The Effects of Liposome Treatment on Red Blood Cells during Hypothermic Storage

by

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Abstract

Red blood cells (RBCs) are the most commonly used components in cell therapy and their transfusion save millions of lives every year. These benefits were only achieved through advances in blood banking storage techniques that guarantee an available supply of blood to support medical emergencies and treatments. Although use of additive solutions extends storage length of RBC units, the quality of stored RBCs progressively decreases during hypothermic storage giving rise to a series of biochemical and biomechanical changes, collectively known as "hypothermic storage lesion" (HSL). Since membrane integrity is an important predictor of RBC survival and function and constitutes one of the targets of HSL, this research has focused on the use of liposomes, synthetic lipid vesicles, to mitigate RBC membrane injury during hypothermic storage.

This thesis tested the hypothesis that liposome treatment of stored RBCs would improve *in vitro* membrane quality resulting in reduced *in vitro* production of proinflammatory and procoagulant markers and a safe transfusion product in an anemic rat model. Investigations were conducted on several levels, from assessing baseline differences between rat and human RBCs and the effect of blood component manufacturing on rat RBCs to transfusion of liposome treated-RBCs in a rat model and evaluation of the impact of liposome treatment on hypothermic storage lesion and consequent effects on hemorheologic, immune and coagulatory profile of human blood banked RBCs.

The work presented here has established a processing method more suitable for use in animal models of transfusion evaluating HSL as well as demonstrated the effect of DOPC liposomes on rat RBC hemorheology and showed for the first time the *in vivo* effects of transfusing liposome-treated RBCs in an animal model. Furthermore, it has verified the benefit

of liposome treatment in human RBCs by fully characterizing the effects of DOPC liposomes on membrane and metabolic *in vitro* quality parameters in human RBCs during hypothermic storage. Finally, it has produced novel information about the potential effects of DOPC-treated RBCs and supernatants on the immune response using different cell types, a comprehensive cytokine panel and endothelial activation markers, relevant to current understanding of *in vivo* inflammatory effects. This thesis has advanced the knowledge of transfusion medicine and biopreservation by offering important insights into the effects of liposome treatment as a tool to mitigate HSL in RBCs that might lead to novel research efforts and unveil the potential of liposomes for biopreservation of other clinically relevant cell types.

Preface

This thesis is an original work by Luciana Da Silveira Cavalcante. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Liposomes in Transfusion Medicine: An Approach for Reducing the Red Blood Cell Hypothermic Storage Lesion", No. Pro00000700, 2014-01-25. It, also, received research ethics approval from the Canadian Blood Services Ethics Board, Project Name "Liposomes in Transfusion Medicine: An Approach for Reducing the Red Blood Cell Hypothermic Storage Lesion", No. 2008-022, 2015-05-06. Experimental protocols were approved by the Animal Use Committee at the University of Western Ontario, Project Name "Modulation of Myocardial Function in Myocardial Infarction, Sepsis, Anemia and Diabetes", No. 2007-011-03, 2012-01-19.

In Chapter 2, potassium supernatants were analyzed by Laboratory Services at the University of Alberta Hospital. The aggregation protocol was developed with the help of undergraduate summer student Jessica Asgarpour under my supervision. Osmotic fragility data collection was performed by HYRS student Diana Yu under my supervision. The remaining data was collected by myself.

Some of the research conducted for this thesis forms part of a research collaboration, led by Professors Dr. Jelena Holovati and Dr. Jason Acker at the University of Alberta, with Professors Dr. Ian Chin-Yee and Dr. Qingping Feng being the collaborators at the University of Western Ontario. Postdoctoral researcher Dr. Dong Kuang, undergraduate student Nan Li and laboratory technician Sharon Lu from Dr. Feng's Lab at the University of Western Ontario performed the *in vivo* experiments described in Chapter 3. The remaining *in vitro* data was collected by myself.

In Chapter 4, potassium supernatants were analyzed by Laboratory Services at the University of Alberta Hospital. Data collection of ATP and 2,3-DPG was performed by MLS honors student Melissa Shyian under my supervision. The remaining data was collected by myself.

Cytokine analysis in Chapter 5 was done in collaboration with Dr. Donald Brach at Canadian Blood Services in Toronto. Multiplex Cytokine analysis was performed by Dr. Trang Duong from The Hospital for Sick Children Research Institute in Toronto. The data collection for the MMA assay was performed by MLS honors student Melissa Shyian under

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In Chapter 6 Rejuvesol was provided by Zimmer Biomet (IN, USA). Hematological indices and deformability data collection was performed with the help of a MSc student Betty Kipkeu and a Doctoral student Ruqayyah Alzmiraq (Lab Medicine and Pathology). The remaining data was collected by myself.

The experimental designs in Chapters 2, 3, 4 and 5 were developed by myself, the experimental design in Chapter 6 was developed with the assistance of Drs. Acker and Holovati. The data analysis in chapters 2, 3, 4, 5 and 6 and concluding analysis in chapter 7 are my original work, as well as the literature review in chapter 1.

Section 1.4 of Chapter 1 of this thesis has been published as Da Silveira Cavalcante L, Holovati JL, Acker JP. Chapter 9: Application of liposomes in biopreservation. In: *Multiscale Techonologies for Cryomedicine 2016, 301-27.* Publisher: World Scientific. And is being reproduced here with permission from the copyright holder World Scientific Publishing Co. Pte. Ltd.

Chapter 2 of this thesis has been published as Da Silveira Cavalcante L, Acker JP, Holovati, JL. Differences in rat and human erythrocytes following blood component manufacturing: the effect of additive solutions. Transfusion Medicine and Hemotherapy, 2015; 42(3):150-7. I was responsible for the data collection and analysis as well as the manuscript composition. Acker JP contributed to concept formation and manuscript edits. Holovati, JL was the supervisory author and was involved with concept formation and manuscript composition.

Chapter 3 of this thesis has been published as Da Silveira Cavalcante L, Feng Q, Chin-Yee I, Acker JP, Holovati, JL. Effect of liposome treated red blood cells in an anemic rat model. Journal of Liposome Research, 2017; 27(1):56-63. I was responsible for the data collection and analysis as well as the manuscript composition. Feng Q assisted with data collection and manuscript edits. Chin-Yee I assisted with data collection and manuscript edits. Acker JP contributed to concept formation and manuscript edits. Holovati, JL was the supervisory author and was involved with concept formation and manuscript composition.

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liposome-treated red blood cells. Journal of Liposome Research. 1-9 (published online). Branch DR contributed to manuscript edits. Duong TT and Yeung RSM assisted with data collection. Acker JP contributed to concept formation and manuscript edits. Holovati, JL was the supervisory author and was involved with concept formation and manuscript composition.

This thesis is dedicated to my beloved parents, Humberto and Ana Maria Cavalcante, my pillars of strength. Thank you for all your love and support.

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List of Abbreviations

ACD	acid citrate-dextrose
AI	aggregation index
Amp	aggregation amplitude
ANOVA	analysis of variance
APC	allophycocyanin
aPTT	activated partial thromboplastin time
ATP	adenosine triphosphate
ASs	additive solutions
AS3	additive solution 3
au	arbitrary units
BSA	bovine serum albumin
BTHC	butyryltrihexylcitrate
Ca^{++}	calcium
$CaCl_2$	calcium chloride
CBS	Canadian Blood Services
CHOL	cholesterol
Cl^-	chloride
C/P ratio	cholesterol/phospholipid ratio
CPD	citrate-phosphate-dextrose
CPDA-1	citrate-phosphate-dextrose-adenine-1
CO_2	carbon dioxide
DEHP	diethylhexyl phthalate
DINCH	1,2-cyclohexane-dicarboxylic acid diisononyl ester
DLS	dynamic light scattering
DPPC	dipalmitoylphosphatidylcholine
DOPC	dioleoylphosphatidylcholine
EBM-2	endothelial basal medium-2
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EI_{max}	maximum elongation index

FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF-2	fibroblast growth factor-2
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
Hb	hemoglobin
Hct	hematocrit
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HiCN	cyanmethemoglobin
HS	hypothermic storage
HSL	hypothermic storage lesion
HUVECs	human umbilical vein endothelial cells
IFN	interferon
IL	interleukin
I/R	ischemia and reperfusion
K^+	potassium
K_{EI}	shear stress required to achieve half of the EImax
LORCA	laser-assisted optical rotational cell analyzer
LPS	lipopolysaccharide
MCF	mean corpuscular fragility
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCP	monocyte chemoattractant protein
MCV	mean corpuscular volume
MDA	malondialdehyde
MDC	macrophage-derived chemokine
MFI	mean fluoresce intensity
MIP	macrophage inflammatory protein
MMA	monocyte monolayer assay
MPs	microparticles

NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NaH_2PO_4	sodium dihydrogen phosphate (anhydrous monobasic sodium phosphate)
NO	nitric oxide
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PC	phosphadylcholine
PE	phycoerythrin (flow cytometry context)
PE	phosphatidylethanolamine (phospholipid context)
PerCP-Cy™ 5.5	peridinin chlorophyll protein complex-cyanine 5.5
PIPA	pyruvate-inosine-phosphate-adenine
PO	polyolefin
pRBCs	packed red blood cells
PS	phosphatidylserine
PT	prothrombin time
PVC	polyvinyl chloride
RBCs	red blood cells
SAGM	saline-adenine-glucose-mannitol
SM	sphingomyelin
SUVs	small unilamellar vesicles
$t_{1/2}$	aggregation half-time
Tc	gel to liquid-crystalline phase transition temperature
TGF	transforming growth factor
TNF	tumor necrosis factor
TRALI	transfusion-related acute lung injury
TTC	triphenyltetrazolium chloride
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
2,3-DPG	2,3-diphosphoglycerate

Chapter 1*

Introduction

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1.1 Red blood cells

Red blood cells (RBCs), or erythrocytes, are anucleate biconcave discs of about 8 μm in diameter that are responsible for transporting oxygen to the tissues. They have an *in vivo* life span of 120 days and obtain energy from anaerobic glycolysis. They are composed of a flexible membrane bilayer that allows them to deform and pass through small capillaries (1). The main component of the cytoplasm portion is hemoglobin (Hb) a protein composed of two α and two β polypeptide chains (globins) each one containing a heme group with an iron molecule in the center, that serves as the binding site for oxygen (2).

1.2 Blood banking and transfusion medicine

Blood transfusion is a lifesaving treatment for patients with massive blood loss (i.e. surgery or trauma), chronic hemolytic anemias (i.e. sickle cell anemia, thalassemia) and decreased erythropoiesis (i.e. cancer, aplastic and chronic disease anemia), as well as a supportive therapy, as it optimizes oxygen delivery and tissue perfusion (3, 4). The number of units can range from 2-5 for surgery patients and up to 50 units for car accident victims. Approximately 85 million units of red blood cells are transfused globally every year (5). In Canada, about 850,000 RBC units were transfused into patients in 2011/2012 (6). The clinical benefits of blood transfusion were only made possible through the development of techniques to preserve *ex vivo* RBC viability, which allowed the blood donor and transfusion recipient to be separated in time and space (7).

The first reports related to blood storage date back to World War I where it was collected and stored in glass bottles developed by Oswald Robertson, containing a citrate-glucose solution discovered by Rous and Turner in 1915 that allowed blood to be stored for a

few weeks after collection (8). The first storage solutions, based on the one of Rous & Turner, composed of citrate (anticoagulant) and dextrose (nutrient) allowed blood to be stored for 3 weeks, such as acid citrate-dextrose (ACD) and citrate-phosphate-dextrose (CPD) (8). In the 70's it was discovered that the addition of adenine would extend the storage time to 5 weeks, and so citrate-phosphate-dextrose-adenine 1 (CPDA-1) and CPD combined with saline-adenine-glucose (SAG) started to be used (8, 9). Additive solutions, such as saline-adenine-glucose-mannitol (SAGM) and additive solutions (AS) 1 and 3 have effectively extended RBC storage for up to six weeks (8, 10). In the period following the discovery of the first storage solution, transfusions where performed using whole blood and only in the 1950's the development of a component therapy was possible, with the introduction of plastic bags by Walter and Murphy that replaced the glass bottles for the collection and storage of blood (11).

1.2.1 RBC manufacturing methods

In Canada, Canadian Blood Services (CBS) is responsible for blood collection, manufacturing and distribution across the country, with the only exception being the province of Quebec that has their blood supply managed by Hema-Quebec. Blood for transfusion is collected as whole blood and processed into different components: packed RBCs (pRBCs), platelets and plasma (12, 13). There are different ways of obtaining the same blood components (apheresis vs. whole blood collection) and variations between processing methods (automated vs. manual), for the purpose of this review, the focus will be on two processing methods currently used by CBS (13).

With the buffy coat production method, a bag of whole blood (450 mL ± 30 mL) is collected in CPD anticoagulant (70 mL). After collection, the blood is cooled to room temperature (18-24 °C) and held overnight. The next step is centrifugation using a moderate

spin (3,000-3,500 x g) to separate the blood into three layers: plasma (top layer), buffy coat (middle layer) and red blood cells (bottom layer) (Figure 1.1A). After separation plasma is extracted to a top satellite bag, packed red blood cells are extracted to a bottom satellite bag and the buffy coat remains in the original bag. Buffy coats (four) are further pooled in plasma to produce a unit of pooled platelet concentrate. The additive solution SAGM (110 mL) is added to packed RBCs and the mixture RBCs/SAGM is further passed through a leukoreduction filter at room temperature, within 24 hours of collection (10, 14-16).

With the whole blood filtration method, a bag of whole blood (450 mL ± 30 mL) is collected in CPD anticoagulant (70 mL). After collection, the blood is cooled and passed through a leukoreduction filter while refrigerated, within 72 hours of collection. The leukoreduced whole blood is then centrifuged using a hard spin (≥ 4500 x g) to separate plasma and red blood cells (Figure 1.1B). The plasma is extracted to a satellite bag and 110 mL of SAGM is added to RBCs in the remaining bag (10, 14-16).

The final product on both processing methods is packed leukoreduced CPD-SAGM RBCs. The volume ranges from 250-350 mL with a 55-65 % hematocrit (15). These RBC units are stored under hypothermic conditions (1-6 °C) up to a maximum of 42 days according to recommendations by regulatory bodies (US Food and Drug Administration and Health Canada). To meet regulatory standards, in Canada, RBC units must have a hematocrit of ≤ 80% throughout storage, hemoglobin of at least 40 g/unit and hemolysis lower than 0.8% at expiry (17). The 24-h post-transfusion survival of RBCs must be at least 75% (18).

Although the final product is theoretically the same, the differences in manufacturing have been shown to produce RBC units with different *in vitro* quality (15, 16). For the purpose of this thesis, all RBCs used were produced using the buffy coat production method, or an

adaptation of it that was developed on Chapter 2 to be applied to rat RBCs for further use in an animal model.

1.3 RBC hypothermic storage lesion

Although the use of additive solutions extended storage length of RBC units, the quality of stored RBCs progressively decreases during hypothermic storage (1-6 °C). RBCs undergo a series of biochemical, biomechanical and oxidative changes, collectively known as "hypothermic storage lesion" (HSL). Characteristics of the HSL include RBC membrane remodeling, decreased metabolites, loss of intracellular potassium, oxidative injury of proteins and lipids, membrane loss, microparticle (MP) release and ultimately hemolysis (19-22) (Figure 1.2).

1.3.1 Effect of storage on RBC metabolome

RBCs obtain energy from the anaerobic breakdown of glucose to lactate that generates high-energy phosphate molecules (i.e. adenosine triphosphate - ATP) (Figure 1.3) (23). Metabolic activities are higher under physiological temperatures (~37 °C), by storing RBCs at 4 °C the metabolic activities are slowed down guaranteeing that cells will have enough nutrients from additive solutions to last through their shelf life (24). Other characteristics of the storage medium, like pH, are also important determinants of metabolic pathways (25). 2,3-diphosphoglycerate (2,3-DPG) is another end product of metabolism that determines the affinity of the hemoglobin molecule for oxygen, and therefore its production is directly associated with RBC's main function of delivering oxygen to the tissues (26). Under physiological conditions there is a balance between the production of ATP and 2,3-DPG, but under storage conditions this balance is lost (27). The pH of packed RBCs at the beginning of storage is around 7.1, combined with the low temperature that slows down glycolysis, this

environment facilitates the breakdown of 2,3-DPG as the main source for obtaining ATP (28). Consequently an increase in ATP will be observed while 2,3- DPG decreases, and will be depleted after 2 weeks of storage (7). The accumulation of lactate and protons will contribute to further acidity of the media that will decrease glycolysis by inhibiting enzyme activity of hexokinase and phosphofructokinase (25), and ATP levels will continue to decline shutting down ion pumps and lipid transporters that are ATP-dependent.

