreased at the expiry testing in both manufacturing methods ($p < 0.05$). The interaction effect and the main effects of manufacturing method and storage length were significantly large ($p < 0.01$) (Table 3.2).

3.3.3. Correlations between water permeability and RBC in vitro quality measures

Table 3.3 shows the linear correlations between water permeability and RBC in vitro quality measures. There was a significant correlation between the Lp data of units processed by RCF method and EI_{max}, %hemolysis, MCV, MCHC, Supernatant K^+, and supernatant Na^+. The water permeability of WBF units was significantly correlated to MCV, Supernatant K^+, and supernatant Na^+. There was no significant correlation with other quality parameters.

3.4.Discussion

Previous research on the factors behind storage lesion has found that, in addition to the storage length, blood manufacturing also has an impact on stored RBCs quality (12, 13, 15, 26-

29). The membrane water permeability and membrane related quality measures can be used to test if there is any significant difference between production methods as the cell membrane of stored RBCs is one of the cell components that are highly affected from the storage lesion (4, 30, 31). This study was undertaken to assess the membrane osmotic, structural, and biochemical parameters of stored RBCs as a function of the manufacturing process and storage length. Findings of the present study confirmed the previously found data on that the quality of RBCs, that are processed using the two CBS manufacturing pathways; RCF and WBF, is influenced by the manufacturing method as well as the storage length. In addition, it adds to the knowledge of the effect of manufacturing process on membrane Lp and the main and interaction effects of storage duration and the processing methods on the Lp and membrane quality measures.

Water permeability (Lp) measurement was used in this study to determine the osmotic behavior of stored RBCs manufactured by the two CBS production process. As it stated in the results section, RBC units manufactured by WBF had significantly higher Lp at all of three testing points compared to RCF units. The most striking result to emerge from the Lp data is that the value of Lp at the fresh testing of WBF units exceeded the value of Lp at the expiry testing of RCF units. These findings increase the possibility that part of the membrane-related storage lesion happens during the processing and even before the start of the hypothermic storage. Correlations of the membrane Lp to other membrane parameters indicated that it significantly correlated to hemolysis, EI_{max}, MCV, MCHC, supernatant K^+, and supernatant Na^+ of the RCF units, while with WBF it correlated with MCV, supernatant K^+, and supernatant Na^+. The results of the correlation analysis, therefore, provide further support for the efficacy of using water permeability to monitor product quality during manufacturing.

It, also, was not surprising that manufacturing process had an impact on the membrane related parameters, as that was described previously in the literature. We have demonstrated that the storage length and the manufacturing methods have a significant main effect on the product measures of hemolysis, supernatant K^+, and supernatant Na^+. WBF units have been shown to have significantly higher hemolysis and supernatant Na^+ and lower supernatant K^+. These results infer that WBF manufacturing method raises the level of membrane damages of stored RBCs. However, some membrane related quality parameters have shown no difference between the two methods such as EI_{max} and K_{EI}.

Results of the membrane quality parameters in this study match those observed in earlier studies which further support the idea of processing whole blood collections using RCF is better for RBCs quality during storage. Various studies in the literature have provided evidence of that RBC products varied based on manufacturing methods. A recent study by Jordan et al. (2016) examined quality control data of over 28 thousand RBC units produced by RCF and WBF methods (32). They found that compared to the WBF units, the RCF units exhibited lower hemolysis at the end of storage (day 42). Another major study was undertaken by Acker et al. (2014) as a part of the quality monitoring of RBC units processed by different processing methods (13). Units prepared by WBF method were found to exhibit higher hemolysis, hemoglobin content, and supernatant K^+ and lower ATP and 2,3-DPG compared to those processed by RCF method. Hansen et al. (2015) compared the in vitro quality parameters of RBC units produced by nine different manufacturing methods including the two methods that we include in this study (RCF and WBF) (15). They reported that RCF units exhibited the lowest hemolysis among all other methods at both fresh and expiry testing points. They also demonstrated that there were no significant differences between in EI_{max} and K_{EI} which is in

agreement with our findings. However, although there are significant differences in most of in vitro quality measures between manufacturing methods, these results should be interpreted with caution as that does not necessarily mean it clinically significant. Future studies on the post-transfusion influences of RBCs prepared by different manufacturing methods are therefore recommended.

It can thus be suggested that the quality of cell membrane of stored RBCs varies in dependent to the manufacturing method that used to prepare products. There are three possible explanations for the significant differences observed between the two manufacturing methods. The first key difference that may have a potential effect on the stored RBC membrane quality is the order by when the leukoreduction occur during manufacturing (33). The major benefit from leukoreduction is to reduce the amount of white blood cells (WBCs) in the bag and therefore minimize the transmission of WBCs-associated viruses, such as cytomegalovirus and herpesviruses, and the immunomodulation effects (34, 35). When looking at the process on which RBCs obtained from WB donation, the RCF units were separated into three components of RBCs, plasma, and platelet followed by RBCs leukoreduction. While in the WBF methods, the WB were leukoreduced at the beginning followed by centrifugation to separate into plasma and RBCs (Figure 3.1). Doing the leukoreduction at the last, which is the case in the RCF method, could filter cell fragments and damaged cells after centrifugation leading to increase the ratio of intact cell in the bag. In a study which set out to determine the effect of buffy coat removal on filtration quality, Sonker et al. found that RCF units have less damaged cells and residual leukocytes (36). Another source of difference between RCF and WBF methods which may affect the cell membrane quality is the centrifugation speed (32). It seems that doing the centrifugation at a high speed may add more shear stress to cells causing cell membrane to break

off. As noticed in the method section and Figure 3.1, the spinning of WB collections was at different situation depend on the components needed to be extracted. In the RCF preparation, the WB were centrifuged at 3493x g for 11 min to separate WB into packed RBCs, platelets, and plasma. However, the leukoreduced WB of the WBF manufacturing were centrifuged at 4552x g for 6 min to have RBCs and plasma. It has been demonstrated that variations in the centrifugation were associated with variability in residual plasma level in RBC units. Hansen et al. found that RCF units had lower residual plasma compared to WBF units (15) which has been demonstrated to cause membrane damages leading to increase the level of hemolysis in RBC product (37). The time of the manufacturing process from the WB collection to RBC unit separation could be, also, a key difference between the two methods. In the RCF method, the WB were handled within 20 h after collection, while it can be up to 72 h (three days) to be processed by the WBF. Data from several sources have identified the extended processing time associated with disrupting metabolic activities of RBCs which has a direct impact on membrane energy-dependent functions such as sodium/potassium pump leading to increased hemolysis level (12, 38).

This study is subject to certain limitations. One source of weakness in this study which could have affected the measurements of Lp and membrane quality measures was that units were collected and processed using three different production sites. This issue is supported by Jordan et al. (2016) who found that hemolysis measurements vary between 14 processing CBS sites in Canada (32). Therefore, there is a possibility that part of the variation is due to the source of units. Another limiting factor is that the fresh testing point was set at day 5 ± 2. It would be more reasonable to detect the manufacturing effect if we had a day 0 or 1 testing point.

In summary, this study set out to assess the impact of blood manufacturing on membrane water permeability and in vitro membrane quality measures. This study has found that water permeability can be used along with other membrane quality parameters to assess RBCs quality during hypothermic storage. The present study has shown that there were some significant differences between RBC units prepared by RCF and WBF methods. This experiment has also confirmed the effect of the manufacturing process by applying the adjusted main effects and interaction between storage length and the processing methods. Future research should, therefore, concentrate on the investigation of other factors that influence the quality of stored RBCs, including the variability of donor characteristics, such as sex, age, frequency of donation, and ABO-Rh blood group.

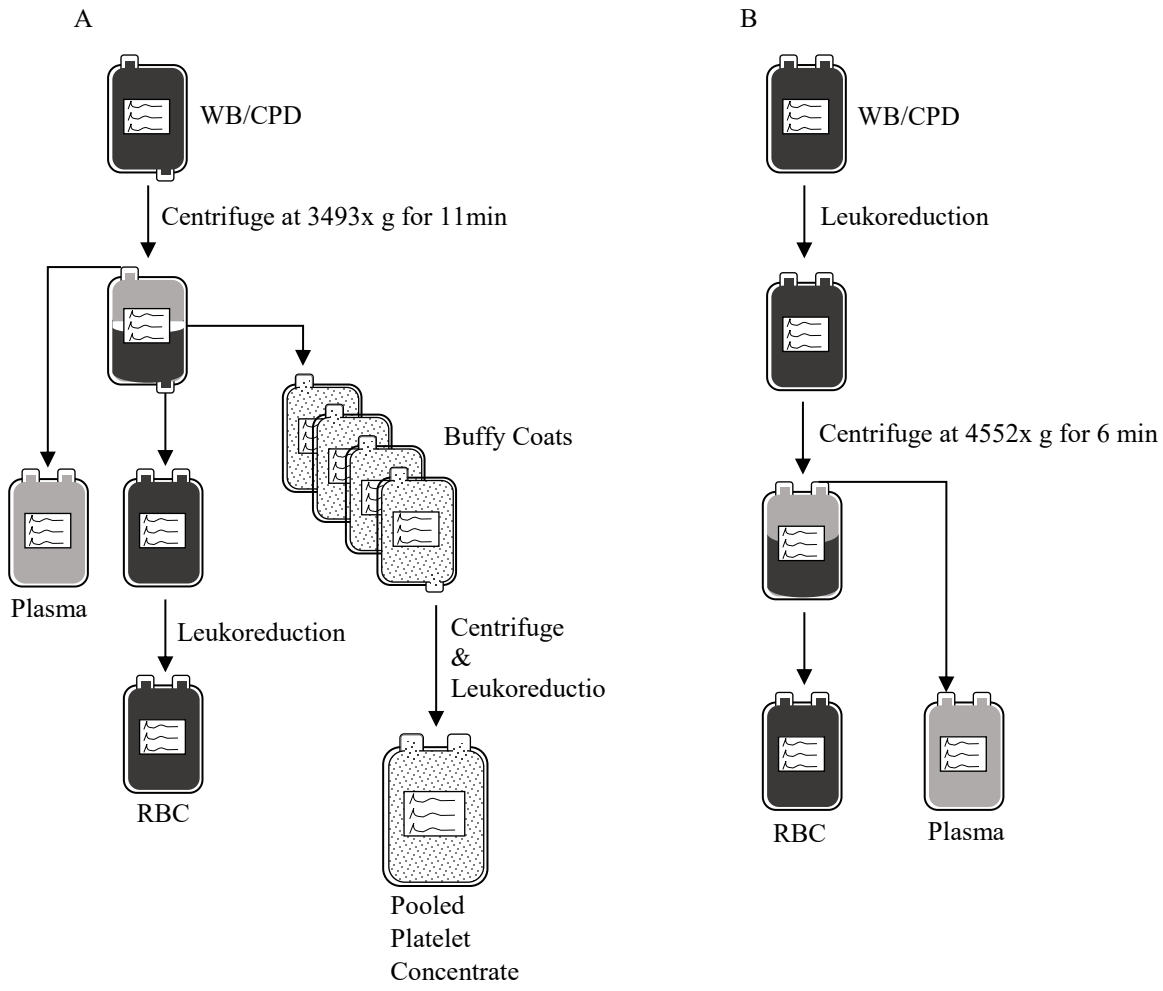

Figure 3.1 Representative diagram for blood manufacturing methods of red cell filtration (RCF, top and bottom) method (A) and whole blood filtration (WBF, bottom and bottom) method (B). Collected RBCs from both methods are mixed with SAGM additive solution and stored at 1-6°C.

