University of Alberta

Identifying and Characterizing Red Blood Cell Microvesiculation, Phosphatidylserine and CD47 Expression As a Predictor of Red Blood Cell *In Vitro* Quality Following Hypothermic Storage

by

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

Master of Science

Medical Sciences - Laboratory Medicine and Pathology

Spring 2013
Edmonton, Alberta

This Achievement Is Dedicated To My Mother Rabab Alkhater, May God Bless and Rest Her Soul.

Abstract

Proper preservation of the quality of stored red blood cells (RBC) is fundamental for successful blood transfusion. RBC microparticles (RMPs) have been shown to be harmful effectors of inflammation and coagulation posttransfusion, as well as *in vitro* indicators of RBC function and integrity throughout *ex vivo* storage. The main objectives of this thesis were to develop a flow cytometric analysis method to identify, characterize and quantify RMPs during hypothermic storage (HS) along with examining the effects of biochemical rejuvenation on RBC microvesiculation and membrane composition. This thesis demonstrated that HS conditions induce RBC microvesiculation and changes in CD47 and PS. This study also showed evidence that ATP restoration following the rejuvenation had no significant effect on RMPs generation or PS and CD47 expression throughout HS. The work presented in this thesis has furthered the field of the biopreservation by outlining techniques to improve blood transfusion.

Acknowledgments

I wish to acknowledge and express my sincere gratitude and appreciation to all of following people who were supportive and cooperative in the successful completion of this thesis. First and foremost, praises and thanks to *Allah* Almighty for the strengths and His blessing s in completing this thesis.

To Dr. Jason Acker, my supervisor: Your full support, guidance and effort in making this thesis a magnificent experience for me. I have been extremely fortunate to have a supervisor whose patience and encouragement guided me to complete this dissertation.

To Dr. Jelena Holovati, my co-supervisor: You gave me the confidant to go on and I will never forget your kind monitoring. I am deeply grateful to your valuable discussions that helped me understand my research area better.

To Ms. Kristi Lew and Dr. Rodrigo Onell: Thank you for serving as my committee member and examiner for my thesis.

To all of the members of Dr. Acker's lab: A great thanks for their friendship and encouragement throughout my graduate studies that has meant to me more than I could ever express. Special thanks to Jayme Tchir and Mariia Zhurova for her kindly assistance and contributions to this thesis

To the Ministry of Higher Education in Saudi Arabia for giving me the opportunity to study abroad and providing financial support

Last but not least I wish to advantage myself of this opportunity to express gratitude and love to my friends and my beloved family for their understanding, endless love, and intensive support through the duration of my studies. I hope that this work makes you proud.

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List of Abbreviations:

ACD	acid citrate dextrose
ATP	adenosine triphosphate
CBS	Canadian Blood Services
CPD	citrate-phosphate-dextrose
CPDA-1	citrate phosphate dextrose adenine
CV	coefficient variation
FC	flow cytometer
FSC	forward scatter
G6PD	glucose-6-phosphate dehydrogenase
H_2O_2	hydrogen peroxide
Hb	hemoblobin
Hct	hematocrit
HiCN	cyanmethemoglobin
HS	hypothermic storage
HSL	hypothermic storage lesion
ICU	intensive care unit
LoB	limit of blank
LoD	limit of detection
LoQ	limit of quantification
MCV	mean corpuscular volume
MCH	mean corpuscular hemoglobin

MPs	microparticles
MCHC	mean corpuscular hemoglobin concentration
NADH	nicotinamide adenine dinucleotide
NEM	N-ethylmaleimide
netCAD	Network Centre for Applied Development
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PIPA	pyruvate-inosine-phosphate-adenine
pRBC	packed red blood cell
PS	phosphatidylserine
PVP	polyvinylpyrrolidone
RBC	red blood cell
RMP	red blood cell microparticle
SAGM	saline-adenine-glucose-mannitol
SD	standard deviation
SM	sphingomyelin
SRA	specific research aim
SSC	side scatter
TRALI	transfusion related lung injury

Chapter 1

Introduction

1.1.RBC Biopreservation

1.1.1. Importance of RBC Biopreservation

Red blood cell (RBC) transfusion is a procedure that is clinically used to save lives. Inclusive categories of injuries and medical disorders resulting in low oxygen–carrying capacity, such as acute anemia, require transfusions to save their lives [1-4]. Since RBCs show damage as soon as they leave the body, it is necessary to find an effective preservative system to maintain the integrity of the RBCs. Historically, the preservation of blood was not an issue because the donor and the recipient were connected vein-to-vein [5]. However, the demand for blood transfusion has significantly increased, where 85 million RBC products are transfused around the world every year [6,7]. The massive need for RBC products in clinical use has motivated many researchers to develop methods and techniques to preserve RBCs [8,9]. Different biopreservation approaches exist for short or long term RBC preservation in order to maintain safe and readily available RBC products for transfusion medicine. The goals of these approaches are to preserve the viability, functionality, and integrity of the stored RBCs in an *ex vivo* environment to expand the safety, efficacy and efficiency of transfusion [8,10]. The RBCs stored in liquid form, at a hypothermic storage at 1-6 ^{o}C, are the most common blood product transfused globally and thus reflect the modern medical practice of blood transfusion [11,12].

1.1.2. Hypothermic Storage of RBC

In the context of cellular biopreservation, hypothermic temperatures can be defined as those above the freezing point of a storage solution, but below the normal physiological temperature [8,13]. The principle of hypothermic preservation is based on the fact that cell metabolism slows down as the temperature is reduced [8,13]. Consequently, the decrease in the metabolic processes resulting in the depletion in the rate of biochemical reactions, slows cellular metabolism, and diminishes the accumulation of waste products [8,14]. Thereby, this reduction in the temperature allows longer *in vitro* preservation for several weeks in the presence of a hypothermic preservative solution [8,14]. The preservative solution provides the cells with the required components these include nutrition, buffer to maintain pH, and sources of metabolic energy to increase the RBC viability and survival during hypothermic storage [15]. The aim of RBC hypothermic preservation is to maintain viable, functional, and good quality RBCs; therefore, it is important to gain knowledge about RBC physiology as well as discuss historical developments as they pertain to RBC hypothermic storage.

In vivo, human RBCs or erythrocytes are derived from multipotential stem cells in the bone marrow [16]. As a response to the signal transduction by erythropoietin (a growth factor), these stem cells go through a maturation and

development process (erythropoiesis) before providing mature erythrocytes to the circulation [16]. The process of erythropoiesis includes several stages that are named based on the essential features of the cells produced throughout the morphological development. These stages are: pronormoblast, basophilic normoblast, polychromatic normoblast, orthochromic normoblast, reticulocyte, and finally the mature RBC or erythrocyte [16]. Along with the maturation process the cells undergo a significant decrease in cell size, chromatin condensation, progressive increase in hemoglobin (Hb) synthesis, decline in the amount of cytoplasmic RNA, decease in mitochondria, and loss of nucleoli [16,17].

The mature erythrocyte, which is a non-nucleated cell lacking organelles, is typically a biconcave discocyte with an approximate diameter of 7 to 8 μm, a thickness of 2 μm, a surface area of 140 μm^2 and an approximate volume of 100 μm^3 [16,18]. Although the RBC has the ability to meet the tissue demands for oxygen and enhance the blood flow by releasing vasodilatory compounds such as adenosine triphosphate (ATP) and nitric oxide, the unique shape of the RBC maximizes the surface area to volume ratio for proper function and optimal O_2 deliver to maintain sufficient oxygenation for tissues [16,19,20]. The RBC membrane consists of 40% lipids, 52% proteins, and 8% carbohydrates [16,21].The lipids, which form a bilayer, mainly consist of equal portions of phospholipids and cholesterol [16]. The cholesterol appears equally distributed between the outer and the inner leaflets of the lipid bilayer while the

phospholipids are asymmetrically dispersed and distributed between the two layers [16,22]. Cholesterol is an important determinant of membrane fluidity and flexibility and it is required for RBC viability and deformability [16,23,24]. An increase in the amount of cholesterol and subsequent alteration in the cholesterol to phospholipid ratio leads to a decrease in the deformability [16,24,25]. Extreme changes in deformability as a result of membrane compositional changes leads to early destruction of the RBC and consequent removal from the circulation [16]. The structure and design of the RBC protein cytoskeleton and its interaction with the membrane lipid bilayer gives the RBC the strength, elasticity and flexibility to deform and maintain RBC functions [16]. In addition, due to its biconcave shape and membrane elasticity the RBC has the ability and flexibility to deform, squeeze and pass through the small vessels and capillaries throughout the circulatory system [18]. These cells, normally circulating for approximately 120 days, undergo several alterations leading to their removal by the reticuloendothelial system [16,26]. Effective preservation techniques are required to maintain the quality of these cells for transfusion medicine.

The hypothermic preservation of RBCs comes out from a number of discoveries and subsequent improvements. During World War I, blood transfusion required a vein to vein connection between the donor and the recipient to prevent blood clotting and maintain viability of the blood components [27]. Hustin in 1914 demonstrated the use of citrate as an anticoagulant which overcame the challenge with coagulation of the blood. Then, in 1915 Rose and Turner

developed their preservative solution which consists of citrate as an anticoagulant and glucose as a nutrient to preserve rabbit RBCs [8,27]. The use of glucose-citrate solutions allows the donor and patient to be separated in space and time. After that, Robertson applied this technique to human RBCs, leading to the establishment of the first blood bank in the world in France during World War I [28] . Robertson illustrated that a citrate-dextrose solution could be used to store blood for up to 26 days; however, the United States Army Medical Corps chose to use only citrate in autoclaved bottles to avoid bacterial contamination as dextrose caramelized when solutions were sterilized with heating [5]. This collection with 3.8% sodium citrate allowed RBC storage for 5 days on ice, becoming the first standard for RBC hypothermic storage [5]. Loutit and Mollison pointed out that citrate-dextrose solutions could be autoclaved without dextrose caramelization if the pH was lower than 5.8 [5]. Later, acid citrate dextrose (ACD) solutions extended the storage period up to 21 days which subsequently became the standard for the national blood systems in both the British Commonwealth and USA during and after World War II [5].

Improvements in preservation followed improvement in understanding RBC metabolic activities and storage-induced injury. In addition of sodium phosphate to preservation solutions resulted in citrate phosphate dextrose (CPD) which decreased the loss of intracellular phosphate during storage. Radiochromium-51 (Cr51) allowed for the labelling of stored RBCs to improve measurement of the *in vivo* posttransfusion recovery (RBCs remaining in

circulation at 24-hour post transfusion) [5,15]. This technique showed that the CPD solution increased the *in vivo* recovery of RBCs after 3 weeks of storage. As a result, the Food and Drug Administration (FDA) licensed this solution since it improved storage [5]. In an attempt to further extend the storage period, adenine was also added. Adenine is the main component of ATP, playing a significant role in maintaining RBC viability during hypothermic storage. This led to the creation of citrate phosphate dextrose adenine (CPDA-1) which was licensed by the FDA for 35 days of storage of pRBCs [5,15]. By the early 1980s, it was discovered that the higher hematocrit associated with RBC concentrates result in a high viscosity of the pRBCs which created a slower flow rate compared to whole blood and therefore dilution before transfusion was needed [15,29]. This problem was solved by the use of additive solutions that were originally designed to provide more nutrients to stored RBCs resulting in a longer storage period [5].

During blood donation, whole blood is collected in a bag containing an anticoagulant solution (CPD or CPDA) then centrifuged to separate the blood components into plasma, RBCs, and the buffy coat [5]. The first additive solution was SAG which consists of saline to overcome the high viscosity of pRBC, adenine for the maintenance of intracellular ATP levels, and glucose as a nutrient for RBCs [15]. SAG was modified to reduce RBC hemolysis during storage by adding mannitol, which lead to saline adenine glucose mannitol (SAGM) and hemolysis of stored RBCs was reduced by 50% [15]. Adsol (AS-1), Nutricel (AS-3), and Optisol AS-5 are other additive solutions similar to SAGM that are

licensed in Europe, Canada, and the USA. Additional improvement in the hypothermic storage has been driven by the removal of leukocytes by leukofiltration techniques that enhanced RBC recovery and reduced hemolysis [5]. Currently, hypothermic storage at 1-6 ^{o}C allows RBCs preservation for 5 to 7 weeks depending on the additive solution used. All of the additive solutions approved by the FDA meet the general standard requirements set by international regulatory agencies where at the end of storage period, a minimal 75% recovery of infused RBCs 24-hour posttransfusion and the percent hemolysis should be less than 1% (less than 0.8 in Canada) [8,30]

1.2.Shortcoming of RBC Hypothermic Storage

1.2.1. RBC Hypothermic Storage Lesion

Although numerous attempts have been made to preserve the viability and quality of RBCs during hypothermic storage in order to provide high quality RBC products before transfusion, hypothermically stored RBCs undergo a wide variety of changes during hypothermic storage in blood banking conditions [1,31,32]. All of the alterations which occur during *ex vivo* storage are collectively known as the "hypothermic storage lesion" (HSL) [8,32]. These alterations have been extensively considered, studied and classified in different ways [1,8,32-34], but

the most common classification to describe the types of alterations occurring is by dividing them into two major categories: biochemical and biomechanical changes.

1.2.1.1. Biochemical Changes During RBC Hypothermic Storage

The most significant biochemical changes observed within stored RBCs are associated with changes in metabolism, illustrated by the depletion of adenosine triphosphate (ATP) and almost complete disappearance of 2,3-diphosphoglycerate (2,3-DPG). ATP levels, significant indicator of the cell viability, decline during storage due to the RBC glycolytic metabolism that not just consuming the ATP to maintain the function and viability of RBCs but also preventing ATP synthesis due to the acidic pH that resulting from the metabolic processes [5,34,35]. Normal ATP concentrations are essential for many cellular processes. Examples include the maintenance of sodium–potassium ATPase activity, which is necessary for cellular osmotic balance, and membrane structure and stability. Intracellular RBC ATP depletion during hypothermic storage has been associated with the inactivity of Na+/K+ cationic pumps which results in to a considerable loss of K+ and an influx of Na+ into the cells [32]. Furthermore, this depletion also influences erythrocyte deformability, hemolytic volume and membrane changes that include lipid loss through microvesiculation leading to a reduced surface area to volume ratio [8,36]. Since RBCs release ATP to stimulate the production of nitric oxide (NO), an important vasodilatory compound that

plays a significant role in regulating blood flow in response to hypoxia, the loss of the ATP may diminish the ability of the transfused RBCs to affect NO- facilitated vasodilation [29,33,37-41].

The rapid loss of 2,3-DPG is considered one of the most remarkable changes during hypothermic storage which significantly affects RBC functions [8,32]. The role of 2,3-DPG is to bind and adapt hemoglobin's affinity for O2, facilitating the release of O2 to tissues [1,32]. Consequently, a decrease in 2,3-DPG increases the hemoglobin affinity for oxygen which raises the concern that stored RBCs may not be able to deliver sufficient oxygen to the tissues [1,8,32]. Even though complete *in vivo* restoration of the 2,3-DPG is observed within 2 to 3 days after transfusion, the duration before the restoration may significantly affect patients in particular clinical situations where maintaining the efficiency of oxygen delivery and the quality of transfused RBCs are critical [8,42-44]. Moreover, it has been shown that S-nitrosothiol (SNO) bioactivity rapidly falls during hypothermic storage [32]. Releasing NO from SNO-hemoglobin is another mechanism used by RBC to regulate the local blood flow in response to pO2 and lost SNO-Hb during storage may alter the ability of RBC to deliver oxygen [32,45-47]. There are also other biochemical injuries which have been detected during hypothermic storage such as a decrease in the pH, increase in intracellular calcium, and oxidative damage which adversely affect the RBC function and viability [14,48,49]. It is important to mention that these biochemical changes contribute to the development of other storage lesions such as morphological

changes, membrane loss and other RBC membrane injuries which represent the biomechanical changes.

1.2.1.2.Biomechanical Changes During RBC Hypothermic Storage

The most notable biomechanical alterations which occur during hypothermic storage focus on the changes and injuries in the erythrocyte membrane and cytoskeleton [14]. Biochemical changes include: protein oxidation, lipid peroxidation, membrane phospholipid redistribution, membrane phospholipid loss through microvesiculation, a reduction in the surface area to volume ratio, and an increase in intracellular viscosity, which all lead to changes in the RBC morphology and deformability [32,50-56].

Oxidative damage that involves cytoskeleton protein oxidation and membrane lipid peroxidation occurs during hypothermic storage is of particular concern. The biological structure of the RBC contains high concentration of oxygen and iron ions in ligand state (in Hb) [57]. The iron ions in ligand state are secured from the oxygen radicals *in vivo* to prevent the Hb oxidation. However, it has been shown that the RBC loses this protection during *ex vivo* storage which results in Hb oxidation [49,57]. Some researchers proposed that the RBC protein oxidation during storage is possibly the mechanism of phospholipid loss through the RBC microvesiculation [14,49,57,58]. In addition, it has been illustrated that membrane lipid peroxidation is a mechanism for cell damage, but it is not well

known [14]. Also, the relationship between these oxidative injuries and the viability of the stored RBC remains uncertain [14].

The highly asymmetric distribution of phospholipids between the inner and the outer layer is determined in RBC plasma membrane. Phospholipids in the RBC membrane typically include *phosphatidylcholine* (PC), and *sphingomyelin* (SM), which are predominantly present on the outer layer, and *phosphatidylserine* (PS) and *phosphatidylethanolamine* (PE) which are present on the inner layer (the cytoplasmic side of membrane bilayer). The asymmetrical distribution of these phospholipids within the RBC membrane is energetically preserved and maintained by ATP-dependent aminophospholipid enzymes (flippases, floppases, and scramblases) [16,59,60]. The loss of phospholipid asymmetry within the plasma membrane is associated with many physiological and pathological circumstances [61]. One manifestation of lost membrane asymmetry is the appearance of PS on the outer leaflet of the plasma membrane [16]. This is an early signal of cellular apoptosis and contributes to the removal of the RBCs with PS exposure from the circulation by the reticuloendothelial system [16,59-61].Therefore, maintenance of the membrane phospholipid asymmetry is fundamental during the erythrocyte lifespan [62]. The appearance of PS on the outer leaflet of the plasma membrane is also observed during hypothermic storage [63]. Recently, it has been shown that RBCs with PS on their surface are removed from circulation leading to a reduction in the number of viable RBCs remaining after transfusion [63].

The phospholipid loss through RBC microvesiculation has been considered one of the most notable and important features of RBC biomechanical storage lesion [1,14]. Microvesicles, or microparticles are small phospholipid particles-less than one micron in size, which are produced as a response to cellular stimulation and aging. These RBC microparticles (RMPs) consist of hemoglobin, phospholipids, proteins and protein antigen of their original cellular source [14]. A small number of MPs are formed *in vivo* in healthy people and they play crucial roles such as facilitating communication between different cells and acting as a defence system for the cell or organ [60,64]. Moreover, RBC microvesiculation might be a protection system for a cell to clear itself from harmful compounds, like the appearance of PS on its surface that would cause removing these cells from the circulation if the cell did not get rid of them [1]. It has been pointed out that RBC microvesiculation increases during hypothermic storage [65-67]. It has also been shown that the progressive morphological transformation of the erythrocytes during hypothermic storage leads to microvesicle formation [68]. Potentially, this increase in the number of RMPs may increase their immunomodulatory and thrombogenic effects which may adversely affect transfusion patient outcomes [32,68].

It is worth mentioning that some of the biomechanical changes remain uncertain between different studies. Some studies show a significant increase in RBC microvesiculation and PS externalization in the older units while other studies indicated that the amount of PS on the surface of the cell remains low even

when the number of the microvesicles gradually increases along with the duration of storage [1]. These differences between studies could be influenced by several variables that include but are not restricted to the assay and techniques used which lead to technician to technician variability, lab to lab (site to site), and experiment to experiment variability.

In addition, it is important mentioning that there are many more biomechanical changes that occur to the red cells during hypothermic storage. For example, the progressive morphological change from a bi-concave shape to echinocytic with spicules or spheroechinocytic, leads to a decrease in deformability, increase in RBC aggregation, and adhesion to endothelial cells [32,34,54]. These biochemical changes likely adversely affect the efficiency and the quality of RBCs, and the clinical outcomes posttransfusion [34].

1.2.2. Clinical Outcomes of RBC Hypothermic Storage Lesion

The importance of RBC transfusions for clinical needs is obvious. Although the development and improvement of the RBC hypothermic storage using different methods and techniques extended the storage period for up to 42 days, prolonged storage has harmful impact on the RBC structure and function. From a safety perspective, questioning the efficiency and quality of the stored RBCs opens the door for extensive clinical studies in order to look at the effect of the stored erythrocytes on the recipient. Specific concern is for those patients who

undergo multiple transfusions and are more likely to get older units such as the patients in intensive care units (ICU), general surgery, or those with various trauma [3,14]. It has been indicated in several studies that the transfusion of older stored RBCs have been associated with a potential increase in the adverse clinical outcomes for chronically ill patients [68-76]. Some of the reported clinical and/or physiological negative effects include but are not limited to: increased length of stay in hospital, multiple organ failure, and increased incidence of postoperative infections [14,77]. In addition, it has been stated that patient who undergo cardiac surgery or suffer from multiple trauma and received transfusions show poorer clinical consequences, involving increased mortality and morbidity rates, compared to the non –transfused patients or patients received fresh RBC transfusion and undergo the same medical disorders [14]. At the present time, there are several recommendations to deal with the undesirable impact after transfusing stored blood. A number of institutions and clinical trials like the "Transfusion Requirements In Critical Care" (TRICC) trial, investigate the safety of blood, provide a quantity of suggestions, and work to promote the safety of blood for transfusion medicine [14]. Currently, a number of other clinical trials have been established such as the National Heart, Lung, and Blood Institute (NHLBI) Red Cell Storage Duration Study (RECESS), the Research Age of Blood Evaluation (ABLE) study that is being planned in Canada, the Age of Red Blood Cells in Premature Infants (ARIPI) and the current large study in U.S.A. is the Red Cell Storage Age Study [78,79].

1.2.3. Rejuvenation Solutions for RBC Preservation

Although additive solutions promote the preservation of RBCs and prolong the storage duration from 5-7 weeks [1,11], still several approaches for better RBC preservation have emerged. RBC rejuvenation is one of these approaches that is based on adding a special solution to reverse some of the storage lesion that occur to RBCs during storage [15,80-82]. The major goal of rejuvenation is to regenerate the metabolic components of the RBCs: ATP and 2,3-DPG [15,36,80]. The first method to rejuvenate the ATP and 2,3-DPG was illustrated by Valeri using a rejuvenation solutions containing pyruvate, inosine, phosphate, and adenine (PIPA) [15,83]. The PIPA is added to the stored RBC at the end of the storage period and then incubated for 1 to 4 hour at 37 ^{o}C which increased the level of the ATP and the 2,3-DPG. Rejuvenated cells could be either stored or transfused within 24-hour after a washing process to remove the inosine [15]. The FDA require transfusion within 24-hour after rejuvenation to minimize the bacterial contamination associated with the open system used during the infusion of the PIPA to the packed RBCs [15]. A second rejuvenation solution, sold by Fenwal Laboratories, named FRES and contains pyruvate, inosine, and phosphate. Even though the FRES has been used to restore ATP and 2,3-DPG before freezing the RBCs, it is not commercially available because of low consumer demand [15]. The third method confirmed by Sohmer and Scott was based on washing the stored RBC and incubating them with an acidic solution of phosphoenolpyruvate

(PEP) at 37 ^{o}C to allow the PEP to enter the cell and increase the level of ATP and 2,3-DPG [15].

1.2.4. RBC Membrane Hypothermic Storage Lesion and *In Vivo* Viability

Current standards for RBC quality is illustrated as less than 1% hemolysis (0.8% in Canada) and more than 75% of cells must remain in circulation 24 hour after transfusion [78]. However, these standards do not properly reflect RBC viability post transfusion. Moreover, 25% of transfused RBCs that are removed from the circulation post-transfusion may significantly adverse patient outcomes, specially the critically ill patients. Therefore, it is important to consider and make use of better predictors for RBC quality and *in vivo* variability.

A recent study shows that the biochemical and biomechanical RBC membrane changes during hypothermic storage can induce post-transfusion removal of transfused RBCs which perhaps act as major predictors of RBC functions and *in vivo* survival [66]. The most significant RBC membrane changes which act as predictors for RBC *in vitro* quality and as features leading to loss of RBCs *in vivo* viability after transfusion are: changes in surface area to volume ratio, decrease in membrane deformability, and alteration in RBC surface properties involving lipid and protein alterations [34]. This suggests that reduced membrane loss and changes including RBC microvesiculation may act as indicator of improved RBC preservation and viability.

1.3.Hypothesis and Thesis Objectives

This thesis will test the following hypotheses: 1) multi-color flow cytometry is an effective method to identify, characterize and quantify RBC microparticles; 2) hypothermic storage induces erythrocyte microvesiculation, PS externalization and decrease CD47 expression on RBC and on RBC MPs; 3) biochemical rejuvenation can be used as an approach to prevent or minimize changes in RBC microvesiculation, PS and CD47 expression.

The main aims of this thesis are to develop a multi-color flow cytometric analysis method to identify, characterize and quantify RBC MPs along with examining the effect of the rejuvenation solution on the RBC microvesiculation during the hypothermic storage period. This thesis is made up of three experimental studies that fit with the specific research aims (SRA) as the following:

SRA (1): to develop a multi-color flow cytometric analysis method to identify, characterize and quantify RBC MPs together with establishing a standard validating protocol for this method (second chapter).

SRA (2): to establish a baseline of quality of RBCs during storage and to determine if hypothermic storage induces erythrocyte microvesiculation and

changes in CD47 and PS on RBCs as well as on RBC MPs using the method from the second chapter (third chapter).

SRA (3): to examine if the use of a rejuvenation solution reduces the exocytic vesiculation of RBC during the hypothermic storage period (fourth chapter).

1.4.Thesis Approach

The literature provides an extensive list of RBC *in vitro* quality changes that occur during hypothermic storage period. Although the current standard for RBC transfusions requires more than 75% of infused RBCs to remain 24-hour post-transfusion, numerous clinical studies show the undesirable impact of the transfusion of older blood on a wide range of patients including an increase in mortality and morbidity. Therefore, it is necessary to have better tools to examine and assess the quality of hypothermic storage that better predict *in vivo* viability. Measuring the alternations of the RBC membrane composition that are more reflective of the *in vivo* viability may hypothetically be novel pointers for RBC quality during the hypothermic storage. Setting up and validating methods and techniques to properly assess the *in vivo* viability of the stored RBCs may lead to better blood banking and safer transfusions.

This thesis will be focus on two parts: first, the development and validation of a multi-color flow cytometric analysis method to identify, characterize, and

quantify RBC microvesiculation during hypothermic storage. Developing and validating a suitable method to assess the RBC microvesiculation, a significant predictor for RBC *in vitro* quality and *in vivo* viability, will contribute to enhancing the quality assessment of stored RBC.

Since studies suggest that better RBC preservation is associated with decreased membrane loss and vesicle formation [14], the second part of this thesis will be to concentrate on a rejuvenation study to assess the effect of rejuvenation on the RBC *in vitro* quality parameters as an approach to reduce the RBC membrane injury and RBC microvesiculation. As is indicated in 1.2.3, it has been shown that the use of PIPA rejuvenation solutions improve the quality of hypothermically stored RBCs by restoring the depleted metabolic components, including ATP and 2,3-DPG concentrations, improved RBC morphology, as well as the post-transfusion survival [83,84]. Reducing membrane phospholipid loss via microvesiculation may potentially be assessed by improving the metabolic and the structural status of the RBC membrane using the rejuvenation solutions. Consequently, improving the quality of the hypothermically stored RBCs will be driven by the development of methods to examine and modify RBC microvesiculation during the hypothermic storage as an approach to promote the sciences of RBC biopreservation and transfusion.

1.5.References:

1. Lion N, Crettaz D, Rubin O, Tissot JD. Stored red blood cells: A changing universe waiting for its map(s). J Proteomics 2010;**73**: 374-85.

2. Lundsgaard-Hansen P. Treatment of acute blood loss. Vox Sang 1992;**63**: 241-6.

3. Lelubre C, Vincent JL. Red blood cell transfusion in the critically ill patient. Ann Intensive Care 2011;**1**: 1-9.

4. Wang JK, Klein HG. Red blood cell transfusion in the treatment and management of anaemia: the search for the elusive transfusion trigger. Vox Sang 2010;**98**: 2-11.

5. Hess JR. An update on solutions for red cell storage. Vox Sang 2006;**91**: 13-9.

6. Takei T, Amin NA, Schmid G, Dhingra-Kumar N, Rugg D. Progress in global blood safety for HIV. J Acquir Immune Defic Syndr 2009;**52 Suppl 2**: S127-31.

7. Carson JL, Grossman BJ, Kleinman S, Tinmouth AT, Marques MB, Fung MK, Holcomb JB, Illoh O, Kaplan LJ, Katz LM, Rao SV, Roback JD, Shander A, Tobian AAR, Weinstein R, Swinton McLaughlin LG, Djulbegovic B, Aabb ftCTMCot. Red Blood Cell Transfusion: A Clinical Practice Guideline From the AABB*. Ann Intern Med 2012;**157**: 49-58.

8. Scott KL, Lecak J, Acker JP. Biopreservation of red blood cells: past, present, and future. Transfus Med Rev 2005;**19**: 127-42.

9. Holovati JL, Acker JP. Emerging Role for Use of Liposomes in the Biopreservation of Red Blood Cells. Transfus Med Hemother 2011;**38**: 99-106.

10. Hogman CF, Meryman HT. Storage parameters affecting red blood cell survival and function after transfusion. Transfus Med Rev 1999;**13**: 275-96.

11. Hogman CF. Liquid-stored red blood cells for transfusion. A status report. Vox Sang 1999;**76**: 67-77.

12. Hess JR. Conventional blood banking and blood component storage regulation: opportunities for improvement. Blood Transfus 2010;**8 Suppl 3**: s9-15.

13. Acker JP. Biopreservation of cells and engineered tissues. Adv Biochem Eng Biotechnol 2007;**103**: 157-87.

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

15. Moore GL. Additive solutions for better blood preservation. Crit Rev Clin Lab Sci 1987;**25**: 211-29.

16. Rodak BF, Fritsma GA, Keohane EM. Hematology : clinical principles and applications. St. Louis, Mo.: Elsevier Saunders, 2012.

17. Chen K, Liu J, Heck S, Chasis JA, An X, Mohandas N. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. Proc Natl Acad Sci U S A 2009;**106**: 17413-8.

18. Khairy K, Foo J, Howard J. Shapes of Red Blood Cells: Comparison of 3D Confocal Images with the Bilayer-Couple Model. Cell Mol Bioeng 2010;**1**: 173-81.

19. Jensen FB. The dual roles of red blood cells in tissue oxygen delivery: oxygen carriers and regulators of local blood flow. J Exp Biol 2009;**212**: 3387-93.

20. Ellsworth ML. The red blood cell as an oxygen sensor: what is the evidence? Acta Physiol Scand 2000;**168**: 551-9.

21. Arbustini E. Total erythrocyte membrane cholesterol: an innocent new marker or an active player in acute coronary syndromes? J Am Coll Cardiol 2007;**49**: 2090-2.

22. Smith JE. Erythrocyte membrane: structure, function, and pathophysiology. Vet Pathol 1987;**24**: 471-6.

23. Ohvo-Rekila H, Ramstedt B, Leppimaki P, Slotte JP. Cholesterol interactions with phospholipids in membranes. Prog Lipid Res 2002;**41**: 66-97.

24. Forsyth AM, Braunmüller S, Wan J, Franke T, Stone HA. The effects of membrane cholesterol and simvastatin on red blood cell deformability and ATP release. Microvasc Res 2012;**83**: 347-51.

25. Kohno M, Murakawa K, Yasunari K, Yokokawa K, Horio T, Kano H, Minami M, Yoshikawa J. Improvement of erythrocyte deformability by cholesterol-lowering therapy with pravastatin in hypercholesterolemic patients. Metabolism 1997;**46**: 287-91.

26. Sparrow RL, Healey G, Patton KA, Veale MF. Red blood cell age determines the impact of storage and leukocyte burden on cell adhesion molecules, glycophorin A and the release of annexin V. Transfus Apher Sci 2006;**34**: 15-23.

27. Mollison PL. The introduction of citrate as an anticoagulant for transfusion and of glucose as a red cell preservative. Brit J Haematol 2000;**108**: 13-8.

28. Robertson OH. Transfusion With Preserved Red Blood Cells. Br Med J 1918;**1**: 691-5.

29. Dietrich HH, Ellsworth ML, Sprague RS, Dacey RG. Red blood cell regulation of microvascular tone through adenosine triphosphate. Am J Physiol-Heart C 2000;**278**: H1294-H8.

30. Sowemimo-Coker SO. Red blood cell hemolysis during processing. Transfus Med Rev 2002;**16**: 46-60.

31. Bessos H, Seghatchian J. Red cell storage lesion: The potential impact of storage-induced CD47 decline on immunomodulation and the survival of leucofiltered red cells. Transfus Apher Sci 2005;**32**: 227-32.

32. Kor DJ, Van Buskirk CM, Gajic O. Red blood cell storage lesion. Bosn J Basic Med Sci 2009;**9 Suppl 1**: 21-7.

33. van de Watering L. Red cell storage and prognosis. Vox Sang 2011;**100**: 36-45.