1.3.2 Effect of storage on RBC membrane and hemorheology

Experimental *in vitro* depletion of ATP has been shown to induce shape changes in RBCs (29). Red cell shape during storage progressively change from biconcave disks to echinocytes and spherocytes (20). It is proposed that ATP modulates cells shape by maintaining the balance of ions between the intracellular and extracellular medium (20), as well as by controlling transmembrane lipid transporters and interactions between membrane bilayer and cytoskeleton (30). Changes in pH and accumulation of potassium and lactate have also been associated with shape changes (20, 31).

Other alterations in RBC membrane during storage include oxidative damage of proteins and lipids and loss of lipids through membrane shedding. Autoxidation of hemoglobin is implicated in the generation of reactive oxygen species that will further contribute to oxidative damage (32, 33), while glycation (HbA1c) could contribute to alterations in hemorheology (34). Malondialdehyde (MDA) a marker of lipid peroxidation has also been detected in stored RBCs units and has been shown to increase with storage time (35). Lipid oxidation and protein aggregation/degradation (i.e. Band 3, Hb) could lead to vesicle formation from RBCs and loss of membrane (36, 37) that could account for the progressive decrease in deformability observed with prolonged storage (33, 38). A decrease in

RBC aggregation has also been reported during storage (33, 39). There are concerns that early removal of RBCs from circulation and/or impaired hemorheology after transfusion could be a consequence of these changes.

1.3.3 Effect of storage on RBC microvesiculation

Microvesiculation is the process by which extracellular vesicles (EVs) are formed and released *in vivo* and *in vitro* by cells in response to a variety of conditions. Cells can release a mixed population of EVs that based on size and origin can be divided into exosomes (~50 to 100 nm) or microparticles (~50 to 1000 nm). Exosomes originate from multivesicular bodies, while MPs originate from the plasma membrane (40). Microparticles are present in blood under physiological conditions and their concentration may increase under pathological conditions and *ex vivo* cold storage (41).

The loss of RBC membrane phospholipid asymmetry ultimately leads to PS externalization, which will culminate in MP formation and release (42). Several mechanisms can be attributed to MP shedding from RBCs during hypothermic storage including protein oxidation (43), ATP depletion (44) and increased intracellular Ca^{2+} concentrations (45). Almizraq and colleagues (2013) have reported increased MP concentration in RBC units with increasing storage time (46). A study by Salzer *et al*. (2008) compared MPs from stored blood to MPs produced *in vitro* by calcium ionophore. They found both MPs to be similar in size, in thrombogenic activity, and in membrane protein composition, with the major difference being the concentration of integral proteins stomatin and flotilin-2 (36). MPs derived from blood cells have been generally shown to exhibit both procoagulant and proinflammatory activities (41). Current strategies to preserve *in vitro* RBC quality prior to transfusion do not adequately address the loss and remodeling of RBC membrane.

1.3.4 Effect of storage on RBC immune and coagulatory profile

The loss of membrane lipid asymmetry that leads to phosphatidylserine (PS) exposure is one of the accepted mechanisms of immunomodulatory and coagulatory properties associated with transfusion of stored RBCs. Phosphatidylserine is a negatively charged phospholipid, that remains in the inner part of the membrane bilayer under physiological conditions (47). Its translocation to the outer part of the bilayer signals RBC removal from the circulation or initiation of the clotting cascade (48-50). During storage, the decrease in ATP levels leads to the shutdown of ATP-dependent lipid transporter (i.e. flippase) that is responsible for maintaining lipid asymmetry (51). Consequently, stored RBCs and MPs will express more PS on their surface and that will cause them to adhere to the endothelium initiating an inflammatory response (52). Since PS is also a signal for removal, the larger number of PS expressing RBCs and MPs might also increase activation of cells from the reticuloendothelial system with release of cytokines (53, 54).

In terms of coagulation, PS provides a structure for the assembly of coagulation factors that will initiate the clotting cascade making PS exposure a significant contributor for hypercoagulatory states (41, 55-57). In addition to the role of PS, it has also been proposed that free hemoglobin and RBC-derived MPs containing hemoglobin can uptake nitric oxide (NO) reducing the bioavailability of NO to the endothelium causing injury and dysfunction that will contribute to changes in homeostasis leading to inflammation and coagulation (58, 59).

Although effects of storage lesion may play a role in immunomodulation, recent studies attribute the immunomodulatory effects of RBC units to residual leukocytes and residual plasma present in RBC units that could vary with difference processing methods and donor characteristics such as gender and age (60).

1.3.5 Approaches to evaluate the impact of HSL on transfusion

Many clinical concerns have led to a call to investigate HSL *in vivo* using animal models. Several animal models have been used in studies trying to show the effects of "fresh" vs. "old" blood transfusion. Hod and colleagues (2010) using a mouse model, showed harmful effects related to inflammation produced by transfusing old stored murine RBCs (61). While Hendrickson *et al.* (2011) showed that transfusing mice with fresh RBCs would reverse the adverse events caused by the transfusion of old RBCs (62). In rats it was shown that microcirculatory oxygenation is not restored with transfusion of old RBCs after hemorrhagic shock (63); the transfusion of fresh but not old RBCs reduced infarct size (64) and transfusion of old RBCs causes neuroinflammation and reduces cognitive function (65). In guinea pigs, old RBC transfusion has led to intravascular hemolysis, hypertension and vascular injury (66). In healthy dogs, it was shown that transfusion of old RBCs produces an inflammatory response (67) and transfusion of old blood increased mortality in canines with pneumonia (68). Old RBCs and RBC supernatants have also been shown to induce acute lung injury in rats (69) and pigs (70) and pulmonary hypertension in sheep (71). Because animal models demonstrated sensitivity to the effects of blood storage, and are regularly used in preclinical safety studies (72), a well-stablished rat model of myocardial ischemia and reperfusion was chosen for this project (64, 73, 74). Considering that, there are similarities between RBC species in terms of membrane lipid and protein composition and membrane lipid asymmetry (75-77).

Animal models provide a great opportunity to understand physiology and investigate mechanisms of diseases and while there is no doubt about their contribution to advance knowledge in medical research (78), there are also many limitations and pitfalls attributed to these studies. Therefore, studies involving animal models to mimic clinical settings in humans,

need to be interpreted with caution due to limitations such as species differences, group sizes, simplified conditions and a possible underestimation of the adaptive response in animals (79, 80).

Given the *in vitro* data on HSL in stored RBCs and the *in vivo* data obtained using animal models, several large clinical trials further investigated the relationship between the age of blood transfused and adverse events in patients. The most recent ones are presented here, with some trials still ongoing.

The first study to be completed was the "Age of Red Blood Cells in Premature Infants" (ARIPI), done in Canada evaluating premature infants. It was a double-blind, randomized controlled trial that evaluated 377 premature infants in a period of six years. The primary outcome measured was neonatal morbidities. In this study, the fresh blood group received RBC units that were stored for 7 days or less (3-7 d), and the old blood group received standard-issue RBCs in accordance with standard blood bank practice (14-22 d). They concluded that the use of fresh RBCs did not improve the outcomes in premature infants requiring blood transfusion, compared to standard-issued RBCs (81).

The "Age of Blood Evaluation" (ABLE) was a multicenter, blinded, randomized controlled trial that evaluated 2,430 patients in 64 centers in Canada and Europe over a period of five years. The study population was critically ill adults that were assigned to receive either RBCs stored for less than 8 days (6.1 ± 4.9 d) or standard-issue RBCs (22.0 ± 8.4 d). The main outcome measure was 90-day mortality. The study concluded that, transfusion of fresh RBCs did not decrease mortality in critically ill patients compared to standard-issue RBCs (82, 83).

The "Red Cell Storage Duration Study" (RECESS) was a multicenter, randomized trial that evaluated 1098 patients in centers across the United States over a period of four years. The study population was patients undergoing complex cardiac surgery. One group of patients

was assigned to receive RBCs stored for 10 days or less and the second group assigned to receive RBCs stored for 21 days or more. The primary outcome measured was the change in multiple organ dysfunction score (MODS: 0-24), with higher score values indicating severe organ dysfunction. This trial concluded that, the duration of RBC storage did not cause significant changes in MODS, and therefore the transfusion of fresh RBCs was not superior to transfusion of older RBCs among this patient population (84, 85).

The "Informing Fresh versus Old Red Cell Management" (INFORM) was a multicenter, randomized controlled trial that evaluated 20,858 patients in six hospitals in Australia, Canada, Israel and the United States over a period of three years. The study population was hospitalized patients who required RBC transfusion (less than 10 units at a time). Patients were assigned to receive RBC units that have been stored for the shortest (average of 13.0 days) or the longest (average of 23.6 days) period. The primary outcome measured was in-hospital mortality. The study found no significant difference in mortality among those who received the freshest available RBCs and those who received the oldest available RBCs (86).