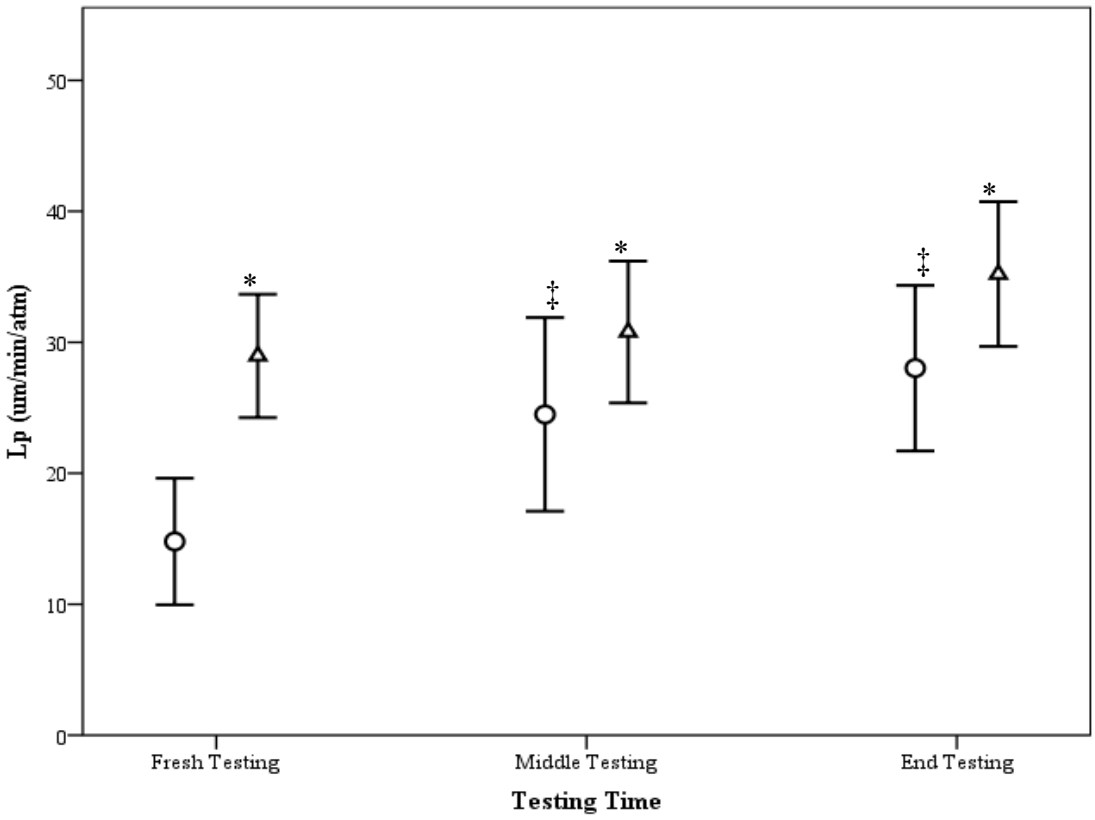

Figure 3.2 The effect blood manufacturing process on water permeability of stored RBCs. Water permeability was measured at three time points of fresh testing (day 5 ± 2), middle testing (day 21), and expiry testing (day 42). The results are mean ± SD. (○) Red cell filtration method, $n_1 = 27$; (△) Whole blood filtration method, $n_2 = 24$. p values of less than 0.05 was considered to be significant.

* Significant mean difference when compared with red cell filtration method at the same testing time point.

‡ Significant results in comparison to fresh testing of the same manufacturing method.

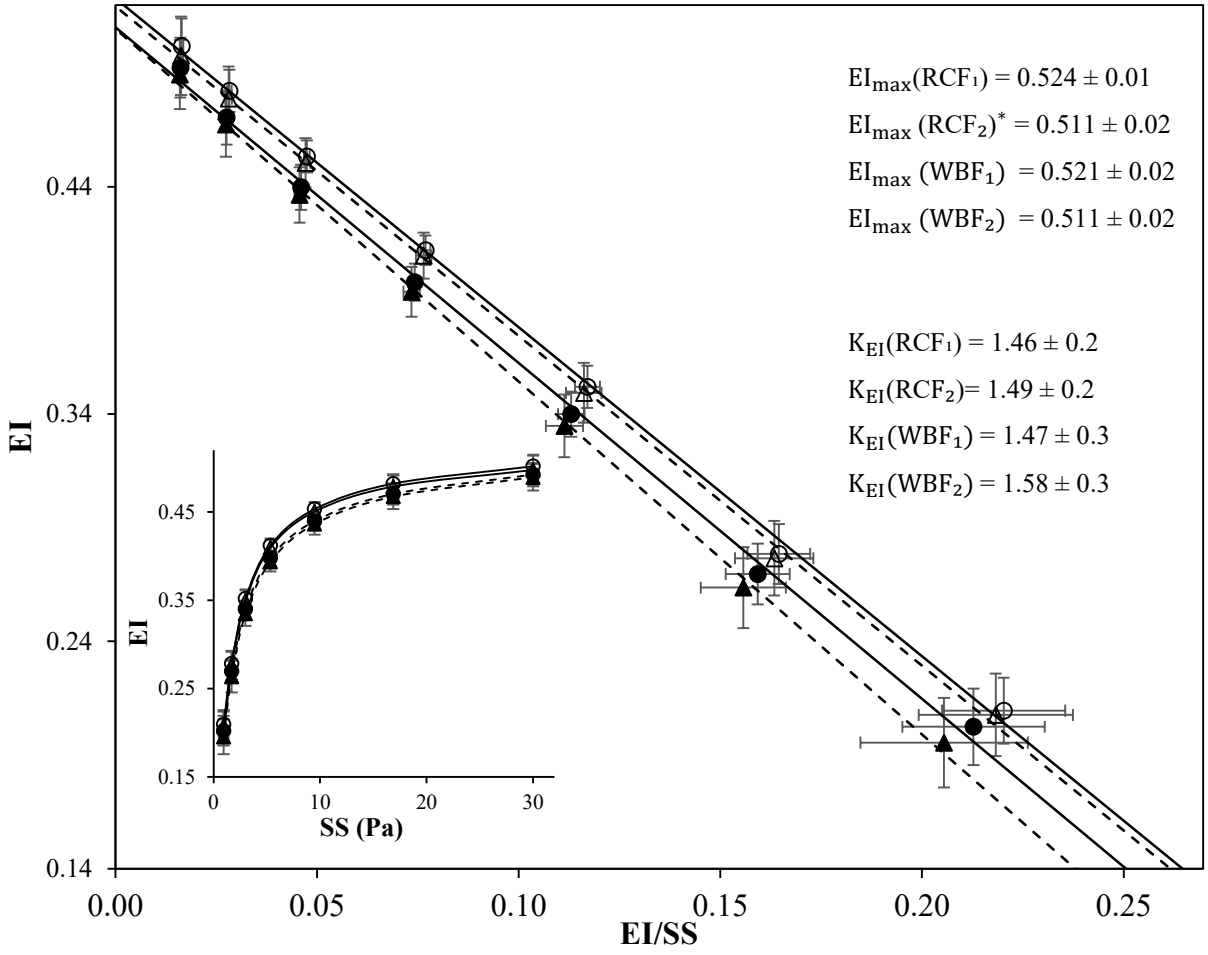

Figure 3.3 Eadie-Hofstee plot of RBCs deformability of red cell filtration units at fresh testing (RCF_1), red cell filtration at expiry testing (RCF_2), whole blood filtration units at fresh testing (WBF_1), and whole blood filtration units at expiry testing (WBF_2). Reported EI_{max} and K_{EI} are mean ± SD. (–○–) RCF_1, (-●-) RCF_2, (–△–) WBF_1, (-▲-) WBF_2.

* Significant mean difference when compared with fresh testing of the same manufacturing method ($p < 0.05$).

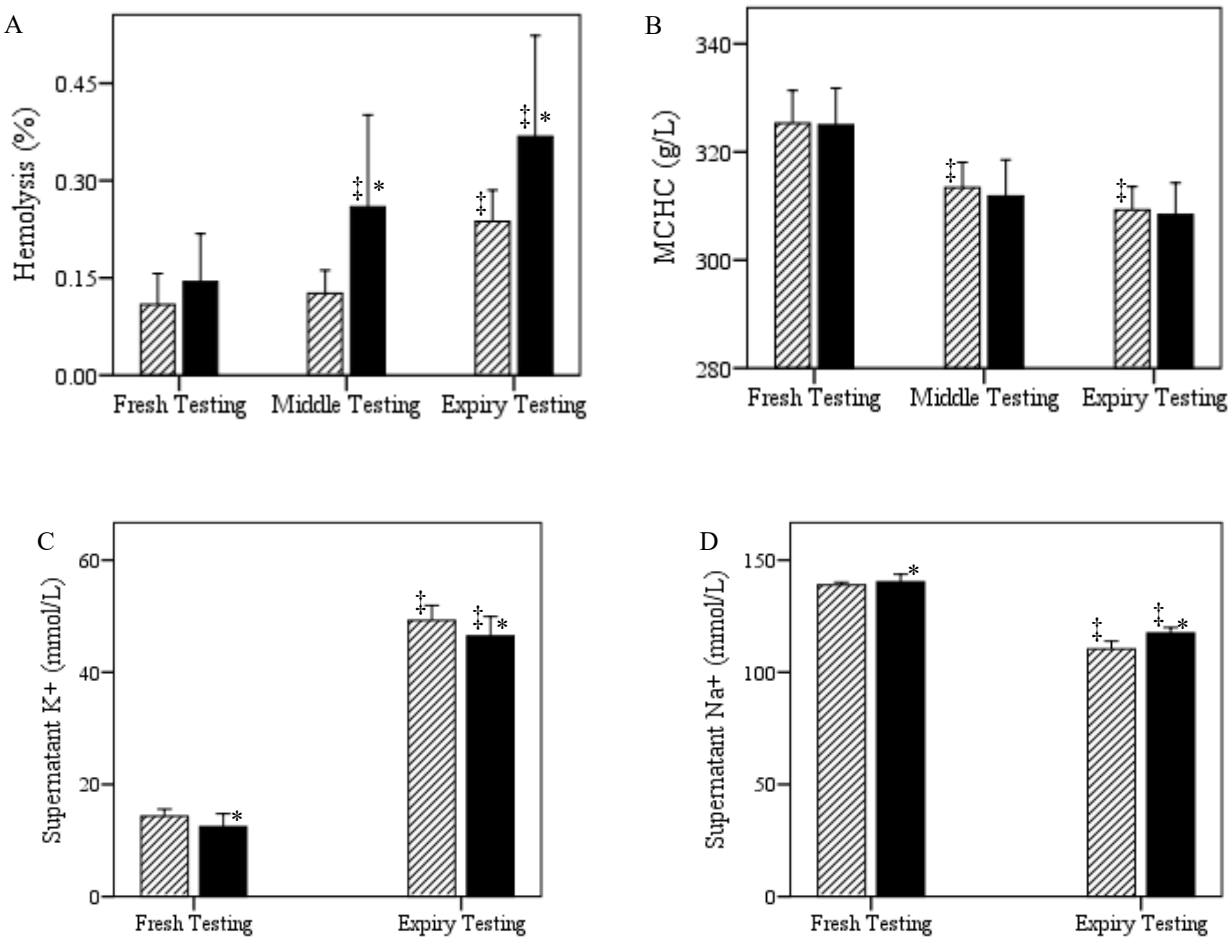

Figure 3.4 Significant membrane quality measures of red cell filtration (RCF) and Whole blood filtration method (WBF) units. Hemolysis (A), MCHC (B), supernatant K^+ (C), supernatant Na^+ (D), were measured for day 5 ± 2 (fresh testing), day 21 (middle testing),if applicable, and day 42 (expiry testing). (▨) RCF; (■) WBF.