34. Wolfe LC. The membrane and the lesions of storage in preserved red cells. Transfusion 1985;**25**: 185-203.

35. Tsuda S, Tomoda A, Minakami S. Intracellular pH (pHi) of red cells stored in acid citrate dextrose medium. Effects of temperature and citrate anions. J Biochem 1975;**78**: 469-74.

36. Dumaswala UJ, Petrosky TL, Greenwalt TJ. Studies in red blood cell preservation. 6. Red cell membrane remodeling during rejuvenation. Vox Sang 1992;**63**: 12-5.

37. Bergfeld GR, Forrester T. Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. Cardiovasc Res 1992;**26**: 40-7.

38. Ellsworth ML, Forrester T, Ellis CG, Dietrich HH. The erythrocyte as a regulator of vascular tone. Am J Physiol-Heart C 1995;**269**: H2155-H61.

39. Sprague RS, Ellsworth ML, Stephenson AH, Lonigro AJ. ATP: The red blood cell link to NO and local control of the pulmonary circulation. Am J Physiol-Heart C 1996;**271**: H2717-H22.

40. McCullough WT, Collins DM, Ellsworth ML. Arteriolar responses to extracellular ATP in striated muscle. Am J Physiol 1997;**272**: H1886-91.

41. Hanson MS, Ellsworth ML, Achilleus D, Stephenson AH, Bowles EA, Sridharan M, Adderley S, Sprague RS. Insulin inhibits low oxygen-induced ATP release from human erythrocytes: implication for vascular control. Microcirculation 2009;**16**: 424-33.

42. Heaton WA. Evaluation of posttransfusion recovery and survival of transfused red cells. Transfus Med Rev 1992;**6**: 153-69.

43. Heaton A, Keegan T, Holme S. In vivo regeneration of red cell 2,3-diphosphoglycerate following transfusion of DPG-depleted AS-1, AS-3 and CPDA-1 red cells. Br J Haematol 1989;**71**: 131-6.

44. Valeri CR, Collins FB. Physiologic effects of 2,3-DPG-depleted red cells with high affinity for oxygen. J Appl Physiol 1971;**31**: 823-7.

45. McMahon TJ, Moon RE, Luschinger BP, Carraway MS, Stone AE, Stolp BW, Gow AJ, Pawloski JR, Watke P, Singel DJ, Piantadosi CA, Stamler

JS. Nitric oxide in the human respiratory cycle. Nature Med 2002;**8**: 711-7.

46. Bennett-Guerrero E, Veldman TH, Doctor A, Telen MJ, Ortel TL, Reid TS, Mulherin MA, Zhu H, Buck RD, Califf RM, McMahon TJ. Evolution of adverse changes in stored RBCs. Proc Natl Acad Sci U S A 2007;**104**: 17063-8.

47. Reynolds JD, Ahearn GS, Angelo M, Zhang J, Cobb F, Stamler JS. S-nitrosohemoglobin deficiency: a mechanism for loss of physiological activity in banked blood. Proc Natl Acad Sci U S A 2007;**104**: 17058-62.

48. Wolfe LC. Oxidative injuries to the red cell membrane during conventional blood preservation. Semin Hematol 1989;**26**: 307-12.

49. Kanias T, Acker JP. Biopreservation of red blood cells--the struggle with hemoglobin oxidation. FEBS J 2010;**277**: 343-56.

50. Salzer U, Zhu R, Luten M, Isobe H, Pastushenko V, Perkmann T, Hinterdorfer P, Bosman GJ. Vesicles generated during storage of red cells are rich in the lipid raft marker stomatin. Transfusion 2008;**48**: 451-62.

51. Rumsby MG, Trotter J, Allan D, Michell RH. Recovery of membrane micro-vesicles from human erythrocytes stored for transfusion: a mechanism for the erythrocyte discocyte-to-spherocyte shape transformation. Biochem Soc Trans 1977;**5**: 126-8.

52. Dumaswala UJ, Dumaswala RU, Levin DS, Greenwalt TJ. Improved red blood cell preservation correlates with decreased loss of bands 3, 4.1, acetylcholinestrase, and lipids in microvesicles. Blood 1996;**87**: 1612-6.

53. Berezina TL, Zaets SB, Morgan C, Spillert CR, Kamiyama M, Spolarics Z, Deitch EA, Machiedo GW. Influence of storage on red blood cell rheological properties. J Surg Res 2002;**102**: 6-12.

54. Hess JR, Greenwalt TG. Storage of red blood cells: new approaches. Transfus Med Rev 2002;**16**: 283-95.

55. Hogman CF, de Verdier CH, Ericson A, Hedlund K, Sandhagen B. Studies on the mechanism of human red cell loss of viability during storage at +4 degrees C in vitro. I. Cell shape and total adenylate concentration as

determinant factors for posttransfusion survival. Vox Sang 1985;**48**: 257-68.

56. La Celle PL. Alteration of deformability of the erythrocyte membrane in stored blood. Transfusion 1969;**9**: 238-45.

57. Yoshida T, Shevkoplyas SS. Anaerobic storage of red blood cells. Blood Transfus 2010;**8**: 220-36.

58. Wagner GM, Chiu DT, Qju JH, Heath RH, Lubin BH. Spectrin oxidation correlates with membrane vesiculation in stored RBCs. Blood 1987;**69**: 1777-81.

59. Wolfe CA, James PS, Mackie AR, Ladha S, Jones R. Regionalized lipid diffusion in the plasma membrane of mammalian spermatozoa. Biol Reprod 1998;**59**: 1506-14.

60. Herrmann A, Müller P. A model for the asymmetric lipid distribution in the human erythrocyte membrane. Biosci Rep 1986;**6**: 185-91.

61. Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood 1997;**89**: 1121-32.

62. Greenwalt TJ. The how and why of exocytic vesicles. Transfusion 2006;**46**: 143-52.

63. Holme S. Current issues related to the quality of stored RBCs. Transfus Apher Sci 2005;**33**: 55-61.

64. Simak J, Gelderman MP. Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers. Transfus Med Rev 2006;**20**: 1-26.

65. Xiong Z, Oriss TB, Cavaretta JP, Rosengart MR, Lee JS. Red cell microparticle enumeration: validation of a flow cytometric approach. Vox Sang 2012;**103**: 42-8.

66. Rubin O, Crettaz D, Canellini G, Tissot JD, Lion N. Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools. Vox Sang 2008;**95**: 288-97.

67. Pankoui Mfonkeu JB, Gouado I, Fotso Kuate H, Zambou O, Amvam Zollo PH, Grau GE, Combes V. Elevated cell-specific microparticles are a biological marker for cerebral dysfunctions in human severe malaria. PLoS One 2010;**5**: e13415.

68. Tissot JD, Rubin O, Canellini G. Analysis and clinical relevance of microparticles from red blood cells. Curr Opin Hematol 2010;**17**: 571-7.

69. Chin-Yee I, Arya N, d'Almeida MS. The Red Cell Storage Lesion and its Implication for Transfusion. Transfus Sci 1997;**18**: 447-58.

70. Head DJ, Lee ZE, Swallah MM, Avent ND. Ligation of CD47 mediates phosphatidylserine expression on erythrocytes and a concomitant loss of viability in vitro. Brit J Haematol 2005;**130**: 788-90.

71. Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. Blood Rev 2007;**21**: 157-71.

72. Jy W, Ricci M, Shariatmadar S, Gomez-Marin O, Horstman LH, Ahn YS. Microparticles in stored red blood cells as potential mediators of transfusion complications. Transfusion 2011;**51**: 886-93.

73. Triulzi DJ, Yazer MH. Clinical studies of the effect of blood storage on patient outcomes. Transfus Apher Sci 2010;**43**: 95-106.

74. Ho J, Sibbald WJ, Chin-Yee IH. Effects of storage on efficacy of red cell transfusion: when is it not safe? Crit Care Med 2003;**31**: S687-97.

75. Corwin HL. Anemia and blood transfusion in the critically ill patient: role of erythropoietin. Crit Care 2004;**8 Suppl 2**: S42-4.

76. Klein HG, Spahn DR, Carson JL. Red blood cell transfusion in clinical practice. Lancet 2007;**370**: 415-26.

77. Hod EA, Spitalnik SL. Harmful effects of transfusion of older stored red blood cells: iron and inflammation. Transfusion 2011;**51**: 881-5.

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

79. Yazdanbakhsh K, Bao W, Zhong H. Immunoregulatory effects of stored red blood cells. Hematology Am Soc Hematol Educ Program 2011;**2011**: 466-9.

80. Usry RT, Moore GL, Manalo FW. Morphology of stored, rejuvenated human erythrocytes. Vox Sang 1975;**28**: 176-83.

81. Barras JP, Moldovanyi A, Wagner S, Koerner K. [Effect of rejuvenation on the rheologic properties of stored erythrocytes]. Vasa 1994;**23**: 305-11.

82. DeVenuto F, Brenneman G, Wilson SM. Rejuvenation of human red blood cells during liquid storage. Transfusion 1974;**14**: 338-44.

83. Valeri CR. Use of Rejuvenation Solutions in Blood Preservation. Crc Cr Rev Cl Lab Sc 1982;**17**: 299-374.

84. Meyer EK, Dumont DF, Baker S, Dumont LJ. Rejuvenation capacity of red blood cells in additive solutions over long-term storage. Transfusion 2011;**51**: 1574-9.

Chapter 2*

A Method for Identifying and Characterizing Red Blood Cell Microvesiculation as a Predictor of Red Blood Cell in *Vitro* Quality following hypothermic storage

* Tracy Turner and Jayme Tchir contributed in the tech to tech assay.

2.1. Introduction:

Red blood cell (RBC) microvesiculation is the process by which microparticles (phospholipid vesicles that are less than 1.5 μm in diameter) are released by red blood cells in response to different stimuli and conditions. These RBC microparticles consist of hemoglobin, phospholipids and proteins from parent RBCs [1]. A small number of microparticles (MPs) are formed *in vivo* in healthy people and they play crucial roles such as facilitating communication between different cells and acting as a defence system for the cell or organ [2,3]. It has been reported that the number of RBC microparticles (RMPs) increase in the patients' circulation who suffer from hemolytic anemia such as thalassemia, sickle cell anaemia and other particular pathological conditions [4-6]. In addition, RBC microvesiculation has been shown to increase throughout hypothermic storage and cryopreservation and may act as a novel marker of phospholipid and membrane loss in *ex vivo* biopreservation [3,7-9].

The identification of RBC microvesiculation is important because RMPs have been shown to be harmful immunomodulators upon transfusion, as well as indicators of RBC function and viability throughout *ex vivo* storage which has led to an increased interest in studying RMPs [4,5,10,11]. Research and clinical importance of cell-derived microparticles required more detailed studies to analyze these MPs. Even though there are several methods and assays used to detect the MPs [3,12], flow cytometry is the most common and ideal method used

to define and quantify MPs [3,13]. Other assays for MPs, such as the microplate affinity assays, are not sufficient to detect specific MPs, are deficient in direct quantification, and do not provide size and granulate information about MPs [3,12]. Therefore, flow cytometry appears to be a superior method to analyze and quantify the MPs due to its ability to identify, characterize, and quantify large numbers of microparticles with rich of information it provides which overcome the disadvantages of the other methods [3,12,14].

Flow cytometry with fluorescence and monoclonal antibodies for a sample test [15] is used for counting cells or particles based on its flow rate method, immunephenotyping for particles, and multi-chromatic ability [3,13]. The principle of the flow cytometer revolves around the measurement of particle properties when cells or particles from a sample pass through a stream of fluid [16]. As the cells or particles flow, light from a laser is either scattered or absorbed (fluorescence), in order to be detected, counted, and characterized [16] (Figure 2.2). Characterizing MPs is done by their size which correlate with the forward scatter (FSC) light and by their relative granularity which is shown by the side scatter (SSC) light of a flow cytometry [3,13]. Usually, the light sources in flow cytometer are lasers [16] (Figure 2.2). Fluorescence-based detection takes place when the cell absorbs light and re-emits it at a different frequency to be collected by the detectors [17,18]. Fluorescence emissions or signals in flow cytometry experiment are obtained by the staining or labelling the test sample with monoclonal fluorescent antibodies, reagents or markers [16,17]. This

fluorescence is used to measure the presence of the fluorescent monoclonal marker on the cell or its fragments. Thus, the amount of fluorescence emissions emitted is dependent on amount of bound fluorescent markers to particles in the sample [17]. Therefore, it is important to ensure an appropriate amount of fluorescent antibodies and incubation time following staining to allow the reaction to occur before analysing the samples.

Flow cytometry utilizing fluorescently-labeled monoclonal antibodies is used to identify, characterize and quantify RBC and RMPs. RBC and RMPs are quantified and discriminated from other particles based on their binding of fluorescently-labeled antibodies directed against glycophorin A [4]. Further characterization of RMPs can be determined through the expression of two other markers, phosphatidylserine (PS) and CD47, as their expression appears to change throughout storage [9,19,20]. Annexin V is a phospholipid-binding protein with a high affinity for PS, which is typically found on the inner leaflet of the plasma membrane [9]. However, in response to cell stress, PS is translocated to the outer leaflet and thereby exposed to the extracellular environment and annexin V mediated binding [7]. CD47 is a surface antigen that has been shown to be a "marker of self" [20,21]. RBCs lacking this antigen on their surface are rapidly cleared from the circulation by macrophages in the spleen [21]. It has been demonstrated that the expression of CD47 on RBCs decreases during storage and may be an important determinant of post-transfusion *in-vivo* survival [20].

Flow cytometry for RBCs and RMPs allows identifying a wide variety of cells and their particles. It plays a significant role in the advancement of the science of RBC biopreservation, and may have a role in clinical medicine. However, even though flow cytometry appears to be the best method to analyze MPs, it lacks standard protocols and validation process to analyze and enumerate MPs [22-24]. Lacking standardization refers to several analytical and pre-analytical variables such as differences in blood collection, preparation, and processing the test sample [22,23,25]. It is important to mention that biomarkers and the biological parameters can be difficult to validate due to the complexity of the biological system. For example, one of the validation parameters is estimating day to day imprecision which requires testing the samples on several days to achieve the long term imprecision for a parameter [26]. However, it is difficult to have consistent measurements throughout a period of time for a biological material where it is expected to change during this time especially if it is out of their original environment. Further validation challenges will be addressed in the discussion section of this chapter.

Since there is no standardized approach to quantitatively and qualitatively analyzing cell-derived microparticles with a validation process [3,4,12,22,23,25] , this chapter presents and validates a multicolor flow cytometric analysis method to identify, characterize and quantify the RBC and their microparticles during hypothermic storage to be used to establish a baseline of this thesis. Moreover,

this method can establish standard protocols that allow for the comparison of results from different laboratories or across different studies.

2.2. Materials and Methods:

2.2.1. RBC Processing

Leukoreduced packed CPD-SAGM RBC units (n=20) were obtained from two Canadian Blood Services (CBS) Centres: Edmonton, Alberta and the Network Centre for Applied Development (netCAD), Vancouver, British Columbia. Whole blood (450 ± 50mL) units were collected using standard blood bank phlebotomy procedures and processed using the standard buffy coat method [27]. All units were rapidly cooled to room temperature (20-25 °C) and held for up to 24 hours. Within the 24 hours, the units were centrifuged in order to obtain the RBCs; buffy coat and platelet poor plasma layers were separated and divided into 2 satellite bags using the top and bottom method [27]. After separation, SAGM (v=110 mL) was added to each of the RBC units, which was then leukoreduced by filtration and sent to the research laboratory. All units were received 2 days after collection and placed in hypothermic storage at 1 to 6 °C.

On the day of testing, all of the pRBC units were tested for RBC microvesiculation, as well as PS and CD47 expression on the RBC and on RMPs.

2.2.2. Flow Cytometry (FC) Assay

Three tagged antibodies were used to label RBCs and RMPs. Fluorescein isothiocyanate (FITC) anti-human CD235a antibody (Invitrogen, MHGLA01 or MHGLA01-4) was used as a marker for RBCs and RMPs, while APC Annexin V (BD Pharmingen) and PE mouse anti-human CD47 antibody (BD Pharmingen) were used to label PS and CD47, respectively. Packed RBCs (5 μL) were diluted with annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM of $CaCl_2$, pH 7.4) and 5 μL of each of the fluorescently labelled monoclonal antibodies (FITC, APC, PE). After 15 minutes of incubation in the dark at room temperature (20-25 °C), the prepared samples were run on a FACSCalibur flow cytometer (BD Sciences, San Jose, CA) equipped with a 488 nm argon laser and computer software (CellQuest BD Sciences, San Jose, CA). Commercial isotype controls directed against glycophorin A (FITC mouse IgG1, k isotype control [BD Pharmingen]) and CD47 (PE mouse IgG1, isotype control [BD Pharmingen]) were used to account for any non-specific binding of the antibodies. N-ethylmaleimide (NEM)–treated (Sigma-Aldrich, St. Louis, MO, USA) RBCs served as the positive control for PS expression and microvesiculation, as described by Stewart et al [28] with some modification where time for centrifuge was 5 minutes in each step rather than 10 minutes. The buffer used for RBC re-suspension, containing only antibodies and no RBCs, served as a negative control. TruCOUNT beads (BD Pharmingen) were used to determine the absolute number

of RMPs per microliter. RBC MPs were further discriminated based on their size: Latex beads (1.01 µm in diameter; Uniform Polystyrene Microspheres- Bangs Laboratories) were used to create a gate around the desired population of microparticles and only MPs less than 1.01 µm in diameter and positive for glycophorin A-FITC were further analyzed. All of the prepared samples for the flow cytometer were incubated at 20-25 °C for 15 minutes in dark before being analyzed. The forward scatter (FSC) and sideward scatter (SSC) of the flow cytometer, measured on a logarithmic scale, was used to distinguish between RBC and RMPs populations.

Flow cytometry analysis:

Labeled RBC samples were analyzed on the FACSCalibur flow cytometer. Samples were acquired for 3 minutes and the RMPs events, which were positive for glycophorin A-FITC and less than 1.01 µm in size, were shown on a FSC-SSC dot plot. TruCOUNT beads were distinct for the RMPs using different instrument settings (Appendix 2.1). Then, according to Nantakomol et al. 2009, absolute numbers of RBC microparticles per unit were calculated by the following formula [29]:

$$\frac{\#\ events\ microparticles}{\#\ events\ TruCOUNT\ beads} \times \frac{Bead\ Count}{Suspension\ Volume}$$

$$= Absolute\ count\ of\ microparticles/unit \qquad \text{Equation (2.1)}$$

Where:

(# events microparticles)= the number of FITC- Glycophorin A positive events in the RMP gate/ 3 minutes as R1 in Figure 2.3, (*# events TruCOUNT beads)* = the number of events in the TruCOUNT bead gate/ 3 minutes, *(Bead Count)* = the bead count from the TruCOUNT package for the specific lot number used, and *(Suspension Volume)* = the volume of buffer used to suspend the TruCOUNT beads.

Then, the following formula used to calculate RBC microparticles/ microliter:

Absolute count of microparticles per unit x dilution factor

=microparticles/ microliter Equation (2.2)

Where:

(Absolute count of microparticles) = determined from the above calculation, *(Dilution factor)* = determined from the ratio of buffer and fluorochromes added to the RBC sample

2.2.3. Validation Methods

2.2.3.1 Quality Control Assay:

The calibration for all of the equipments and materials was performed following the criteria established by our laboratory or recommended by the manufacturer [30]. Pipettes ware calibrated every 3 months. Centrifuges were sent to Bio-Med every 6 months for calibration. The flow cytometer was calibrated daily following the Cross Cancer Institute protocol. In this protocol, CaliBRITE 3 beads suspension (unlabelled and mixed labeled) were used for 4 color calibration following the instructions of the FASComp program of the flow cytometer. Under the high mode condition, the vortex "unlabelled" bead suspension was run to perform and complete the time delay calibration. Then, the "mixed labeled" bead suspension was run to perform the compensation and the sensitivity test. A successful calibration was identified via a "PASS", while a "FAIL" result mandated troubleshooting was performed [31].

2.2.3.2 Determining the size sensitivity for RMPs

Sensitivity of the method was determined by acquiring latex beads ranging from 0.12 µm to 1.01 µm in diameter. For each size of beads (0.12, 0.20, 0.40, 0.80, and 1.01 µm), the vial was vortexed for at least 5 minutes and 1 µL of each

size bead was separately diluted with Annexin V binding buffer. Specific instrument settings with the flow cytometry for size beads were adjusted as shown in (Appendix 2.1 A). The prepared size beads were vortexed for at least 60 seconds prior to being analyzed. Each of the prepared size beads was run on the flow cytometer for 10 seconds on high mode.

2.2.3.3 Imprecision

2.2.3.3.1 Intra-run imprecision:

One RBC unit was prepared to be run twenty times over a short period of time (2 hours) and under the same conditions by same researcher. 20 identical samples were prepared for flow cytometer from the same unit with the three tagged antibodies, which were used to label RBCs and RMPs. The coefficient of variation (CV) for the intra-run assay, run to run, was calculated according to according to Westgard, 2008 using the mean and standard deviation as the following[32]:

$$\ddot{X} = \Sigma X / N \qquad \text{Equation (2.3)}$$

$$SD = \sqrt{\frac{\Sigma(X-\ddot{X})^2}{N-1}} \qquad \text{Equation (2.4)}$$

$$CV = (SD/\ddot{X})100 \qquad \text{Equation (2.5)}$$

Where:

(Ẍ) represents the mean; (*N*) is the number of individual measurements; (*SD)* Standard deviation; *(X)* Individual measurement; and *(CV)* Coefficient of variation.

Mean ± SD used to show the results for run-run assay. The absolute number of RMPs/µL, the number of RMPs and RBCs expression PS or CD47, and well as the MFI of CD47 and PS on RBCs or RMPs were measured for this assay.

2.2.3.3.2 Inter-run imprecision or tech to tech variability

Three researchers/technicians prepared the samples for the flow cytometer (n=20) and ran them on the same day following the same protocol. Mean ± SD used to show the results for inter-run assay. As shown in the run –run assay, the mean, SD and CV calculated for the same parameters of each technician (1, 2, and 3) as described in section 2.2.3.3.1. The final %CV for all of the technicians was calculated as the following:

$$Final\ CV\ (\%) = (SD_{all} / mean_{all}) * 100 \qquad \text{Equation (2.6)}$$

Where:

SD_{all} = SD of (mean of tech 1; mean of tech 2; mean of tech 3)

$Mean_{all}$ = Average of (mean of tech 1; mean of tech 2; mean of tech 3)

Total variance was calculated from the intra-run and inter-run variances of tech to tech assay as the following [33]:

$$[(N-1)/N] * \text{Average of intra-run variance} + (\text{inter-run } SD_{all})^2 = \text{Total variance} \quad \text{Equation (2.7)}$$

2.2.3.4 Linearity

Serial dilutions of TruCOUNT beads were prepared to determine the lowest and highest results for a test which led to the linearity for the experiment. TruCOUNT beads tubes were labelled as the amount of the Annexin V binding buffer which that was added to each tube: 200 µL, 400 µL, 600 µL, 800 µL, 1000 µL (1 ml), 2 ml, 3 ml, 4 ml, 5 ml, 6 ml, 7 ml, and 8 ml. All of the samples were incubated at room temperature for at least 15 minutes in the dark before being analyzed with the flow cytometer. Replicate measurements were done and the results represented for this assay as mean ± SD.

2.2.3.5 Limit of Detection and Qualification

Serial dilutions of NEM-treated RBCs from the same unit were prepared to estimate the limit of detection (LoD) and limit of quantification (LoQ) for the RMPs, PS and CD47 expression on RBC and on RMPs. Six tubes of each dilution were prepared and labelled with the antigens. The buffer used for RBC re-suspension, only antibodies were added and no RBCs (buffer cocktail), served as the blank control to estimate the limit of blank (LoB). LoB, LoD, and LoQ calculated as the following [34];

$$LoB = Mean\ blank + 1.645\ (SD_{blank}) \qquad \text{Equation (2.8)}$$

$$LoD = LoB + 1.645\ (SD_{Low\ concentration\ sample}) \qquad \text{Equation (2.9)}$$

$$LoQ = LoD \qquad \text{Equation (2.10)}$$

Where:

Mean $_{blank}$ = the mean of blank samples, SD $_{Low\ concentration\ sample}$ = the standard deviation or a test replicate of a sample known to contain low concentration of analyte where a change in the pattern of values was observed.

2.2.4. Statistical analyses

SPSS 11.0 Software (Lead Technologies, Charlotte, NC) and Microsoft Excel 2011 were used to calculate descriptive statistics (mean, SD, CVs, and ranges). A paired t-test was used for the tech to tech variability. P-values less than 0.05 were considered significant for all comparisons unless stated otherwise.

2.3. Results

2.3.1. Sensitivity for RMP Size:

Results for the size sensitivity of the RBC microparticles method are shown in figure 2.4. Sample prepared with a 1.01 µm size beads were used to create a gate around the desired population of microparticles and only MPs less than 1.01 µm in diameter were further analyzed (Figure 2.3). The sensitivity method was shown to be able to detect events as small as 0.20 µm in diameter to be served as the lowest size can be detected. The level of noise started (CV over 100%) with the samples that prepared with less than 0.20 µm such as 0.12 µm size beads used in this experiment (Figure 2.4 A).

2.3.2 Imprecision:

2.3.2.1 Intra-run imprecision:

Table 2.1 summaries the intra-run impressions for all of the measured parameters: absolute number of RMP/µl, RMPs and RBCs with CD47 or PS expression (% and MFI). The coefficient of variations for the measured factors yielded less than 20%. While the number of RBC expressing the CD47 results showed the lowest intra-run CV at 2%, the MFI of CD47 expression on RMPs showed the highest intra-run CV at 19.3% measured.

2.3.2.2 Inter-run imprecision or tech to tech variability

The box plots of figure 2.5 shows the data obtained from inter run precision assay that was used to determine the tech to tech variability for all measured parameters. Figure 2.5F shows a significant difference between the results for MFI of CD47 on RBC obtained by tech 1 in comparison to tech 2 and tech 3 where the CV equal 44.5%. Furthermore, there was a statistically significant increase in the number of RBC expressing PS values between tech 1 and tech 2 values which resulted in a high inter-run CV= 81.9% (Figure 2.5, Box plot I). Table 2.3 shows the impression and the total variation for all or the measure parameter which was calculated from inter run assay.

2.3.3 Linearity:

The linearity of the RBC and RMPs quantitation was determined by examining the linearity of TruCOUNT beads diluted in buffer to various concentrations. The range of the linearity experiments was from 592 ±11 events to 30412 ±1962 events (figure 2.6 A). The linear relationship between the means of TruCOUNT beads concentrations and number of RMPs acquired events was established (R^2= 0.925, Figure 2.6 B).

2.3.4 Limit of Detection and Qualification

Table 2.3 show the LoB calculated form the blank control samples (buffer cocktail) as well as the LoD for all of targeted parameters at different dilutions. Most of the LoD for the measured parameters observed with a dilution of 1/128. The LoQ for this study equals the LoD.

2.4 Discussion:

A multi-color flow cytometric analysis method was validated to determine its fitness to study the effect of the hypothermic storage on the structure of RBCs and their microparticles for the following chapter.

Identification and Specificity:

To detect and to confirm the identity of RBCs and their microparticles, FITC-anti human antibodies directed against glycophorin A was used. Due to the possibility of non-specific binding, a commercially available isotype control antibody against glycophorin A, which has no specificity for the target particles, was used to help determine the level of background of staining with binding antibody. This excluded the non-specific binding characteristics of the glycophorin A-FITC antibody used in this experiment (negative events) and validated the specificity of glycophorin A-FITC antibody (positive events) [35]. Further identification of the RMPs according to their size was obtained by their light scatter properties using size beads and only microparticles less than 1.01 μm were targeted for further investigation. However, even though flow cytometry was appropriate to detect and identify microparticles less than 1.01 μm, the findings of this study shows that flow cytometry as we used it cannot detect microparticles less than 200 nm or 0.20 μm in size. A very high CV (over 100%) was observed with the size beads 0.12 μm in diameter. On the other hand, it has been shown that the size of MPs is very heterogeneous [36], and there are small RBC microparticles less than 0.20 μm [14,37]. Even though this fact is known, most researchers use the common size limit (less than 1 μm) with no lower size limit [25]. Thus, the use of size beads as it is shown in this study may afford a strategy for standardizing flow cytometry analysis of microparticles and set up a globalized limit for the microparticles size that can be detected.

Imprecision:

After the method has been successfully developed to identify and characterize the RBC and the RMPs, assays were established to determine the precision of intra-run and inter-run assays. While the within run or intra run CV assay was less than 20% (table 2.1) for all of the measured parameters, which is the acceptable range of precision for bio-analytical method validation [38], the imprecision of tech to tech assay (inter-run, figure 2.5) was more than 20% for some of measured parameters. The highest inter-run CV was determined for the number of RBC with PS exposure (CV= 81.9 %, figure 2.5, Box plot I). Furthermore, there was a significant difference between tech 1 and tech 2 values of % RBC-PS ($p<0.05$). A similar pattern was observed with the MFI of CD47 expressed on RBC where inter run CV was 44.5% and tech 1 values were significantly higher than tech 2 and tech 3 values ($P< 0.5$, figure 2.5 F). There are several possible causes for this high imprecision and the significant differences between technicians. In this assay it is more likely to be due to incubation time and/or preparation error. According to a number of studies, after preparing flow cytometry samples and labelling them with the fluorescent antibodies they should be incubated for 15 minutes and analyzed within one hour [23,36]. However, this was not the case for the tech to tech variability assay because all the technicians prepared the samples for the flow cytometry almost at the same time and went to

flow cytometer where tech 2 started running the sample within an hour of the preparation time and tech 1 ran the last sample after 3.5 hour after preparing the samples. Consequently, this may explain the higher values and range of tech 1 samples which observed in almost all of the measured parameters (figure 2.5). Therefore, the length between the preparation time and analysis of the samples for each technician could be the cause of the very high imprecision and significant differences in this assay.

Another explanation for the high inter run imprecision has to do with the preparation time and pipetting error. Although all of the technicians followed the same protocol for this assay, there are many variables to consider including, but not limited to, expertise, precision and speed in samples preparation. Technician(s), with more experience may require less time for sample preparation.. Also, any of the technicians could fall into unintended mistake like staining error for one sample or more because of the large number of samples or because of the materials used for staining. For example, APC Annexin V antibody used in this assay to label PS provided by the manufacturer is a colorless antibody and is used in very small volume used to stain each sample. As a result, significant negative impacts on the final results might occur. Further variations could be possible when the samples are out of the storage temperature during sampling by three technicians and when the prepared samples are exposed to light after the staining process for different period of time. Also, although all

technicians follow the same sampling protocol, having the units sampled more than once may create another source of variation.

Typically, it is important to measure the accuracy of the assay but in our study this was a challenge. Accuracy, which is expressed as the bias or the systematic error, is used with random error (imprecision) to calculate the total error for analytic method [39,40]. In this study, the challenge with estimating the accuracy was due to the lack of reference standards and an alternative procedure that could be used.

Limits of blank, detection and quantification (LoB/LoD/LoQ):

The limit of blank or LoB is the highest expected value to be observed for repeated measurements of samples that contain no analyte of interest [34]. Table 2.2 shows the calculated LoB from replicates of buffer cocktail samples for all of targeted parameters. The results from the blank samples are used to provide a threshold for this study with values above the LoB were considered as a positive measurement. In view of that, the LoD was calculated from the LoB and SD of the low concentration sample values that show a detectable change in the pattern of values and exceed the LoB (Table 2.2 Highlighted values). For instance, table 2.2-B shows that the absolute number of RMPs/µL decrease as the concentrations of the sample decreases (positive correlation), but after 1/128 dilution the number of RMPs increase rather than being decrease by lower concentration. Therefore,

this point of concentration (1/128 dilution) used to be the “cut off” point for detection and quantification for RMPs. Similarly, the LoD was calculated and detected for all of the measured parameters in table 2.2. As it shown in table 2.2, most of the LoD for the measured parameters are realized when the sample is diluted more than 16 time of the original concentration. The difference in the LoD for the these parameters is more likely to be due to the type and the strength of the florescent antibodies used where the amount of florescence from PE might be different than APC depends on their brightness [41] on the flow cytometry. Moreover, detecting the florescence for large particles (RBC) is different than small particles (PMPs) and the effectiveness of the florescent antibodies to bind and label the specific antigen may be diverse. Similarly, it has been suggested by Lacroix et al.(2010) that there is difficulty in detecting an antigen on the surface of very small particles due to its low density [12]. They also proposed that the detected florescence signals with flow cytometry are varied not just because of the fluorochromes, but also because of antigens on different size of particles [12].

In addition, it is important to mention that the limits (LoB, LoB, and LoQ) were obtained from RBCs treated with NEM, which has been shown to be a gold standard for PS exposure and microvesiculation. As the amount of MPs and PS in fresh or untreated RBC samples is very low, further dilution of the sample would prevent assessing the limits. Therefore, the treatment with NEM could be a reason behind the different detection limit for the measured parameters. Furthermore, it was difficult to determine the LoQ because NEM treatment for RBC was used for

limits assay. LoQ is defined as the lowest amount of concentration that can be quantified with acceptable precision and trueness (less than or equal 20% CV) [34,38]. In this study, the imprecision assay was done using fresh samples (not NEM- treated sample) and estimating the trueness of the assay was not possible as mentioned earlier in the discussion. Therefore, the LoQ for this assay was decided to be equal to LoD since it not possible to be based on a lower concentration than LoD [34].