The "Red Cell Storage Duration and Outcomes in Cardiac Surgery" is a single-center, randomized controlled trial that intends to evaluate 2,800 patients in The Cleveland Clinic in Ohio (USA). The study population is cardiac surgery patients receiving RBCs stored for 14 days or less and 20 days or more. The outcome measured is postoperative morbidity (30 days post surgery) (ClinicalTrials.gov No.: NCT00458783) (87).

The "Standard Issue Transfusion Versus Fresher Red Blood Cell Use in Intensive Care" (TRANSFUSE) is a multi-center, double blind, randomized controlled trial that intends to evaluate 5,000 patients in Australia, New Zealand, Europe and the Middle East. The study population is critically ill intensive care unit adult patients receiving standard of care blood

transfusions (oldest RBCs) or freshest available RBCs. The primary outcome measured is 90-day mortality (ClinicalTrials.gov No.: NCT01638416) (88, 89).

So far, all these large clinical trials seemed to come to the same conclusion: that fresh blood is not superior to standard-issue blood. However because what they considered "old" was blood stored for only 21-28 days, they were not able to answer the question of whether old blood (35-42 days) is harmful. This question is difficult to be addressed by clinical trials because of ethical concerns of assigning patients to receive only extremely old blood, based on the *in vitro* data showing the presence of the storage lesion. Therefore, more research is needed to understand the impact of HSL in transfused patients.

1.3.6 Approaches to minimizing HSL

Additive solutions maintain some aspects of RBC quality and function but many remain unaddressed as previously described in section in 1.3. Alternate solutions have been investigated in order to decrease cell injury during storage, such as anaerobic storage, the use of plasticizers, rejuvenating solutions and a new generation of additive solutions.

The anaerobic storage proposition is based on the fact that RBCs stored under aerobic conditions undergo oxidative damage, that affects lipids and proteins. Therefore, by removing oxygen at the beginning of storage and maintaining the oxygen-free environment throughout storage, would stop hemoglobin denaturation pathways and other oxidative reactions facilitated by oxygen and result in improved cell quality (90). The anaerobic condition is achieved by equilibrating RBC with argon gas prior to refrigeration, then the blood bag is stored in a canister containing argon and hydrogen gases in a proportion of 9:1 (90). Anaerobic storage has been shown to extent RBC storage time up to 9 weeks (91, 92). A clinical study using anaerobic stored RBCs have shown superior *in vivo* recovery at 6 weeks

compared to control aerobic stored RBCs and comparable recovery values at 9 weeks of storage, after autologous transfusion (93).

Plastic collection bags became commercially available in the 1960's and replaced glass bottles for storage of blood components (8). Bags are made of the synthetic plastic polymer polyvinyl chloride (PVC), which is very stiff and therefore requires the addition of a plasticizer to confer flexibility (94, 95). The plasticizer most commonly used is diethylhexyl phthalate (DEHP), and can make up to 40% of the total weight of a PVC film (94). DEHP has also been shown to interact with RBCs by entering their membrane and reducing hemolysis and membrane loss through microvesiculation (8). However, concerns about the toxicity and carcinogenicity of DEHP (96) have led to investigations of new plasticizers that could possibly replace DEHP in terms of positive interactions with RBCs without the potential health hazard (95). Possible replacements being investigated include: butyryltrihexylcitrate (BTHC) and 1,2-cyclohexane-dicarboxylic acid diisononyl ester (DINCH). An earlier study evaluating BTHC have shown protection against hemolysis, attributed to the BTHC component hexanol, and similar levels of ATP and lipid membrane loss compared to DEHP (97). A more recent study comparing DEHP, BTHC and DINCH bags showed that BTHC conferred less protection against vesiculation, osmotic stress and hemoglobin leakage compared to DEHP and DINCH bags (98). Another study also confirmed the lower performance of BTHC bags and added that DINCH bags maintained better ATP levels compared to DEHP (99). The use of PVC-free bags has also been investigated with the use of polyolefin (PO) that does not require a plasticizer. One study evaluating PO bags found no superior effects in terms reducing hemolysis and maintaining ATP levels compared to regular PVC bags (100), while a more recent study using similar bags did not include a comparison with regular PVC bags and therefore results are difficult to interpret (101).

Building on the improved understanding of additive solutions and the impact its components have on RBC quality, rejuvenating solutions were created. A better understanding of the conditions that modulated metabolic pathways of RBCs as well as the role of 2,3-DPG in oxygen affinity in the late 60's (102, 103) led to studies investigating methods to restore metabolites in stored RBCs, especially 2,3-DPG, in the early 70's using pyruvate, inosine and phosphate (PIP) (104-106). Valeri & Zaroulis from the Naval Blood Research Laboratory (Chelsea, MA, USA) first used the term "rejuvenation" in a publication of 1972 (106). Another military research followed in 1974 by DeVenuto and colleagues from the Army Medical Research Laboratory (Fort Knox, KY, USA), continuing with the term rejuvenation and adding adenine to the PIP mix (107), originating the PIPA solutions that are still used nowadays. Rejuvenation solutions aim to boost RBC metabolism by providing substrates needed for the Embden-Meyerhof glycolytic pathway (generation of ATP) and the Rapoport-Luebering shunt (generation of 2,3-DPG) (Figure 1.3) (108). Many studies have documented the effects of rejuvenation on stored RBCs including restoration of 2,3-DPG and ATP levels, improved morphology and decreased adhesion to the endothelium (109-111).

Rejuvenation is a time consuming process and RBCs can only be stored for up to 24 h after rejuvenation, therefore logistically it would be more advantageous if the additive solutions could provide similar benefits. There are several ASs currently licensed for use in different countries including ADSOL (AS1), Nutricel (AS3), Optisol (AS5) and SAGM, with slightly variations in their composition but generally hypertonic solutions, which focus on maintaining the metabolic function of RBCs during storage, but fail to prevent membrane loss and consequent MP formation (46, 112). Recently, next-generation ASs have been developed (i.e. Erythro-Sol 5 and SOLX or AS7) and they differ from conventional ASs in the sense that they are buffered and more hypotonic (112-114). So far, SOLX is the only next-generation AS

to obtain approval from US Food and Drug Administration (FDA). The new solution has been shown to reduce storage lesion (115), to limit the effect of overnight room temperature-hold of whole blood on RBC biochemical variables (116) and to extend shelf-life from 6 to 8 weeks (although the FDA approval is limited to the 6 week period) (114). The beneficial effects of this new additive solution seem to be provided by metabolic modulation as a study by D'Alessandro and colleagues suggests that the new formulation improves energy and redox metabolism in stored RBCs (117).

1.4 Liposomes as an approach to HSL*

As highlighted in section 1.3.2, membrane remains a prominent target of HSL not completely addressed by current preservation approaches. In the meantime previous studies have shown that liposomes conferred membrane protection during freezing (118, 119) and lyophilization (120). Liposomes are defined as microscopic lipid vesicles composed of a central aqueous core surrounded by one or more phospholipid bilayers (Figure 1.4). They can be classified in terms of size, lamellarity and preparation method (121).

Liposomes constitute an important tool for preservation of RBCs not only because of their delivery capacity, but also because the lipids present in the liposomes themselves can act as membrane stabilizers. Mature RBCs lose the capacity to synthesize phospholipids, so they rely on lipid exchange with plasma lipoproteins to renew their membrane phospholipids and acylation of lysophospholipids (122-124). In the blood bank processing of whole blood into a packed RBC unit, the lipoproteins remain in the plasma fraction, which is separated from RBCs. Therefore, during the next 42 days of storage following manufacturing, RBCs no

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longer have a source of lipid exchange relying exclusively on acylation reactions that are normally reduced during storage (125).

1.4.1 Structural components and characteristics

Composition

Liposomes are mainly composed of phospholipids and cholesterol. Phospholipid molecules are composed of a hydrophilic head group (e.g. phosphatidylcholine, phosphatidylethanolamine), a glycerol backbone and two hydrophobic fatty acids (e.g. palmitic, oleic, stearic) (Figure 1.5) (126). They can be extracted from natural sources (i.e. egg, soy, tissues) or artificially synthesized (127).

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) phospholipids are the major constituents of cell membranes and therefore the major constituents of liposomes designed for biological applications (128, 129). The fatty acid portion can vary in number of carbon atoms and degree of unsaturation, and are important determinants of the liposome-cell interaction.