* Significant mean difference when compared with red cell filtration method at the same testing time point.

‡ Significant results in comparison to fresh testing of the same manufacturing method.

Table 3.1 Effect of manufacturing processes and storage length on the water permeability and quality parameters

Parameters	manufacturing x storage length *	Manufacturing ‡	Storage length †
Water Permeability	< 0.01 (0.28)	< 0.01 (0.58)	< 0.01 (0.74)
EI_{max}	> 0.05 --	> 0.05 --	< 0.01 (0.43)
% Hemolysis	< 0.01 (0.36)	< 0.01 (0.32)	< 0.01 (0.80)
MCHC	> 0.05 --	> 0.05 --	< 0.01 (0.93)
Supernatant K^+	> 0.05 --	< 0.01 (0.24)	< 0.01 (0.99)
Supernatant Na^+	< 0.01 (0.40)	< 0.01 (0.44)	< 0.01 (0.98)

The numbers in the table represent the p-value for the possibility of effect (the effect size in brackets), that show the main effect for both manufacturing methods and storage length and the interaction between them. The effect size of less than 0.06 represents a small effect, between 0.06 and 0.14 represent a moderate effect, greater than 0.14 represent a large effect (39).

* Interaction effect between the manufacturing process and storage length.

† Main effect of manufacturing.

‡ Main effect of storage length

Table 3.2 Non-significant effect on the remaining membrane quality measures for red cell filtration (RCF) and Whole blood filtration method (WBF) units.

Quality parameter	Red cell filtration (n=27)			Whole blood filtration (n=24)		
	Fresh	Mid	Expiry	Fresh	Mid	Expiry
MCF, g/L	4.86 ± 0.2	4.96 ± 0.2	4.99± 0.2	4.81 ± 0.3	4.90 ± 0.3	4.94 ± 0.3
MCV, fL	93.4 ± 4.4	95.1 ± 4.7	95.9 ± 4.7	91.4 ± 3.7	92.8 ± 3.8	94.1 ± 4.0
MCH, pg	30.3 ± 1.6	29.7 ± 1.7	29.6 ± 1.6	29.7 ± 1.5	28.9 ± 1.5	29.0 ± 1.4

Data are reported as mean ± SD

Table 3.3 Correlation between water permeability of two manufacturing process and quality measures

Quality Measure	Water Permeability	
	Red cell filtration method (n = 27)	Whole blood filtration (n = 24)
EI_{max}	-0.273 *	-0.213
K_{EI}	-0.040	-0.024
Hemolysis	0.493 **	0.157
MCF	0.205	0.017
MCV	0.310 **	0.297 *
MCH	0.082	0.207
MCHC	-0.389 **	-0.052
Supernatant K^+	0.734 **	0.538 **
Supernatant Na^+	-0.772 **	-0.513 **

The values of Pearson correlation coefficients (r) are shown in the table in which the sign of r represents the direction and its absolute value indicate the strength of the relationship between Lp and other quality parameter.

* Correlation is significant at the 0.05 level.

** Correlation is significant at the 0.01 level.

3.5.References

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Chapter 4[1]

Membrane water permeability as a function of donor characteristics

4.1.Introduction

A growing body of literature suggests that donor characteristics are among the most important factors affecting stored red blood cell (RBC) quality. Donor-related characteristics refer to a wide range of variabilities among donor's population including biological factors of sex, age, and ABO-Rh blood group, genetic-related variability such in thalassemia, and behaviors and lifestyle differences such as frequency of donations, smoking, and alcoholic consumptions. A recent review was done by Tzounakas et al. that looked at the potential impact of these determinants on blood donations (1). This current study will address the role of donor-biological variations on stored RBCs quality.

During hypothermic storage of RBCs, the cell membrane is one of the main cell structures that is progressively damaged (2). In fact, preserving the cell membrane structural and functional properties during hypothermic storage is not only crucial for in vitro storage quality but also for in vivo quality of transfused RBCs as RBCs with impaired cell membranes are unable to withstand the post-transfusion mechanical and osmotic stresses (3). Various in vitro quality parameters have been used to assess membrane integrity concerning the biomechanical, structural, and biochemical properties during storage. The osmotic properties of stored RBCs can be monitored by Lp (4) and osmotic fragility (5) assessments. Membrane deformability also has been measured through the principle of ektacytometry to determine the ability of cell membrane

[1] This chapter is in preparation to be submitted for publication.

to rapidly deform and undergo reversible shape changes when subjected to various shear stresses (6). Measuring the amount of free hemoglobin through the Drabkins hemolysis method, monitoring the hematologic indices of RBCs, and assessing the extracellular potassium and sodium concentrations can also help in the prediction of how structurally intact is the cell membrane during the storage. These parameters have shown promising results on predicting the effect storage length and manufacturing methods on the quality of stored RBCs.

In this study, the effect of donor characteristics on measured values for Lp and membrane quality parameters was undertaken. Specificlly, this paper is to assess the influence of donor age and sex on the Lp and membrane quality measures during hypothermic storage of RBCs to determine whether the effect of donor characteristics is enhanced by the storage length. Since the Lp is the center of interest for this thesis and the hemolysis testing is one of the most experimental and clinical standards to assess the quality of RBCs (11), multiple regression models were also applied to test how much of variability in these parameters can be explained by the storage length, manufacturing process, and donor characteristics.

4.2.Materials and methods[1]

4.2.1. Blood collection and processing

Whole blood units were collected from healthy volunteers and processed by the Canadian Blood Services (CBS). Units were mixed with citrate phosphate dextrose (CPD) anticoagulant and processed using red cell filtration (RCF, top and bottom system) method or whole blood

[1] This study was a part of research collaboration called the Quality Monitoring Program (QMP), led by Dr. Jason Acker and Anita Howell at Canadian Blood Services. The experimental design and data analysis referred to in this chapter were developed by me.

filtration (WBF, top and top system) methods. All red blood units were leukoreduced and mixed with saline adenine glucose mannitol (SAGM) preservative solution. Red blood cell units were categorized according to donor's age and sex (Table 4.1). Twenty-seven red blood cell units were obtained from male donors, including 15 units from male over 50 years old donors and 12 units of male less than 30 years old. Twenty-four additional units were obtained from age-matched female volunteers, in which 12 units were derived from female over 50 years old and 12 units were derived from female less than 30 years old. All units were stored at 1 to 6ºC for six weeks and sampled[1] at day 5 ± 2 (fresh testing), day 21 (middle testing), and day 42 (expiry testing) for the water permeability and quality parameters. Ethics approval for this study were obtained from the University of Alberta Research Ethics Board and the Canadian Blood Services Research Ethics Board.

4.2.2. Water permeability and quality parameters

Water permeability (Lp)[2], percent hemolysis (% Hemolysis)[3], and hematologic indices[4] of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were determined at fresh, middle, and expiry testing time points for each group. The experimental methods were described in detail in sections 2.2.2. and 2.2.3 of chapter two. In short, to prepare the RBC suspension for Lp determination, RBCs were diluted 1 : 50 in 1x phosphate buffered saline (PBS) solution (285 ± 1 mOsmol/kg). The intensity of RBCs intrinsic hemoglobin fluorescence upon mixing equal amount of RBC

[1] Sampling was performed by Anita Howell and Angela Hill.
[2] I performed the water permeability measurements.
[3] Hemolysis fresh and expiry measurements were performed by Tracey Turner. The middle hemolysis testing was done by me.
[4] RBCs indices measurements were performed by April Xu and Angela Hill.

suspension with 0.5x PBS (217 ± 1 mOsmol/kg) was then determined using an SX18 MV stopped-flow analyzer (Applied Photophysics, Leatherhead, UK). RBCs intrinsic hemoglobin fluorescence was then converted to volume as a function of time, and Lp was then calculated using an Excel fitting solver software (4, 7). %Hemolysis was determined using Drabkin's method by spectrophotometric measurement of total and supernatant hemoglobin (8). Hematologic indices of the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH), and the mean corpuscular hemoglobin concentration (MCHC) were also determined using automated cell counter (Beckman Coulter ACT 8, Fullerton, CA).

At fresh and expiry testing time points, supernatant potassium (K^+), supernatant sodium (Na^+)[1] were measured for all units using a biochemistry analyzer (Beckman Coulter DXC800, Fullerton, CA) (9). Membrane biomechanical parameters of elongation index (EI_{max}), half of elongation index (K_{EI})[2], and mean corpuscular fragility (MCF)[3] were determined. EI_{max} and K_{EI} were analyzed using ektacytometry principle through a Laser-assisted Optical Rotational Cell Analyzer (LORCA, Mechatronics, the Netherlands) (6). MCF were determined from the concentration of salt required to cause 50% hemolysis in a series of decreasing concentration from the normal physiological saline ranging from 0.9 g/L to 0.0 g/L in dH2O (5).

4.2.3. Statistical analysis[4]

Statistical analysis was performed using SPSS statistical software (Version 23, IBM, NY). Results are reported as mean ± standard deviation (SD). In all the following statistical

[1] Sample were spun (2200x g, 10 min, 4°C), and an aliquot of supernatant was prepared by Anita Howell and April Xu.
[2] EI_{max} and K_{EI} were performed by April Xu and Angela Hill.
[3] MCF measurements were performed by me.
[4] Data analysis was performed by me.

analysis, a p value of less than 0.05 was considered significant. One-way analysis of variance (ANOVA) and the following Tukey post hoc was used to identify significance among pairwise comparisons of donors' age and sex group. Storage length effect at fresh, middle, and expiry testing time points was assessed using two-way ANOVA. Mixed between-within subjects analysis of variance (mixed ANOVA) was then used to show the main effect of donor characteristics, and whether these results were affected by the interaction with the storage length. Donor specifications of age (> 50 or < 30) and sex (male or female) were considered the independent between-subjects' variables. The storage length of fresh, middle, or expiry testing time points were considered the within-subjects' variables. Multiple regression was then conducted to provide a model to test the possible contribution for each of storage length, manufacturing process, and donor characteristics on the prediction of water permeability and hemolysis.

4.3. Results

The water permeability (Lp) measurements for different donor age and sex groups are shown in Figure 4.1. Measurements were taken at fresh testing (day 5 ± 2), middle testing (day 21), and expiry testing (day 42) for units prepared from red cell filtration (RCF) and whole blood filtration (WBF). Units obtained from > 50-year-old male donors and processed by RCF had a significantly lower Lp than other donors group at the fresh testing point of day 5 ± 2 from the collection. However, there were no other significant differences between donors' groups at other testing points of units prepared by either RCF or WBF. The Lp in RCF units from > 50-year-old male were significantly increased at middle and expiry testing points compared to the fresh testing of the same group. RCF units of other donors' groups were significantly increased at the

expiry testing point, but not significant at the middle testing points. For groups prepared by WBF, there were no significant increase throughout the storage except for > 50-year-old female group at the expiry testing point. The mixed ANOVA results are presented in Table 4.2. There was a large significant interaction effect between donors' age and sex and storage length for RCF units, but to a nonsignificant degree for WBF units. The main effects of age and sex were nonsignificant in both RCF and WBF units. However, the main effect of storage length was large and significant in both RCF and WBF units.