2.5 Conclusion:

The main limitation of the flow cytometry method, the preferred method used, is the lack of standardized and validated protocols for quantitatively and qualitatively analyzing cell-derived microparticles. Although there are a number of published studies utilizing the same method and targeting the same parameters, most of these studies are difficult to compare [3]. This established method not only facilitate the work in subsequent chapters, but also may facilitate establishing standard protocols that allow comparing results from different laboratories or different studies. This study can be a useful approach that contributes in the advance of biopreservation science and clinical practice. Additional studies are recommended to overcome the validation challenges associated with the pre-analytical and bio-analytical assays.

Table 2.1: Intra-run assay; one RBC unit was prepared to be run 20 times within 2 hours and under the same conditions by same researcher as explained in the method and material section. The table show the mean± SD, CV and the % CV for the run to run assay.

	Mean ± SD	**CV**	**% CV**
Number of RMPs /µl	33079 ± 15557	0.05	4.71
% of RMPs-CD47	19.5 ± 3.0	0.16	15.73
% of RMPs-PS	20.9 ± 2.3	0.11	10.88
MFI of RMP-CD47	1283 ± 247	0.19	19.26
MFI of RMP-PS	47.8 ± 6.2	0.13	12.92
% of RBC-CD47	99.5 ± 2.3	0.02	2.33
% of RBC-PS	0.65 ± 0.06	0.1	9.67
MFI of RBC-CD47	494.3 ± 67.5	0.14	13.64
MFI of RBC-PS	27.6 ± 2.2	0.08	8.07

Table 2.2 (A and B): Shows the LoB calculated from the blank control samples (buffer cocktail) as well as the LoD for all of targeted parameters at different dilution concentrations where a change in the pattern of values was observed. (D=Dilution)

A) RBCs		Glycophorin A +	CD47 MFI	PS MFI	% of RBC-CD47	% of RBC -PS
Buffer cocktail	Mean	75.17	798.48	124.66	59.58	36.51
	SD	16.33	258.58	28.88	4.12	10.83
	LoB	**102.02**	**1223.84**	**172.18**	**66.35**	**54.33**
D 1	Mean	66700.83	553.39	46.38	97.43	8.01
	SD	21585.75	163.68	5.41	5.42	1.42
D 1/2	Mean	12353.50	640.04	73.53	96.77	11.58
	SD	11585.19	106.89	11.05	1.51	1.34
D 1/4	Mean	4671.83	570.09	88.86	97.96	15.49
	SD	3184.99	137.24	7.91	2.17	1.69
D 1/8	Mean	2581.67	553.93	74.59	98.99	16.02
	SD	1014.15	43.71	6.71	0.38	1.11
D 1/16	Mean	1650.33	598.82	78.97	97.35	18.31
	SD	957.51	114.48	10.66	1.21	2.67
D1/32	Mean	1567.67	944.09	94.62	97.62	17.95
	SD	522.25	73.56	7.29	1.92	1.04
	LoD	**961.12**	**1344.85**	**184.18**	**69.51**	**56.03**
D 1/64	Mean	501.67	853.73	92.82	93.43	20.95
	SD	162.79	65.69	12.13	2.42	3.08
	LoD	**369.82**	**1331.91**	**192.12**	**70.33**	**59.40**
D 1/128	Mean	327.83	698.59	115.73	91.56	28.50
	SD	170.78	151.07	18.25	3.77	3.06
	LoD	**382.96**	**1472.35**	**202.19**	**72.55**	**59.36**
D 1/256	Mean	219.50	912.31	164.01	74.75	27.26
	SD	58.14	157.74	38.21	7.41	5.49
	LoD	**197.67**	**1483.31**	**235.02**	**78.54**	**63.37**
D 1/512	Mean	184.67	928.78	150.87	67.49	28.96
	SD	68.95	308.84	45.95	7.42	7.33
	LoD	**215.45**	**1731.88**	**247.76**	**78.55**	**66.39**
D 1/1024	Mean	186.67	784.37	161.42	71.61	27.43
	SD	34.4	237.19	36.81	3.26	6.01
	LoD	**158.64**	**1614.03**	**232.72**	**71.71**	**64.21**

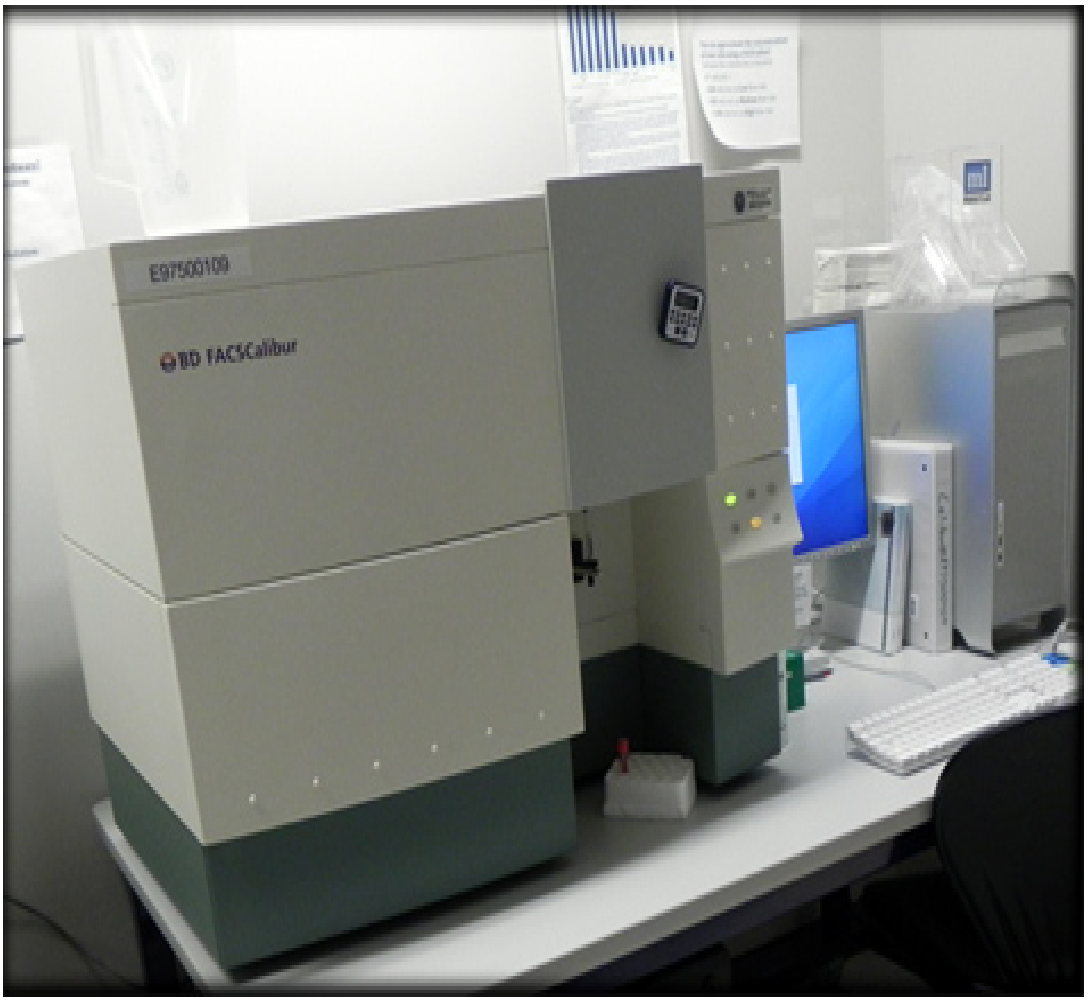
B) RMPs		Glycophorin A +	Absolute # of RMPs/µL	CD47 MFI	PS MFI	% of MP-CD47	% of MPs-PS
Buffer cocktail	Mean	520.50	8412.09	807.12	40.06	31.51	12.50
	SD	152.46	2464.05	279.62	4.65	5.34	1.50
	LoB	**771.30**	**12465.45**	**1267.08**	**47.71**	**40.29**	**14.98**
D 1	Mean	154846.67	2502563.62	173.77	43.51	90.71	37.41
	SD	6983.69	112867.47	22.43	3.83	5.15	7.58
D 1/2	Mean	59958.00	969014.79	169.55	60.29	85.07	38.53
	SD	2000.45	32330.34	12.98	8.39	2.83	9.59
D 1/4	Mean	25149.83	406460.52	185.43	70.92	90.53	62.36
	SD	580.29	9378.45	19.62	2.52	3.38	3.20
D 1/8	Mean	13572.50	219352.78	206.89	79.24	92.96	80.40
	SD	501.26	8101.16	16.99	7.94	1.12	3.29
D 1/16	Mean	7033.67	113675.02	213.15	107.99	89.47	86.82
	SD	171.91	2778.32	42.99	16.08	2.93	3.03
D1/32	Mean	4060.17	65618.63	573.24	124.61	81.29	79.81
	SD	285.93	4621.09	813.63	14.26	3.36	4.37
	LoD	**1241.66**	**20067.14**	**2605.50**	**71.17**	**45.82**	**22.17**
D 1/64	Mean	2097.67	33901.57	247.83	102.86	79.56	73.95
	SD	104.76	1693.147	20.39	22.91	2.11	5.67
	LoD	**943.64**	**15250.68**	**1300.64**	**85.39**	**43.77**	**24.31**
D 1/128	Mean	1264.67	20438.99	326.91	89.10	76.81	65.17
	SD	205.73	3324.85	27.59	2.58	4.54	5.59
	LoD	**1109.72**	**17934.82**	**1312.46**	**51.95**	**47.76**	**24.17**
D 1/256	Mean	1972.50	31878.68	350.57	46.99	35.57	27.56
	SD	526.53	8509.57	45.71	2.69	8.34	6.25
	LoD	**1637.45**	**26463.69**	**1342.28**	**52.12**	**54.02**	**25.26**
D 1/512	Mean	1402.00	22658.51	418.03	44.15	40.29	26.29
	SD	273.13	4414.15	83.91	1.99	2.89	2.59
	LoD	**1220.60**	**19726.72**	**1405.11**	**50.99**	**45.06**	**19.24**
D 1/1024	Mean	1349.17	21804.64	465.58	43.46	37.71	24.57
	SD	183.71	2969.09	31.24	1.66	2.18	1.36
	LoD	**1073.51**	**17349.61**	**1318.48**	**50.44**	**43.88**	**17.21**

Table 2.3: Total variance calculated from the tech to tech assay following equation (2.7) in the method section

Impression

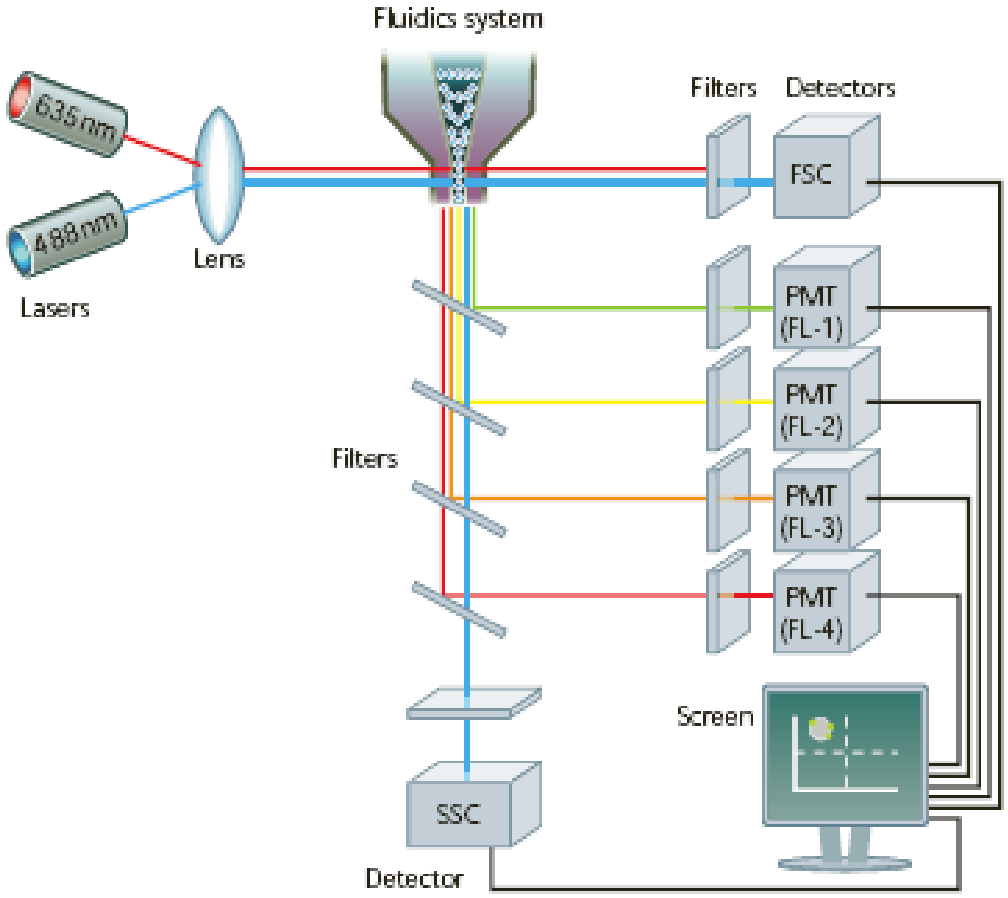
	% of RBC-CD47	% of RBC-PS	CD47 MFI on RBC	PS MFI on RBC	# of MPs with dilution factor	% of RMP-CD47	% of RMP-PS	CD47 MFI on RMPs	PS MFI on RMPs
Mean of Tech 1	99.05	1.05	947.60	146.07	150802	24.40	20.02	645.43	73.11
Mean of Tech 2	99.94	0.14	407.51	152.73	134820	15.13	12.02	515.29	35.49
Mean of Tech 3	98.79	0.49	540.42	138.61	159562	17.66	15.10	481.38	39.82
Mean all	99.26	0.56	631.84	145.80	148395	19.06	15.71	547.37	49.47
SD all	0.60	0.46	281.41	7.07	12545	4.79	4.04	86.60	20.58
% CV	0.61	81.93	44.54	4.85	8.45	25.15	25.69	15.82	41.61
Inter-run variance	0.36	0.21	79191.55	49.93	157381351.05	22.98	16.30	7499.82	423.64
Variance of tech 1	9.61	0.14	6508.10	409.14	504859823.84	63.91	45.69	8096.12	657.98
Variance of tech 2	0.00	0.00	5886.27	613.47	123391899.44	9.34	7.97	7102.35	23.15
Variance of tech 3	21.48	0.05	5617.38	390.97	1739769935.69	36.09	16.09	2805.01	45.31
Average of tech Variances	10.36	0.06	6003.92	471.19	789340552.99	36.45	23.25	6001.16	242.15
Total Variance	10.21	0.27	84895.27	497.56	907254876.39	57.61	38.38	13200.92	653.67

Figure 2.1: The FACSCalibur flow cytometer is equipped with a laser and diode laser, which motivate suitable fluorochromes conjugated to antibodies used to stain cells. The FACSCalibur runs the software program on workstations with Cell Quest, which can also be used for the analysis of flow cytometry data.



http://www.georgiahealth.edu/cancer/shared/flow/equipment.html

Figure 2.2: A liquid flow moves the suspended cells in the tube through the flow cytometer instrument where the cells are exposed to a laser. This is examined by different detectors. Forward scatter (FSC) and side scatter (SSC) are the most important detectors that are installed in the flow cytometer. Then, the cells finish in waste container [16,42].



http://static.abdserotec.com/uploads/Flow-Cytometry.pdf

Figure 2.3: Scatter Plot, as it is displayed on the screen of the flow cytometer, showing the RBCs and the microparticles gates.

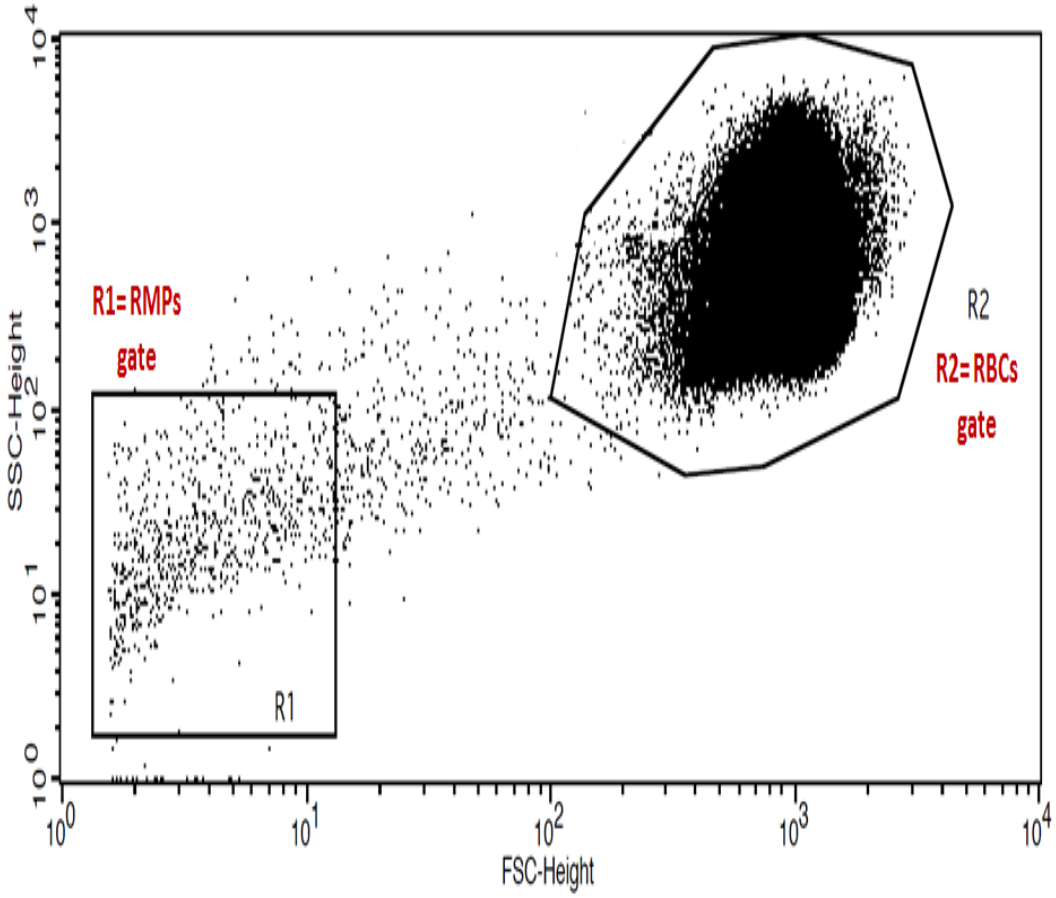
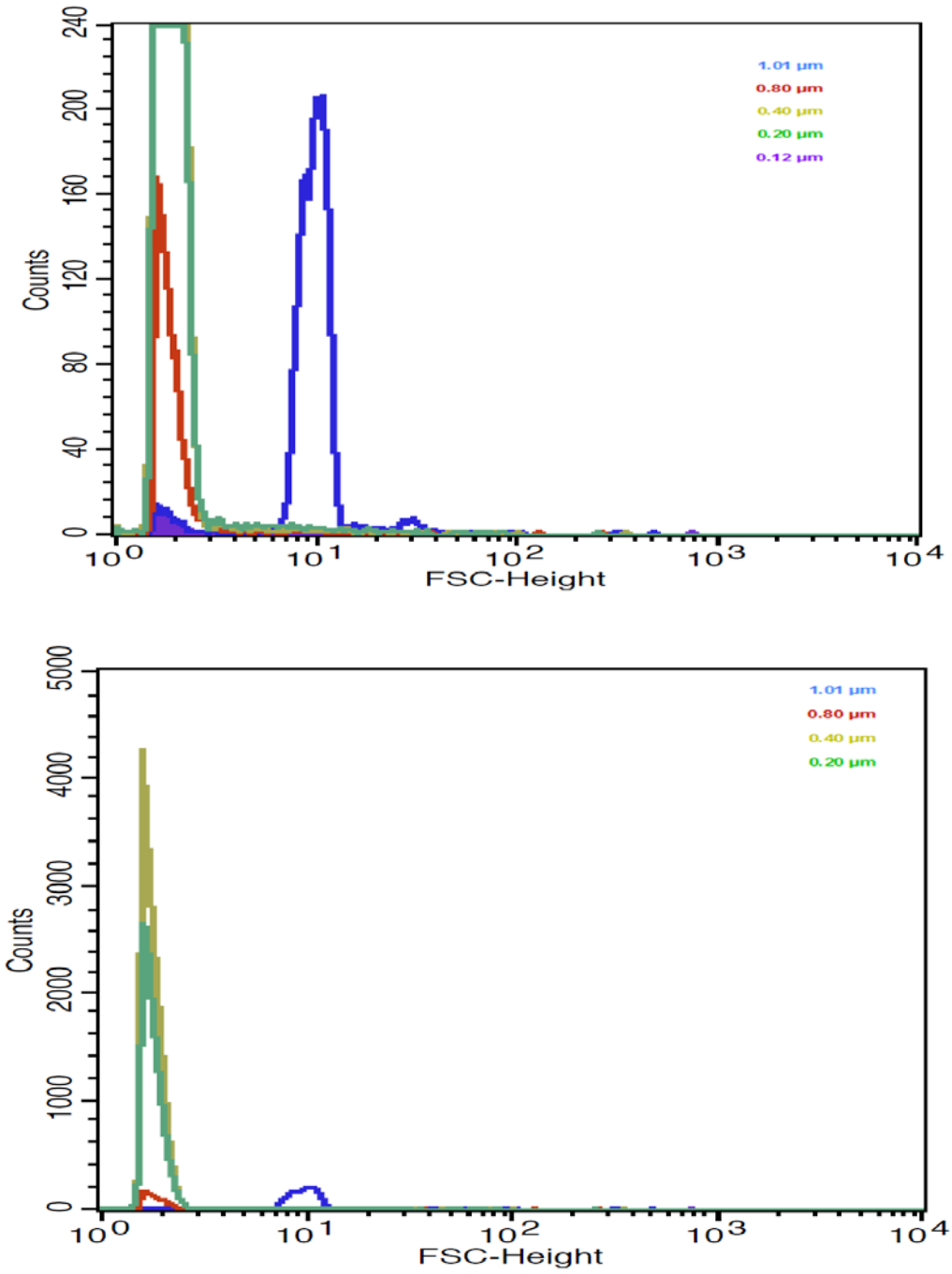
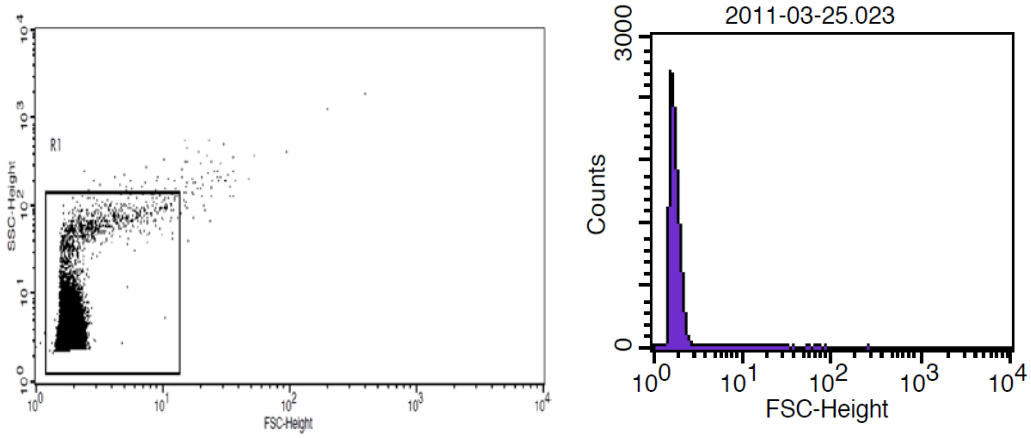


Figure 2.4.: Sensitivity of flow cytometry to resolve beads of different sizes. Off axis data in top figure is resolved in the lower figure. (A); Size beads 0.20 µm represents the lower limit of size used to detect the RMPs (B), and Size beads 1.01 µm represents the upper limit of size should be used to detect the RMPs(C).

A)



Size beads 0.20 µm (B)



Size beads 1.01 µm (C)

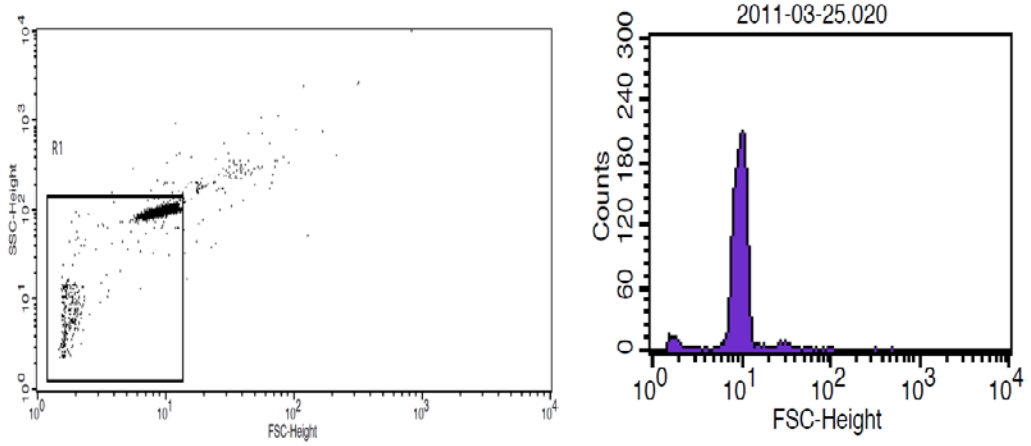
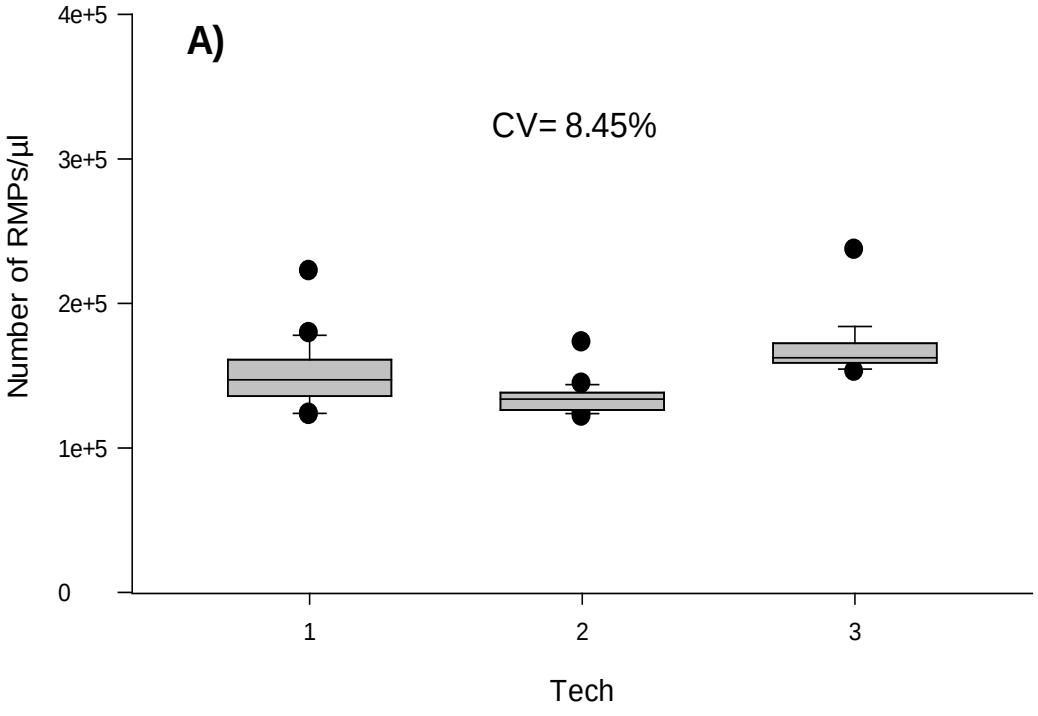
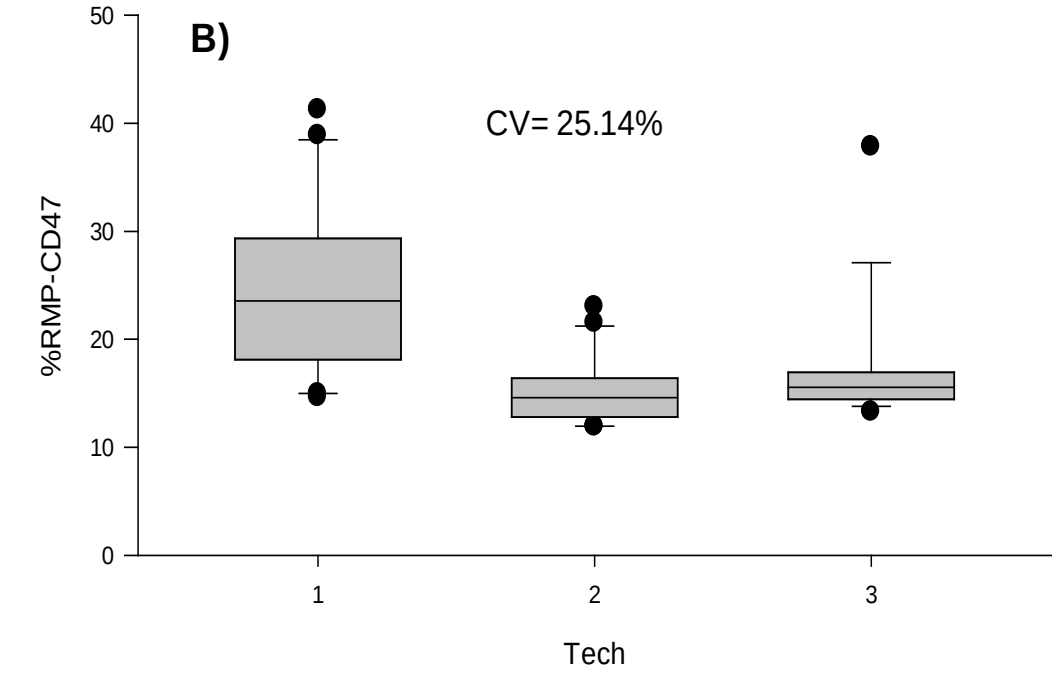
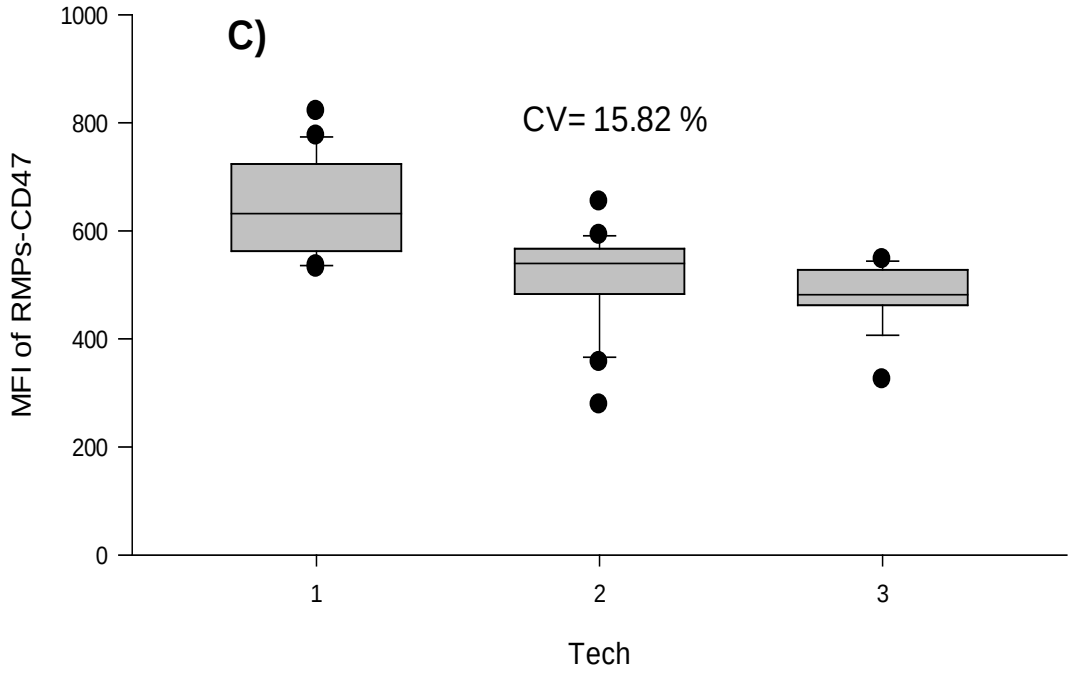


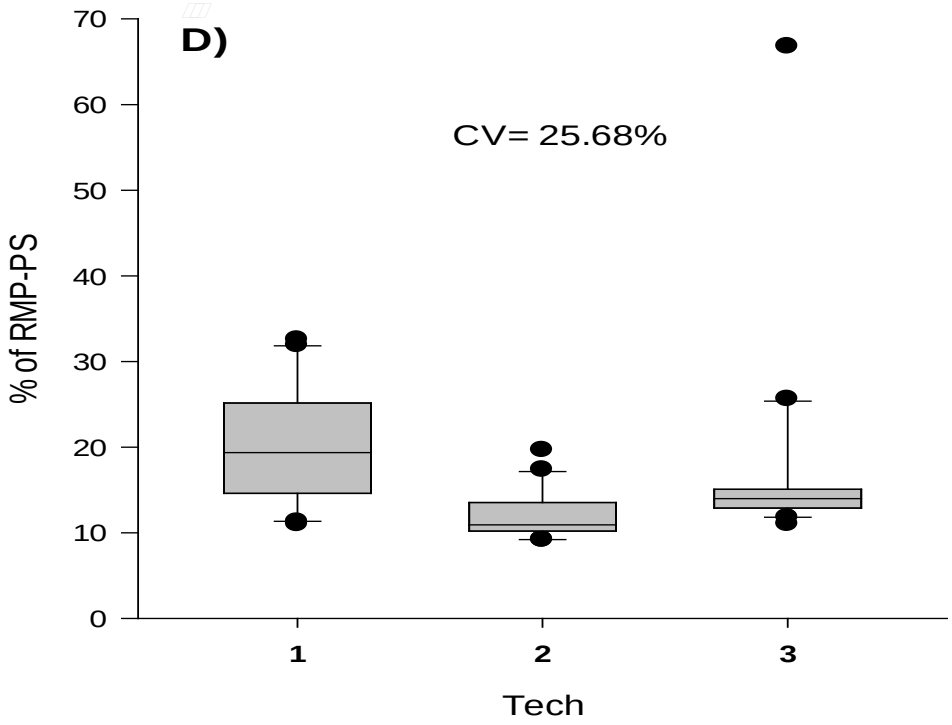
Figure 2.5: Precision –Inter run

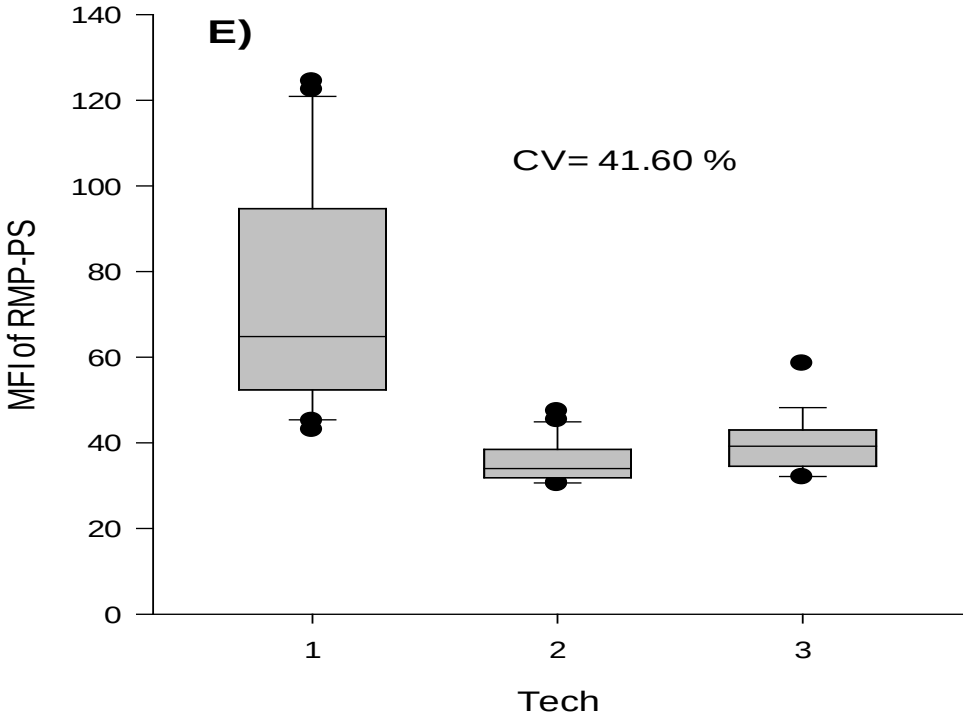
Box plots created by SigmaPlot 12.0. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. Asterisk indicates a significant value ($p<0.05$).