Liposomes containing phospholipids with short fatty acid chains (12–14 carbons) have been shown to induce cell lysis, probably due to extraction of cholesterol from the cell membrane or rapid uptake and replacement of the native phospholipid by the short chain phospholipid. Either mechanism results in destabilization of the cell membrane with consequent lysis (130-132).

Unsaturation refers to the presence of double bonds on the fatty acid chain, numbers can range from zero (saturated) up to six double bonds in each chain. Unsaturated fatty acids (i.e. oleate-18:1 and linoleate- 18:2) have been shown to be less toxic to bilayer membranes than saturated ones (i.e. palmitate- 16:0) (133). Although literature reports have shown that

liposomes composed of unsaturated fatty acids had better preservation effects for freeze-dried and freezing of human red blood cells, while liposomes composed of saturated fatty acids conferred protection for hypothermic storage (1-6 °C) of human red blood cells (120, 134).

Cholesterol (another major component of cell membranes) is also normally included in the liposome preparation as it improves the fluidity and stability of the bilayer (135).

Size and lamellarity

Size and lamellarity are the most common properties used to classify liposomes, and less frequently, the preparation method. In terms of size, they can be divided into small (~ 100 nm), large (~ 400 nm) and giant (> 1 μm) vesicles. In terms of lamellarity they can be divided into unilamellar (ULV), multilamellar (MLV) and multivesicullar (136, 137).

Size should be considered for delivery purposes, if a substance has protective effects in a dose-dependent manner, large liposomes are able to entrap a larger volume of the substance to be delivered. Liposome size has also been implicated in interactions with the immune system. Liposomes larger than 100 nm can induce secretion of various types of cytokines (138-140).

Lamellarity, on the other hand, is related to the release capacity, where more layers means slower release. The characteristics of the molecules to be encapsulated also play a role in deciding between ULVs and MLVs. ULVs are more efficient for encapsulation of hydrophilic molecules due to increased internal aqueous volume, while MLVs having more lipid content are capable of encapsulating larger amounts of hydrophobic molecules (141).

Surface charge

The head group composition of the phospholipid and pH will determine the surface charge of liposomes. Liposomes composed of PC, sphingomyelin (SM) and PE (pH 7–7.4) will have a neutral charge. Liposomes containing phosphatidic acid (PA), PS and

phosphatidylglycerol (PG) (pH 7–7.4) will have a negative charge. Liposomes containing trimethylammonium (i.e. DOTAP) or sterylamine (SA) (pH 7–7.4) will have a positive charge (126, 142).

Surface charge is particularly important for fusion of liposomes and cells, which will increase the delivery of intra-liposomal content. Holovati and colleagues (2008) reported a 100 times increase in the delivery of trehalose from negatively charged liposomes (containing PS) compared to neutral liposomes, under the same incubation conditions (143). Charge can also determine the toxicity as well as the interaction of liposomes with the immune system *in vivo*, which will determine how long they stay in the circulation (144). Cationic liposomes interact with plasma proteins causing turbidity and have been shown to cause dose-dependent cytotoxicity (145). They are also rapidly cleared from the circulation by macrophages due to activation of the complement system (146). Anionic liposomes are taken up by phagocytic cells at faster rates than neutral liposomes in an unclear mechanism (147). Neutral liposomes are reported as not inducing any detectable toxicity. The issue of reduced half-life has been addressed by coating the liposomes with polyethylene glycol (PEGylated or stealth liposomes) to suppress the uptake and increase circulation time (148).

1.4.2 Mechanisms of liposome-RBC interaction

Four types of liposome-cell interactions have been described in the literature: endocytosis, lipid transfer, adsorption and fusion (149, 150). Endocytosis involves the internalization of vesicles by cells (i.e. pinocytosis or phagocytosis) (151). Since is it generally accepted that mature RBCs do not exhibit phagocytic activity, endocytosis as a mechanism of interaction would not be possible, nor the most desired for successful membrane manipulation (152). The remaining possible mechanisms have been previously reported for liposome-RBCs

using unilamellar vesicles (134, 153) and are usually dependent on liposome characteristics such as surface charge, composition and incubation temperature (Figure 1.6). Lipid transfer is characterized as the transfer of cholesterol and/or phospholipids from liposomes to cell membranes and vice-versa, the specific molecular process is not well-understood but it might involve transfer proteins (154). Adsorption is defined as the binding of intact liposomes to the cell surface and it could be receptor-mediated or nonspecific (155, 156). Fusion occurs when liposome and plasma membrane merge, following the release of their content (if loaded) into the cytoplasm (153, 157).

1.4.3 Previous studies on liposomal delivery system for cryopreservation of red blood cells

Our research group has conducted several studies on liposomal delivery for cryopreservation of RBCs that served to guide the liposome work presented on this thesis. The main findings are summarized below.

Stress conditions are associated with ice formation that accompanies low temperatures, our group has proposed that delivering of liposome phospholipids through liposome incorporation into RBC membranes may provide an effective approach for reducing cell cryoinjury. The studies showed that liposome treatment significantly improved recovery and membrane integrity of human RBCs following low temperature exposure (119, 158). Liposomes modulated the RBC freezing response, resulting in cell cryoprotection and, thus, behaving as a novel cryoprotectant. The extent of liposome-RBC interaction was shown to play an essential role for cryoprotection as charged trehalose-containing liposomes were more effective at protecting RBCs against cryoinjury than uncharged liposomes (119, 158). While both trehalose- and salinecontaining liposomes improved RBC post-thaw recovery, there was no cryoprotection when negatively charged lipids in the free and multilamellar form were

incubated with RBCs (119). These results suggested that liposomes in the form of defined unilamellar vesicles are necessary to confer cryoprotection to RBCs. Liposome-treated RBCs showed an improved post-thaw survival if extracellular freezing solutions contained trehalose or liposomes. Furthermore, important evidence was shown that the liposome cryoprotective effect is not due to the delivery of intracellular trehalose, but is likely related to modification and/or preservation of the RBC membrane (119).

In a follow-up study, they have evaluated the effects of DMPC, DOPC, and DPPC liposomes on *in vitro* quality of RBCs cryopreserved with known extracellular cryoprotectants, hydroxyethyl starch (HES) and trehalose (159). This approach also involved combining cryoprotectants with different mechanisms of action to maximize potential cryoprotection: large unilamellar liposomes composed of DPPC, DMPC or DOPC lipids, trehalose and HES (159). This study demonstrated that DMPC liposomes (short fatty acid chain) caused hemolysis even before the cryopreservation step, negatively affecting hemorheologic properties of RBCs. RBCs treated with DPPC and DOPC liposomes significantly increased cell membrane permeability, resulting in loading of intracellular trehalose (159). In addition to the membrane stabilizing benefits of the liposome treatment, HES conferred additional cryoprotection during cryopreservation (159). The synergistic protective effects of trehalose, DOPC liposomes and HES potentially hold promise for the development of new cryopreservation methods for RBCs.

1.4.4 Liposomal delivery system for hypothermic storage of red blood cells

Numerous studies using RBCs as membrane models have utilized liposomes as means for modifying the composition of membranes and have documented the effect of these manipulations (152, 160, 161).

Liposomes containing only phospholipids (162) or cholesterol-poor formulations (less than 1.0 C/P ratio) are known to deplete cholesterol from RBC membranes, destabilizing the membrane, increasing the permeability to ions and resulting in hemolysis. The addition of cholesterol to the formulation can prevent these effects. However, cholesterol-rich formulations are also not ideal (more than 1.0 C/P ratio) since cholesterol will then be transferred from liposomes to RBC membrane and can result in decreased red cell deformability (163).

Short-chain phospholipids and lysophospholipids are also known to cause hemolysis probably due to a rapid insertion and accumulation into the RBC membrane that leads to destabilization (131, 132, 164). Kuypers and colleagues (1984) observed that shape changes were induced by replacement of native PC of human RBCs by liposomes composed of other PC species containing different fatty acid compositions. They concluded that the RBC membrane structure and the overall discoid cell shape are optimally stabilized by PC species that contain one saturated and one mono- or diunsaturated fatty acid, and that the cell tolerates only limited variations in the species composition of its PC (165). Acker and colleagues (2010) have examined the effect of liposome treatment on RBC membrane quality during hypothermic storage and the results indicated that RBCs react differently to liposomes with varied fatty acid compositions.