Hemolysis analysis revealed that there were no significant differences between age and sex groups for RCF and WBF units (Figure 4.2). However, all donors' groups were significantly increased at the expiry testing point compared to the fresh point of each group. The age, sex, and storage length interaction effect and the main effects of age and gender were not significant in RCF and WBF units (Table 4.2). The main effect of storage length was large and significant.

Hematologic indices for units obtained from > 50-year-old male, < 30-year-old male, > 50-year-old female, and < 30-year-old female are shown in Table 4.3. There were no significant differences between age and sex groups in all of the hematologic indices for RCF and WBF units. MCV and MCH were nonsignificantly affected by the storage. MCHC analysis showed that it significantly decreased at the middle and expiry testing points for all groups. The mixed ANOVA (Table 4.2) for MCHC revealed that the interaction between age, sex, and storage length and the mean effect of age were not significant. The mean effect of sex on MCHC was significant for donors' groups prepared by RCF units but not with WBF units. The mean effect of storage length was large and significant for all donors' groups.

The fresh and expiry measurements of the supernatant K^+ and supernatant Na^+ for units obtained from all for groups are presented in Figure 4.3 and Figure 4.4, respectively. The

concentration of supernatant K^+ for < 30-year-old female group prepared by RCF was significantly lower than other donors' groups. However, there were no other significant differences between donors' groups in both measurements of supernatant K^+ and supernatant Na^+ of units prepared by RCF and WBF. Supernatant K^+ was significantly increased as a function of storage time for all of the donors' groups. Supernatant Na^+, however, significantly decreased at the expiry testing point. The interaction effect between age, sex, and storage length was not significant (Table 4.2). The main effect of storage length was significant and high. The main effect of sex for groups prepared by RCF was, also, large and significant. There was no other significant main effect was detected.

Membrane biomechanical parameters of EI_{max}, K_{EI}, and MCF indicated no significant differences between donors' groups (Figure 4.5). The value of EI_{max} was significantly reduced at the end of testing for < 30-year-old female units prepared through RCF. The mixed ANOVA for EI_{max} revealed that the mean effect of storage length was large and significant for all donors' groups (Table 4.2). No significant effect of the interaction between age, sex, and storage length or the mean effects of age and gender.

Results of regression analysis provided a conformation of significant contribution of three factors (storage length, manufacturing process, and donor characteristics) on the water permeability (Lp) and % hemolysis. 53.1% of the Lp model can be predicted by measuring these three factors ($R^2 = 0.531$, $p < 0.001$). The evaluation of the contribution of each of the three factors revealed that storage length ($\beta = 0.470$) and manufacturing process ($\beta = 0.549$) make a statistically significant ($p < 0.001$) contribution to the model. The donors' age ($\beta = 0.056$) and sex ($\beta = - 0.085$) do not make a significant contribution in prediction of Lp. Similarly, the hemolysis regression model can predict 48.1% of the measurement variabilities ($R^2 = 0.481$, $p <$

0.001). The storage length (β = 0.544) and manufacturing methods (β = 0.402) have a statistically significant effect ($p < 0.001$) on the prediction of the hemolysis model. The beta value of donor's sex (β = 0.130) indicated that it had a lesser degree but significant ($p < 0.05$) contribution. The contribution of donor's age (β = - 0.067) was not significant in the model.

4.4.Discussion

There are several studies which have focused on factors causing stored RBCs to undergo storage lesion. The vast majority of these studies have been concentrated on the storage length as the sole contributor to the storage lesion. However, there is a gradual awareness following recent studies that other factors may influence the quality of RBCs during hypothermic storage (9, 10). One of these factors is donor-related characteristics as obtaining blood from a variety of age and sex groups may affect the quality of products. It is crucial to generate more studies that describe if these two donor-specific factors, age and sex, are significantly affecting RBCs quality. The membrane water permeability has shown promising results in the proceeding chapters in detecting factors that impact RBCs quality during storage. As an extension, this study was designed to determine the effect of donors' age and sex on the membrane permeability and membrane quality parameters of RBCs at three testing time points during storage.

Except for the Lp in the fresh testing and the supernatant K^+ in the fresh and expiry testings of RCF units, the results of membrane permeability and membrane quality parameters did not show any significant differences between any of the donor groups. Age had a nonsignificant impact on all parameters. However, the ANOVA (mixed ANOVA) showed that donors' sex has a significant effect on MCHC and supernatant K^+ for units prepared by RCF, but to a nonsignificant degree for the rest of parameters. The main effect of storage was,

unsurprisingly, large and significant for Lp, % hemolysis, MCHC, supernatant K^+, supernatant Na^+, and EI_{max}. Thus, this study is rather disappointing as it has unable to clearly demonstrate the significant effect of donors' age and sex on the membrane water permeability and membrane in vitro quality parameters.

The second aim of this study sought to answer the question of which variable(s) of storage length, manufacturing process, and donor characteristics is (are) the best in predicting the Lp and % hemolysis. As the influence of storage length was shown in chapter 2, the manufacturing methods was presented in chapter 3, and donor-specific factors is in this chapter, having all of these factors in one regression model will add to the knowledge of the contribution of each factor on the storage lesion. Results of the regression analyses seem to be consistent with those of previous studies in this thesis. The storage length and manufacturing process were found to have a significant contribution to both the Lp and the hemolysis models. The donor's sex was making a significant contribution to the hemolysis model but not to the Lp model. However, the age was considered not a significant predictor in both models. The results of hemolysis model seem to contradict with ANOVA findings, as the sex factor gave significant contribution with the hemolysis regression model but not with mixed ANOVA. A potential explanation, therefore, for the results of regression models is that it is likely to find a link between storage lesion with the sex-related variations but not with the age-related variations.

These findings are contrary to that of Jordan et al. and Kanias et al. who have examined the impact of donor variations on quality control data of more than 16 thousand male and 11 thousand female donors obtained from CBS (10, 11). They reported that both sex and age had significant impact on hemolysis measure at the end of 42 day of storage. The current study are unable to detect such differences between groups for unclear reasons, but it may have something

to do the fact that this study is limited by the small sample size. Having six participants in the majority of groups did not allow detecting significant results. It is statistically known that small sample size increases the possibility of type II error of accepting a false null hypothesis (12). Therefore, with such a small sample size, caution must be applied when drawing interpretation as findings might not be suitable to be generalized to the general population of donors. Another possible explanation for why the current study was unable to find significant differences between age groups is the way of data entered for statistical analysis. In the present study, we considered the age variables as a nominal scale and it was entered in a dichotomous way by assigning 0 to the > 50 years old category and 1 for the < 30 years old donors. On the contrary, Kanias et al. entered their variables as a continuous interval scale (11) due to the large sample size.

In fact, when looking to the literature, donor variation is still considered as highly debated aspect and more investigations need to be undertaken. Daly et al. in his recent study had tested the effects of donor sex of 12 males and 12 females on stored RBCs (13). They showed that there were no significant rheological differences between RBCs during storage which is in agreement with the current findings. The majority of previous reports, however, are inconsistent with the findings of this study. It has been reported that stored female RBCs exhibit lower hematocrit (10, 14) and mechanical fragility (15) compared to stored male RBCs. These studies suggested that female RBCs have a more intact cell membrane and less susceptible to be affected during storage. Male RBCs, on the other hand, has been reported to exhibit significantly lower deformability and higher fragility compared to female RBCs (23). These variation between male and female RBCs probably because of the high level of young to old RBCs ratio due to the regular loss of blood during menstruation of female group (16, 17). Collectively, these

observations may support the hypothesis of the existence of considerable donor sex- dependent differences which have impact of RBCs quality during hypothermic storage.

The impact of donor's age on storage of RBCs has been even less explored in the literature. The average age of RBCs among donors' subgroups was reported to be 50.7 ± 7.2 days (Mean ± SD) at the time of donation which means it almost halfway toward the 120 days of their lifespan (18). However, it has been demonstrated that donors' age may influence the average ratio of old to fresh RBCs in the circulation (16). To specify, elder male and female possess more old RBCs and less fresh RBCs compared to younger ages. This could be because of that the ability of bone marrow to generate new RBCs through erythropoiesis process becomes limited as the age proceeds (16, 17). During hypothermic storage, donor's age has been associated with the level of hemolysis in the bag as younger male and female tend to have less hemolysis (11). Thus, even though we did not detect any significant effect of age on membrane quality of stored RBC, a number of lines of evidence in the literature suggest that donor biological variation is a confounding factor that need to be taken into account when investigating the quality of stored RBCs in future studies.

Taken together, research on the role of donors' age and sex variation in quality of stored RBCs is inadequate and needs to be expanded to cover more quality parameters. This study aimed to assess the impact of the variations in donors' biological characteristics on the stored RBCs membrane Lp and quality measures. Although the results of this study should be interpreted with caution due to the small sample size, it is possible to hypothesize, therefore, that the factor of donor's age is less likely to cause any variation during storage of RBCs, and a further study with more focus on donor's sex is suggested.

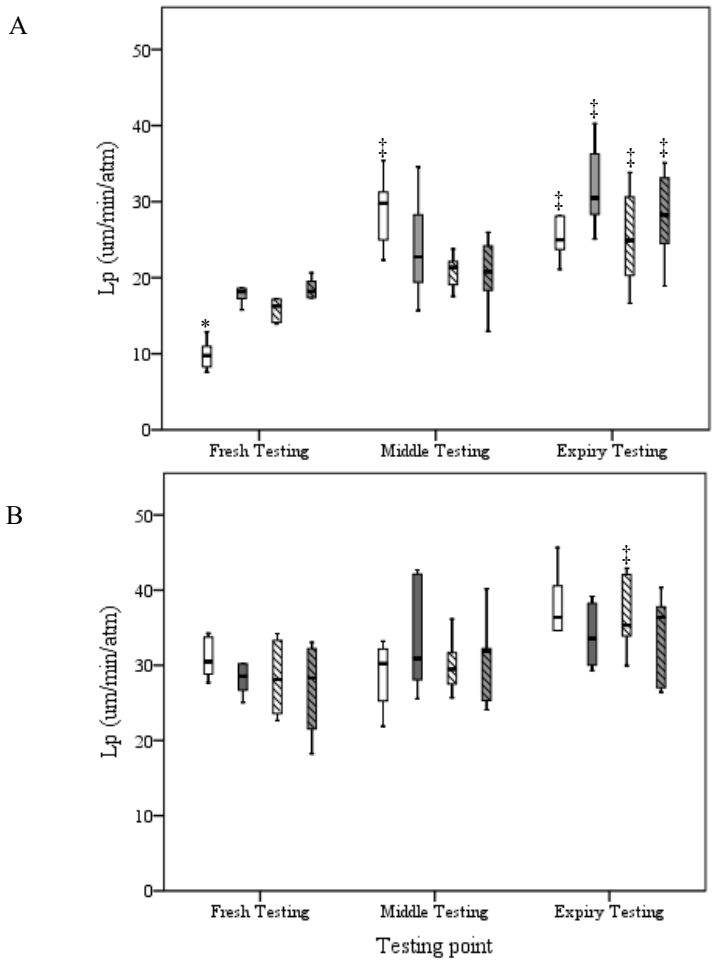


Figure 4.1 Water permeability (Lp) testing for donor groups at fresh testing (day 5 ± 2), middle testing (day 21), and expiry testing (day 42) for units preparing from red cell filtration (A) and whole blood filtration (B). Boxes represents the first and third quartiles of the Lp data for > 50-year-old male (□), < 30-year-old male (■), > 50-year-old female (▨), and < 30-year-old female (▨). The band inside each box is the median, and the top and bottom of the whiskers represent the full range of the minimum and maximum limit of all of the data. p values of less than 0.05 was considered to be significant.