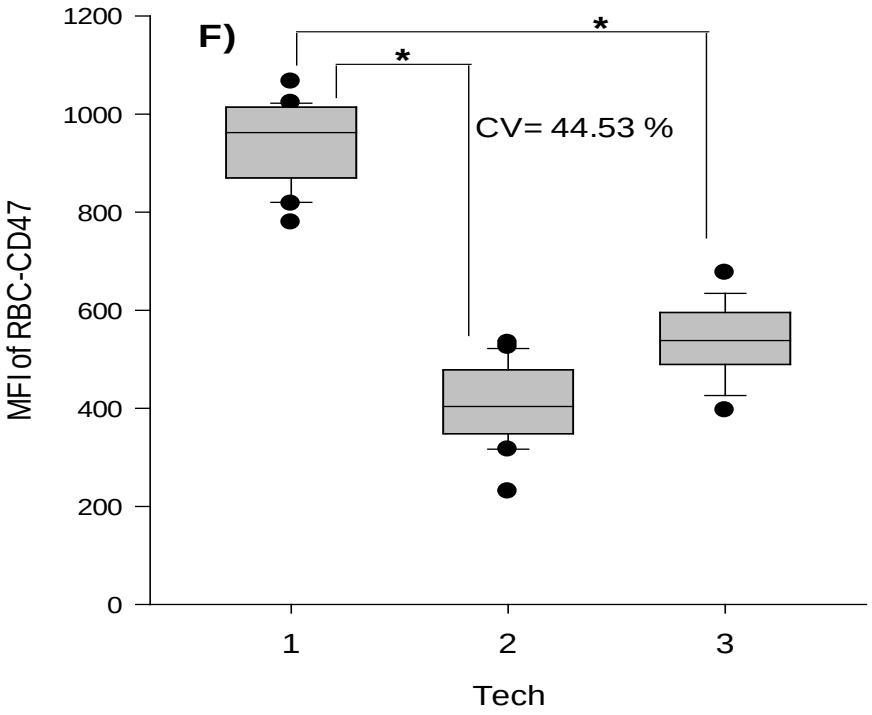


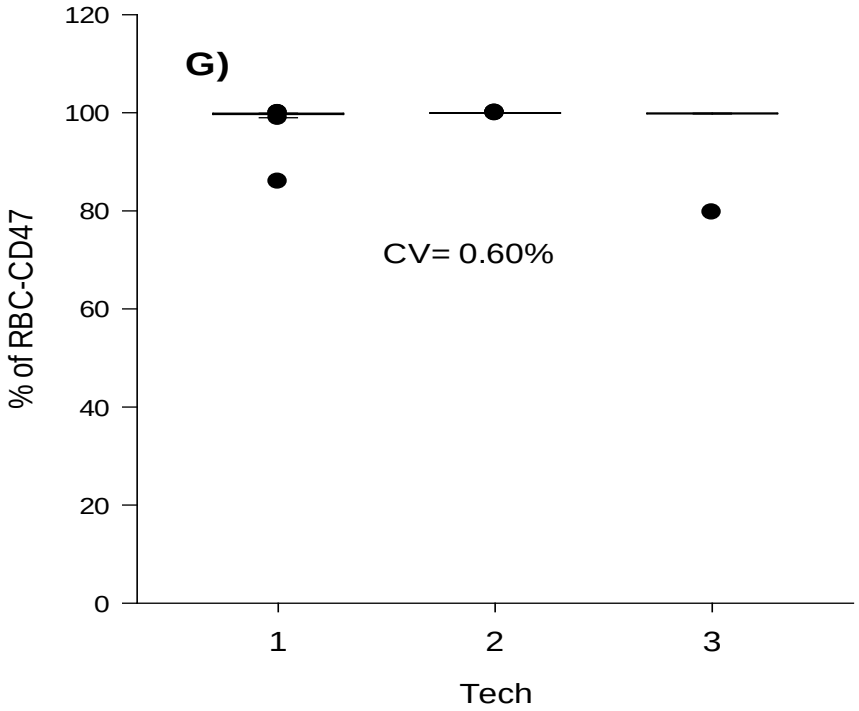

B)
CV= 25.14%
%RMP-CD47
50
40
30
20
10
0
1
2
3
Tech

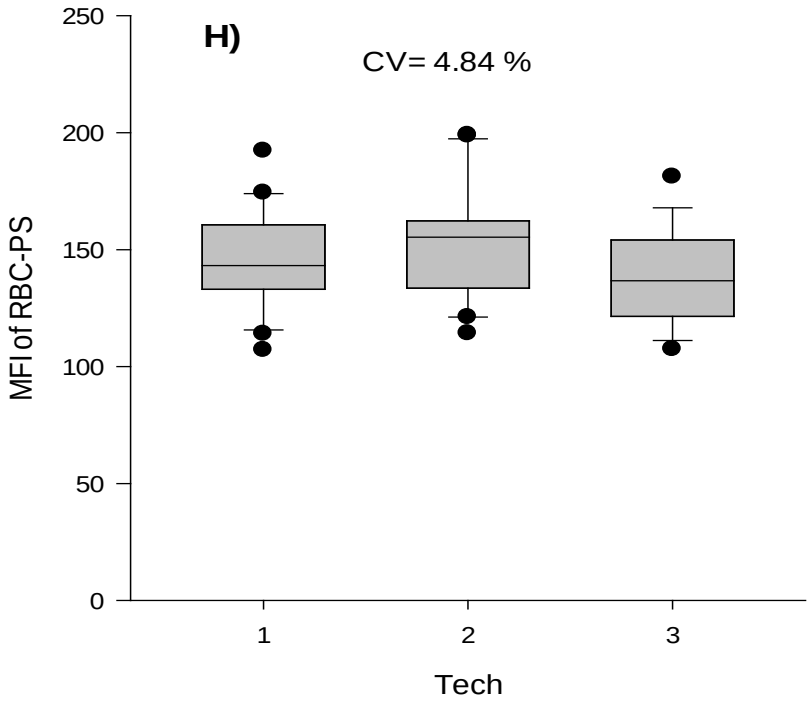

C)
CV= 15.82 %
MFI of RMPs-CD47
1000
800
600
400
200
0
1
2
3
Tech


D)
CV= 25.68%
% of RMP-PS
70
60
50
40
30
20
10
0
1
2
3
Tech


E)
CV= 41.60 %
MFI of RMP-PS
140
120
100
80
60
40
20
0
1
2
3
Tech


F)
*
*
CV= 44.53 %
MFI of RBC-CD47
1200
1000
800
600
400
200
0
1
2
3
Tech


G)
CV= 0.60%
% of RBC-CD47
120
100
80
60
40
20
0
1
2
3
Tech


H)
CV= 4.84 %
MFI of RBC-PS
250
200
150
100
50
0
1
2
3
Tech

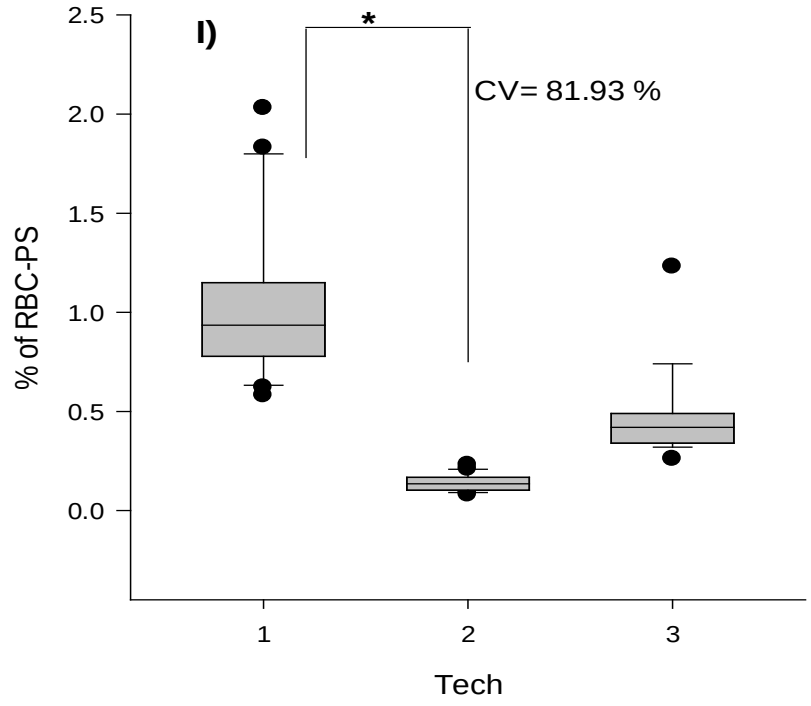
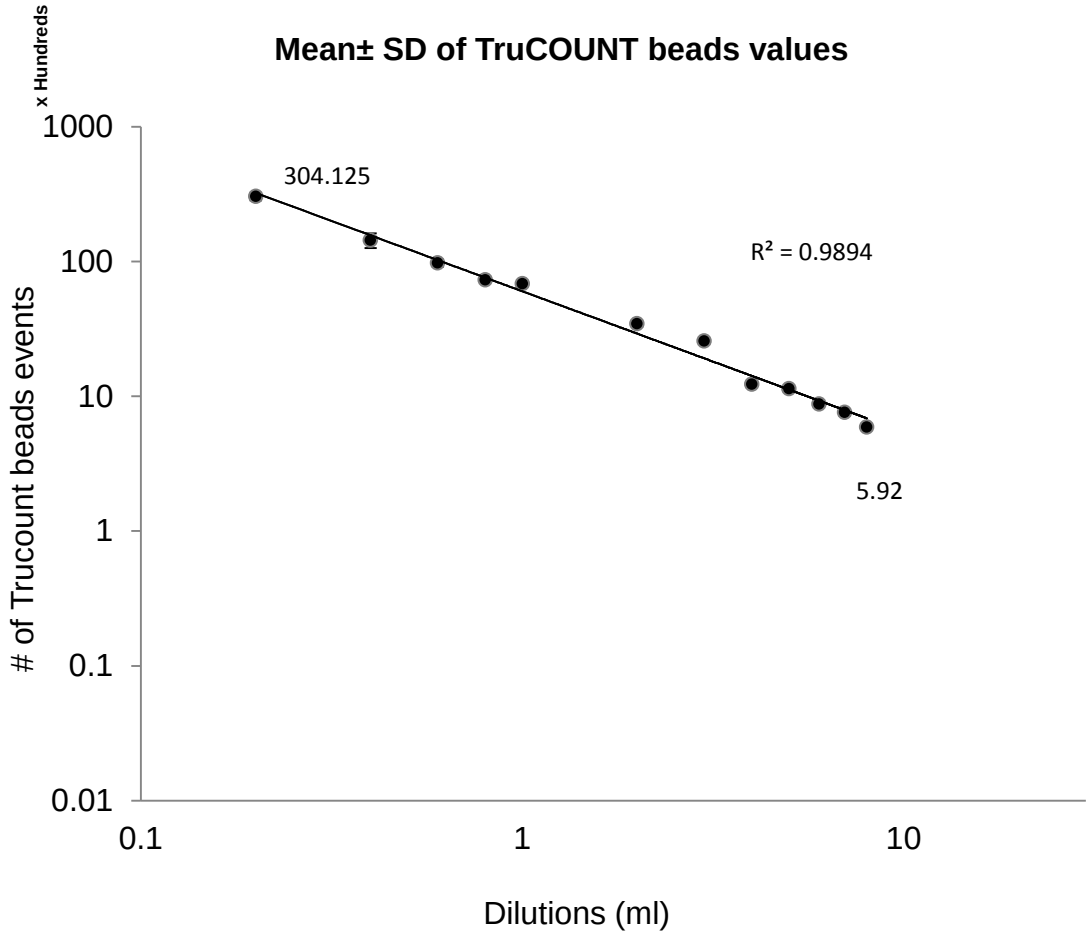
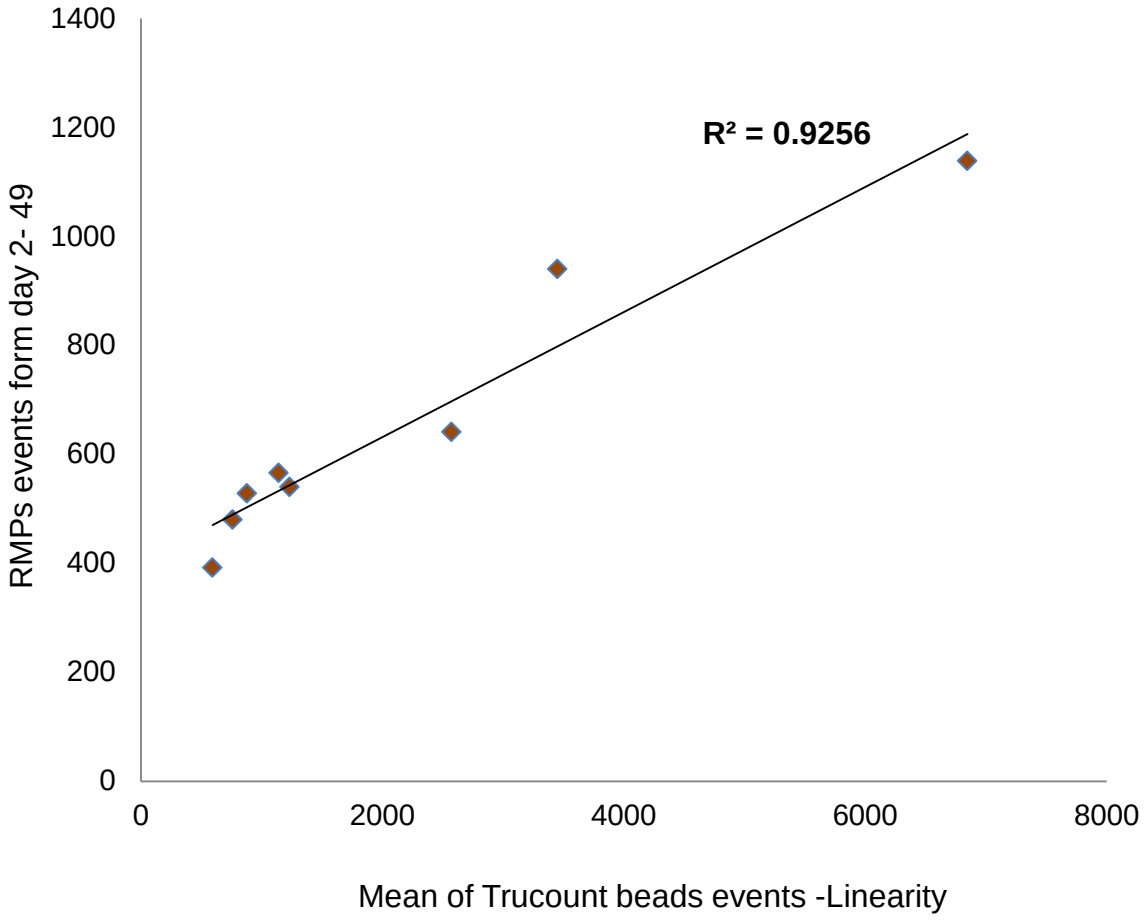

I)
*
CV= 81.93 %
% of RBC-PS
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
Tech

Figure 2.6: A series of concentrations or dilutions of TruCOUNT beads was prepared to determine the lower and the higher results for a test which led to linearity for the experiment.

A)



B)


1400
1200
1000
800
600
400
200
0
RMPs events form day 2- 49
R² = 0.9256
0
2000
4000
6000
8000
Mean of Trucount beads events -Linearity

Appendix 2.1: Flow Cytometry setting for: (A) unstained sample, isotype control and size beads; (B) stained sample with one or the three monoclonal fluorescent antibodies; (C) TruCOUNT beads samples.

A) Instrument setting for unstained sample, isotype control and size beads:

Cytometer Type: FACSCalibur
Detectors/Amps:

Parameters	Detector	Voltage	Amp	Mode
P1	FSC	E00	9.00	Log
P2	SSC	405	1.00	Log
P3	FL1	707	1.00	Log
P4	FL2	670	100	Log
P5	FL3	810	1.00	Log
P6	FL1-A		1.00	Lin
P7	FL4	720		Log

Threshold:
Primary Parameter: FSC
Value: 40
Secondary Parameter: SSC
Value: 100

Compensation:
FL1 – 1.0% FL2
FL2 – 12.1% FL1
FL2 – 0.0% FL3
FL3 – 0.0% FL2
FL3 – 0.0% FL4
FL4 – 0.0% FL3

B) Instrument setting for stained sample with one or three monoclonal fluorescent antibodies used to label RBCs and RMPs:

Cytometer Type: FACSCalibur
Detectors/Amps:

Parameters	Detector	Voltage	Amp	Mode
P1	FSC	E00	9.00	Log
P2	SSC	405	1.00	Log
P3	FL1	707	1.00	Log
P4	FL2	670	100	Log
P5	FL3	810	1.00	Log
P6	FL1-A		1.00	Lin
P7	FL4	720		Log

Threshold:

Primary Parameter: FSC
Value: 40
Secondary Parameter: FL-1
Value: 512

Compensation:
FL1 – 1.0% FL2
FL2 – 12.1% FL1
FL2 – 0.0% FL3
FL3 – 0.0% FL2
FL3 – 0.0% FL4
FL4 – 0.0% FL3

C) Instrument setting for TruCount beads sample:

Cytometer Type: FACSCalibur
Detectors/Amps:

Parameters	Detector	Voltage	Amp	Mode
P1	FSC	E00	9.00	Log
P2	SSC	405	1.00	Log
P3	FL1	505	1.00	Log
P4	FL2	490	100	Log
P5	FL3	810	1.00	Log
P6	FL1-A		1.00	Lin
P7	FL4	720		Log

Threshold:
Primary Parameter: FSC
Value: 40
Secondary Parameter: FL-1
Value: 512

Compensation:
FL1 – 1.0% FL2
FL2 – 12.1% FL1
FL2 – 0.0% FL3
FL3 – 0.0% FL2
FL3 – 0.0% FL4
FL4 – 0.0% FL3

2.5 References:

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

2. Herrmann A, Müller P. A model for the asymmetric lipid distribution in the human erythrocyte membrane. Biosci Rep 1986;**6**: 185-91.

3. Simak J, Gelderman MP. Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers. Transfus Med Rev 2006;**20**: 1-26.

4. Xiong Z, Oriss TB, Cavaretta JP, Rosengart MR, Lee JS. Red cell microparticle enumeration: validation of a flow cytometric approach. Vox Sang 2012;**103**: 42-8.

5. Canellini G, Rubin O, Delobel J, Crettaz D, Lion N, Tissot JD. Red blood cell microparticles and blood group antigens: an analysis by flow cytometry. Blood Transfus 2012;**10 Suppl 2**: s39-45.

6. van Beers EJ, Schaap MC, Berckmans RJ, Nieuwland R, Sturk A, van Doormaal FF, Meijers JC, Biemond BJ, group Cs. Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease. Haematologica 2009;**94**: 1513-9.

7. Chin-Yee I, Arya N, d'Almeida MS. The Red Cell Storage Lesion and its Implication for Transfusion. Transfus Sci 1997;**18**: 447-58.

8. Greenwalt TJ. The how and why of exocytic vesicles. Transfusion 2006;**46**: 143-52.

9. Holovati JL, Wong KA, Webster JM, Acker JP. The effects of cryopreservation on red blood cell microvesiculation, phosphatidylserine externalization, and CD47 expression. Transfusion 2008;**48**: 1658-68.

10. Willekens FLA, Werre JM, Groenen-Dop YAM, Roerdinkholder-Stoelwinder B, de Pauw B, Bosman GJCGM. Erythrocyte vesiculation: a self-protective mechanism? Brit J Haematol 2008;**141**: 549-56.

11. Kriebardis AG, Antonelou MH, Stamoulis KE, Economou-Petersen E, Margaritis LH, Papassideri IS. RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components. Transfusion 2008;**48**: 1943-53.

12. Lacroix R, Robert S, Poncelet P, Dignat-George F. Overcoming limitations of microparticle measurement by flow cytometry. Semin Thromb Hemost 2010;**36**: 807-18.

13. Orozco AF, Lewis DE. Flow cytometric analysis of circulating microparticles in plasma. Cytometry A 2010;**77**: 502-14.

14. Horstman LL, Ahn YS. Platelet microparticles: a wide-angle perspective. Crit Rev Oncol Hematol 1999;**30**: 111-42.

15. Spivak JL. Fundamentals of clinical hematology. Hagerstown, Md.: Harper & Row, 1980.

16. Nunez R. Flow Cytometry: principles and instrumentation. Curr Issu Mol Biol 2001;**3**: 39-45.

17. Jaroszeski MJ, Radcliff G. Fundamentals of flow cytometry. Mol Biotechnol 1999;**11**: 37-53.

18. Brown M, Wittwer C. Flow cytometry: principles and clinical applications in hematology. Clin Chem 2000;**46**: 1221-9.

19. Sparrow R, Healey G. Reduced expression of CD47 on stored red blood cells. Transfusion 2006;**46**: 1263.

20. Anniss AM, Sparrow RL. Expression of CD47 (integrin-associated protein) decreases on red blood cells during storage. Transfus Apher Sci 2002;**27**: 233-8.

21. Oldenborg P, Zheleznyak A, Fang Y, Lagenaur CF, Gresham HD, Lindenberg FP. Role of CD47 as a marker of self on red blood cells. Science 2000;**288**: 2051-4.

22. Ayers L, Kohler M, Harrison P, Sargent I, Dragovic R, Schaap M, Nieuwland R, Brooks SA, Ferry B. Measurement of circulating cell-derived microparticles by flow cytometry: Sources of variability within the assay. Thromb Res 2011;**127**: 370-7.

23. Dey-Hazra E, Hertel B, Kirsch T, Woywodt A, Lovric S, Haller H, Haubitz M, Erdbruegger U. Detection of circulating microparticles by flow cytometry: influence of centrifugation, filtration of buffer, and freezing. Vasc Health Risk Manag 2010;**6**: 1125-33.

24. Holovati JL, Acker JP. Emerging Role for Use of Liposomes in the Biopreservation of Red Blood Cells. Transfus Med Hemother 2011;**38**: 99-106.

25. Shet AS. Characterizing blood microparticles: technical aspects and challenges. Vasc Health Risk Manag 2008;**4**: 769-74.

26. Westgard JO, Barry PL. Basic method validation 10. How is the imprecision of a method determined? Madison, WI: Westgard QC, 2008:113-22.

27. Levin E, Culibrk B, Gyöngyössy-Issa MIC, Weiss S, Scammell K, LeFresne W, Jenkins C, Devine DV. Implementation of buffy coat platelet component production: comparison to platelet-rich plasma platelet production. Transfusion 2008;**48**: 2331-7.

28. Stewart A, Urbaniak S, Turner M, Bessos H. The application of a new quantitative assay for the monitoring of integrin-associated protein CD47 on red blood cells during storage and comparison with the expression of CD47 and phosphatidylserine with flow cytometry. Transfusion 2005;**45**: 1496-503.

29. Nantakomol D, Imwong M, Soontarawirat I, Kotjanya D, Khakhai C, Ohashi J, Nuchnoi P. The Absolute Counting of Red Cell-Derived Microparticles with Red Cell Bead by Flow Rate Based Assay. Cytom Part B-Clin Cy 2009;**76B**: 191-8.

30. Westgard JO, Barry PL. Basic method validation 4. What are the regulatory requirements for method validation? Madison, WI: Westgard QC, 2008:37-50.

31. FACS Calibur 4 Color Calibration using CaliBRITE Beads Protocol. Flow Cytometry Facility in Cross Cancer Institute. University of Alberta, Edmonton, Canada, 2009:1.

32. Westgard JO, Barry PL. Basic method validation 8. How are the statistics calculated? Madison, WI: Westgard QC, 2008:83-100.

33. Westgard JO, Barry PL. Basic method validation 18. How do you use statistics in the real world? Madison, WI: Westgard QC, 2008:211-20.

34. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev 2008;**29 Suppl 1**: S49-52.

35. Trummer A, De Rop C, Tiede A, Ganser A, Eisert R. Isotype controls in phenotyping and quantification of microparticles: A major source of error and how to evade it. Thromb Res 2008;**122**: 691-700.

36. Rubin O, Crettaz D, Canellini G, Tissot JD, Lion N. Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools. Vox Sang 2008;**95**: 288-97.

37. Rubin O, Delobel J, Prudent M, Lion N, Kohl K, Tucker EI, Tissot JD, Angelillo-Scherrer A. Red blood cell-derived microparticles isolated from blood units initiate and propagate thrombin generation. Transfusion 2012.

38. Wille SMR, Peters FT, Di Fazio V, Samyn N. Practical aspects concerning validation and quality control for forensic and clinical bioanalytical quantitative methods. Accredit Qual Assur 2011;**16**: 279-92.

39. Hartmann C, Massart DL, McDowall RD. An analysis of the Washington Conference Report on bioanalytical method validation. J Pharm Biomed Anal 1994;**12**: 1337-43.

40. Westgard JO, Barry PL. Basic method validation 3. What is the purpose of a method validation study? Madison, WI: Westgard QC, 2008:27-36.

41. Maecker H, Trotter J. Selecting Reagents for Multicolor Flow Cytometry. BD Application Note 2009.

42. Luther E, Kamentsky L, Henriksen M, Holden E. Next-Generation Laser Scanning Cytometry. In: Zbigniew Darzynkiewicz MR, Hans T, eds. Methods Cell Biol: Academic Press, 2004:185-218.

Chapter 3 *

Hypothermic Storage of Red Blood Cells Affects Membrane Composition, Microvesiculation, and In Vitro Quality

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*This chapter with modifications has been published as: Almizraq R, Tchir JDR, Holovati JL, Acker JP. Storage of red blood cells affects membrane composition, microvesiculation, and in vitro quality. Transfusion 2013. doi: 10.1111/trf.12080. [Epub ahead of print].

*ATP concentration and percent hemolysis were performed by Jayme Tchir. * Dr. Qilong did the statistical analysis of this study.

3.1 Introduction

Growing literature on the adverse effects of blood storage and the possible clinical implications for transfusion recipients has recently identified the quality of stored blood as "the most critical issue facing transfusion medicine."[1] At present, the most widely used approach for RBC biopreservation involves the collection of donor blood in anticoagulant solution, preparation of packed RBC concentrates by plasma removal, leukoreduction by filtration and storage at hypothermic temperatures (1-6 °C) in an additive solution. As mentioned in the first chapter, this approach is based on the principle that biochemical processes and molecular reactions can be suppressed by reducing the storage temperature to below physiological but above freezing, while maintaining additive supplementation with nutrient media. Although hypothermic storage (HS) minimizes RBC injury, cellular metabolism is not completely suppressed under these conditions, resulting in nutrient depletion and accumulation of cell waste [2,3]. All the detrimental biochemical and biomechanical effects of *in vitro* RBC HS can be summarized by the term "hypothermic storage lesion (HSL)".

One of the major structures affected by the HSL is the RBC membrane, the organization and integrity of which is critical for *in vivo* RBC circulation and function. The RBC membrane is a lipid bilayer consisting of asymmetrically-distributed phospholipids, cholesterol and proteins. Changes throughout HS have the ability to affect membrane composition and integrity, which are both integral

to the maintenance of RBC function**.** Compositional membrane changes can affect membrane fluidity, lipid disorder, bilayer thickness, and the packing efficiency of membrane components, factors which have the potential to affect RBC deformability. Compositional changes may also lead to changes in passive membrane permeability, which may affect the RBCs ability to respond to different osmotic stresses [4-6]. Membrane loss leads to critical, irreversible changes in shape and surface area:volume ratio, which affect the gross morphology of RBCs, and have serious implications on RBC deformability [7].

Even though the effects of microvesiculation are discussed in the previous chapters, it is important to emphasize that the harmful effects of microvesiculation is not referring to the formation of the microvesicles but to the subsequent consequences such as the loss of the membrane integrity, cell quality, viability and fluidity as a reflection of the changes in surface to volume ratio, and changes in the phospholipid to protein ratio [8-10].

In addition to membrane loss, *in vitro* metabolic and mechanical stresses can lead to disruption of the asymmetric phospholipid distribution of the cell membrane, resulting in PS externalization, which then acts as a signal for erythrophagocytosis by macrophages of the reticulo-endothelial system [11-15]. Furthermore, PS exposure has been shown to enable RBCs to participate in blood coagulation and cell adhesion [16,17]. Another signal for erythrophagocytosis is thought to be decreased expression of CD47 antigen. Since CD47 in normal RBCs activates signal regulatory protein-a, which leads to the inhibition of

macrophage activation for phagocytosis, injured and/or senescent RBCs lacking CD47 are quickly recognized and removed from circulation [12-14,18,19]. In addition to promoting RBC clearance, this type of RBC membrane lesion is also believed to initiate shedding of membrane microparticles, which are thought to result from a transient overload of the outer membrane leaflet at the expense of the inner one [20-24]. Although long considered innocuous cell debris, membrane microparticles have more recently been shown to have physiologic and pathophysiologic significance by playing a role in inflammation, hemostasis, and vascular (dys-) function [23,25,26]. Although the exact nature of PS externalization, decreased CD47 expression and membrane microvesiculation in relation to the quality and safety of RBC concentrates is not well understood, recent studies have identified these markers to be potentially significant determinants of *in vivo* RBC survival and function, and these may also act as harmful immunomodulators [12-14,19,23,24,26-28].