RBCs incubated with liposomes containing phospholipids with higher degree of unsaturation and shorter acyl chain length had higher percent hemolysis after 42 days of storage. In terms of deformability, RBCs treated with liposomes containing phospholipids with saturated acyl chains maintained better membrane deformability during hypothermic storage (166). A curious fact is that RBCs from different species will have different preferences in terms of fatty acid saturation. In the previous works mentioned above, the

liposomes were not loaded with any active compounds, so the improvements observed in hemolysis and deformability were attributed solely to lipid exchange or adsorption of liposomes to the RBC membrane that could result in either membrane lipid renewal or a "coating" effect (119).

1.5 Thesis approach

Several *in vitro* changes of stored RBCs have been documented in literature and although large clinical trials have not shown differences between fresh and standard-issue blood the question of whether long stored blood is harmful or not, remains. Since reducing the shelf life of RBCs would cause many logistical challenges, it is important to invest in efforts that lead to better preservation of the current transfusion products that we have available. Since the cell membrane plays an important role in post transfusion survival and oxygen delivery capacity of RBCs (167), this research will focus on the use of liposomes to mitigate RBC membrane injury during hypothermic storage.

The first part of this thesis focus on evaluating liposomes as strategy to preserve RBCs using an animal model as means of detecting any undesired effects that would compromise the use of liposomes for the purpose of RBC preservation for transfusion. The second part focus on applying the liposome treatment to blood banked human RBCs while tracking the *in vitro* effects on hemorheology, immune and coagulation profile using a comprehensive panel of assays. The last part of this work evaluate combining the liposome approach with the rejuvenation approach introduced on section 1.2.5, as PIPA solutions have demonstrated benefits on restoring metabolism. The National Heart, Lung and Blood Institute (NHLBI) Working Group on Strategies to Optimize RBC Products has established that "development of improved RBC storage/rejuvenation solutions" among others as a scientific priority (168).

This thesis will contribute to this effort by analyzing thoroughly a novel approach to RBC HSL, as well as the combination of this new approach to already established ones and will discuss this strategy and its potential for use and implementation in biopreservation.

1.6 Hypothesis and thesis objectives

This thesis will test the hypothesis that liposome treatment of stored RBCs will improve *in vitro* membrane quality resulting in reduced *in vitro* production of proinflammatory and procoagulant markers and a safe transfusion product in an anemic rat model. The thesis consists of experimental studies with five specific research aims (SRAs):

SRA 1: To evaluate baseline differences between rat and human RBCs in SAGM and investigate the impact of component manufacturing and additive solutions on rat RBCs (Chapter 2)

SRA2: To evaluate different liposome formulations on *in vitro* rat RBC quality parameters, as well as the safety of transfusing liposome-treated rat RBCs in an anemic rat model (Chapter 3)

SR3: To evaluate the impact of liposome treatment time on human RBCs and to assess the impact of liposome treatment on MP release, hemolysis and hemorheology in human RBCs during hypothermic storage (Chapter 4)

SR4: To examine the effect of fresh and stored liposome-treated RBCs and RBC-derived MPs on *in vitro* markers of inflammation and coagulation compared to non-treated RBCs (Chapter 5)

SR5: To investigate a possible synergistic effect of liposome treatment combined with rejuvenation treatment (Chapter 6)

Figure 1.1: Blood processing methods. Schematic representation of blood processing (A) buffy coat production and (B) whole blood filtration methods. WB = whole blood, LR = leukoreduction.

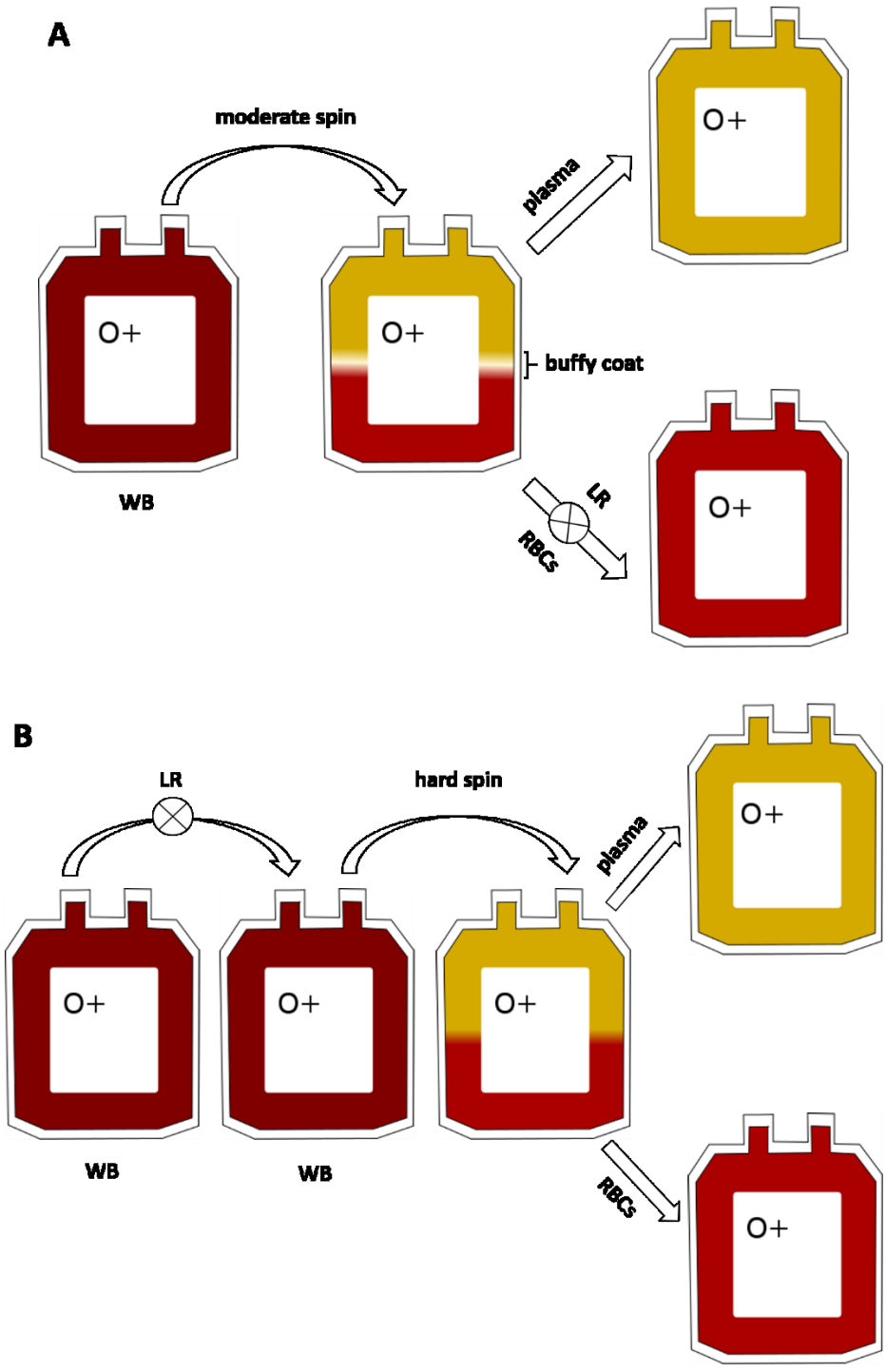


Figure 1.2: Hypothermic storage lesion. Main elements of RBC hypothermic storage lesion.