* Significant mean difference when compared to other age/sex groups at the same testing time point.

‡ Significant results in comparison to fresh testing of the same age and sex group.

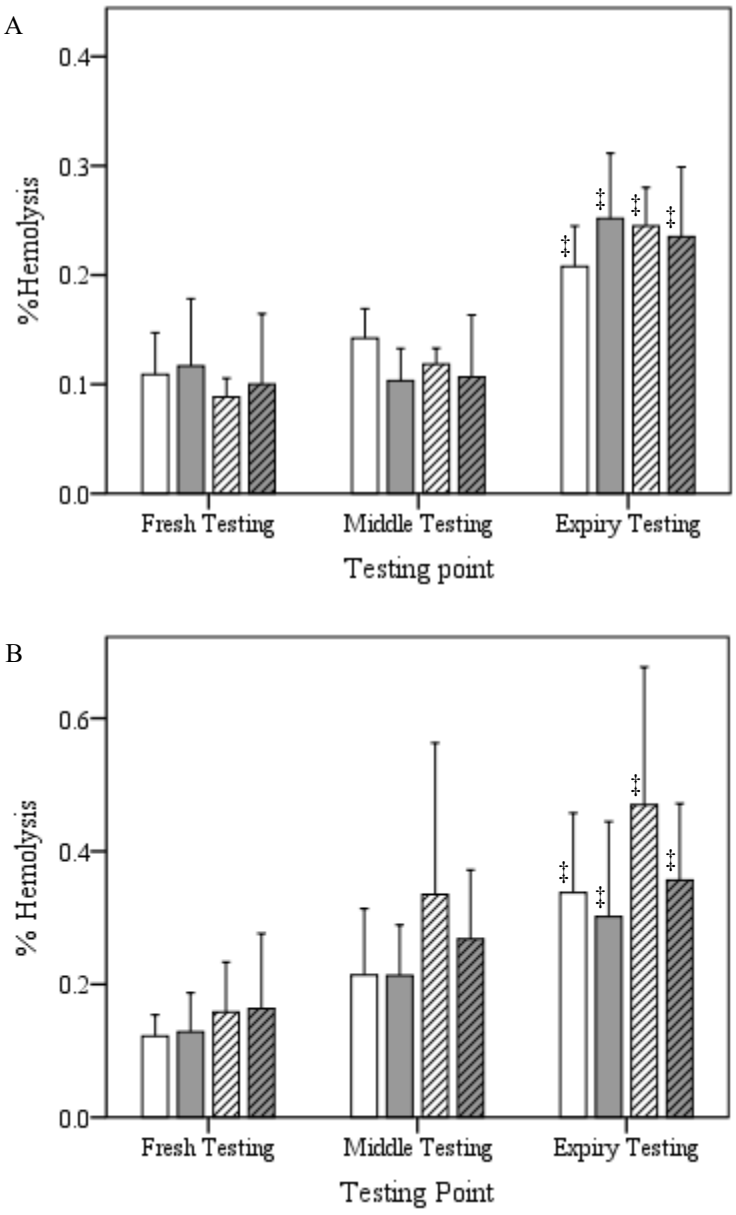
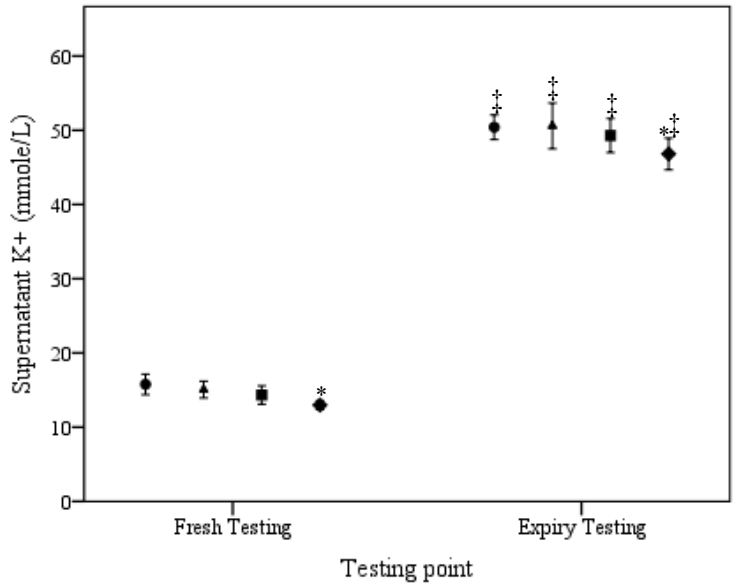


Figure 4.2 %hemolysis for donors' age and sex groups of units preparing from red cell filtration (A) and whole blood filtration (B) at fresh testing (day 5 ± 2), middle testing (day 21), and expiry testing (day 42). Bars represent % hemolysis measures for > 50-year-old male (□); < 30-year-old male (■); > 50-year-old female (▨); and < 30-year-old female (▩).

‡ Significant results in comparison to fresh testing of the same age and sex group.

A



B

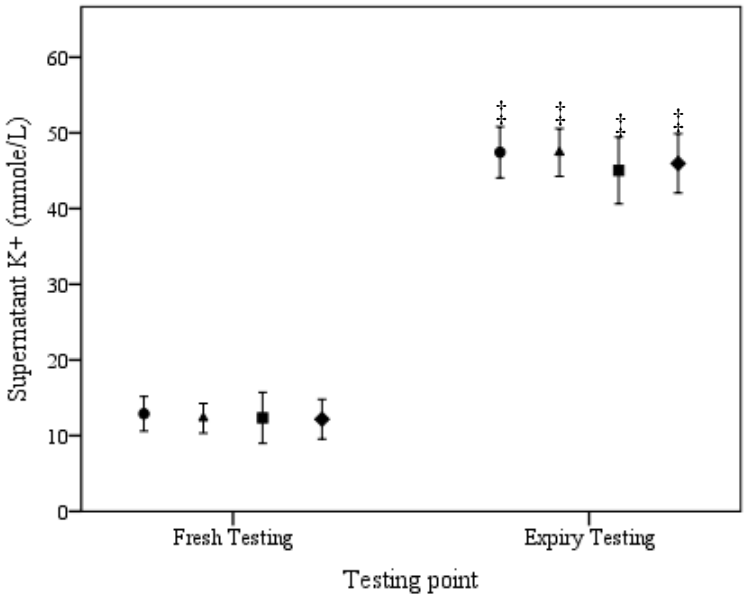


Figure 4.3 Fresh and expiry measurements of supernatant potassium (K^+) for red cell filtration units (A) and whole blood filtration units (B) obtained from (●) > 50-year-old male group; (▲) < 30-year-old male group; (■) > 50-year-old female group; (◆) < 30-year-old female group.

* Significant mean difference when compared to other age/gender groups at the same testing time point.

‡ Significant results in comparison to fresh testing of the same age and sex group.

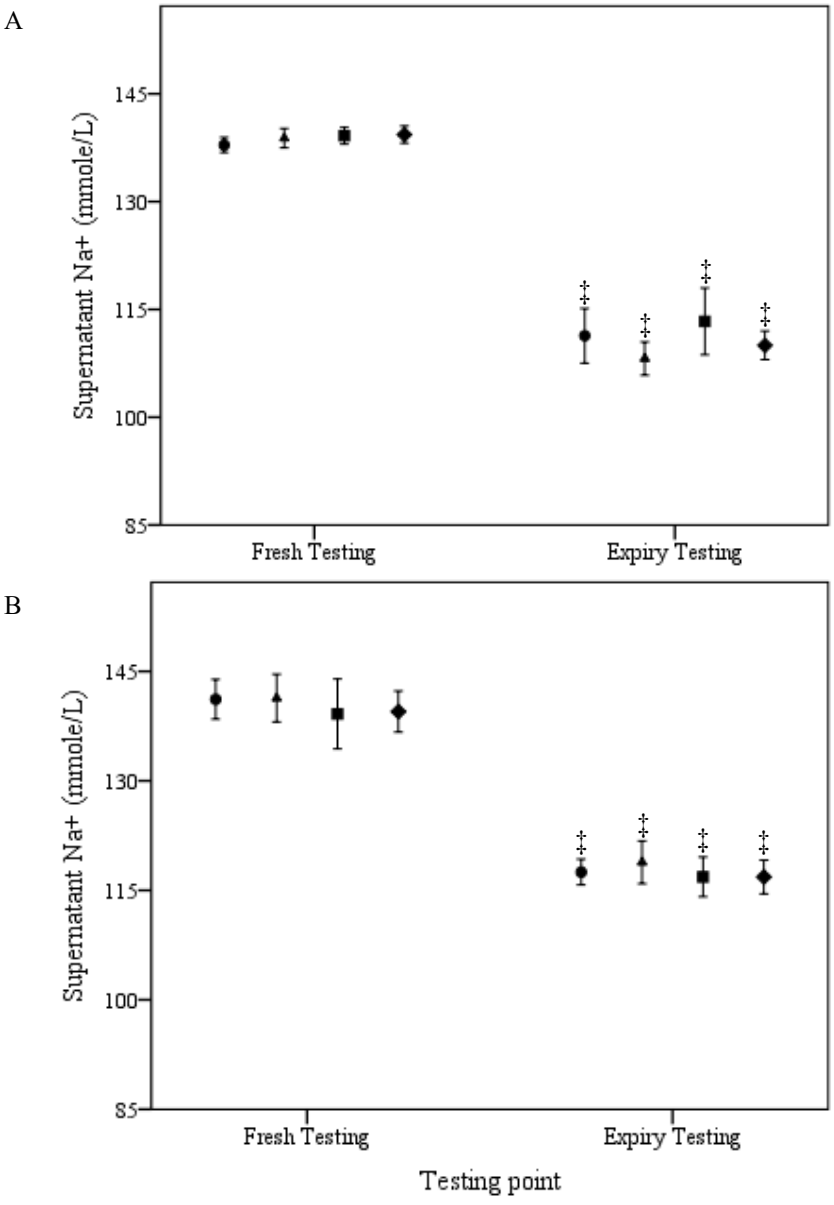


Figure 4.4 Fresh and expiry measurements of supernatant sodium (Na^+) for red cell filtration units (A) and whole blood filtration units (B) obtained from > 50-year-old male (●); < 30-year-old male group (▲); > 50-year-old female group (■); and < 30-year-old female group (◆).

‡ Significant results in comparison to fresh testing of the same age and sex group.