In light of the important clinical implications of deteriorating quality of stored RBCs, the objective of this study was to quantitatively and qualitatively clarify the effect of HS on the integrity of RBC membrane and other *in vitro* quality parameters. The focus was on both the compositional changes within the RBC membrane throughout HS, and the temporal effects of RBC quality during HS measured using *in vitro* quality assays.

3.2 Materials and Methods

3.2.1 Sample Collection

Ethics approval was granted by both Canadian Blood Services (CBS) and the University of Alberta Research Ethics Boards prior to initiation of the study. Leukoreduced CPD-SAGM RBC units (n=30) were obtained from two Canadian Blood Services Centres: Edmonton, Alberta and the Network Centre for Applied Development (netCAD), Vancouver, British Columbia. Whole blood (450 ± 50mL) units were collected using standard blood bank phlebotomy procedures and processed using the standard buffy coat method [29]. All units were rapidly cooled to room temperature and held for up to 24 hours. Within the 24 hours, the units were centrifuged in order to obtain the RBCs; buffy coat and platelet poor plasma layers were separated and divided into 2 satellite bags using the top and bottom method [29]. After separation, SAGM (v=110 mL) was added to each of the RBC units, which was then leukoreduced by filtration and shipped to Edmonton. All units were received 2 days after collection and placed in hypothermic storage at 1 to 6 °C for up to 49 days. At each testing point (day 2, day 7, day 14, day 21, day 28, day 35, day 42, and day 49) units were thoroughly mixed and 5 mL of RBCs were aseptically drawn from the bag, and used for RBC *in vitro* quality assays.

3.2.2 RBC *In Vitro* Quality Assays

All packed RBC units were assessed for percent hemolysis, ATP concentration, microvesiculation, as well as PS and CD47 expression. RBC microvesiculation, PS and CD47 expression was assessed by flow cytometry which is described in section 2.2.2 of chapter 2.

3.2.2.1 Percent Hemolysis

Percent hemolysis was determined by comparing the supernatant hemoglobin (Hb_S) to the total hemoglobin (Hb_T) concentrations of a sample using a Drabkin's-based method [30] . A small volume of the RBCs were diluted (1:25 for Hb_S and 1:200 for Hb_T) in Drabkin's reagent (0.61 mM potassium ferricyanide, 0.77 mM potassium cyanide, 1.03 mM potassium dihydrogen phosphate, and 0.1 % Triton X-100). As a result, most forms of hemoglobin convert to cyanmethhemoglobin (HiCN). Consequently, a two-step reaction takes place converting hemoglobin to methemoglobin. Methemoglobin then reacts with the cyanide to form HiCN [31]. HiCN absorbs light maximally at 540 nm with the measured absorbance being directly proportional to the hemoglobin concentrations present in the sample. The resultant hemoglobin concentration can be calculated directly for the absorbance of the solution using the following equation [32]:

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

Where:

c = concentration of hemoglobin (g/L), A_{540} = absorbance of the solution at 540 nm, M = molecular mass of hemoglobin monomer (16114.5 mg/mmol), F = dilution factor, ε_{540}= millimolar absorptivity of HiCN at 540 nm (11.0 $cm^{-1} \cdot mM^{-1}$), and l = light path (cm)

Once supernatant and total hemoglobin values have been determined, the % hemolysis is calculated from the following equation [33]:

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

Where:

Hct = hematocrit (%), Hb_S = supernatant hemoglobin concentration (g/L), Hb_T = total hemoglobin concentration (g/L)

Percent hemolysis was calculated as a ratio of the supernatant hemoglobin to the total hemoglobin, with the Hct being used to account for the volume of the supernatant in the sample [33].

3.2.2.2 ATP Concentration

ATP concentrations were determined enzymatically using a commercially available kit and controls (DiaSys Diagnostic Systems GmbH). The RBC samples were added to 10% trichloroacetic acid, vortexed and placed on ice. The supernatants were then combined with substrates (glucose, and NAD^+) and enzymes (hexokinase and glucose-6-phosphate dehydrogenase) required for the enzymatic reaction to occur. The following chemical equation summarized the chemical reactions occurring during preparation of the samples:

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

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

The resulting amount of NADH produced, which is proportional to the amount of ATP within the sample, was measured spectrophotometrically @ 340 nm. The

amount of ATP in the sample was calculated as µmol/dL using the following equation [34]:

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

Where:

ΔA= (absorbance of the samples solution at 340 nm)-(absorbance of the blank at 340 nm), V= total volume of the sample, F=dilution factor of the sample preparation, ε= extinction coefficient of NADH (6.3@340 nm), v =sample volume used in ATP assay, d= light path (cm)

The concentration of ATP is further normalized using the total hemoglobin concentration (µmol/g Hgb) as the following [34]:

$$C\ (\mu mol/g\ Hb) = \frac{10^{*}\ C\ (\mu mol/dL)}{Hb\ (g/L)} \qquad \text{Equation 3.6}$$

3.2.3 Statistical analyses

Statistical analysis was completed using SPSS 12.0 Software (Lead Technologies, Charlotte, NC). ANOVA analyses were used to identify significant differences within the HS period for the individual assays, followed by a Scheffe

Post-hoc test, to identify significance among pair-wise comparisons of testing time points throughout the storage period. Probabilities less than 0.05 were considered significant.

3.3 Results

3.3.1 RBC *In Vitro* Quality Assays

The amount of ATP in RBCs stored hypothermically for up to 49 days is shown in Figure 3.1 A., During early storage, the ATP concentration remained relatively stable (day 2 and day 7), with a slight increase at day 7. Following this, the ATP concentration began to decline. Overall, the ATP concentration decreased significantly throughout storage ($p<0.001$), with significant decreases in ATP in comparison to day 2 identified at day 28 ($p<0.001$) and all subsequent testing points ($p<0.001$).

Hemolysis remained relatively stable during early storage (day 2 and day 7, Figure 3.1 B), prior to increasing at each subsequent testing point. Overall, hemolysis increased significantly throughout HS ($p<0.001$). Significant increases were identified at day 35 ($p=0.023$) and throughout the remaining HS testing period (day 42 and day 49, $p<0.001$), in comparison to day 2 and day 7 hemolysis values. Although significant changes in percent hemolysis were identified

throughout HS, all of the samples tested fell within the Canadian Standards Association (CSA) criteria for acceptable hemolysis of <0.8%.

3.3.2 Flow Cytometry

Flow cytometric analysis showed that the number of RMPs/µL gradually increased during the HS period (Figure 3.2). At the end of storage period (day 42 and 49), the increase in RMPs/µL became statistically significant, in comparison to the amount in RBC units at day 2 of HS ($p<0.05$). Further analysis of RMPs showed statistically significant decreases in the mean fluorescence intensities (MFI) for the expression of both PS and CD47 after day 28 of HS (Figure 3.3 A and 3.3 B, respectively $p<0.05$). Although there were no statistically significant changes in the % of RBCs expressing PS throughout HS (Figure 3.4 A), a significant decrease in the MFI of the PS on the stored RBCs was observed on day 35, 42 and 49 of HS ($p<0.05$). In contrast to RMPs, RBCs did not show statistically significant changes in the expression of CD47 marker throughout the HS (Figure 3.4 B).

3.4 Discussion

In this study, the *in vitro* quality of RBCs throughout HS was measured using classical assays like percent hemolysis, while more detailed assessments of

RBC metabolism and quality, including ATP and microvesiculation were also used. Significant changes occurred in many of the RBC *in vitro* quality measures throughout 49 days of HS, suggesting a significant decline in RBC quality during HS in the current additive solution, SAGM.

From current blood banking standards and Canadian Blood Services quality control perspectives, all of the tested RBC units were suitable for transfusion. Throughout the 42 days of storage, the current outdate for RBCs stored in SAGM in Canada, the percent hemolysis for the tested units was less than 0.8% in 100% of tested units, with the highest average percent hemolysis being identified at day 49 (0.35 ± 0.12 %). However, these minimum quality control standards for hemolysis do not take into account the significant changes that occurred in many of the other RBC *in vitro* quality assays. For instance, the ATP concentrations for stored RBCs significantly declined during storage to a concentration of 1.92 ± 0.56 µmol/g Hgb at day 42. ATP concentrations fell below the minimum recommended range of 2.3-2.7 µmol/g Hgb, which has been shown to correlate with 75% of transfused RBCs remaining in circulation 24 hours post-transfusion [35,36,37]. According to this criterion, the majority of our units would have met the target ATP criteria up until day 21 of storage, after which a significant decrease in ATP was recorded.

Our study has shown that changes in RBC membrane throughout storage have the potential to influence many other *in vitro* quality parameters. There was a strong correlation identified between the ATP concentration and number of

RMPs (R^2=0.49) throughout storage (Figure 3.5). Data from this study support the work of others that show the negative correlation between the ATP concentration and RMP generation during storage, with the greatest number of RMPs observed at the end of the storage period when the ATP concentration was at its lowest [26,38]. These findings showed that the ATP depletion during storage modified the ability of RBC membrane to release microparticles.

Flow cytometric analysis showed that the number of RMPs/µL gradually increased during the storage period. Recent studies suggest that changes during storage including increased osmotic fragility, reduced ATP concentration, and deformability contribute to the formation of the RMPs [26,39-41]. In addition, it has been shown that changes in RBC morphology during storage are associated with RBC fragmentation and microvesiculation [42]. *In vivo,* it has been demonstrated that microparticles generated by RBCs during storage scavenge the vasodilator, nitric oxide (NO), from blood vessels faster than intact RBCs [43]. This type of scavenging increases the consumption of the NO which leads to reduction in its bioavailability and the vasodilator activity [44]. This may cause reduced blood flow, tonic vasodilation, and decreased tissue oxygenation post-transfusion [39,43,45]. In addition, RBC membrane loss and microparticle formation are probable causes for pro-coagulant, pro-inflammatory and thrombogenic effects [45,46]; whereas RMPs released during storage have been shown to interact with the recipient's platelets post-transfusion, leading to chemokine release and stimulation of an inflammatory response [45].

Flow cytometry analysis also showed that there was no statistically significant change in the expression of the RBC-CD47 during the storage period. It is well known that RBCs lacking CD47 antigen on their surface are rapidly cleared from the circulation [47,13]. It has been suggested that the RBCs are taken up by macrophages when the expression of CD47 decreases to less than 50% [48]. However, our results contrast with other studies which reported a significant reduction in of RBC-CD47 during the storage period [13]. This contrast suggests that storage with CPD-SAGM solution is more likely to maintain the RBC-CD47 expression during storage time (over 98%, Figure 3.4 B) which may further suggest that the stored CPD-SAGM RBCs are of a superior quality and could be expected to circulate and survive longer post-transfusion.

The RBC-PS data presented in this chapter show that there was no statistically significant changes in the percentage of RBC expressing PS while a significant decline in the MFI of RBC-PS was detected after day 28 of storage. As previously mentioned in the introduction and in the chapter 1, loss of the asymmetric phospholipid distribution of the plasma membrane leads to PS externalization which is an effective removal indicator that plays an important role in the clearance for the damaged or senescent cells and their fragments from the circulation [2,14,40,49]. It has been shown that PS expression on the RBC increase significantly by the end of storage time and potentially contribute in the storage lesion [14]. In contrast, our flow cytometry results show that there was a decrease in the MFI-PS on the stored RBC throughout the storage time (Figure

4B). The decrease in the MFI of RBC-PS become statistically significant on day 35 of hypothermic storage and remained significantly reduced for rest of storage duration. Taking into consideration the significant increase in the number of RMPs during storage, this data strongly support other studies which indicate that most lipids are lost during storage through microvesiculation [50]. Therefore, while there was a significant increase in the number of RMP by the end of storage time, a significant decrease in the MFI of PS on RBC was observed (Figure 3.2 and 3.4 A). The phospholipid loss, microvesicles generation, and membrane lipid disorganization during *ex vivo* storage is consistent with similar alterations occurring *in vivo* during cell ageing [51].

RBC microvesicles carry the same antigens on their surface as their cells of origin and have similar recognition, phagocytosis and injury patterns as the old erythrocytes [40,52,53]. Flow cytometry was also used for further characterizations of the surface of RMPs which was performed by analysing the expression of PS and CD47 (Figure 3 A and 3 B). Even though there were similar amounts of PS florescence expression on RBCs and RMPs throughout the storage time, over 30% of RMPs expressed PS on their surface while less than 1% of RBCs expressed this removal signal. This data support the theory that RBCs use microvesiculation to get rid of particularly harmful components or markers from their surfaces, such as PS and denatured hemoglobin, in order to prevent them being removed from the circulation by macrophages [46,52,54,55]. In addition, a significant decline in the expression of CD47 on RMPs was observed on day 28,

35, and day 42 of storage (Figure 3.3 B). Furthermore, the MFI of CD47 on RMPs significantly decreases from 1560.4 ± 409.4 on day 2 to 697.5 ± 287.6 on day 42 and (580.3 ± 269.6) on day 49 of storage. The high percentage of RMPs that expose PS with significant decline in the CD47 expression could be an additional important cause for the rapid removal of these particles from the circulation post-transfusion.

3.5 Conclusion:

In this study, we were able to characterize and quantitate membrane changes occurring throughout HS. The concern may not be the actual RBC membrane changes themselves during HS, but the effect that increasing concentrations of microvesicles and continuous RBC membrane loss throughout HS might have on transfusion outcomes. It is important to note that the membrane changes observed in this study are specific to the manufacturing method and additive solutions used by Canadian Blood Services. There is growing evidence to suggest that the manufacturing and storage system used in blood component preparation has a significant impact on pre-hemolytic injury to RBC. Future preservative technologies need to address the deteriorating condition of the membrane with the ultimate goal of stabilizing the membrane and decreasing microvesiculation throughout HS.

Table 3.1: **ATP and hemolysis for RBCs stored for up to 49 days**

	Length of Storage						
	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49
Hemolysis (%)	0.10 ± 0.03	0.11 ± 0.02	0.13 ± 0.02	0.14 ± 0.03*	0.17 ± 0.03*	0.23 ± 0.06*	0.35 ± 0.12*
ATP (µmol/g hgb)	4.48 ± 0.51	4.10 ± 0.57	3.70 ± 0.59	3.16 ± 0.46	2.58 ± 0.62**	1.92 ± 0.56**	1.73 ± 0.60**
Ratio ATP Day 1 / Day 7 (%)	100	90 ± 1	79 ± 1	70 ± 1	55 ± 11	42 ±11	37 ± 11

Mean ± 1SD

* ($p < 0.05$) in comparison to day 2 mean percentage hemolysis values
** ($p < 0.05$) in comparison to day 2 and 7 mean ATP values

Figure 3.1: A. ATP values for RBCs stored hypothermically for up to 49 days. The asterisk indicates statistically significant results ($p<0.005$) in comparison to day 2 HS values. **B.** Percentage hemolysis values for RBCs stored hypothermically for up to 49 days. The asterisk indicate statistically significant results ($p<0.05$) in comparison to day 2 and day 7 values.

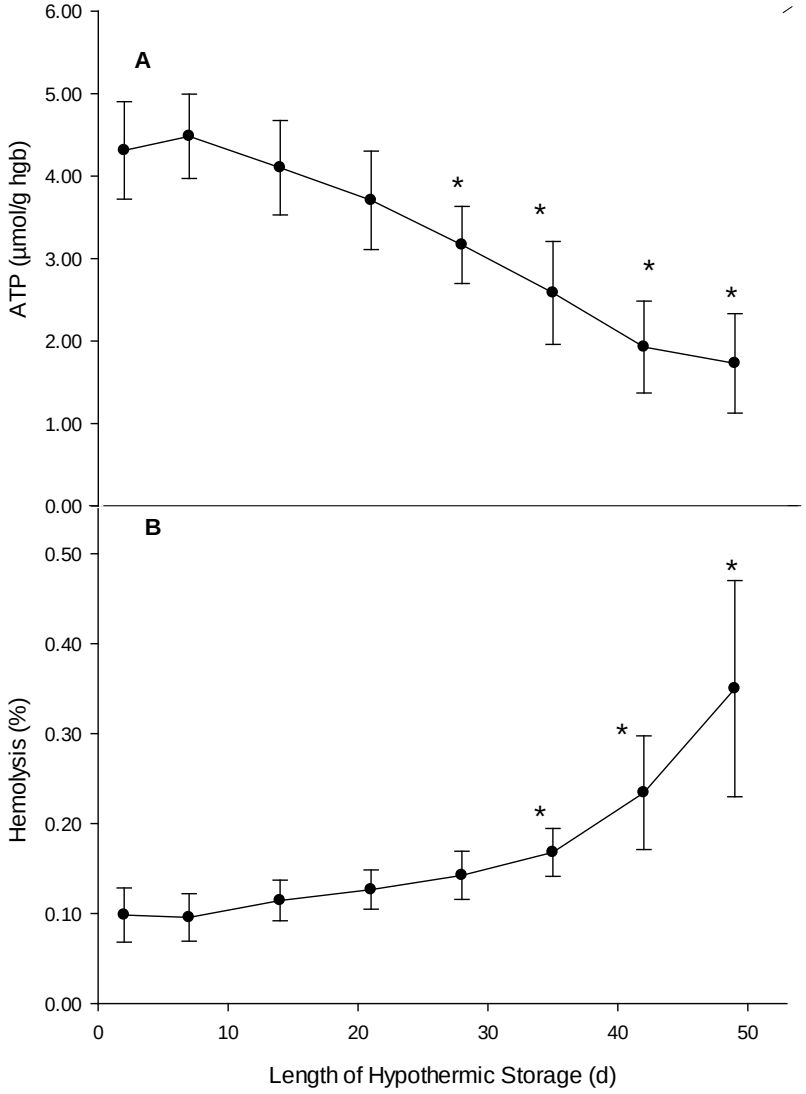


Figure 3.2: Number of red blood cell microparticles per microliter (RMPs/µL) during the hypothermic storage for up to 49 days (n=10). The values are expressed as mean number of RMPs/µL ± 1 SD. Absolute numbers of RMPs/µL were calculated as it mentioned in chapter 2 section 2.2.2. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.

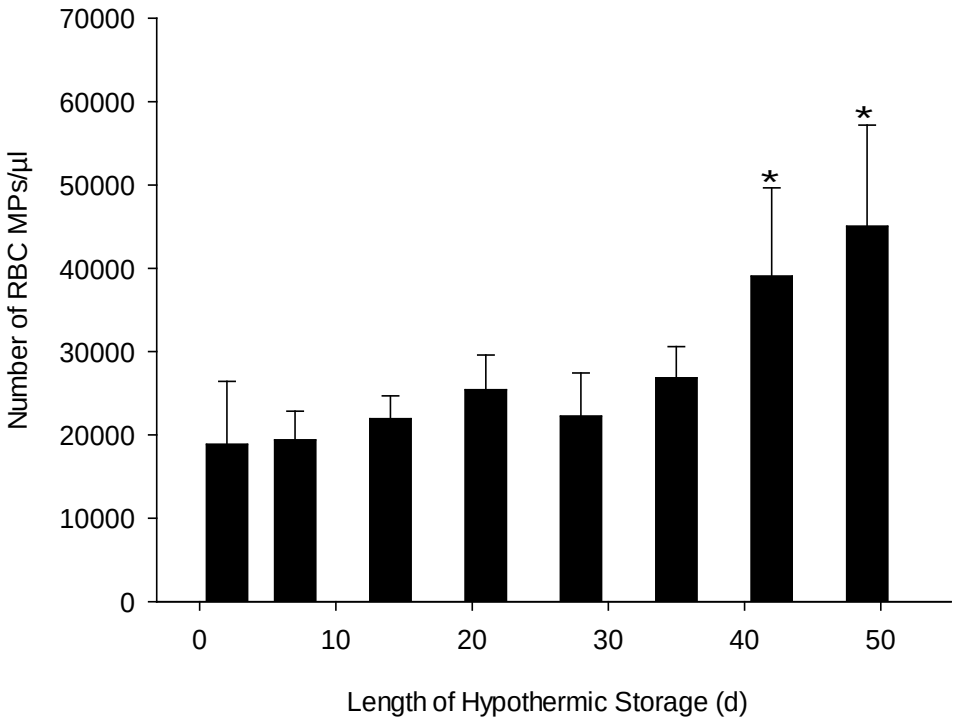
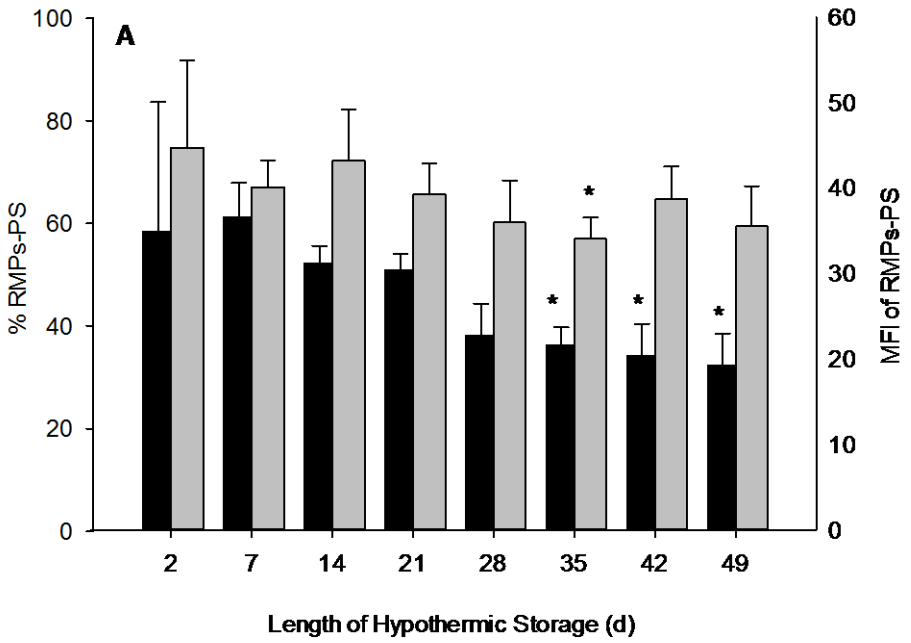


Figure 3.3: Percent (■) and mean fluorescence intensities (▭MFI) of the expression of PS (A) and CD47 (B) on RMPs during the hypothermic storage for up to 49 days. The values are expressed as mean ± 1 SD. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.



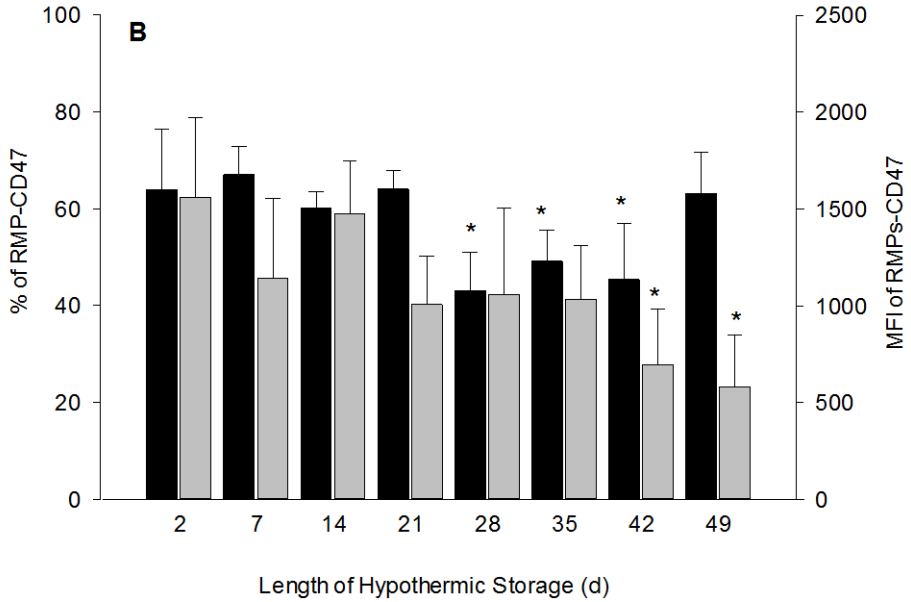
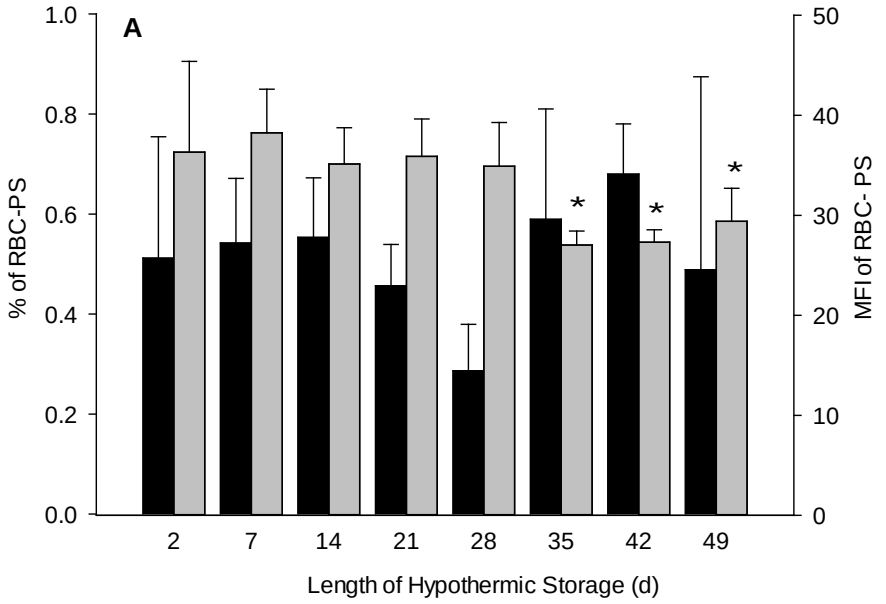


Figure 3.4: Percentage (■) and mean fluorescence intensities (▭MFI) of PS and CD47 expression on RBC during hypothermic storage for up to 49 days. The values are expressed as mean ± 1 SD. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.



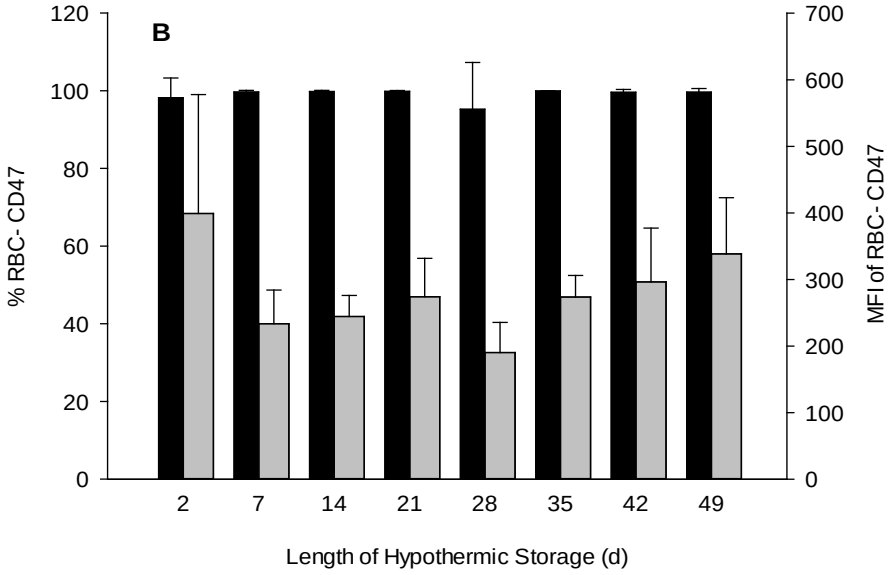
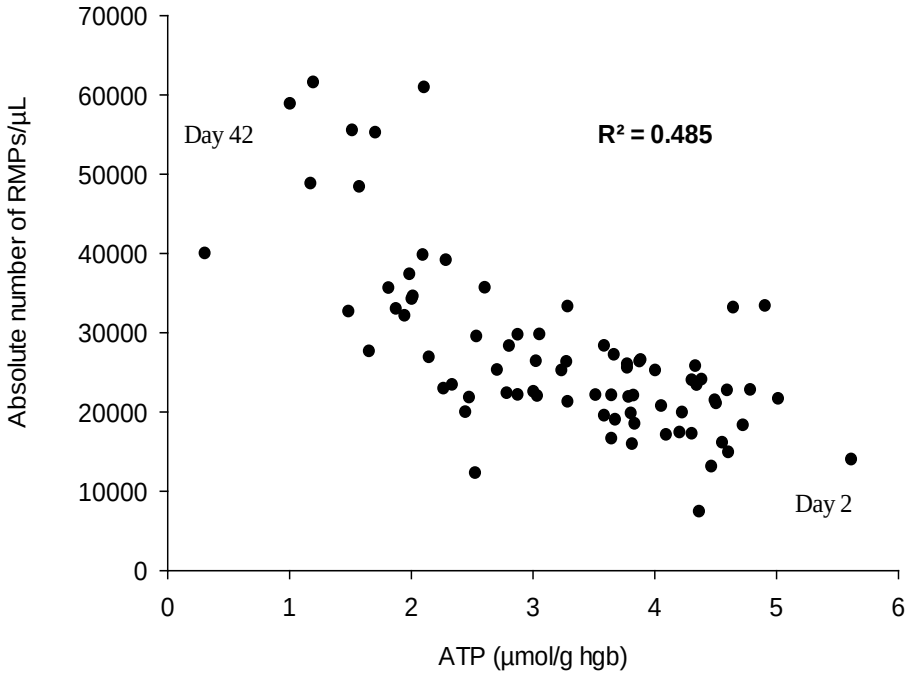


Figure 3.5: A scatter plot comparison of the RBC ATP concentration (µmol/g Hgb) and the number of RMPs/µL during storage for up to 42 days (n=10). The R^2 value, which provides a measure of goodness-of-fit of linear regression, indicates a strong negative correlation between the measured ATP concentration (µmol/g Hgb) and number of RMPs/ µL.



3.6 References:

1. Ness PM. Does transfusion of stored red blood cells cause clinically important adverse effects? A critical question in search of an answer and a plan. Transfusion 2011;**51**: 666-7.

2. Bosman GJ, Cluitmans JC, Groenen YA, Werre JM, Willekens FL, Novotny VM. Susceptibility to hyperosmotic stress-induced phosphatidylserine exposure increases during red blood cell storage. Transfusion 2011;**51**: 1072-8.

3. Bratosin D, Leszczynski S, Sartiaux C, Fontaine O, Descamps J, Huart JJ, Poplineau J, Goudaliez F, Aminoff D, Montreuil J. Improved storage of erythrocytes by prior leukodepletion: flow cytometric evaluation of stored erythrocytes. Cytometry 2001;**46**: 351-6.

4. Stoll C, Stadnick H, Kollas O, Holovati JL, Glasmacher B, Acker JP, Wolkers WF. Liposomes alter thermal phase behavior and composition of red blood cell membranes. Biochim Biophys Acta 2011;**1808**: 474-81.

5. Cooper RA. Influence of Increased Membrane Cholesterol on Membrane Fluidity and Cell-Function in Human Red Blood-Cells. J Supramol Str Cell 1978;**8**: 413-30.

6. Wolkers WF, Crowe LM, Tsvetkova NM, Tablin F, Crowe JH. In situ assessment of erythrocyte membrane properties during cold storage. Mol Membr Biol 2002;**19**: 59-65.

7. Gov N, Cluitmans J, Sens P, Bosman GJCGM. Chapter 4 Cytoskeletal Control of Red Blood Cell Shape: Theory and Practice of Vesicle Formation. In: Liu AL, Aleš I, eds. Advances in Planar Lipid Bilayers and Liposomes: Academic Press, 2009:95-119.

8. Wagner GM, Chiu DT, Yee MC, Lubin BH. Red cell vesiculation--a common membrane physiologic event. J Lab Clin Med 1986;**108**: 315-24.

9. Butikofer P, Lin ZW, Kuypers FA, Scott MD, Xu CM, Wagner GM, Chiu DT, Lubin B. Chlorpromazine inhibits vesiculation, alters phosphoinositide turnover and changes deformability of ATP-depleted RBCs. Blood 1989;**73**: 1699-704.

10. Dumaswala UJ, Dumaswala RU, Levin DS, Greenwalt TJ. Improved red blood cell preservation correlates with decreased loss of bands 3, 4.1, acetylcholinestrase, and lipids in microvesicles. Blood 1996;**87**: 1612-6.

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

12. Anniss AM, Sparrow RL. Expression of CD47 (integrin-associated protein) decreases on red blood cells during storage. Transfus Apher Sci 2002;**27**: 233-8.

13. Stewart A, Urbaniak S, Turner M, Bessos H. The application of a new quantitative assay for the monitoring of integrin-associated protein CD47 on red blood cells during storage and comparison with the expression of CD47 and phosphatidylserine with flow cytometry. Transfusion 2005;**45**: 1496-503.

14. Bessos H, Seghatchian J. Red cell storage lesion: The potential impact of storage-induced CD47 decline on immunomodulation and the survival of leucofiltered red cells. Transfus Apher Sci 2005;**32**: 227-32.

15. Holme S. Current issues related to the quality of stored RBCs. Transfus Apher Sci 2005;**33**: 55-61.

16. Chung S-M, Bae O-N, Lim K-M, Noh J-Y, Lee M-Y, Jung Y-S, Chung J-H. Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. Arterioscler Thromb Vasc Biol 2007;**27**: 414-21.