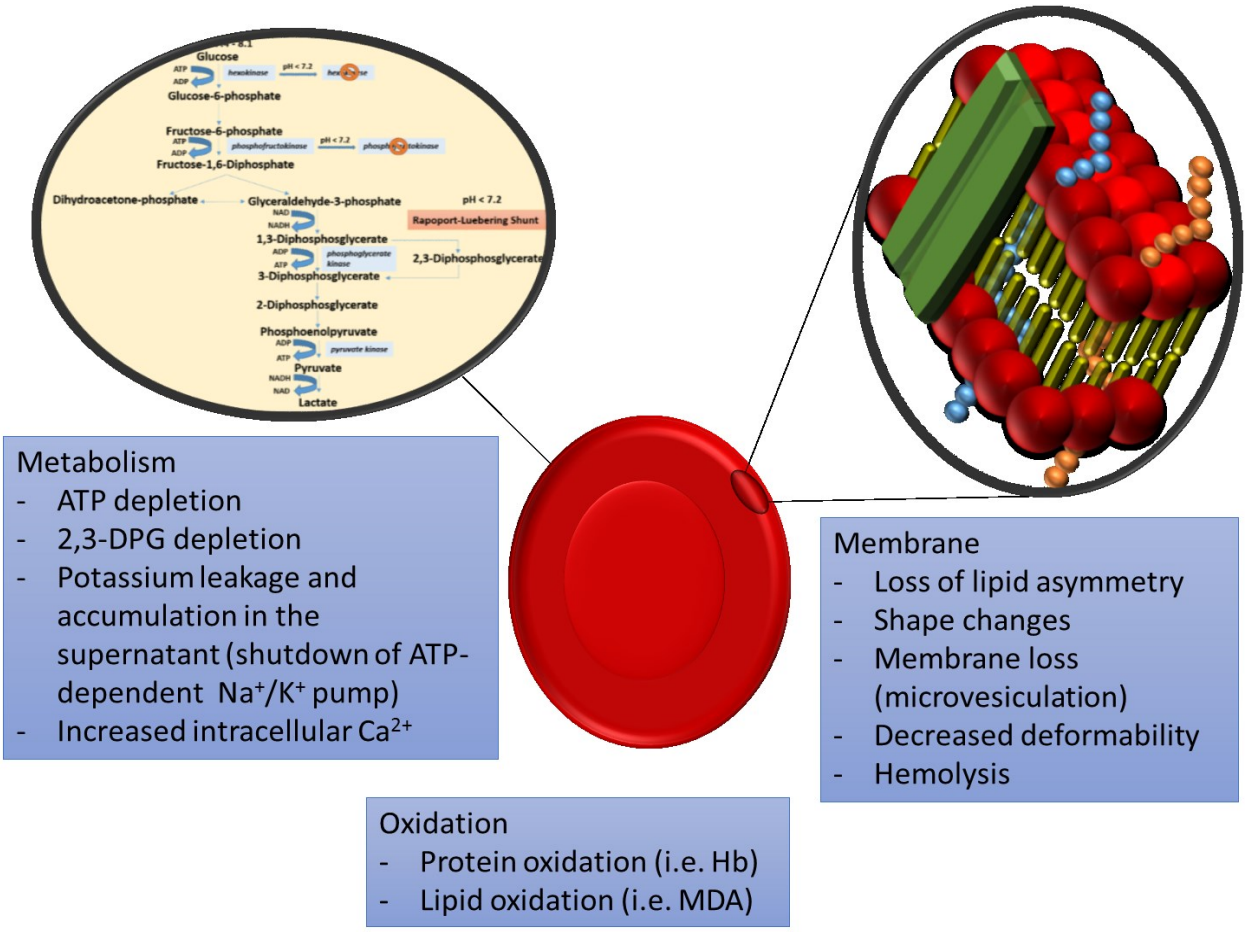


Figure 1.3: Energy metabolism in red blood cells. Overview of the pathways of glucose metabolism.

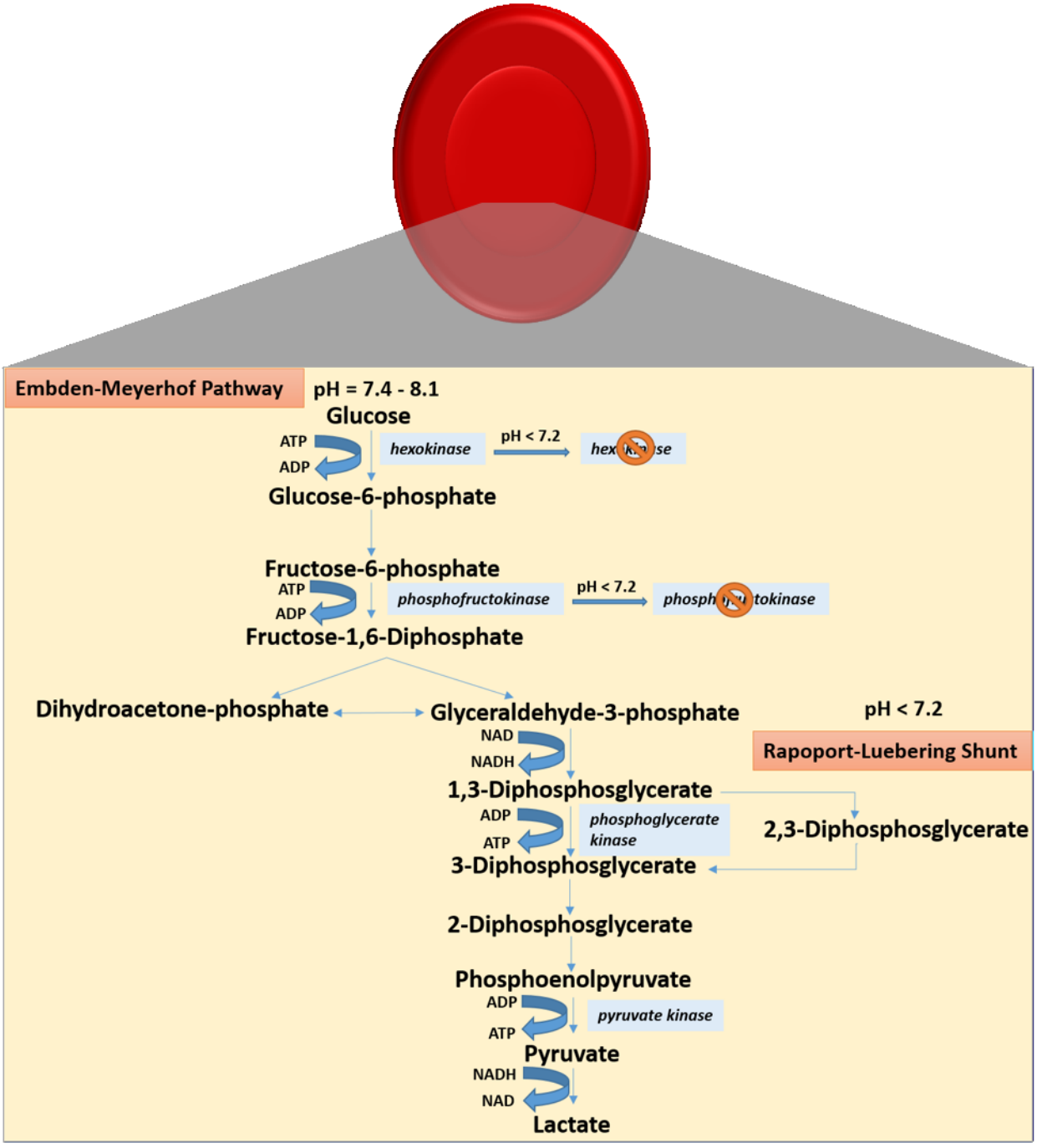


Figure 1.4: Liposome and phospholipid bilayer structure. Representation of the phospholipid bilayer structure in liposomes.

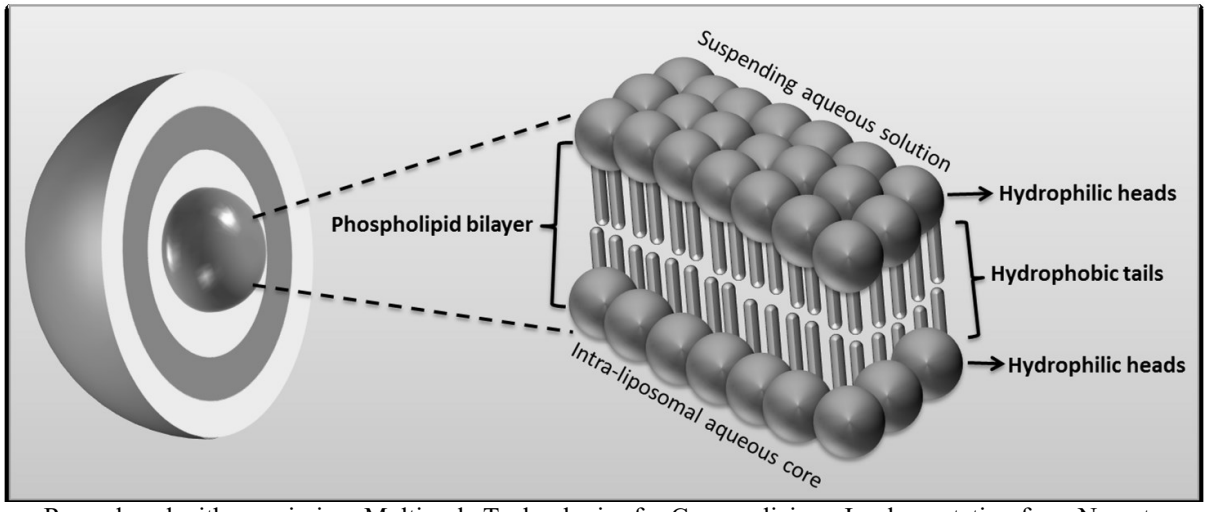


Figure 1.5: Phospholipid structure. Schematic structure of phospholipid composed of fatty acid chains, glycerol backbone and the headgroup (choline).