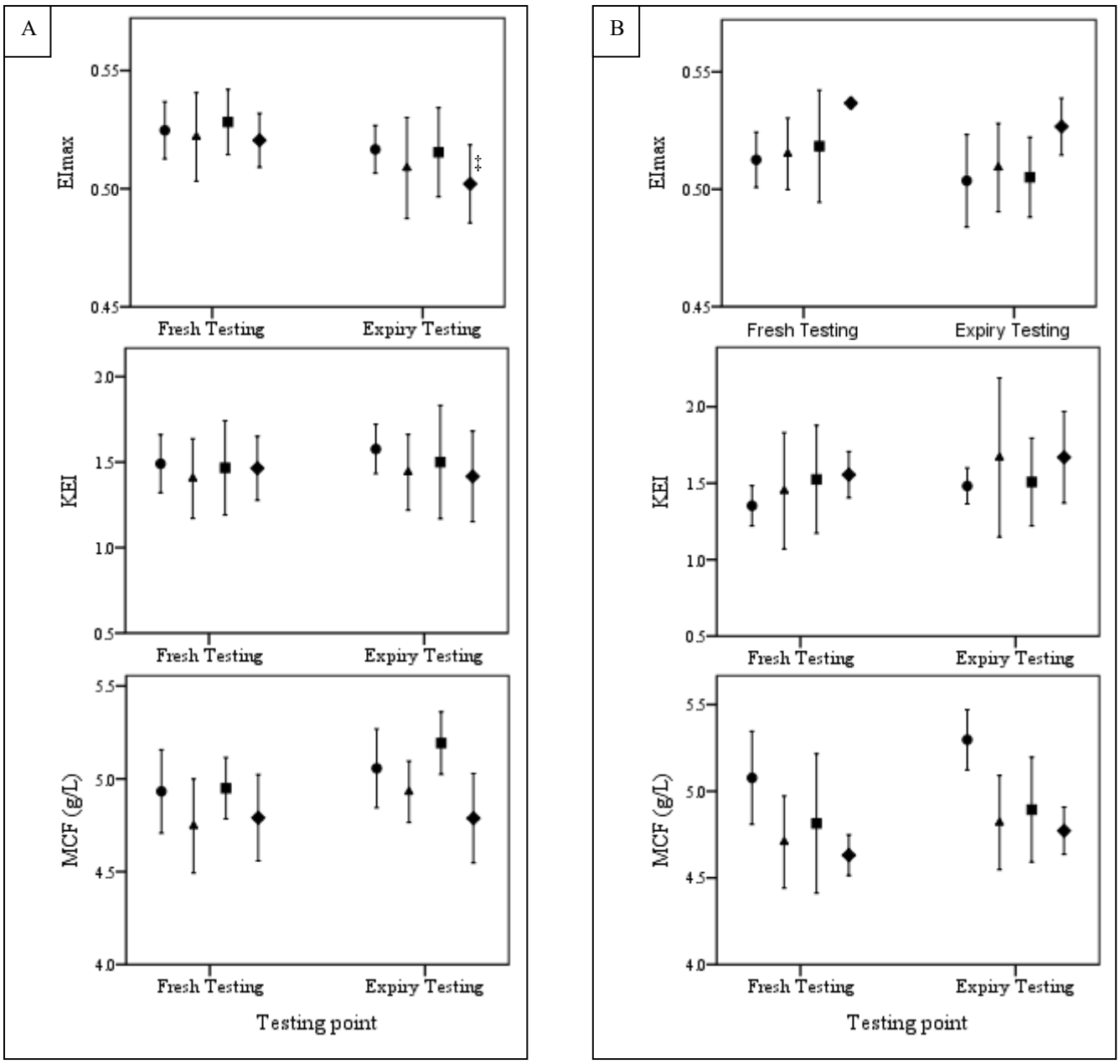


Figure 4.5 RBCs biomechanical parameters of EI_{max}, K_{EI}, and MCF for red cell filtration units (A) and whole blood filtration units (B) obtained from (●) > 50-year-old male group; (▲) < 30-year-old male group; (■) > 50-year-old female group; (◆) < 30-year-old female group. Reported marker data are mean ± SD. p values of less than 0.05 was considered to be significant.

‡ Significant results in comparison to fresh testing of the same age and sex group.

Table 4.1 Donors' characteristics

Variable		> 50-year-old male	< 30-year-old male	> 50-year-old female	< 30-year-old female
Sample size (# of units)	RCF	9	6	6	6
	WBF	6	6	6	6
Average age ± SD		64.2 ± 6.5	25.6 ± 3.1	65.5 ± 3.7	24.1 ± 3.5
Production site (# of units)	BC&Y	9	4	2	4
	Calgary	2	4	2	5
	Dartmouth	4	4	8	3

RCF: red cell filtration manufacturing method. WBF: whole blood filtration manufacturing method. BC&Y: British Columbia & Yukon production site.

Table 4.2 Effect of age, sex, and storage length on the water permeability and quality parameters of units prepared by red cell filtration (RCF) and whole blood filtration (WBF)

Parameters	Age x gender x storage length *		Age ‡		sex †		Storage length "	
	RCF	WBF	RCF	WBF	RCF	WBF	RCF	WBF
Water Permeability	< 0.05 (0.29)	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	< 0.01 (0.89)	< 0.01 (0.59)
%Hemolysis	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	< 0.01 (0.88)	< 0.01 (0.86)
MCHC	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	< 0.05 (0.17)	> 0.05 --	< 0.01 (0.93)	< 0.01 (0.94)
Supernatant K^+	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	< 0.05 (0.34)	> 0.05 --	< 0.01 (0.99)	< 0.01 (0.99)
Supernatant Na^+	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	< 0.01 (0.98)	< 0.01 (0.99)
EI_{max}	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	> 0.05 --	< 0.01 (0.55)	< 0.01 (0.34)

The numbers in the table represent the p-value for the possibility of effect (the effect size in brackets), that show the main effect for both manufacturing methods and storage length and the interaction between them. The effect size of less than 0.06 represents a small effect, between 0.06 and 0.14 represent a moderate effect, greater than 0.14 represent a large effect.

* Interaction effect between the donor's age/sex and storage length.

† Main effect of donor's age.

‡ Main effect of donor's sex.

" Main effect of storage length.

Table 4.3 Hematologic indices of units prepared by red cell filtration (RCF) and whole blood filtration (WBF)

Group	Testing point	MCV (fL)		MCH (pg)		MCHC (g/L)	
		RCF	WBF	RCF	WBF	RCF	WBF
> 50-year-old male (n = 9)	fresh	94.2 ± 3.7	92.6 ± 2.8	30.8 ± 1.4	30.4 ± 1.5	327.4 ± 5	328.0 ± 7
	middle	96.9 ± 3.8	94.2 ± 2.8	30.6 ± 1.4	29.7 ± 1.4	315.3 ± 3 ‡	315.3 ± 6 ‡
	expiry	97.4 ± 4.1	95.6 ± 3.4	30.1 ± 1.3	29.5 ± 1.3	309.3 ± 2 ‡	309.3 ± 4 ‡
< 30-year-old male (n = 6)	fresh	92.8 ± 3.9	89.4 ± 4.0	30.3 ± 1.2	29.2 ± 1.0	326.6 ± 4	327.3 ± 5
	middle	94.3 ± 3.8	90.7 ± 3.8	29.5 ± 1.0	28.4 ± 1.1	313.0 ± 2 ‡	313.5 ± 4 ‡
	expiry	95.3 ± 4.1	91.7 ± 4.1	29.6 ± 1.3	28.4 ± 0.9	311.0 ± 2 ‡	310.5 ± 7 ‡
> 50-year-old female (n = 6)	fresh	94.2 ± 5.4	93.3 ± 2.9	30.3 ± 1.9	29.9 ± 1.2	321.5 ± 6	320.5 ± 6
	middle	95.4 ± 5.5	94.3 ± 2.7	29.5 ± 1.8	29.2 ± 1.4	309.1 ± 5 ‡	309.3 ± 8 ‡
	expiry	96.8 ± 5.5	95.8 ± 2.5	29.6 ± 1.8	29.2 ± 1.1	305.8 ± 5 ‡	305.5 ± 6 ‡
< 30-year-old female (n = 6)	fresh	91.9 ± 5.3	90.4 ± 4.2	29.4 ± 1.9	29.3 ± 1.9	320.6 ± 7	324.1 ± 6
	middle	92.7 ± 5.7	91.9 ± 5.0	28.8 ± 2.2	28.4 ± 1.8	310.6 ± 8	309.1 ± 6 ‡
	expiry	93.3 ± 5.1	93.0 ± 5.0	28.9 ± 1.8	28.7 ± 1.9	310.5 ± 5	308.1 ± 5 ‡

Reported marker data are mean ± SD.

‡ Significant results in comparison to fresh testing of the same age and sex group.

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Chapter 5

General discussion and conclusions

5.1.Summary of thesis objectives and results

The overall purpose of this thesis was to assess the membrane water permeability of red blood cells (RBCs) during the six weeks of hypothermic storage. It also aimed to test the role of storage length, blood component manufacturing method, and donor characteristics on the water permeability and in vitro membrane quality parameters of RBCs throughout storage. Due to the dependence of the water permeability on the structure and biomechanical function of the cell membrane of stored RBCs, this thesis presented the hypothesis that the membrane water permeability can be used as a quality predictor to assess membrane quality throughout the storage. In addition, membrane water permeability along with the other membrane quality measures, can be used as an experimental indicator for factors affecting RBC quality during the hypothermic storage. These include storage length, blood component manufacturing method, and donor characteristics. In this thesis, investigating the red blood membrane permeability and membrane quality parameters during the hypothermic storage was carried out in three experiments beginning from assessing the effect of the six weeks storage length in chapter two, determining the impact of two different manufacturing methods in chapter three, and ending with investigating the influence of donor biological characteristic of age and sex in chapter four.

The first experimental study set out in chapter two with the aim of assessing the red blood membrane water permeability by using a newly established method that uses the intensity of intracellular hemoglobin fluorescence to measures the kinetics of osmotically induced volume

(1). It was my goal to monitor the two other osmotic parameters that characterize the movement of water through the cell membrane which are the osmotically inactive fraction and the Arrhenius activation energy. The third aim was to test the effect of storage length on RBC membrane quality parameters. This study was also attempted to test correlations between water permeability measures at different testing time points of storage with the previously mentioned quality parameters. The results of this experiment demonstrated that the membrane water permeability measurements at day 3, 14, 21, 28, 35, and 42 of storage were significantly increased at the day 21 of storage which remains significantly elevated for the rest of 42 days of storage. This was accompanied by significant alterations in the water permeability-associated parameters of osmotically inactive fraction and Arrhenius activation energy. Data was also revealed that there is a strong relationship between water permeability and quality measures, such as hemolysis and MCV, which increases the possibility of that the water permeability is a good predictor of the osmotic quality of stored RBCs. In chapter two, I validited use of a newly developed method to look at membrane osmotic injuries during storage. These results indicated that measurement of the RBC water permeability changes is appropriate for monitoring early storage damage that affect cell membrane quality which supports the potential use of water permeability measurments as quality control metric to monitor RBC products during storage. Based on these findings, moreover, identifying all of the membrane quality parameters along with the membrane osmotic parameters prove the significant damages and quality loss of the cell membrane as a function of six week storage length.

The first aim of the chapter three sought to determine the impact of RBC manufacturing methods on the membrane water permeability. The RBCs were processed by the two manufacturing methods that are commonly used by Canadian Blood Services to separate RBC

from whole blood collection. These are the red cell filtration method (RCF, top and bottom) and the whole blood filtration method (WBF, top and top). This was to evaluate which of the two manufacturing processes has more impact on the RBC membrane as determined by measurements of water permeability. In addition to investigating the water permeability as a function of manufacturing methods, the effect of the storage length was also assessed in this study. This allowed testing whether the effect of blood manufacturing methods was enhanced by storage length. This study found that units processed by WBF exhibited significantly higher membrane water permeability from the start of storage until the end of 42 days of storage compared to RCF units. Moreover, this experiment demonstrated that the water permeability significantly increased as a function of storage length in both manufacturing methods. The statistical analysis, therefore, found that both storage length and manufacturing process significantly affect the membrane water permeability with a larger effect from the storage length. The WBF units, also, have been shown to have significantly higher hemolysis, supernatant K^+, and supernatant Na^+ than units prepared by RCF manufacturing method. Lastly, correlations of membrane water permeability to other membrane quality measures revealed that it has significant correlation to hemolysis, EI_{max}, MCV, MCHC, supernatant K^+, and supernatant Na^+ of the RCF units, while with WBF it correlated with MCV, supernatant K^+, and supernatant Na^+. Hence, chapter three showed the role that manufacturing methods could have in RBCs quality during storage. Using membrane water permeability as a quality measure to look at manufacturing effects on membrane osmotic properties had not previously been reported. Considering that water permeability test was successful as it was able to characterize the depth of damage to stored RBCs, this would open abundant room to use membrane water permeability in future studies as predictor for damaged cells.