17. Andrews DA, Low PS. Role of red blood cells in thrombosis. Curr Opin Hematol 1999;**6**: 76-82.

18. Oldenborg P, Zheleznyak A, Fang Y, Lagenaur CF, Gresham HD, Lindenberg FP. Role of CD47 as a marker of self on red blood cells. Science 2000;**288**: 2051-4.

19. Sparrow R, Healey G. Reduced expression of CD47 on stored red blood cells. Transfusion 2006;**46**: 1263.

20. Ahn YS, Jy W, Jimenez JJ, Horstman LL. More on: cellular microparticles: what are they bad or good for? J Thromb Haemost. 2004;**2**: 1215-6.

21. Schlegel RA, Williamson P. Phosphatidylserine, a death knell. Cell Death Differ 2001;**8**: 551-63.

22. Kuypers FA, Lewis RA, Hua M, Schott MA, Discher D, Ernst JD, Lubin BH. Detection of altered membrane phospholipid asymmetry in subpopulations of human red blood cells using fluorescently labeled annexin V. Blood 1996;**87**: 1179-87.

23. Seghatchian J, Krailadsiri P. Red cell storage lesion assessed by the levels of potassium, haemoglobin and Annexin V in supernatants. Transfus Apher Sci 2002;**26**: 139-43.

24. Freyssinet JM. Cellular microparticles: what are they bad or good for? J Thromb Haemost 2003;**1**: 1655-62.

25. VanWijk MJ, VanBavel E, Sturk A, Nieuwland R. Microparticles in cardiovascular diseases. Cardiovasc Res 2003;**59**: 277-87.

26. Greenwalt TJ. The how and why of exocytic vesicles. Transfusion 2006;**46**: 143-52.

27. Head DJ, Lee ZE, Swallah MM, Avent ND. Ligation of CD47 mediates phosphatidylserine expression on erythrocytes and a concomitant loss of viability in vitro. Brit J Haematol 2005;**130**: 788-90.

28. Chin-Yee I, Arya N, d'Almeida MS. The Red Cell Storage Lesion and its Implication for Transfusion. Transfus Sci 1997;**18**: 447-58.

29. Levin E, Culibrk B, Gyöngyössy-Issa MIC, Weiss S, Scammell K, LeFresne W, Jenkins C, Devine DV. Implementation of buffy coat platelet component production: comparison to platelet-rich plasma platelet production. Transfusion 2008;**48**: 2331-7.

30. Drabkin DL. The standardization of hemoglobin measurement. Am J Med Sci 1949;**217**: 710.

31. Zijlstra WG. Standardisation of haemoglobinometry: History and new challenges. Comp Hematol Int 1997;**7**: 125-32.

32. Zwart A, van Assendelft OW, Bull BS, England JM, Lewis SM, Zijlstra WG. Recommendations for reference method for haemoglobinometry in

human blood (ICSH standard 1995) and specifications for international haemiglobinocyanide standard (4th edition). J Clin Pathol 1996;**49**: 271-4.

33. Sowemimo-Coker SO. Red blood cell hemolysis during processing. Transfus Med Rev 2002;**16**: 46-60.

34. Heaton WAL. The Quality of Red-Blood-Cells. Immunol Invest 1995;**24**: 371-90.

35. van der Meer PF, Pietersz RN. Overnight storage of whole blood: a comparison of two designs of butane-1,4-diol cooling plates. Transfusion 2007;**47**: 2038-43.

36. Brunskill S, Thomas S, Whitmore E, McDonald CP, Dorée C, Hopewell S, Staves J, Cardigan R, Murphy MF. What Is the Maximum Time That a Unit of Red Blood Cells Can Be Safely Left Out of Controlled Temperature Storage? Transfus Med Rev 2012;**26**: 209-23.

37. Heaton WA. Evaluation of posttransfusion recovery and survival of transfused red cells. Transfus Med Rev 1992;**6**: 153-69.

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

39. Lee JS, Gladwin MT. Bad Blood The risks of red cell storage. Nature Med 2010;**16**: 381-2.

40. Holovati JL, Wong KA, Webster JM, Acker JP. The effects of cryopreservation on red blood cell microvesiculation, phosphatidylserine externalization, and CD47 expression. Transfusion 2008;**48**: 1658-68.

41. Simak J, Gelderman MP. Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers. Transfus Med Rev 2006;**20**: 1-26.

42. Snyder LM, Fairbanks G, Trainor J, Fortier NL, Jacobs JB, Leb L. Properties and Characterization of Vesicles Released by Young and Old Human Red-Cells. Brit J Haematol 1985;**59**: 513-22.

43. Donadee C, Raat NJH, Kanias T, Tejero J, Lee JS, Kelley EE, Zhao XJ, Liu C, Reynolds H, Azarov I, Frizzell S, Meyer EM, Donnenberg AD, Qu LR, Triulzi D, Kim-Shapiro DB, Gladwin MT. Nitric Oxide Scavenging

by Red Blood Cell Microparticles and Cell-Free Hemoglobin as a Mechanism for the Red Cell Storage Lesion. Circulation 2011;**124**: 465-76.

44. Kim-Shapiro DB, Lee J, Gladwin MT. Storage lesion: role of red blood cell breakdown. Transfusion 2011;**51**: 844-51.

45. Yazdanbakhsh K, Bao W, Zhong H. Immunoregulatory effects of stored red blood cells. Hematology Am Soc Hematol Educ Program 2011;**2011**: 466-9.

46. Jy W, Ricci M, Shariatmadar S, Gomez-Marin O, Horstman LH, Ahn YS. Microparticles in stored red blood cells as potential mediators of transfusion complications. Transfusion 2011;**51**: 886-93.

47. Sparrow RL, Healey G, Patton KA, Veale MF. Red blood cell age determines the impact of storage and leukocyte burden on cell adhesion molecules, glycophorin A and the release of annexin V. Transfus Apher Sci 2006;**34**: 15-23.

48. van de Watering L. Red cell storage and prognosis. Vox Sang 2011;**100**: 36-45.

49. Frey B, Gaipl US. The immune functions of phosphatidylserine in membranes of dying cells and microvesicles. Semin Immunopathol 2011;**33**: 497-516.

50. Wolfe LC. The membrane and the lesions of storage in preserved red cells. Transfusion 1985;**25**: 185-203.

51. Antonelou MH, Kriebardis AG, Papassideri IS. Aging and death signalling in mature red cells: from basic science to transfusion practice. Blood Transfus 2010;**8 Suppl 3**: s39-47.

52. Bosman GJCGM, Willekens FLA, Werre JM. Erythrocyte aging: A more than superficial resemblance to apoptosis? Cell Physiol Biochem 2005;**16**: 1-8.

53. Rubin O, Canellini G, Delobel J, Lion N, Tissot JD. Red Blood Cell Microparticles: Clinical Relevance. Transfus Med Hemother 2012;**39**: 342-7.

54. Kriebardis AG, Antonelou MH, Stamoulis KE, Economou-Petersen E, Margaritis LH, Papassideri IS. RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components. Transfusion 2008;**48**: 1943-53.

55. Willekens FLA, Werre JM, Groenen-Dop YAM, Roerdinkholder-Stoelwinder B, de Pauw B, Bosman GJCGM. Erythrocyte vesiculation: a self-protective mechanism? Brit J Haematol 2008;**141**: 549-56.

Chapter 4*

Assessment of the effects of biochemical rejuvenation on red blood cell microvesiculation, phosphatidylserine and CD47 expression during hypothermic storage

*Portions of this chapter have been submitted for publication. Almizraq R, Bicalho B, Tchir JDR, Acker JP, Holovati JL. Assessment of the effects of rejuvenation on red blood cell membrane lipids, microvesiculation, phosphatidylserine and CD47 expression during hypothermic storage. Transfusion 2013.

*Portions of this chapter have been accepted for publication. Jayme D.R Tchir, Jason P. Acker, Jelena L. Holovati· Rejuvenation of ATP during storage does not reverse effects of the hypothermic storage lesion. Transfusion 2013.

*Rejuvenation /ATP assay (Performed by Jayme Tchir).

4.1 Introduction:

Several methods and techniques have been established with different preservation solutions to develop an effective biopreservation strategy for RBCs to ensure safe and readily available products for transfusion medicine [1-4]. Currently in Canada, RBCs are leukoreduced, and stored in CPD-SAGM at 1-6 ^{o}C for up to 42 days prior to transfusion [3,5]. Even though hypothermic storage allows for the preservation of RBC products for up to 6 weeks [5], biophysical and biochemical alterations and damage occur during this time [4,6-9]. These include: the biochemical depletion of ATP and 2,3-DPG, cholesterol and phospholipid loss through microvesiculation, and several membrane changes [10] as pointed out in chapter 3. Moreover, the effects of prolonged hypothermic storage lead to measurable *in vitro* quality changes which have the potential to lead to adverse clinical consequences post transfusion such as infection, renal failure, respiratory failure, multiple organ failure, and mortality [7,11]. Therefore, despite the advances in the preparation or preservation of RBC products, more research and novel methods are needed to promote further RBC preservation and provide high quality products for patients.

As mentioned in chapter 1 and 3, prolonged hypothermic storage induces several changes to the stored RBC membrane as demonstrated by significant increases in RBC microvesiculation and changes in PS and CD47 expression. Microvesiculation is the process by which MPs are shed from the RBC membrane

in response to different stimuli and conditions including hypothermic storage conditions [12,13]. RBC microvesiculation during storage leads to the loss of significant parts of the RBC membrane which leads to a significant decrease in the surface area to volume ratio [14]. Furthermore, the shedding of the microvesicle plays a significant role in RBC morphologic changes [15-17]. Increasing numbers of RMPs have been shown to be harmful effectors of inflammation and coagulation post transfusion [18-20], as well as indicators of RBC viability, quality and integrity throughout *ex vivo* storage [13,21,22].

Previous studies demonstrated that ATP, the RBC's main energy source, is depleted during hypothermic storage, and this is implicated in producing the effects of the hypothermic storage lesion such as shape changes, lipid loss, and microvesiculation [7,9]. ATP is essential for proper RBC functioning and the maintenance of metabolic processes which maintain electrolyte balance and phospholipid asymmetry in the RBC [7,23]. Recent studies suggest that microvesiculation during storage results from biochemical depletion and/or cell senescence [24]; therefore, several studies with varying methodologies have been performed to manipulate the level of metabolic components, ATP and 2, 3-DPG [23,25]. Studies have shown that using a pyruvate-inosine- phosphate- adenine (PIPA) based rejuvenation solution is one of the most effective methods for restoring the intracellular ATP and 2, 3-DPG levels in hypothermically stored RBCs [1,10,26]. Moreover, it has been demonstrated that rejuvenation solutions can improve morphology, oxygen transport function, quality and reverse storage

induced injury to the stored RBCs [1,27]. Even though several studies have shown that the addition of rejuvenation solutions at the end of the hypothermic storage period are capable of improving the biochemical components of stored RBCs, limited studies have been performed to investigate the effect of the rejuvenation solutions on the biomechanical parameters of the storage lesion, including the RBC membrane. Since PIPA rejuvenations solutions are FDA-licensed for use, more research is needed to investigate the effect of rejuvenation solutions on the biomechanical properties and other membrane quality parameters of the RBC.

The third chapter of this thesis showed evidence that hypothermic storage induces RBC microvesicle generation, and changes in the PS and CD47 surface expression on both RBCs and RMPs. In addition, negative correlation between the ATP concentration and number of RMPs throughout storage was identified in chapter 3. Therefore, in this chapter we will investigate the effect of biochemical rejuvenation on the ATP concentration, RBC microvesiculation, as well as PS and CD47 expression. Moreover, we will investigate the effect of different treatment times within the hypothermic storage period with PIPA rejuvenation solution on the biochemical and the biomechanical parameters of stored RBCs will be also investigated.

4.2 Materials and Methods:

4.2.1 RBC Processing

Leukoreduced packed CPD-SAGM RBC units (n= 21) were obtained from the Network Centre for Applied Development (netCAD), Canadian Blood Services (CBS) Centre, Vancouver, British Columbia. Fourteen of the units were MacoPharma collection sets and 7 units were Fenwal collection sets (Appendix 4.1). There are two main differences between the MacoPharma and Fenwel collection sets. First, the length of the Fenwal RBC storage container is 3 cm shorter than MacoPharma container. Second, there is a different method to access the port of the RBC storage container where the MacoPharma container has "pull-apart tabs", which are identified by arrows on the top of the port protectors, while the Fenwal container has peel apart port protectors. Whole blood (450 ± 50 mL) was collected using standard blood bank phlebotomy procedures and processed using the standard buffy coat method [28]. All units were stored at room temperature for up to 24 hours. Within 24 hours, the units were centrifuged in order to obtain the RBCs; buffy coat and platelet poor plasma layers were separated and divided into 2 satellite bags using the top and bottom method [28]. SAGM (110 mL) was added to each of the RBC units, which were then leukoreduced by filtration and then placed in storage at 1 to 6 °C for 49 days.

On day 2 after collection, 3 sets of 7 units were received (total n=21). Each set (n=7), with matched ABO-group and collection date were sterilely connected to a large pooling bag with a multi-lead set using a sterile connection device with a hand sealer (*Hematron*® III bench device, Baxter, Sweden). The sterile

connections were opened and the blood units were elevated to allow the blood to flow and empty all of the 7 units into the pooling bag. The pooling bag was mixed well then held upright and gently squeezed to return the blood to the original blood bags. The clamps and spiked leads, which allow flexibility for open or closed system processing, used to control the pooling and ensure the volume in each of the 7 blood bags was equal according to weight (± 5 g). The tube between that connecting the pooling bag was sealed off and the empty pooling bag was disconnected.

After the pooling and splitting process, the 7 units of each set (pool A, B and C) were labeled from 1 to 7. Unit 1 of each pool were tested weekly up to 49 days and served as non-treated controls. Units 2, 4, and 6 of each pool served as sham control while the rest of the units (3, 5, and 7 of each pool) acting as rejuvenated group. Furthermore, the sham and rejuvenated units were divided into groups based on the day of treatment: units 2 and 3 were treated on day 28, units 4 and 5 treated on day 35, and units 6 and 7 treated on day 4 (Appendix 4.2, flowchart for the rejuvenation study)

4.2.2 RBC Rejuvenation

As shown in the flowchart (Appendix 2), units of each pool were divided into 3 different experimental groups: untreated control (1), sham control (2, 4, and 6) and rejuvenated group (3, 5, and 7). On days 28, 35, and 42 of hypothermic

storage the rejuvenated group was incubated at 1 to 6 °C for one hour with 50 mL of PIPA solution containing containing 0.1 M sodium pyruvate, 0.1 M inosine, 5.0 mM adenine, 70 mM dibasic sodium phosphate anhydrous, and 30 mM monobasic sodium phosphate monohydrate (pH =7.0, osmolality = 500 mOsm/kg H_2O), while the sham control group was incubated at 1 to 6 °C for one hour with 50 mL of 1X phosphate buffered saline (PBS, pH 7.4, 297 mOsm/kg H_2O, Mediatech Inc., Manassas, Virginia)

4.2.3 Biochemical and Biomechanical RBC Quality Assessments:

Following the pooling process, the incubation after treatments, and all the subsequent weeks post-treatment, 6 mL was aseptically sampled from the units by an appropriate syringe. Then, samples were tested for ATP concentration, quantity of RMPs, and PS and CD47 expression as described previously in the material and methods of chapter 2 section 2.2.2 and chapter 3 section 3.2.2.2.

4.2.4 Statistical Analysis:

A mixed model with the treatment time was adjusted used to examine the effect of time on the treated group with saline or with rejuvenation solution. Due to the small sample size of each experimental group (n=3), a paired t-test was used throughout storage time to determine statistical differences in comparison to

day 2 of storage. Values were considered significant when p values were less than 0.05.

4.3 Results

4.3.1 Spectrophotometer Measurement of ATP Concentration:

Figure 1 indicates that ATP concentration (µmol/g Hgb) significantly decreased throughout the hypothermic storage in untreated and sham RBC units ($p<0.05$) in comparison to day 2 of the matched group. Significant decreases in ATP levels of the untreated group, in comparison to day 2, were identified at day 28 and all subsequent testing points ($p<0.05$) (Figure 4.1). On day 2, the mean ATP concentration of the untreated group was 4.53 ± 0.38 µmol/g Hgb while the lowest mean concentration at day 49 was 1.69±0.26 µmol/g Hgb (Table 4.1 and Figure 4.1). On the other hand, the rejuvenated samples on day 28, 35, and 42 showed significant increase in ATP concentration one week after treatment ($p<0.05$). Furthermore, significant interactions were identified between treatment day with rejuvenation solution and treatment time ($p<0.0001$). This data indicates that one week post-rejuvenation, rejuvenated RBC units on day 28 had higher ATP concentration than rejuvenated RBC units on day 35 or day 42.

4.3.2 Flow Cytometry Measurements for the RBC Biomechanical Indicators:

The flow cytometric analysis showed that the number of RMP/µL gradually increased during hypothermic storage, regardless of experimental group or treatment day (Figure 4.2). In comparison to the number of RMP/µL on day 2 of storage, the increase in the number of RMP/µL became statistically significant on the day of treatment for the rejuvenated group on day 28. Moreover, a significant increase at the end of storage period (day 49, $p<0.05$) was observed on the sham-treated RBCs on day 35.

Figures 4.3 to 4.6 show the number of RMPs expressing PS and CD47 (%) and the mean fluorescence intensity (MFI) of PS and CD47 on RMPs during the hypothermic storage. There was no statistically significant change observed in any of these parameters with or without treatment except the MFI of CD47 on RMPs (figure 4.6) in the rejuvenated group day 42 which showed a significant decline on the day of treatment (day 42) and the week after (day 49) ($p<0.05$). Furthermore, there was no statistically significant interaction between the treatment and the treatment time in the PS or CD47 expression on RMPs regardless of the different time points of treatment with saline or rejuvenation solution.

The number of RBCs expressing PS and CD47 as well as the overall MFI for both PS and CD47 on stored RBCs did not change during hypothermic storage in the untreated group (Figure 4.7 to 4.10). Moreover, the treated samples, with

saline or rejuvenation solution, did not show a statistically significant difference in the expression of PS or CD47 on RBCs in comparison to the untreated control. However, there was a significant increase in the number of RBCs expressing PS observed at the end of the storage period (day 49, $p<0.05$) for the sham and rejuvenated group treated on day 42 (Figure 4.7). There was no significant interaction between treatment and the treatment time in any of PS or CD47 expression on RBC.

4.4 Discussion:

Even though storage temperature (1-6 ^{o}C) with anticoagulant and additive solutions are developed to maintain sufficient quality of RBCs during storage time, there are many changes and damage that still occur during hypothermic storage. In chapter 3, we showed strong evidence that hypothermic storage of RBC induces several membrane injuries including changes in PS and CD47 expression and phospholipid loss due to microvesiculation. RBC membrane loss through microvesiculation is a crucial and a major contributing cause of the hypothermic storage lesion of RBC [29]. These changes are clinically important because destroying the RBC membrane integrity may potentially result in an increase in post-transfusion complications. Several studies show that a reduction of RBC microvesiculation may improve the quality of stored RBC since the

accumulation of these vesicles is correlated with multiple negative storage consequences [29,30].

The reduction in the intracellular ATP concentration in RBCs during hypothermic storage has been shown to be a significant cause of RMP generation and disruption of phospholipid asymmetry [23,30,31]. Some studies, including our findings in chapter 3, have shown that there is a significant negative correlation between ATP concentration and the number of RMPs [13,30]. Therefore, in this study we used biochemical rejuvenation to restore ATP as an approach to reverse or minimize the biomechanical storage lesion of the RBC membrane.

Figure 1 shows the mean of ATP concentration (µmol/g Hgb) in RBCs stored hypothermically for up to 49 days. Similar to what was mentioned in section 3.4 of chapter 3, ATP concentrations for the untreated control group decreased by 50 % after 35 days of storage. The concentrations fell below the minimum recommended range of 2.3-2.7 µmol/g Hgb, which has been shown to correlate with 75% of transfused RBCs remaining in circulation 24 hours post-transfusion [32, 33]. Based on this condition, the majority of our units would have met the target ATP concentration up until day 35 of hypothermic storage.

The progressive decline of ATP throughout hypothermic storage is more likely to lead to other effects of storage lesion as it is the energy source for red blood cells. ATP is required to preserve the balance of the cell ions on both sides

of the cell [7,34,35]. It has been indicated in a number of studies that the decrease in the intracellular ATP concentrations of RBC during storage leads to a series of consequences such as the shutdown of the trans-membrane ion pump activity [34,35]. For example, an increase in the level of intracellular calcium accumulation, caused by the shutdown of the pump and resulting in osmotic unbalance is observed [7,34,35]. Moreover, RBCs undergo shape changes from biconcave disks to echinocytes because of ATP reduction [36,37]. The shape changes could be explained by the previous statement where the increase of the intercellular ions causes cell swelling and lipid loss through microvesiculation (as shown to be correlated with the ATP depletion in figure 3.5 in chapter 3 of this thesis). This can lead to the non-deformable spheroechinocytes that are observed during long period of storage and may result in other storage injuries such as hemolysis [11,38,39,40].

The rejuvenated group in this study, which was treated with PIPA solution on day 28, 35, or day 42, showed a significant increase in the intracellular ATP a week after treatment (Figure 4.2). The increase in the ATP concentration following RBC rejuvenation supports quite number of studies that have demonstrated the effect of rejuvenation solutions on RBC biopreservation [1,10,26,27,41-43]. Just about all of these studies showed the ability of the rejuvenation solutions to restore the depleted ATP and the 2, 3-DPG during the storage time. In addition, some of these studies go further than the restoration of the metabolic components and examined the effect of the metabolic rejuvenation

on other elements of the RBC storage lesion. For instance, it has been demonstrated that under the blood banking conditions the adherence of the stored RBCs to the vascular endothelial cell increase with prolonged storage time [44]. Some studies have stated that the RBC adhesion to the vascular endothelial cell correlates with the expression of PS on the RBC surface where more PS exposure on RBC results in an increase RBC to endothelial cell adhesion [44,45]. However, it has been shown that increase adherence of the stored RBCs to the vascular endothelial cells are reversible post treatment with rejuvenation solutions [27]. Similarly, it has been demonstrated that the morphology changes occur to the RBC during cold storage are also reversible with ATP restoration following treatment with rejuvenation solutions [46,47].

Even though studies have examined the effect of the rejuvenation solutions on the RBC storage lesion, the novelty of this study revolves around using biochemical rejuvenation to treat stored RBC in CPD-SAGM at different time points of storage to observe the effect of the rejuvenation on the significant markers of the RBC membrane storage lesion. This study was an approach to prevent the changes during the hypothermic storage before they occur or at least to minimize these changes using biochemical rejuvenation at different time points. PIPA rejuvenation solution is licensed by the FDA to be used any time between 3 days after collection and 3 days after the expiration and washing should take place before transfusion [48]. In this study, units were treated at several time points to demonstrate the effect of the rejuvenation solution on stored RBCs throughout the

early hypothermic storage period, and one week post-expiration. The first treatment point within the hypothermic storage was chosen to be day 28 because most significant changes were shown to occur at this time as it mentioned in the previous chapter. Following treatment, testing points were chosen to determine the effect of treatment time and to find out which time point would be the best to treat the stored RBC for better preservation.

Interestingly, there was a significant interaction between the treatment with rejuvenation solution and the treatment time ($p<0.0001$). RBC units treated with rejuvenation solution showed no significant difference on the day of treatment in comparison to pre-treatment weeks of storage for the same units. However, a week post-treatment showed a significant increase in the intracellular ATP concentrations in comparison to treatment days. Mainly there are two types of rejuvenation treatment; the stored RBCs treated and incubated with a rejuvenation solution at 4 ^{o}C during the storage period (cold rejuvenation) or at 37 ^{o}C post storage time (warm rejuvenation) [1]. The energy production for RBCs is depend on glycolysis and it has been demonstrated that the metabolism of glucose is about 10 times slower in cold temperature (1-6 ^{o}C) than at warm temperature (25 ^{o}C) [49-51]. Therefore, since a cold rejuvenation was used in this study to treat the stored RBCs throughout hypothermic storage, the significant elevation of ATP concentrations were observed a week after treatments with biochemical rejuvenation and not on the day of the treatment as other studies that used warm rejuvenation [47,52].

After successful restoration of the depleted ATP concentration during the storage, the effect of the biochemical modification (rejuvenation) on the RBC microvesiculation, PS and CD47 expression were examined. The flow cytometric analysis showed that regardless of experimental group or treatment day, the hypothermic storage continues to induce microparticle generation (Figure 4.2). Even though there was no significant interaction between the treatments and the treatment time, the rejuvenated group day 28 showed a statistically significant increase in the number of RMP/μL in the day of treatment in comparison to day 2 of storage ($p=0.0312$). An explanation for the increase in the RBC vesiculation can be based on the basic science of the formation of RBC vesicles *in vivo.* It has been shown that lipid loss and vesicles formation play an important role in remodelling the RBC membrane during the maturation process [53-55]. *In vivo,* membrane loss through microvesiculation in immature RBCs result in a decrease in the mean cell volume and modification in the surface to volume ratio which lead to promote the function and maintain the viability of these mature RBCs [10]. According to this principle, the increase in vesicle formation during storage is apparently an important process to maintain the RBC membrane related functions and viability. This assumption in solidarity with the other importance of microvesiculation when the cells get rid of the harmful markers on their surface such as PS to survive as mentioned in the discussion of the third chapter. This could also partly explain the significant increase in the number of RBCs expressing PS on at the last day of testing the group rejuvenated on day 42 ($p= 0.024$) while no significant change was detected in the number of RBC with PS

expression in the group rejuvenated on day 28 (figure 4.7) when a significant increase in the number of RMPs was observed on the day of treatment (figure 4.2). However, this opinion or pattern does not negate the fact that microparticle generation is a significant indicator of the collapse of the cell. Although the biochemical rejuvenation was sufficient to restore the ATP concentration, it did not reverse or prevent the RBC microvesiculation throughout the hypothermic storage time. This work is in agreement with other studies which show that RBC membrane loss through vesiculation is irreversible by rejuvenation solutions [10,29,56].

Further quality assessment of the RBC membrane focused on PS and CD47 expression using flow cytometry to investigate the effect of the rejuvenation treatment at different time points. While the rejuvenation treatment had no significant effect on the RBC- CD47 expression regardless of the different treatment time points, a significant increase in the number of RBC expressing PS was observed on the last day of storage (day 49) with the group that was rejuvenated on day 42 as explained above. In addition, even though there was no significant effect on the number of RMPs expression PS or CD47 and the MFI of PS during the storage in all of the groups and at all of the time points (figure 4.3 and 4.5), a statistically significant decline in the MFI of CD47 was observed at the end of storage time for the rejuvenated group on day 42 (figure 4.5). The importance of the CD47 expression and how lacking this surface antigen on RBCs or their fragments can enhance their rapid clearance from the circulation post

transfusion is addressed in the introductory chapter and in chapter 3. The expression of CD47 on RMPs for the group rejuvenated on day 42 fell form (905.1±191.9) at day 2 to (558.5± 252.6, p=0.015) on day 42 and (522.6 ± 86.8, p=0.030) on day 49. Although these changes observed with the rejuvenated group on day 42 was statistically significant in comparison to day 2 of storage for the matched group, the mixed model analysis used in this study showed that there was no significant difference between treatments with rejuvenation solution or saline and no significant interaction between the treatment and the treatment time for all of measurements with the flow cytometer. Hence, despite the fact that the rejuvenation solution was sufficient to regenerate the depleted ATP concentration during the storage, it did not prevent or reverse the RBC microparticles generation and changes in CD47 or PS expression.

It is necessary to mention that the pool-spilt study design was used in this study to eliminate or minimize variability between units and variances brought by different donors. The disadvantages that might be associated with the pool-split design is that the process of this method may increase the chance of bacterial contamination [57]. Also, it is possible that the pooled units may have different storage characteristics than non-pooled units which would make it difficult to compare the final result of the work with other works which study the same targeted parameters. However, it has been shown that the pool-split design is a good approach to study the effect of a parameter or a technique on samples while minimizing other variables [57,58] which is the main aim of this chapter.

Although a pool and split design method was used in this study to prevent donors and units' variation, another source of difference was observed with the two different blood bag types. As explained in the methodology section 4.2.1, fourteen of the units were MacoPharma collection sets and seven units were Fenwal collection sets (Appendix 4.1). There are two main differences between the MacoPharma and Fenwal collection sets which may contribute to the observed variability in the data. For instance, the number of RMPs observed in Fenwal units was higher than the number of RBC microparticles in the MacoPharma units which were tested on day 2. On the other hand, the number of RBC with PS exposure (Mean ±SD) was very low in Fenwal collection sets (0.5± 0.1) in comparison to the MacoPharma one (1.5± 0.5). Therefore, MacoPharma units (n=14) were studied separately to minimize the errors and eliminate the unit set variation associated with the 3 pools. As a result, acceptable error was observed but the statistical analysis did not give a real view of the data due to the small sample size (n=2) for each subsequent groups (data not shown). In addition, relative normalization has been done for this study with all of the pools but the problems with error were observed again and were even higher than without normalization (data not shown). Therefore, the results of this study shown in this chapter are not normalized or separated. As a consequence, the occurrence of changes with or without rejuvenation may be affected by the difference between the blood bags types used in this research. Small differences between the samples may not be detected due to the small sample size in this study since sufficient

statistical power normally require a larger sample. Therefore, further studies with higher samples size and using the same type of collection sets are recommended.

4.5 Conclusion:

RBC ATP depletion during storage has been shown to be a significant cause of RMPs generation, disruption of phospholipid asymmetry leading to many aspects of the hypothermic storage lesion [23,30,31,59]. This may lead to adverse clinical outcomes post transfusion as previously mentioned in chapters 2 and 3. In light of this, biochemical rejuvenation was used to treat the stored RBCs at different time points of hypothermic storage in order to reverse or prevent the storage lesion. The result of this study has shown that biochemical rejuvenation for stored RBCs was sufficient to restore the depleted ATP during storage but does not reverse the negative effects of hypothermic storage on RBC membrane structure or composition regardless of the experimental group and the different points of treatments. Due to the fact that some of the RBC storage lesion, such as RBC microvesiculation, are irreversible, more advanced techniques focusing on stabilizing the RBC membrane during the hypothermic storage are required to improve the RBC *in vitro* quality parameters and to achieve better quality RBC products for transfusion.

Table 4.1: Absolute ATP values (µmol/g hgb) and ratio / Day 2 for stored RBC up to 49 days (untreated and treated in different time points throughout the storage with saline or rejuvenation solution)

	Length of Storage				
Untreated Group	Day 2	Day 28	Day 35	Day 42	Day 49
ATP (µmol/g hgb)	4.53± 0.38	3.06± 0.44*	2.81± 0.35*	2.14± 0.39*	1.69± 0.26*
Ratio ATP Day 1/ Day2 (%)	1.00± 0.00	0.68± 0.03	0.62± 0.02	0.48± 0.01	0.38± 0.00
Sham-treated Day 28					
ATP (µmol/g hgb)	4.16±0.21	3.37±0.32	2.94±0.47*	2.32±0.70*	1.5±0.43*
Ratio ATP Day 1/ Day2 (%)	1.00± 0.00	0.81±0.12	0.71±0.15	0.56±0.19	0.36±0.12
Rejuvenated Day 28					
ATP (µmol/g hgb)	4.13±0.32	4.07±0.24	6.38±0.72*	6.47±0.22*	4.57±0.56
Ratio ATP Day 1/ Day2 (%)	1.00± 0.00	0.99±0.02	1.55±0.29	1.57±0.07	1.12±0.22
Sham-treated Day 35					
ATP (µmol/g hgb)	4.15±0.31		2.86±0.46	2.54±0.57*	1.71±0.14*
Ratio ATP Day 1/ Day2 (%)	1.00± 0.00		0.69±0.16	0.62±0.18	0.41±0.06
Rejuvenated Day 35					
ATP (µmol/g hgb)	4.18±0.27		4.12±0.77	5.89±0.11*	4.61±0.10
Ratio ATP Day 1/ Day2 (%)	1.00± 0.00		0.98±0.12	1.41±0.06	1.12±0.10
Sham-treated Day 42					
ATP (µmol/g hgb)	4.26±0.50			2.63±0.90	1.76±0.41*
Ratio ATP Day 1/ Day2 (%)	1.00± 0.00			0.63±0.29	0.42±0.15
Rejuvenated Day 42					
ATP (µmol/g hgb)	3.83±0.84			4.08±0.89	4.78±0.53*
Ratio ATP Day 1/ Day2 (%)	1.00± 0.00			1.07±0.41	1.26±0.14

Mean ± SD

*($p < 0.05$) in comparison to day 2 mean ATP values

Figure 4.1: ATP concentration µmol/g Hgb (Mean ± SD) for untreated, sham and rejuvenated groups throughout 49 days of storage. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.