The fourth chapter of this thesis examined the role of donor characteristics on water permeability and membrane quality measures of stored RBCs. Donor-associated characteristics in this chapter were related to biological differences among donors including age and sex. The previous chapters indicated that there were certain variabilities in each of the membrane water permeability and quality parameters. Therefore, assessing theses parameters in groups based on donors' age and sex could minimize the variability and indicate the possible influence of donor biological factors. Another objective from this study was to assess if there is a possible effect of the storage length that leads to enhancing the impact of donor characteristics. Surprisingly, no differences were found between donors' groups on the results of membrane permeability measures. The statistical analysis revealed that donor's sex had a significant effect on some of the membrane quality parameters including MCHC and supernatant K^+ for units prepared by RCF. However, the age had no significant contribution to any of the membrane water permeability and quality parameters. In addition, I used multiple regression models to test how much of variability in water permeability and hemolysis testings can be predicted by all of the previous factors of the storage length, manufacturing process, and donor characteristics. Results were that only the storage length and manufacturing process had significant contributions to the water permeability model. On the contrary, in addition to these two factors, the donor's sex was found to have a significant contribution to the hemolysis model. However, the age was considered not a significant predictor in both models. Findings obtained from the regression models were in agreement with the results of aim one and two of this chapter which imply that there was a potential influence from the sex-related factor but not with the age-related factor on the quality of stored RBCs. In chapter four, I showed influences of donor sex on RBCs

membrane structural and biomechanical properties with recommending further studies to develop a full picture of the effect of donor sex on the quality of stored RBCs.

5.2.Significance of this thesis

This thesis contributes to existing knowledge in the field of RBCs biopreservation for transfusion purposes. The area of novelty of this thesis lies in the development and evaluation of membrane osmotic parameters as quality predictors for RBCs during hypothermic storage. In this thesis, key membrane osmotic parameters including the hydraulic conductivity of water permeability, osmotically inactive fraction, and Arrhenius activation energy have been shown to change during hypothermic storage. To date, much uncertainty still exists in the literature about the osmotic properties of stored RBCs. This thesis comes with the idea that monitoring the stored RBCs osmotic properties can serve as a predictor of the biophysical and chemical changes that affect the quality of RBCs. As there is no previous study has investigated how these parameters behave throughout the storage, this thesis used validated techniques to characterize red cell membrane osmotic parameters in order to identify the flexibility as well as the ability of the cell membrane to undergo quick shrinkage or swelling to overcome the osmotic stresses needed to reach equilibrium volume. This thesis demonstrated the osmotic differences between the fresh and old stored RBCs by reporting that the 21 and more days stored RBCs exhibit higher permeability, inactive fraction, and Arrhenius activation energy compared to the new stored RBCs.

Although looking at membrane permeability properties during storage adds to limited data in the literature for the osmotic behavior of stored RBCs, it also helps us to understand the mechanism and depth of RBC injuries. In this thesis, we demonstrated that any change in stored

RBCs volume, membrane, and internal contents would have an immediate impact on the rate of water crossing the cell membrane to reach the osmotic equilibrium with the external solution. In this thesis, we successfully used the water permeability test to determine the source of membrane damages in stored RBCs from the storage length, manufacturing method, and donor characteristics. This would make it possible to apply water permeability test as a diagnostic procedure for many of membrane-related RBC disorders such as hereditary spherocytosis, sickle cell anemia, and malaria infectious disease.

5.3.General conclusions and future direction

Taken together, this thesis set out to characterize the membrane osmotic properties as a function of storage time, to determine the effect of blood component manufacturing method as a key factor for water permeability and quality parameters changes during storage, and to define the role of donor-related variability on the quality of stored RBCs. The following conclusions can be drawn from this thesis. This work has identified the osmotic changes of RBCs as a part of functional damages that affect the cell membrane during the hypothermic storage. Three factors influencing the quality of stored RBCs were characterized as a function of membrane water permeability and quality parameters starting from storage length, manufacturing method, and donor factors. Findings of this work may imply that the quality of the red cell membrane decrease dramatically as the storage comes to the end of the six-week length. They also could indicate that partial membrane damage occurs during the manufacturing of RBCs and this appears clearly with the higher membrane permeability and hemolysis measurements of units prepared by WBF method which may suggest that the membrane is damaged in these RBCs.

More research in this area is required to verify these findings and to determine other reasons leading to osmotic variability of stored RBCs.

This work has set up the importance of complete understanding of membrane alterations of RBCs during the hypothermic storage as the structure and integrity of the cell membrane is crucial for RBCs functionality. In the same way that alterations in the osmotic properties are sign for the cell membrane remodeling and damages during the hypothermic storage, it may imply that the cell membrane is not functional anymore which could affect the ability of cells to resist circulation mechanical and osmotic stresses leading to shortening cell survival and increase the incidence of cells removal from the circulation.

This work will, hopefully, open the way for future studies to work on achieving the ultimate goal of the RBC biopreservation of creating an effective hypothermic biopreservation protocol that provides RBCs which are experimentally qualified and clinically safe and efficient for those patients who are in need. Considerably more work should be undertaken in order to better understanding other factors that may have effects on storage quality such as storage bag plasticizer and additive solution. More research is, also, needed to better understand the mechanism of water permeability in term of biological properties of the RBC cell membrane, and why water permeability increases during storage. Moreover, it would be interesting to use membrane water permeability test to evaluate new storage technologies and methods such as anaerobic storage of RBCs. The membrane water permeability measurements could also be applied to extensive quality control program to evaluate a large number of blood products such as in the quality monitoring program (QMP) such works conducted for chapters three and four. It might be possible to use membrane permeability properties in more advanced research of microfluidic device in order to evaluate individual cell permeability to select damaged RBCs and

remove them. Lastly but not least, this thesis will serve as a base for future studies in the field of membrane osmotic properties to help optimized the hypothermic storage of RBCs.

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Appendix 1

Modifications from the original method for measuring membrane water permeability

The purpose of this section is to report changes that I applied to the original method of water permeability test using intracellular fluorescence. The following changes were suggested by Dr. Matthew Pope, during the Applied Photophysics installation and training visit on April 22nd 2014, to improve detection of the emitted light and to remove unwanted excitation light (1).

The stopped flow system consists of the power supply, lamp, monochromator, sample handling unit, and the electronic unit as shown in Figure A.1. The arrangement and assembly of the stopped flow main components depend on how the instrument is to be used to measure rapid changes in cell response. In Zhurova et al., one of the principal goal was to scan the fluorescent emission light that is generated after mixing red blood cells with experimental solutions as it was not known for this experiment (2). In order to do that, another monochromator was connected to the sample handling unit to detect the emission light in which the excited light of the first monochromator goes through the observation cell of the sample handling unit into the second monochromator where the fluorescence detector is located (1) as displayed in Figure A.2. Results of the fluorescence scan revealed that the optimal emission of red blood cells can be measured at 314 nm (2).

In the current thesis, however, scanning of the emitted light of the red blood cells intrinsic fluorescence was not an issue as it was reported previously. Therefore, there are some technical modifications which were made to the original membrane water permeability method which was introduced in 2014 by Zhurova et al. (2). The second monochromator was taken out,

and a bandpass filter (APCF360 U-360, Applied Photophysics, UK) was used instead to remove scattered excitation light and allow passing of emitted light of intrinsic fluorescence after mixing cells with experimental solutions. The bandpass filter used in this experiment was inserted directly into the rear port of the observation cell of the sample handling unit. The fluorescence detector was then connected to the rear port of the observation cell to detect of the emitted light fluorescent of cells after passing through the filter as displayed in Figure A.3. By performing these changes, it is clear that we were able to reduce the variability in the intrinsic fluorescence intensity upon mixing red blood cells with various experimental solutions. Figure A.4 shows the kinetics of intracellular fluorescence before and after applying these modifications upon mixing red blood cell suspension to various experimental solutions. After applying these modifications, I evaluated whether they impacted the measurement of RBC water permeability characteristics. I was able to reproduce the original method with acceptable variations as shown in Table A.1.

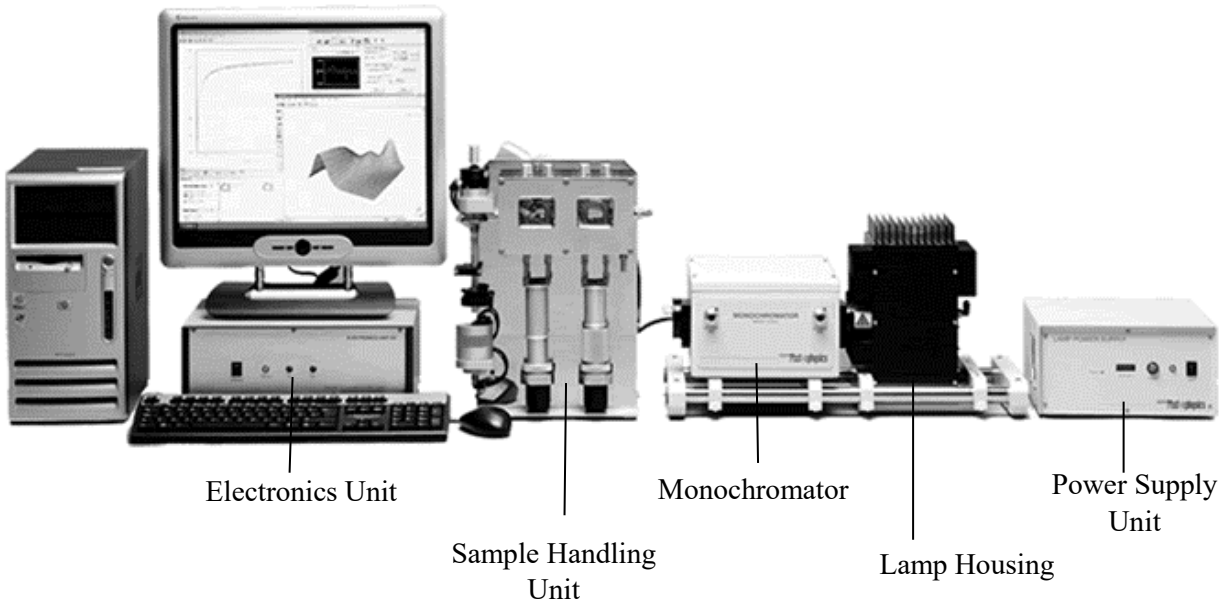


Figure A.1 The main components of the stopped flow analyzer. The image was taken from the Applied Photophysics Ltd. (3). Permission to reproduce this item was provided by the Customer Support Manager at the Applied Photophysics, Dr. Matthew Pope (November 3rd, 2016). Abdulrahman Alshalani has done some modifications.

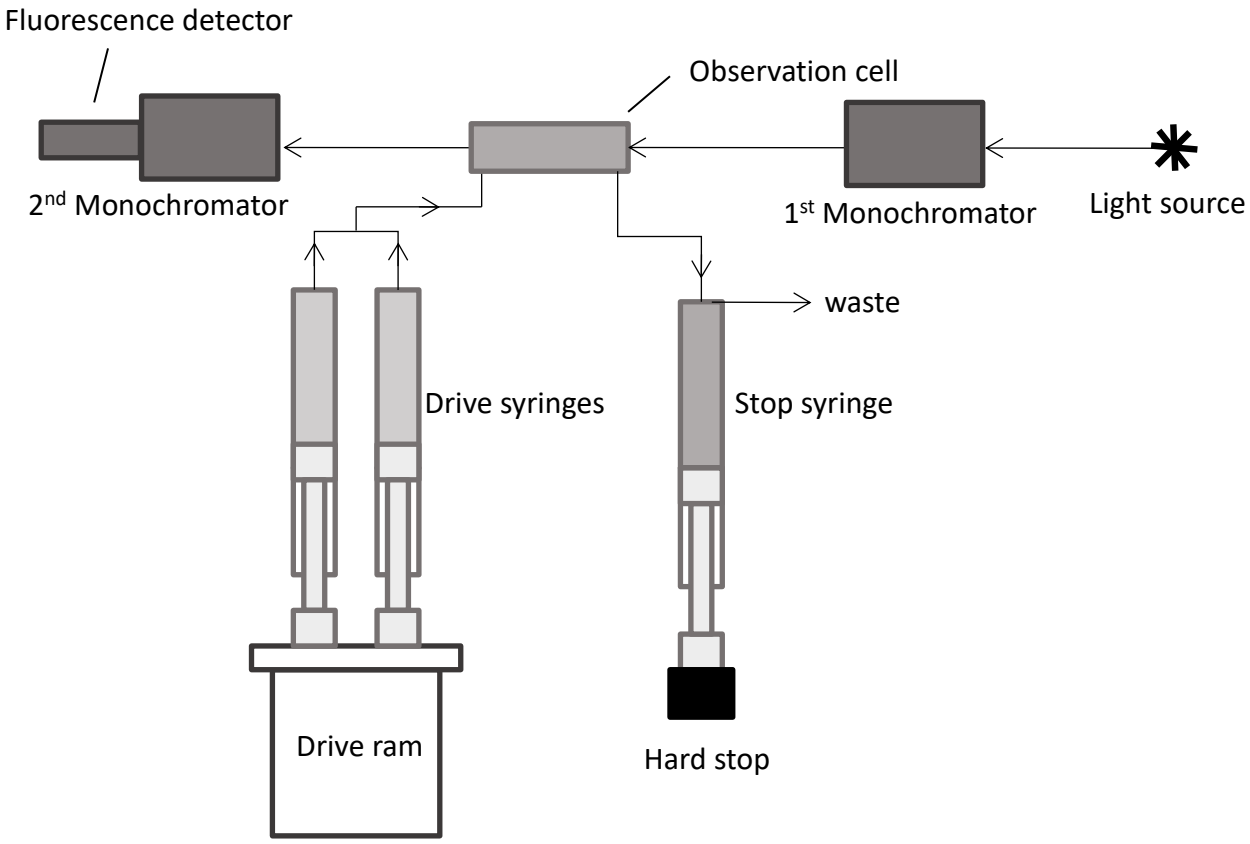


Figure A.2 Schematics for the original arrangement of the major components of the stopped flow analyzer. Permission to reproduce this item was provided by the Customer Support Manager at the Applied Photophysics, Dr. Matthew Pope (November 3rd, 2016). Abdulrahman Alshalani has done modifications from the original item (3).

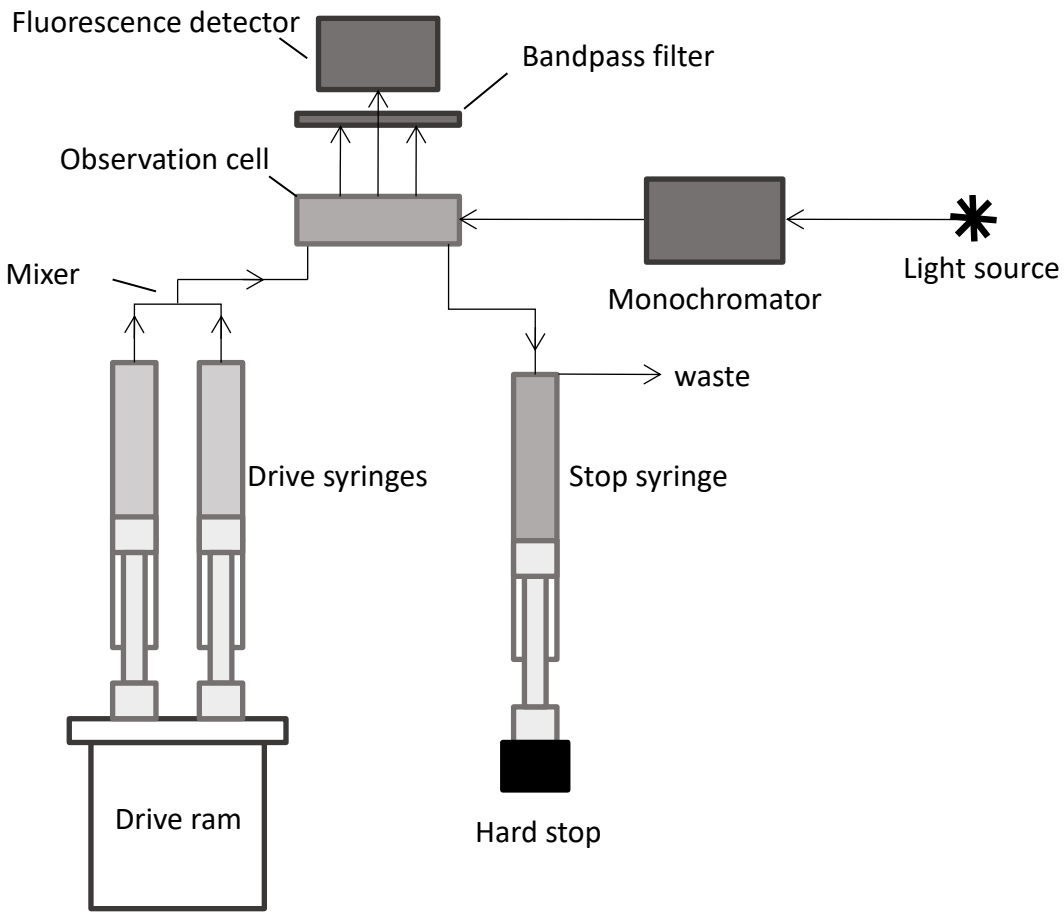
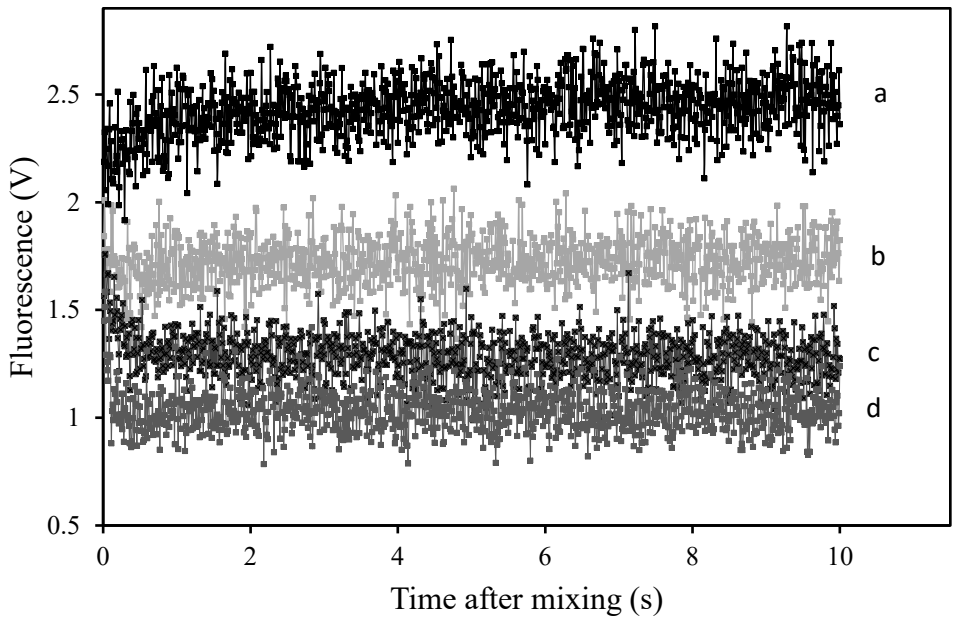


Figure A.3 Schematics for the arrangement of the stopped flow analyzer major components which used in this thesis. Permission to reproduce this item was provided by the Customer Support Manager at the Applied Photophysics, Dr. Matthew Pope (November 3rd, 2016). Modifications from the original item (3) have been done by Abdulrahman Alshalani.

1



2

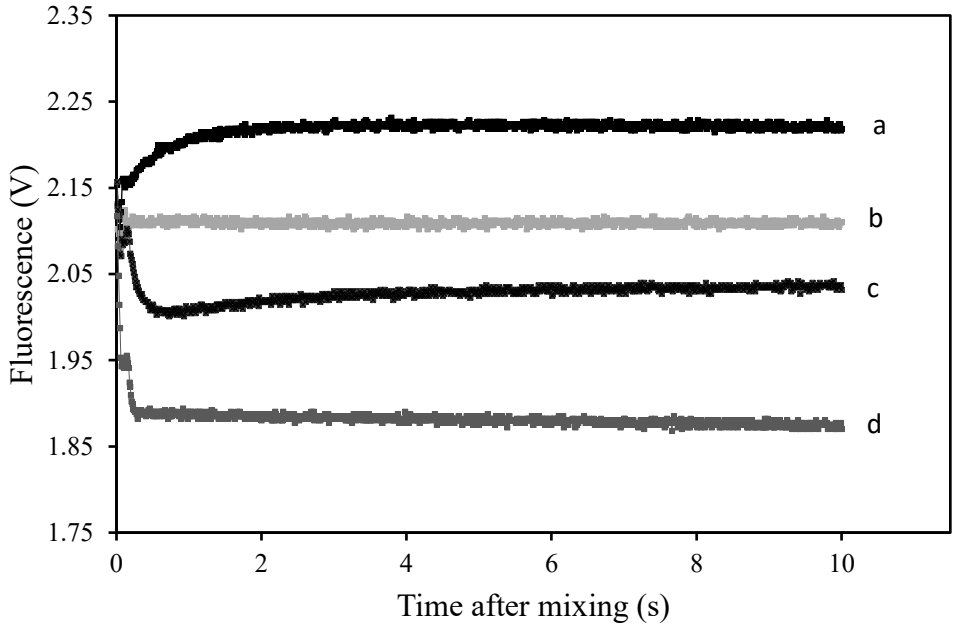


Figure A.4 Kinetics of intracellular fluorescence before (1) and after (2) applying these modifications upon mixing red blood cell suspension with 0.68% NaCl (a), 0.9% NaCl (b), 1.6 NaCl (c), and 3.5 % NaCl (d).

Table A.1 Water permeability (Lp) measurements before and after applying modifications in the Lp technique

	Before modifications *	After modifications ‡
Sample size	n = 2	n = 9
Anisotonic solution	0.68% NaCl	0.75x PBS
Osmolality (mOsmol/Kg)	215 ± 1	217 ± 0.5
Lp at Fresh testing (day)	11.7 ± 2.2 (day 9)	9.9 ± 1.9 (day 3)
Lp at expiry testing (day)	25.9 ± 4.1 (day 44)	27.05 ± 5.7 (day 42)

Samples were measured in triplicate. Data are reported as mean ± SD

* Data was collected as a preliminary data and was used during designing of the hypothesis and objectives of this thesis

‡ Data was collected for chapter two of this thesis

Appendix 1 References

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