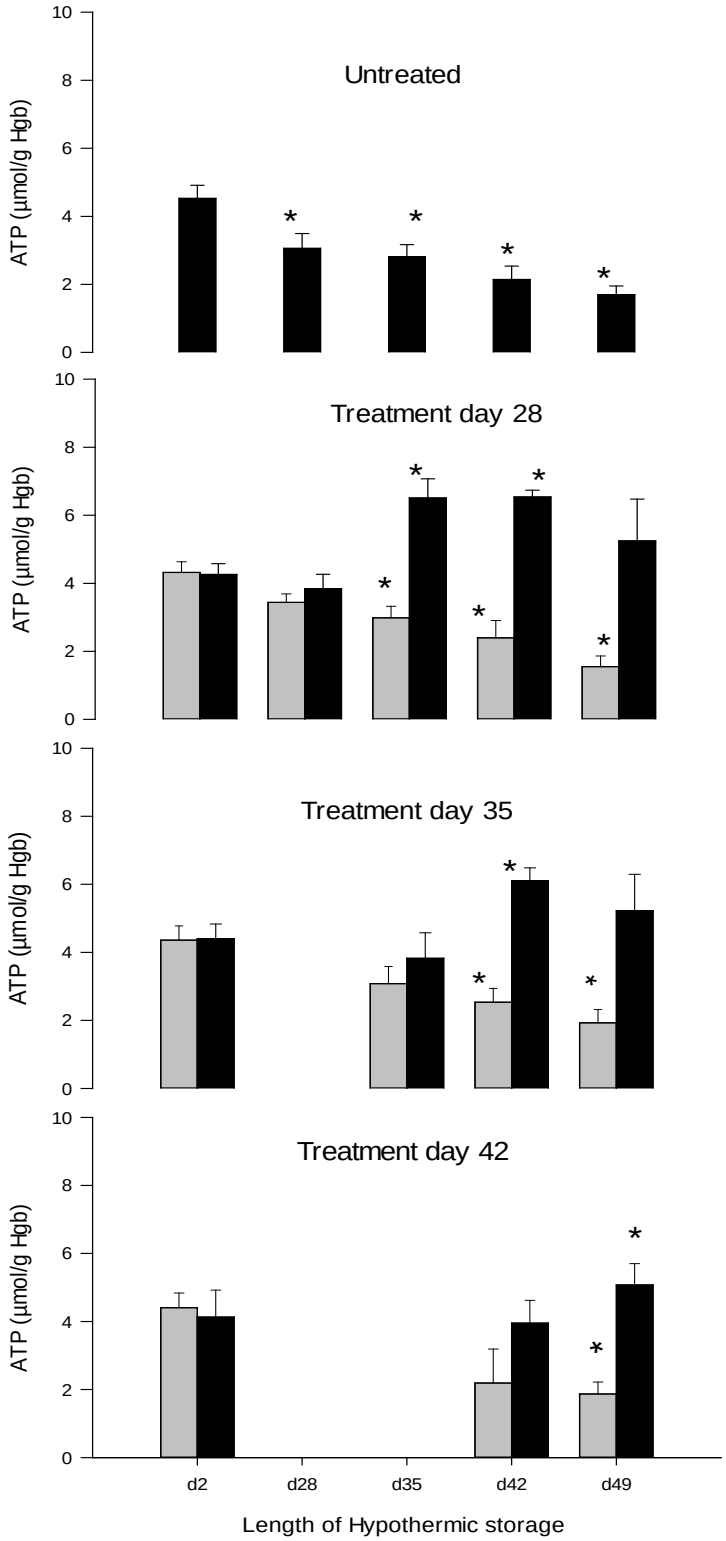


Figure 4.2: Absolute number of RMP/µl (Mean ± SD) for untreated, sham and rejuvenated groups throughout 49 days of storage. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.

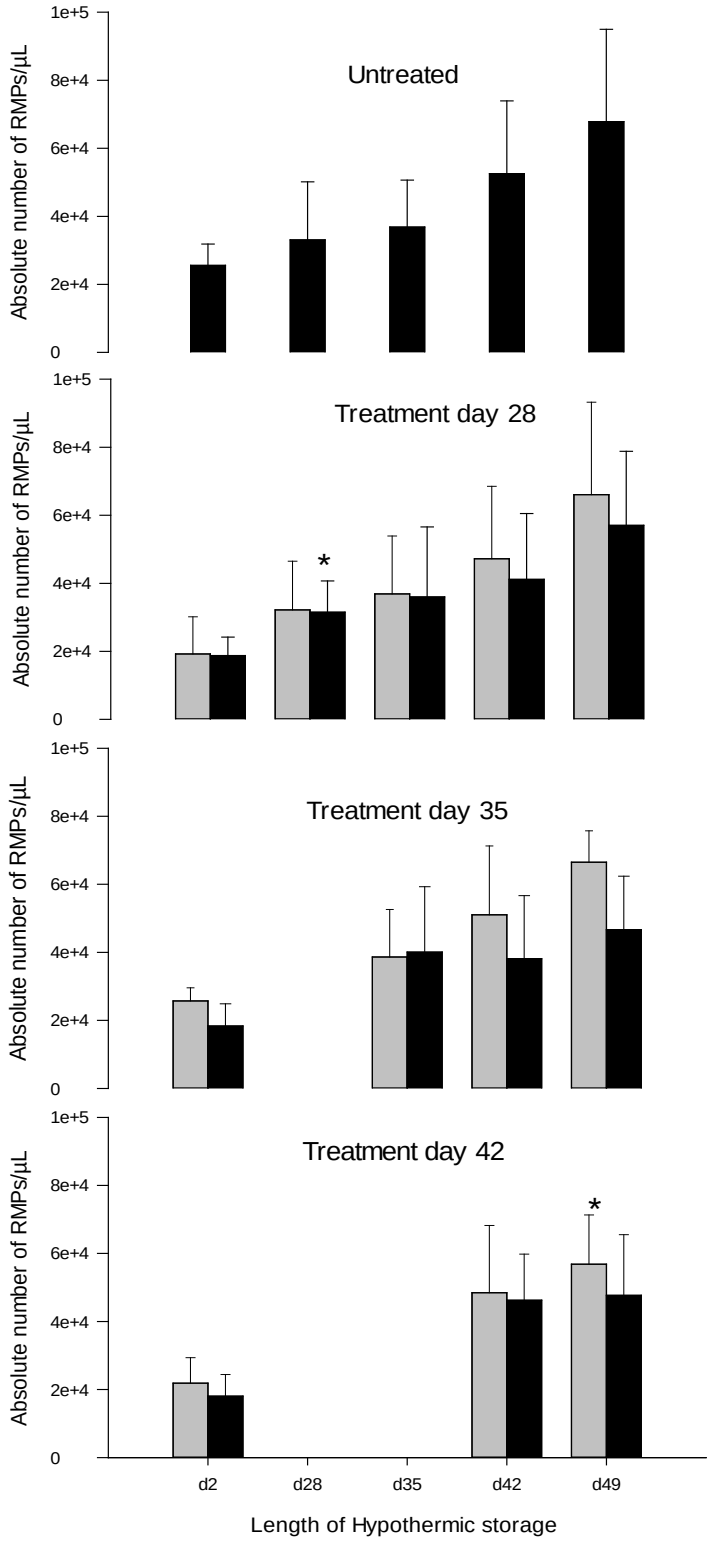


Figure 4.3: Percent of RMP-PS (Mean ± SD) for untreated, sham ▭ and rejuvenated ▬ groups throughout 49 days of storage. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.

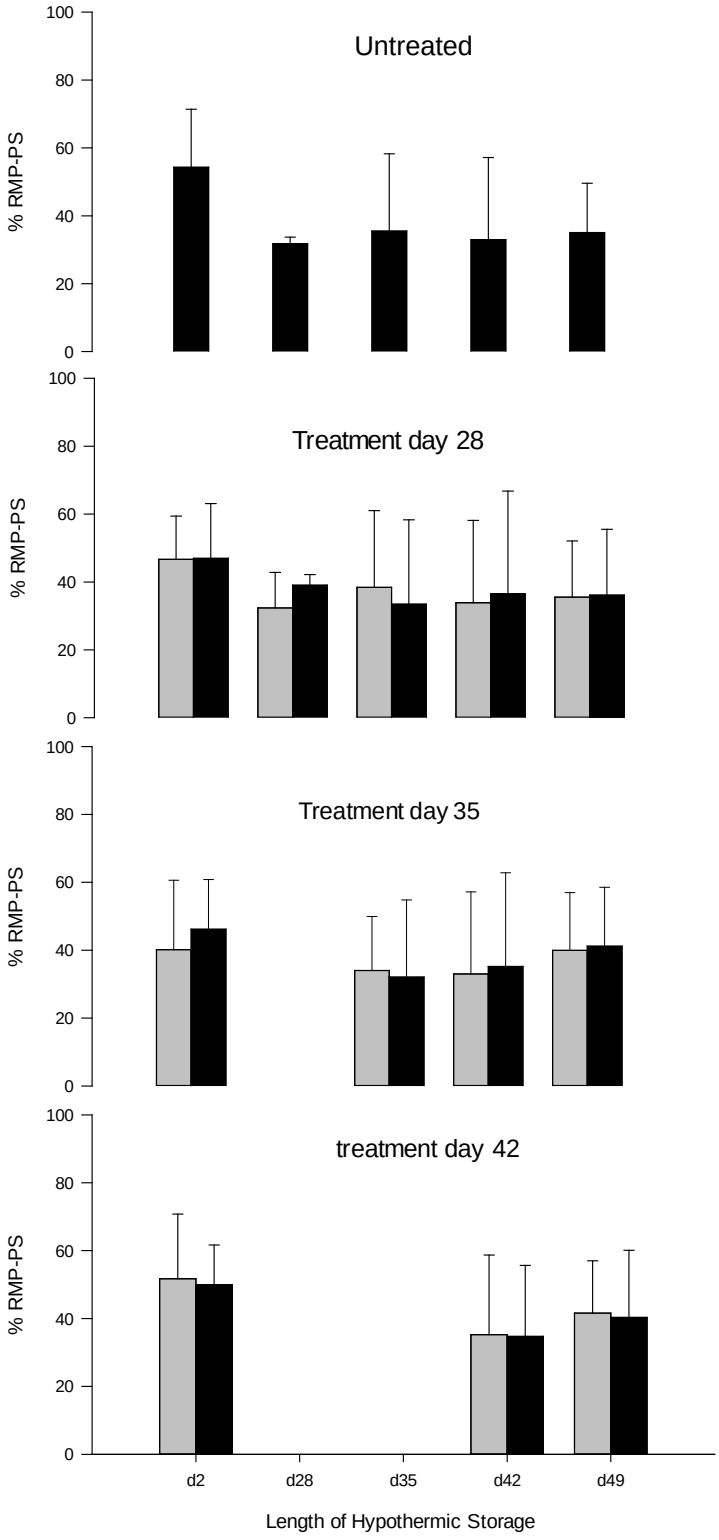


Figure 4.4: MFI of RMP-PS (Mean ± SD) for untreated, sham ▭ and rejuvenated ■ groups throughout 49 days of storage. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.

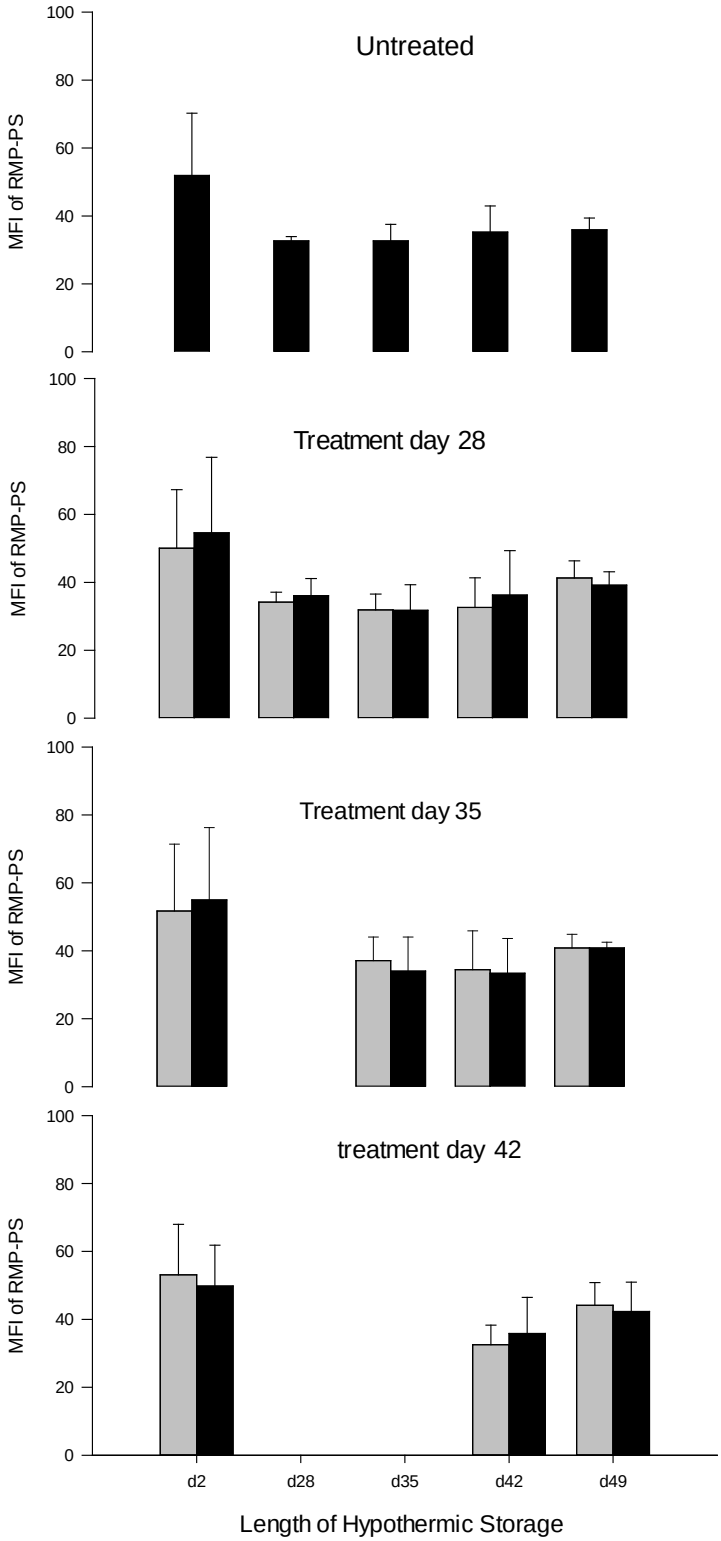


Figure 4.5: Percent of RMP-CD47 (Mean ± SD) for untreated, sham ▭ and rejuvenated ▬ groups throughout 49 days of storage. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.

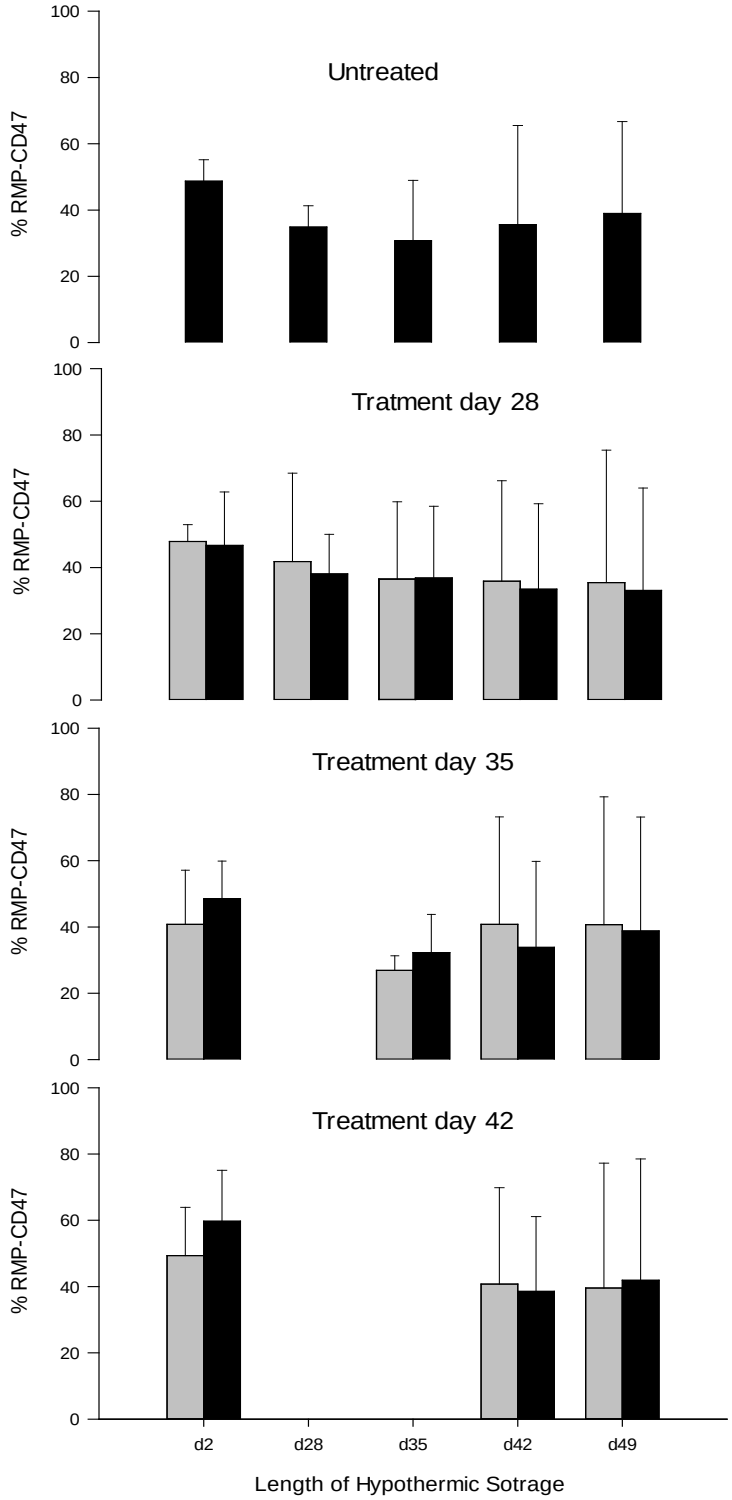


Figure 4.6: MFI of RMP-CD47 (Mean ± SD) for untreated, sham ▭ and rejuvenated ▬ groups throughout 49 days of storage. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.

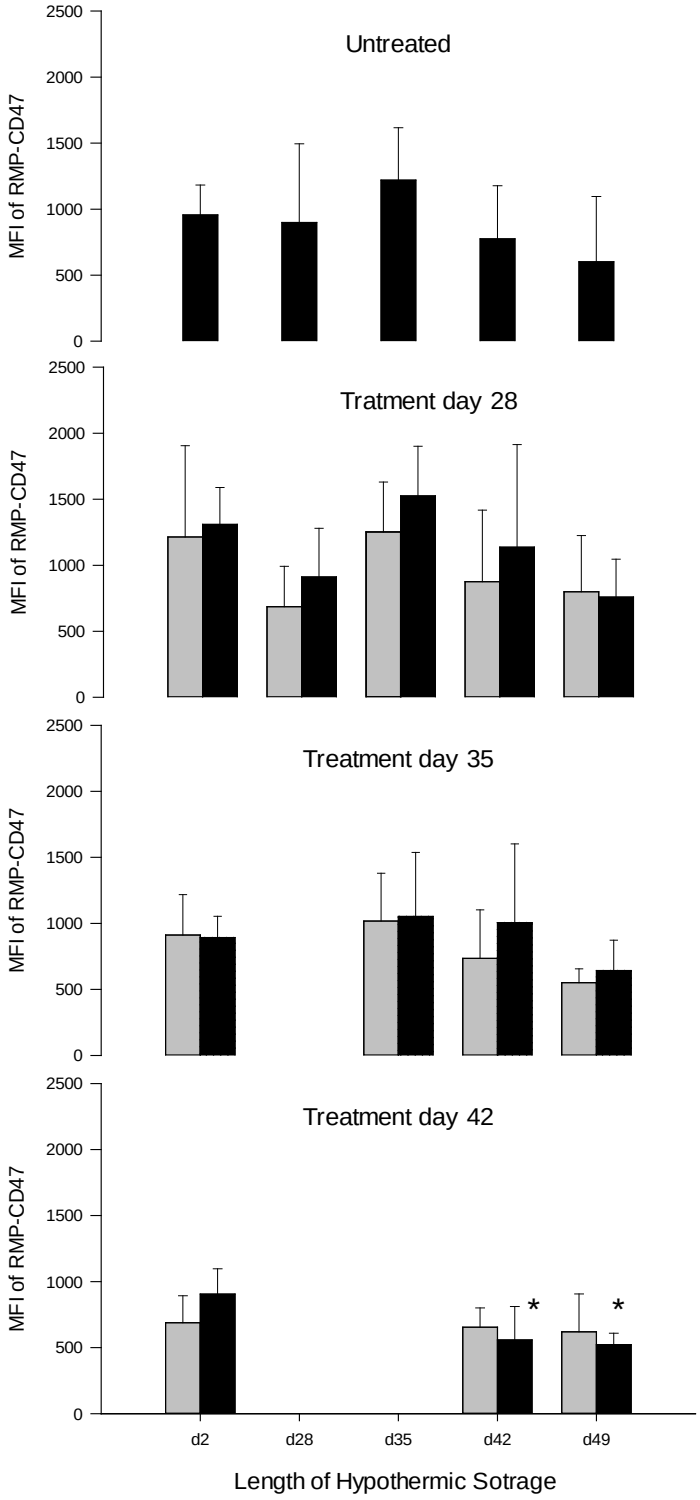


Figure 4.7: Percent of RBC-PS (Mean ± SD) for untreated, sham ▭ and rejuvenated ■ groups throughout 49 days of storage. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.

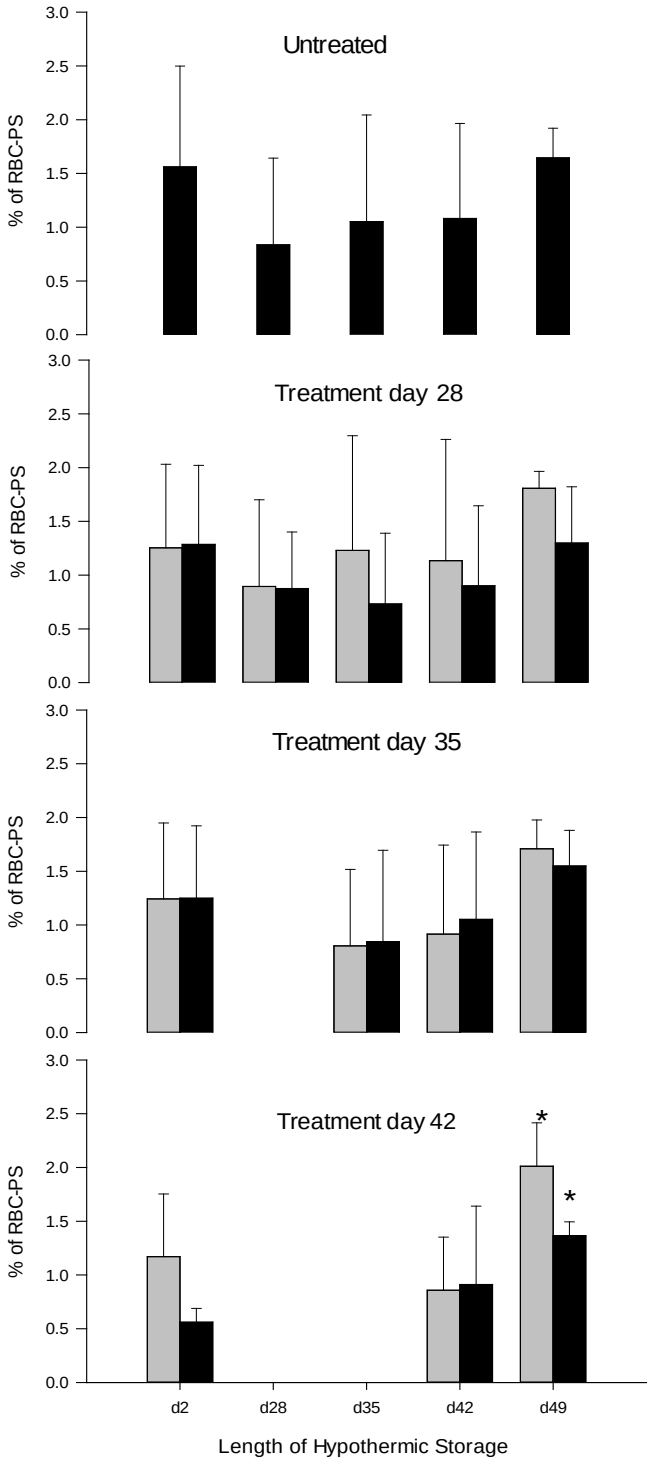


Figure 4.8: MFI of RBC-PS (Mean ± SD) for untreated, sham ▭ and rejuvenated ▬ groups throughout 49 days of storage. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.

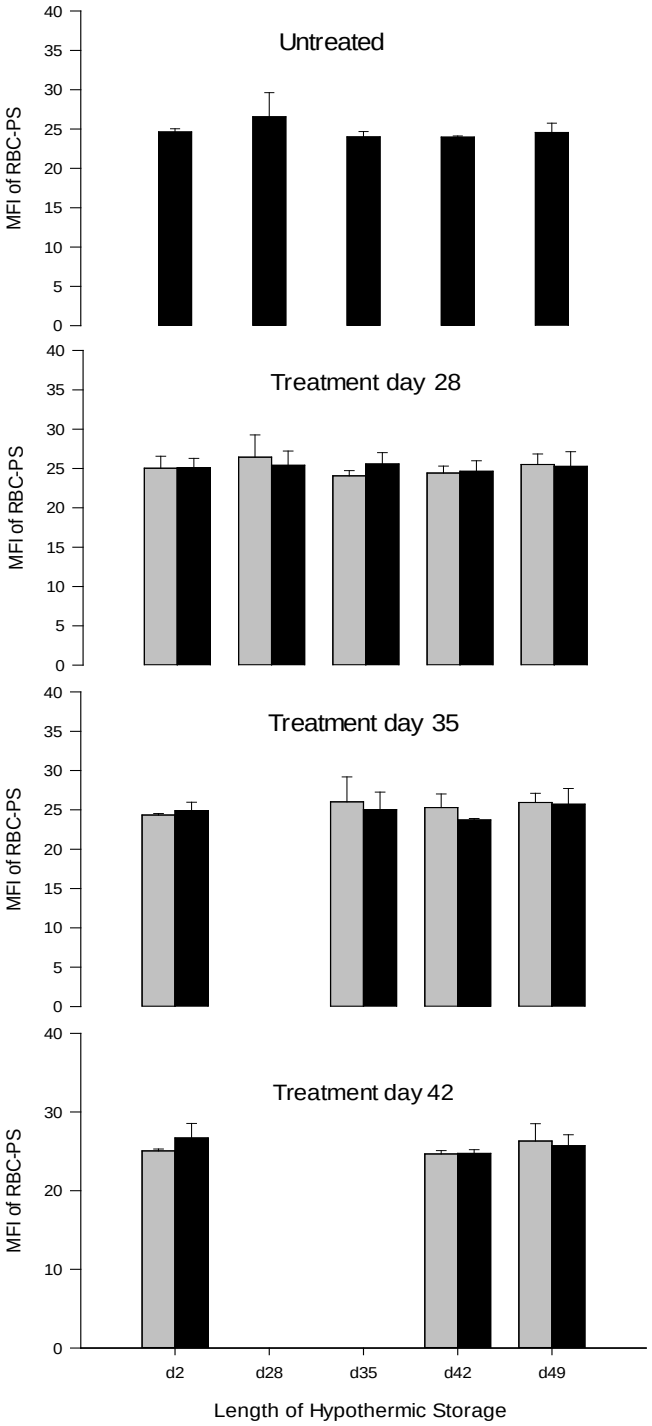


Figure 4.9: Percent of RBC-CD47 (Mean ± SD) for untreated, sham ▭ and rejuvenated ▬ groups throughout 49 days of storage. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.

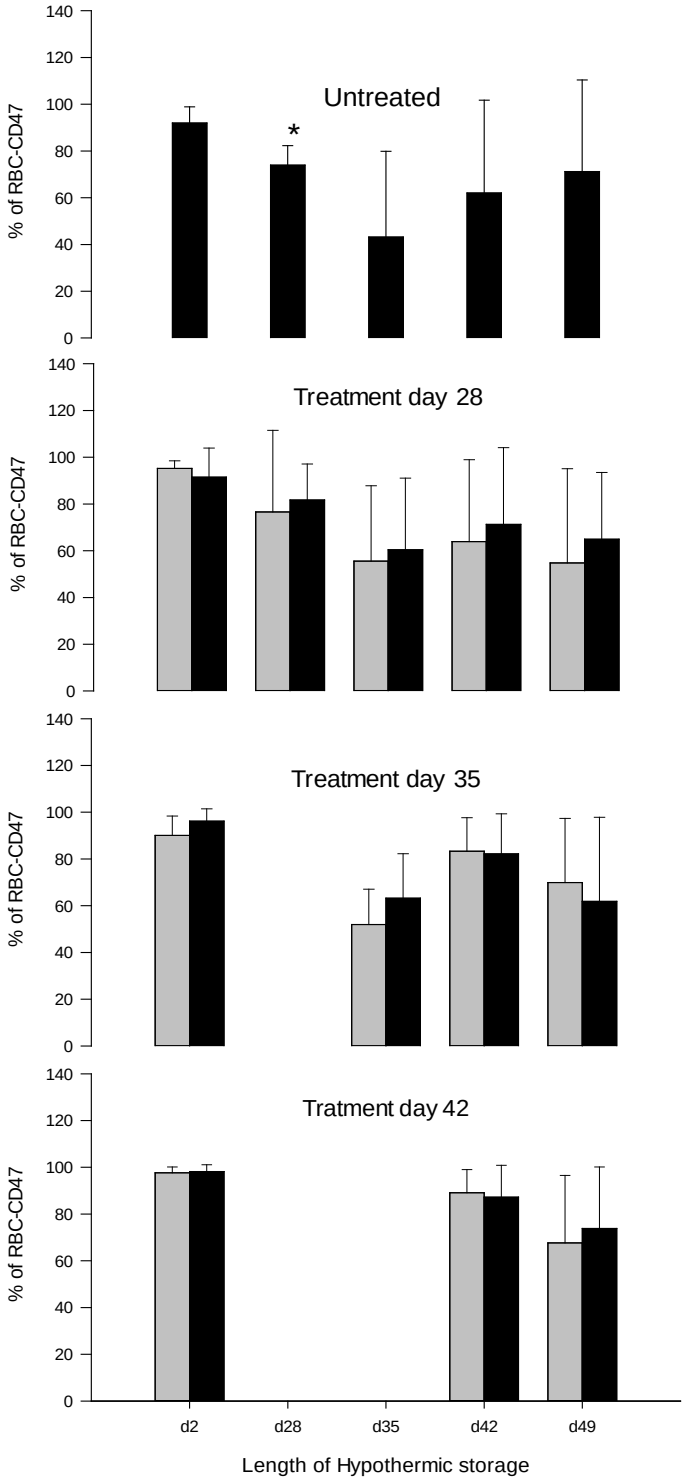
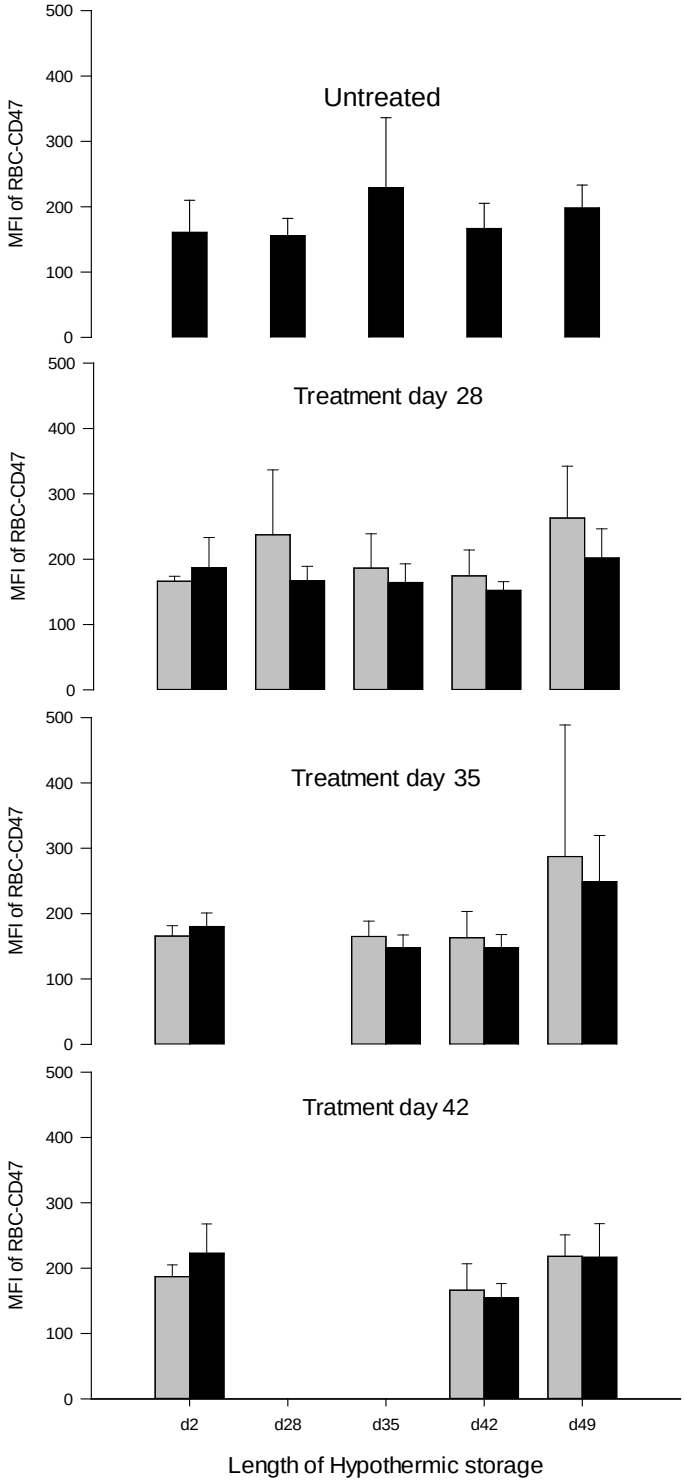
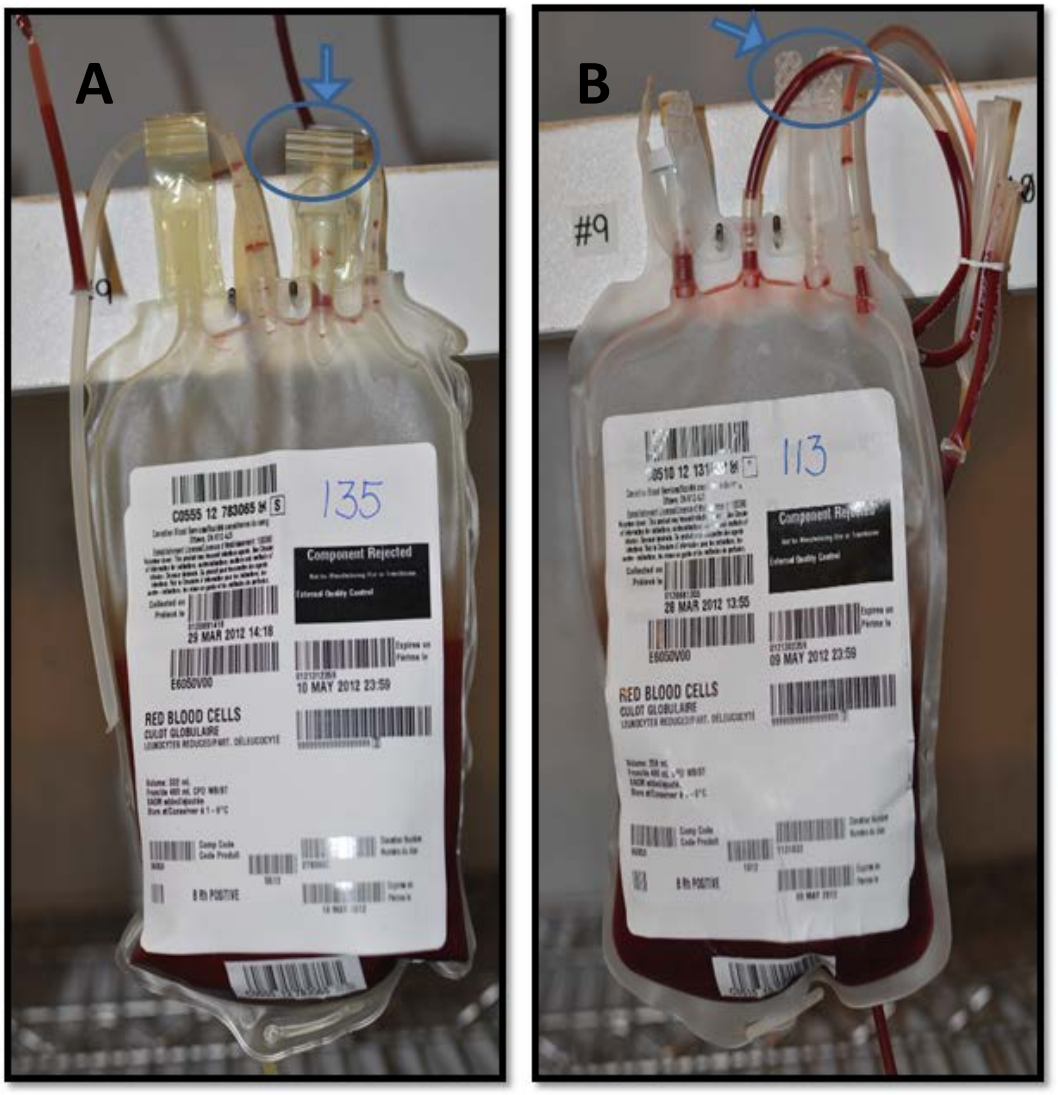


Figure 4.10: MFI of RBC-CD47 (Mean ± SD) for untreated, sham ▭ and rejuvenated ▬ groups throughout 49 days of storage. Asterisk indicated significant change ($p < 0.05$) in comparison to day 2.



Appendix 4.1: Fenwal (A) vs Macopharma (B) blood bag.

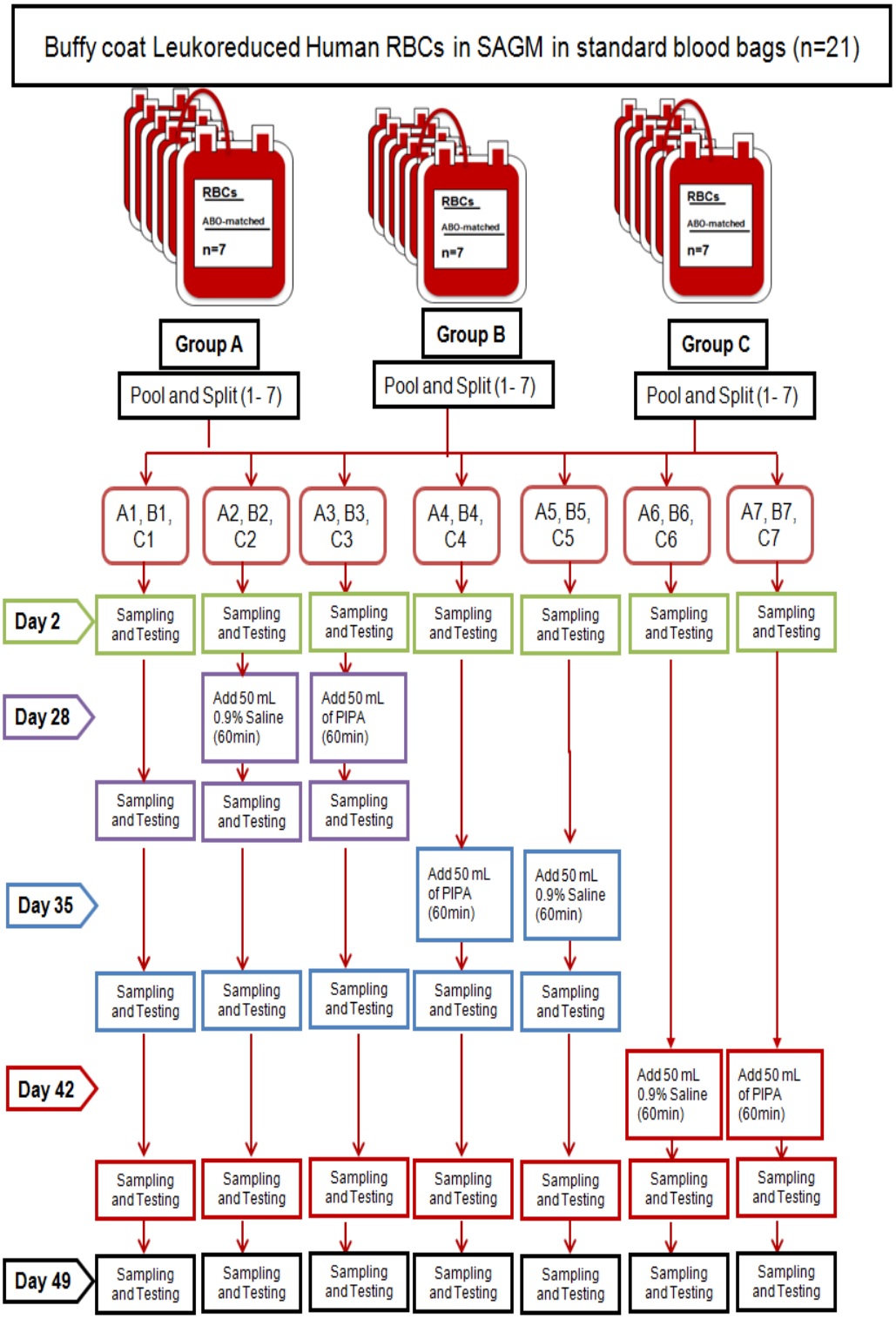


- The length of the Fenwal RBC storage container is 3 cm shorter than MacoPharma container.

- There is a different method to access the port of the RBC storage container where the MacoPharma container has “pull-apart tabs”, which identified by arrow on the top of the port protectors, while the Fenwal container has peel apart port protectors

[Pictures were taken by Tracey Turner in Dr. Acker’s Lab, Canadian Blood services, Edmonton]

Appendix 4.2: Flowchart for RBC Rejuvenation during Hypothermic Storage.



4.6 References:

1. Valeri CR. Use of Rejuvenation Solutions in Blood Preservation. Crc Cr Rev Cl Lab Sc 1982;**17**: 299-374.

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

3. D'Alessandro A, Liumbruno G, Grazzini G, Zolla L. Red blood cell storage: the story so far. Blood Transfus 2010;**8**: 82-8.

4. Hess JR. An update on solutions for red cell storage. Vox Sang 2006;**91**: 13-9.

5. Acker JP. Biopreservation of cells and engineered tissues. Adv Biochem Eng Biotechnol 2007;**103**: 157-87.

6. Sparrow RL, Healey G, Patton KA, Veale MF. Red blood cell age determines the impact of storage and leukocyte burden on cell adhesion molecules, glycophorin A and the release of annexin V. Transfus Apher Sci 2006;**34**: 15-23.

7. Scott KL, Lecak J, Acker JP. Biopreservation of red blood cells: past, present, and future. Transfus Med Rev 2005;**19**: 127-42.

8. Ho J, Sibbald WJ, Chin-Yee IH. Effects of storage on efficacy of red cell transfusion: when is it not safe? Crit Care Med 2003;**31**: S687-97.

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

10. Dumaswala UJ, Petrosky TL, Greenwalt TJ. Studies in red blood cell preservation. 6. Red cell membrane remodeling during rejuvenation. Vox Sang 1992;**63**: 12-5.

11. Kor DJ, Van Buskirk CM, Gajic O. Red blood cell storage lesion. Bosn J Basic Med Sci 2009;**9 Suppl 1**: 21-7.

12. Greenwalt TJ, Dumaswala UJ. Effect of Red-Cell Age on Vesiculation Invitro. British Journal of Haematology 1988;**68**: 465-7.

13. Salzer U, Zhu R, Luten M, Isobe H, Pastushenko V, Perkmann T, Hinterdorfer P, Bosman GJ. Vesicles generated during storage of red cells are rich in the lipid raft marker stomatin. Transfusion 2008;**48**: 451-62.

14. Wolfe LC, Byrne AM, Lux SE. Molecular defect in the membrane skeleton of blood bank-stored red cells. Abnormal spectrin-protein 4.1-actin complex formation. J Clin Invest 1986;**78**: 1681-6.

15. Hess JR. Red cell changes during storage. Transfus Apher Sci 2010;**43**: 51-9.

16. Rumsby MG, Trotter J, Allan D, Michell RH. Recovery of membrane micro-vesicles from human erythrocytes stored for transfusion: a mechanism for the erythrocyte discocyte-to-spherocyte shape transformation. Biochem Soc Trans 1977;**5**: 126-8.

17. Shukla SD, Coleman R, Finean JB, Michell RH. The use of phospholipase C to detect structural changes in the membranes of human erythrocytes aged by storage. Biochim Biophys Acta- Biomemb 1978;**512**: 341-9.

18. Burnier L, Fontana P, Kwak BR, Angelillo-Scherrer A. Cell-derived microparticles in haemostasis and vascular medicine. Thromb Haemost 2009;**101**: 439-51.

19. Horstman LL, Jy W, Bidot CJ, Nordberg ML, Minagar A, Alexander JS, Kelley RE, Ahn YS. Potential roles of cell-derived microparticles in ischemic brain disease. Neurol Res 2009;**31**: 799-806.

20. Rubin O, Crettaz D, Tissot JD, Lion N. Microparticles in stored red blood cells: submicron clotting bombs? Blood Transfus 2010;**8 Suppl 3**: s31-8.

21. AuBuchon JP, Estep TN, Davey RJ. The effect of the plasticizer di-2-ethylhexyl phthalate on the survival of stored RBCs. Blood 1988;**71**: 448-52.

22. Kim-Shapiro DB, Lee J, Gladwin MT. Storage lesion: role of red blood cell breakdown. Transfusion 2011;**51**: 844-51.

23. de Korte D, Kleine M, Korsten HG, Verhoeven AJ. Prolonged maintenance of 2,3-diphosphoglycerate acid and adenosine triphosphate in red blood cells during storage. Transfusion 2008;**48**: 1081-9.

24. Chernitskii EA, Fedorovich IE, Slobozhanina EI. [Vesiculation and change of erythrocyte membrane proteins in storage of preserved blood and erythrocyte mass at 4 degrees C]. Gematol Transfuziol 1993;**38**: 18-21.

25. Moore GL. Additive solutions for better blood preservation. Crit Rev Clin Lab Sci 1987;**25**: 211-29.

26. Meyer EK, Dumont DF, Baker S, Dumont LJ. Rejuvenation capacity of red blood cells in additive solutions over long-term storage. Transfusion 2011;**51**: 1574-9.

27. Koshkaryev A, Zelig O, Manny N, Yedgar S, Barshtein G. Rejuvenation treatment of stored red blood cells reverses storage-induced adhesion to vascular endothelial cells. Transfusion 2009;**49**: 2136-43.

28. Levin E, Culibrk B, Gyöngyössy-Issa MIC, Weiss S, Scammell K, LeFresne W, Jenkins C, Devine DV. Implementation of buffy coat platelet component production: comparison to platelet-rich plasma platelet production. Transfusion 2008;**48**: 2331-7.

29. Greenwalt TJ. The how and why of exocytic vesicles. Transfusion 2006;**46**: 143-52.

30. Veale MF, Healey G, Sparrow RL. Effect of additive solutions on red blood cell (RBC) membrane properties of stored RBCs prepared from whole blood held for 24 hours at room temperature. Transfusion 2011;**51**: 25s-33s.

31. Holovati JL, Wong KA, Webster JM, Acker JP. The effects of cryopreservation on red blood cell microvesiculation, phosphatidylserine externalization, and CD47 expression. Transfusion 2008;**48**: 1658-68.

32. van der Meer PF, Pietersz RN. Overnight storage of whole blood: a comparison of two designs of butane-1,4-diol cooling plates. Transfusion 2007;**47**: 2038-43.

33. Brunskill S, Thomas S, Whitmore E, McDonald CP, Dorée C, Hopewell S, Staves J, Cardigan R, Murphy MF. What Is the Maximum Time That a Unit of Red Blood Cells Can Be Safely Left Out of Controlled Temperature Storage? Transfus Med Rev 2012;**26**: 209-23.

34. Messana I, Ferroni L, Misiti F, Girelli G, Pupella S, Castagnola M, Zappacosta B, Giardina B. Blood bank conditions and RBCs: the progressive loss of metabolic modulation. Transfusion 2000;**40**: 353-60.

35. Rapoport TA, Heinrich R, Rapoport SM. The regulatory principles of glycolysis in erythrocytes in vivo and in vitro. A minimal comprehensive model describing steady states, quasi-steady states and time-dependent processes. Biochem J 1976;**154**: 449-69.

36. Nakao M, Nakao T, Yamazoe S. Adenosine triphosphate and maintenance of shape of the human red cells. Nature 1960;**187**: 945-6.

37. Park Y, Best CA, Auth T, Gov NS, Safran SA, Popescu G, Suresh S, Feld MS. Metabolic remodeling of the human red blood cell membrane. Proc Natl Acad Sci U S A 2010;**107**: 1289-94.

38. Watanabe S, Yamamoto R, Ogata M, Murakami T. Adenosine triphosphate restoration and discocytic transformation of stored human erythrocytes. Acta Med Okayama 1985;**39**: 239-46.

39. Lichtman MA, Williams WJ. Preservation and clinical use of erythrocytes and whole blood Williams hematology. New York: McGraw-Hill, Medical Pub. Division, 2006.

40. Hess JR, Greenwalt TG. Storage of red blood cells: new approaches. Transfus Med Rev 2002;**16**: 283-95.

41. Lockwood WB, Hudgens RW, Szymanski IO, Teno RA, Gray AD. Effects of rejuvenation and frozen storage on 42-day-old AS-3 RBCs. Transfusion 2003;**43**: 1527-32.

42. Szymanski IO, Teno RA, Lockwood WB, Hudgens R, Johnson GS. Effect of rejuvenation and frozen storage on 42-day-old AS-1 RBCs. Transfusion 2001;**41**: 550-5.

43. Yoshida T, AuBuchon JP, Dumont LJ, Gorham JD, Gifford SC, Foster KY, Bitensky MW. The effects of additive solution pH and metabolic

rejuvenation on anaerobic storage of red cells. Transfusion 2008;**48**: 2096-105.

44. Relevy H, Koshkaryev A, Manny N, Yedgar S, Barshtein G. Blood banking-induced alteration of red blood cell flow properties. Transfusion 2008;**48**: 136-46.

45. Barshtein G, Ben-Ami R, Yedgar S. Role of red blood cell flow behavior in hemodynamics and hemostasis. Expert Rev Cardiovasc Ther 2007;**5**: 743-52.

46. Nakao M, Nakao T, Tatibana M, Yoshikawa H, Abe T. Effect of inosine and adenine on adenosine triphosphate regeneration and shape transformation in long-stored erythrocyts. Biochim Biophys Acta 1959;**32**: 564-5.

47. Usry RT, Moore GL, Manalo FW. Morphology of stored, rejuvenated human erythrocytes. Vox Sang 1975;**28**: 176-83.

48. Brecher ME, American Association of Blood B. Technical manual. Bethesda, Md.: Amer Assn of Blood Banks, 2002.

49. Solheim BG, Hess JR. Red Cell Metabolism and Preservation Rossi's Principles of Transfusion Medicine: Wiley-Blackwell, 2009:54-68.

50. Ruddell JP, Lippert LE, Babcock JG, Hess JR. Effect of 24-hour storage at 25 degrees C on the in vitro storage characteristics of CPDA-1 packed red cells. Transfusion 1998;**38**: 424-8.

51. Rodak BF, Fritsma GA, Keohane EM. Hematology clinical principles and applications Energy metabolism and membrane physiology of the erythrocyte. St. Louis (Mo.): Elsevier Saunders, 2012:103-14.

52. Raat NJ, Hilarius PM, Johannes T, de Korte D, Ince C, Verhoeven AJ. Rejuvenation of stored human red blood cells reverses the renal microvascular oxygenation deficit in an isovolemic transfusion model in rats. Transfusion 2009;**49**: 427-4.

53. Ganzoni A, Hillman RS, Finch CA. Maturation of the macroreticulocyte. Br J Haematol 1969;**16**: 119-35.

54. Shattil SJ, Cooper RA. Maturation of macroreticulocyte membranes in vivo. J Lab Clin Med 1972;**79**: 215-27.

55. Gasko O, Danon D. Endocytosis and Exocytosis in Membrane Remodelling during Reticdocyte Maturation. Brit J Haematol 1974;**28**: 463-70.

56. Greenwalt TJ, Bryan DJ, Dumaswala UJ. Erythrocyte membrane vesiculation and changes in membrane composition during storage in citrate-phosphate-dextrose-adenine-1. Vox Sang 1984;**47**: 261-70.

57. van der Meer P, Pietersz R, Reesink H. Leucoreduced platelet concentrates in additive solution: an evaluation of filters and storage containers. Vox Sang 2001;**81**: 102-7.

58. Gyongyossy-Issa MIC, Weiss SL, Sowemimo-Coker SO, Garcez RB, Devine DV. Prestorage leukoreduction and low-temperature filtration reduce hemolysis of stored red cell concentrates. Transfusion 2005;**45**: 90-6.

59. Mokken FC, Kedaria M, Henny CP, Hardeman MR, Gelb AW. The clinical importance of erythrocyte deformability, a hemorrheological parameter. Ann Hematol 1992;**64**: 113-22.

Chapter 5

General Discussion and Conclusion

5.1 Review of Thesis Objectives

Human RBCs have been studied extensively in the last few decades in order to improve RBC preservation [1]. Developing RBC preservation strategies is important for clinical applications and to meet the growing demands of transfusion medicine [1-3]. At the present time, hypothermic storage is the most common method used to preserve the function, quality and integrity of RBCs in a liquid form for blood transfusion. Even though contemporary hypothermic storage temperatures are sufficient to store RBCs up to 5-7 weeks, significant biochemical and biomechanical alterations occur throughout the hypothermic storage period [1,4-11]. In light of recent studies, the effect of storage on the RBC membrane has become a focus of interest. Alterations in the red blood cell membrane, such as microvesiculation and changes in PS and CD47 expression, are important not only as a markers of storage lesion, but also as potential indicators of RBC *in vivo* survival and function as these changes enhance erythrophagocytosis after transfusion [12-16].

The objectives of this thesis were to improve our understanding of the effect of hypothermic storage on the RBC membrane *in vitro* quality parameters by investigating novel markers, including phospholipid loss through microvesiculation, and PS and CD47 expression using a developed multi-color flow cytometric analysis method. In addition, the second aim of this thesis was to

examine the effect of biochemical rejuvenation at various time points during hypothermic storage.

The results of the experimental studies have been presented to test the following hypotheses: 1) Multi-color flow cytometry is an effective method to identify, characterize and quantify RBC microparticles; 2) Hypothermic storage induces erythrocyte microvesiculation, PS externalization and reduction in the CD47 expression on RBCs and on RBC MPs; 3) Biochemical rejuvenation can be used as an approach to prevent or minimize changes in the RBC microvesiculation, PS and CD47 expression during hypothermic storage.

The objective of the first experimental study in this thesis was to determine if the hypothermic storage induced RBC microvesiculation could be identified, characterized and quantified using a multi-color flow cytometry analysis method (Chapter 2). Even though there are several methods and assays used to detect microparticles [17-19], flow cytometry method is the most common and ideal method to identify and quantify MPs [17,20]. Therefore, in Chapter 2, flow cytometry was used for a quantitative and qualitative analysis for the RBC and the RMPs after labelling the test samples with fluorescence monoclonal antibodies. RBC and RMPs were further characterized by the expression of the PS and CD47 on their surface. The sensitivity of RMP size method was shown to be able to detect RMP events as small as 0.20 μm in diameter. The results of this study demonstrate that the developed multi-color flow cytometry method is suitable to detect and characterize the changes in the RBC and their microparticles. A linear

relationship between the TruCOUNT beads concentration and number of acquired events was established. The developed method allows more in-depth monitoring of RBC quality during preservation and gives insight into the surface expression, and potentially the function, of RMPs *in vivo.* This method was used in subsequent chapters to study the effect of hypothermic storage and the rejuvenation on the composition of RBC and their microparticles.

The aim of chapter 3 of this thesis was to apply the flow cytometry analysis method to measure the hypothermic-storage induced changes in CD47 and PS on RBCs as well as quantitatively and qualitatively evaluate RMPs using flow cytometry**.** In addition to establishing a baseline of RBC quality during storage, other *in vitro* quality assays were measured, including ATP concentration and hemolysis. This allowed us to examine the relationship between these controversial *in vitro* quality parameters and RBC microvesiculation. This relationship was to determine if increased hemolysis or/and depletion of ATP is responsible for the microparticle accumulation during hypothermic storage. The results of this study showed that the number of RMPs/µL significantly increased and statistically significant changes of both PS and CD47 was observed by the end of storage period. Moreover, the data from this chapter demonstrated that there was a negative correlation identified between the ATP concentration and number of RMPs throughout storage where the greatest number of RMPs observed at the end of the storage period when the ATP concentration was at its lowest. It was shown that the changes in RBC membrane throughout storage have

the potential to influence other *in vitro* quality parameters. This could be explained in support with other studies where the ATP depletion during storage resulted in a shutdown of the trans-membrane ion pump activity [21,22] leading to an increase in the level of intracellular ions such as calcium [21-23]. Accordingly, the accumulation of the intracellular calcium may enhance the morphological changes and microvesiculation. These findings showed that the ATP depletion during storage modified the ability of the RBC membrane to release microparticles which have been shown to be harmful effectors of inflammation and coagulation post-transfusion. This study demonstrated that the use of the developed flow cytometry method is suitable to detect and analyze RBCs and their microparticles. Also, this study raised the concern that the membrane changes during storage may not be the actual harmful consequences but the effect of microvesicles and membrane loss might have on transfusion.

The purpose of chapter 4 was to examine the effects of biochemical rejuvenation on red blood cell microvesiculation, as well as PS and CD47 expression during hypothermic storage. Previous studies have suggested that ATP depletion during hypothermic storage is implicated in several processes of hypothermic storage lesion [9,24]. Rejuvenation solutions have been shown to be able to restore ATP and 2, 3-DPG levels in hypothermically stored RBCs [7,25-29]. The results of chapter 4 demonstrated that the biochemical rejuvenation used in this thesis was sufficient to restore the depleted ATP during the hypothermic storage but there was no significant effect in preventing or reversing RBC

microvesiculation. This study showed evidence that the RBC membrane lesions during storage are 2 types; reversible changes such as the metabolic alternations including the depletion of the ATP concentration, and irreversible changes such as the biomechanical alterations including RBC microvesiculation. Since ATP restoration did not prevent RMP generation or changes in PS and CD47 expression during storage, this suggested that there are other factors and mechanisms that contribute to these biomechanical alterations, not just ATP depletion. Even though biochemical rejuvenation did not prevent the biomechanical membrane lesion, the different time points of treatment with rejuvenation solution throughout hypothermic storage showed that the earlier biochemical rejuvenation (day 28) for the stored RBC had better improvement for the level of ATP versus late rejuvenation. These results suggested that improvement in the intracellular ATP concentration is also dependent on the storage time and not just on biochemical rejuvenation treatment.

5.2 Contributions to Science and Future Directions

This thesis has made a number of valuable contributions to the fields of the biopreservation and transfusion medicine. The development of a validated and effective flow cytometric method to identify and characterize RBCs and their microparticles throughout the hypothermic storage time, which reflects measurements of significant indicators of the quality of the RBC, is an important

contribution as standardized and validated protocols for quantitatively and qualitatively analyzing to the cell-derived microparticles are currently lacking. This work allows more in-depth monitoring of RBC quality during the current practice preservation for RBC and gives insight into the surface expression, and potentially the function, of RMPs *in vivo*. In light of the potential inflammation, coagulation, immunomodulation and other the observed changes to hypothermically stored RBC, further research into their clinical implications is required. In addition, this thesis has demonstrated that the use of biochemical rejuvenation was sufficient to restore the depleted ATP during hypothermic storage but there was no significant effect on the RMP generation or PS and CD47 expression throughout hypothermic storage. This has confirmed that some of the RBC hypothermic storage lesions, such as RBC microvesiculation, are irreversible. More advance techniques and methods focusing on stabilizing the RBC membrane during storage to improve the RBC *in vitro* quality parameters and to achieve better quality RBC products for transfusion are required. Furthermore, the novelty of different time points of treatment with rejuvenation solution throughout hypothermic storage showed that earlier biochemical rejuvenation for stored RBCs had better improvement for the level of ATP versus late rejuvenation. These results suggested that improvement in ATP levels is dependent not only on rejuvenation treatment but also on the storage time. The use of this strategy of rejuvenation treatment demonstrated that RBC rejuvenation is more effective in earlier stages of storage that late stages where the accumulation and the effects of storage lesion have already occurred. Further *in*

vivo and *in vitro* studies on the effects of biochemical rejuvenation on the quality and the integrity of the RBCs are warranted.

New preservative technologies to address the deteriorating condition of the membrane with the ultimate goal of stabilizing the membrane and decreasing microvesiculation throughout storage are strongly needed. The findings of this thesis provided knowledge that will contribute to have a better understanding of the RBC membrane lesion that occurs during the hypothermic storage and may lead to future developments in the biopreservation of the RBCs. Optimistically, the results, ways and means of this thesis will lead to further advancements in preserving RBCs and maintaining the integrity of the membranes for a readily available products for transfusion medicine.

5.3 References

1. Hogman CF, Meryman HT. Storage parameters affecting red blood cell survival and function after transfusion. Transfus Med Rev 1999;**13**: 275-96.

2. Acker JP. Biopreservation of cells and engineered tissues. Adv Biochem Eng Biotechnol 2007;**103**: 157-87.

3. Hogman CF. Liquid-stored red blood cells for transfusion. A status report. Vox Sang 1999;**76**: 67-77.

4. Kor DJ, Van Buskirk CM, Gajic O. Red blood cell storage lesion. Bosn J Basic Med Sci 2009;**9 Suppl 1**: 21-7.

5. Wolfe LC. The membrane and the lesions of storage in preserved red cells. Transfusion 1985;**25**: 185-203.

6. D'Alessandro A, Liumbruno G, Grazzini G, Zolla L. Red blood cell storage: the story so far. Blood Transfus 2010;**8**: 82-8.

7. Dumaswala UJ, Petrosky TL, Greenwalt TJ. Studies in red blood cell preservation. 6. Red cell membrane remodeling during rejuvenation. Vox Sang 1992;**63**: 12-5.

8. Antonelou MH, Kriebardis AG, Papassideri IS. Aging and death signalling in mature red cells: from basic science to transfusion practice. Blood Transfus 2010;**8 Suppl 3**: s39-47.

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

10. Holme S. Current issues related to the quality of stored RBCs. Transfus Apher Sci 2005;**33**: 55-61.

11. Hess JR, Greenwalt TG. Storage of red blood cells: new approaches. Transfus Med Rev 2002;**16**: 283-95.

12. Bessos H, Seghatchian J. Red cell storage lesion: The potential impact of storage-induced CD47 decline on immunomodulation and the survival of leucofiltered red cells. Transfus Apher Sci 2005;**32**: 227-32.

13. Greenwalt TJ. The how and why of exocytic vesicles. Transfusion 2006;**46**: 143-52.

14. Smith JE. Erythrocyte membrane: structure, function, and pathophysiology. Vet Pathol 1987;**24**: 471-6.

15. Stewart A, Urbaniak S, Turner M, Bessos H. The application of a new quantitative assay for the monitoring of integrin-associated protein CD47 on red blood cells during storage and comparison with the expression of CD47 and phosphatidylserine with flow cytometry. Transfusion 2005;**45**: 1496-503.

16. Chin-Yee I, Arya N, d'Almeida MS. The Red Cell Storage Lesion and its Implication for Transfusion. Transfus Sci 1997;**18**: 447-58.

17. Simak J, Gelderman MP. Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers. Transfus Med Rev 2006;**20**: 1-26.

18. Lacroix R, Robert S, Poncelet P, Dignat-George F. Overcoming limitations of microparticle measurement by flow cytometry. Semin Thromb Hemost 2010;**36**: 807-18.

19. Jy W, Horstman LL, Jimenez JJ, Ahn YS, Biro E, Nieuwland R, Sturk A, Dignat-George F, Sabatier F, Camoin-Jau L, Sampol J, Hugel B, Zobairi F, Freyssinet JM, Nomura S, Shet AS, Key NS, Hebbel RP. Measuring circulating cell-derived microparticles. J Thromb Haemost 2004;**2**: 1842-51.

20. Orozco AF, Lewis DE. Flow cytometric analysis of circulating microparticles in plasma. Cytometry A 2010;**77**: 502-14.

21. Messana I, Ferroni L, Misiti F, Girelli G, Pupella S, Castagnola M, Zappacosta B, Giardina B. Blood bank conditions and RBCs: the progressive loss of metabolic modulation. Transfusion 2000;**40**: 353-60.

22. Rapoport TA, Heinrich R, Rapoport SM. The regulatory principles of glycolysis in erythrocytes in vivo and in vitro. A minimal comprehensive model describing steady states, quasi-steady states and time-dependent processes. Biochem J 1976;**154**: 449-69.

23. Scott KL, Lecak J, Acker JP. Biopreservation of red blood cells: past, present, and future. Transfus Med Rev 2005;**19**: 127-42.

24. Kiartivich S, Talalak P, Chandanayingyoung D. The effect of rejuvenation on 2, 3-DPG level of preserved red blood cells. J Med Assoc Thai 1986;**69**: 665-71.

25. Koshkaryev A, Zelig O, Manny N, Yedgar S, Barshtein G. Rejuvenation treatment of stored red blood cells reverses storage-induced adhesion to vascular endothelial cells. Transfusion 2009;**49**: 2136-43.

26. Szymanski IO, Teno RA, Lockwood WB, Hudgens R, Johnson GS. Effect of rejuvenation and frozen storage on 42-day-old AS-1 RBCs. Transfusion 2001;**41**: 550-5.

27. Lockwood WB, Hudgens RW, Szymanski IO, Teno RA, Gray AD. Effects of rejuvenation and frozen storage on 42-day-old AS-3 RBCs. Transfusion 2003;**43**: 1527-32.

28. Valeri CR. Use of Rejuvenation Solutions in Blood Preservation. Crc Cr Rev Cl Lab Sc 1982;**17**: 299-374.

29. Verhoeven AJ, Hilarius PM, Dekkers DWC, Lagerberg JWM, de Korte D. Prolonged storage of red blood cells affects aminophospholipid translocase activity. Vox Sang 2006;**91**: 244-51.