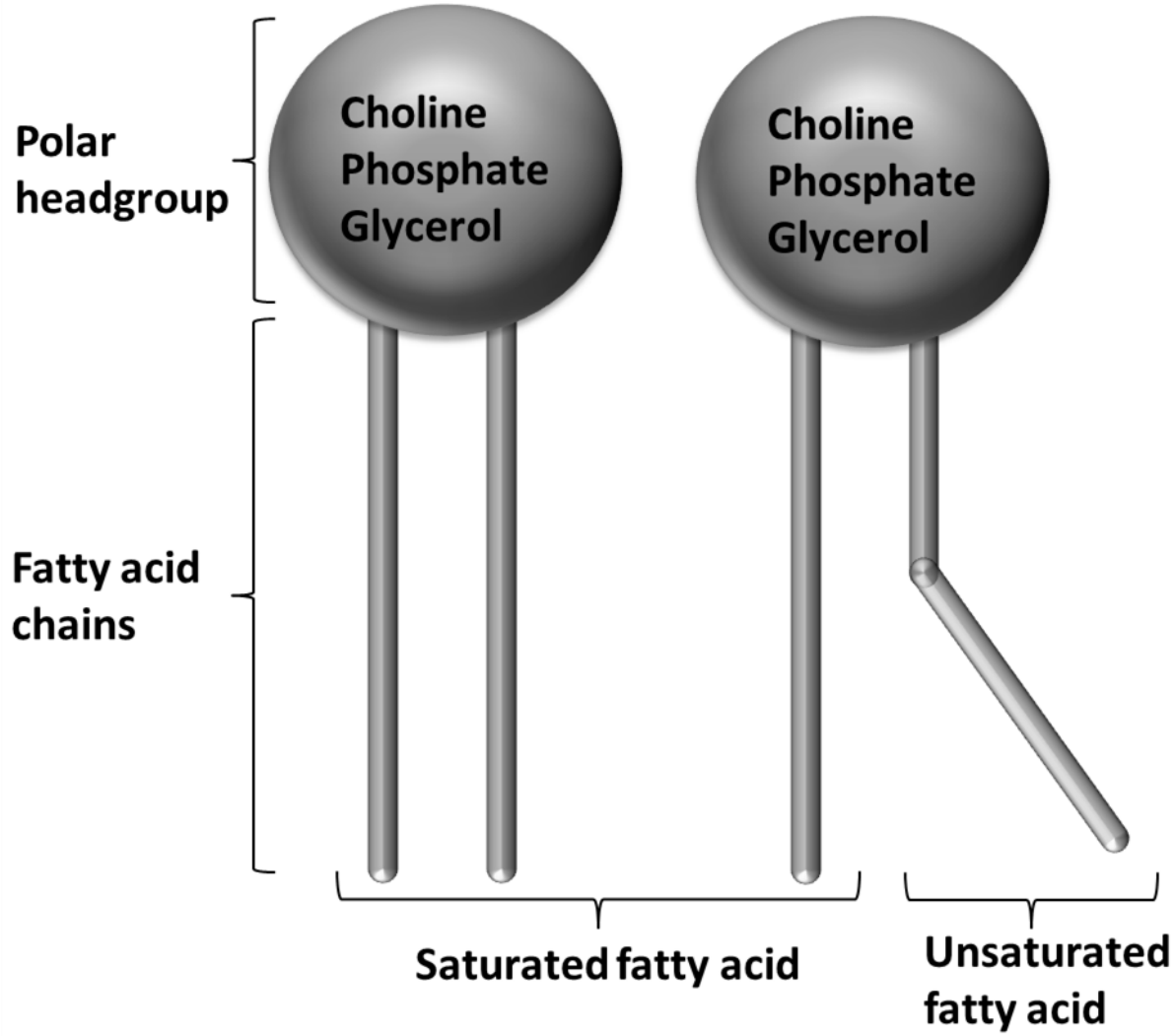
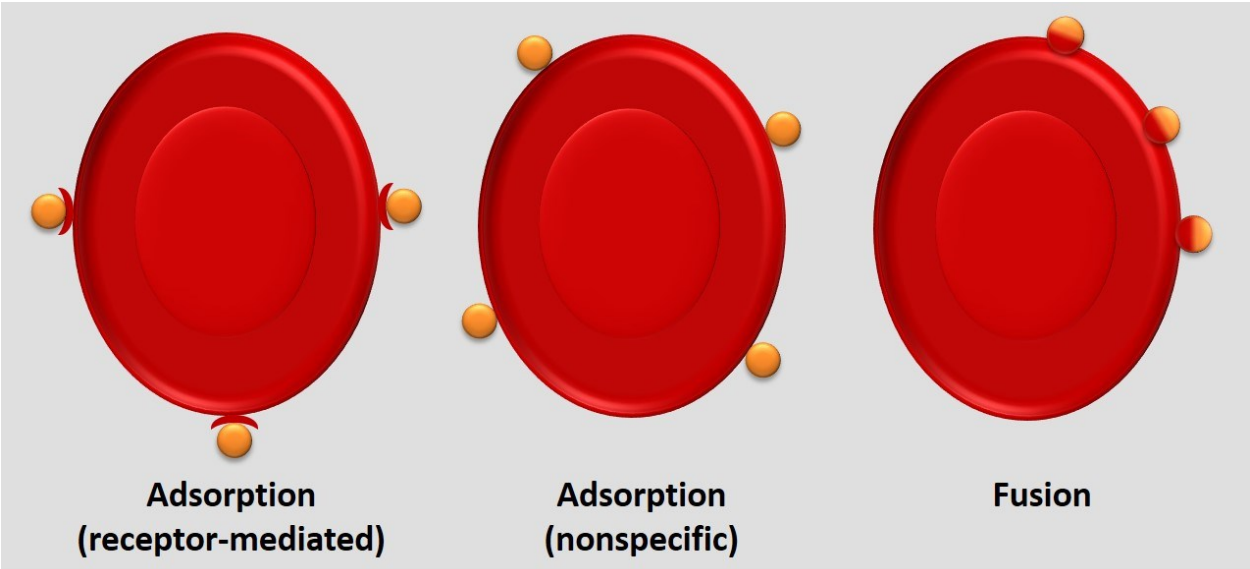


Figure 1.6: Liposome interactions with red blood cells. Schematic representation of possible liposome-RBC interactions.



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Chapter 2*

Differences in rat and human erythrocytes following blood component manufacturing: the effect of additive solutions

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2.1 Introduction

Blood transfusion is a lifesaving treatment for patients with massive blood loss and chronic anemia and a supportive therapy to optimize oxygen delivery and tissue perfusion in critical illness (1, 2). The clinical benefits of blood transfusion were made possible through the development of techniques to preserve cell viability *ex vivo*, allowing the blood donor and transfusion recipient to be separated in time and space (3). In the 1960's, with the introduction of plastic blood bags (4), whole blood transfusion was replaced with specific blood component therapy - RBCs, platelets, plasma and plasma components - translating the life-saving benefits of one whole blood donation to up to four transfusion recipients (5). Currently packed RBCs (pRBCs), the most highly used blood component, are produced by two common component manufacturing methods: the whole blood filtration method and the buffy coat method (6, 7). The general procedure is similar for both techniques: whole blood is centrifuged, plasma and RBCs are separated and RBCs are resuspended in an additive solution, commonly accompanied by leukoreduction (7). Additive solutions, such as SAGM and AS3, contain nutrients RBCs need to survive *ex vivo* and have effectively extended RBC storage for up to six weeks (4). SAGM is widely used in blood banks in Europe, Australia and Canada, while AS3 is mainly used in the United States (8).

Although use of additive solutions extends the storage length of pRBC units, the quality of stored RBCs progressively decreases during hypothermic storage. RBCs undergo a series of biochemical and biomechanical changes, collectively known as the "hypothermic storage lesion" (9). Characteristics of the HSL includes RBC membrane remodeling, decreased metabolites, such as ATP and 2,3-DPG, loss of intracellular potassium, oxidative injury of protein structures and lipid peroxidation, membrane loss, vesiculation and ultimately

hemolysis (10-12). There are increasing concerns regarding the effect of the HSL on hemorheology, including RBC aggregability, deformability and membrane remodeling, effects that could potentially lead to impairment of the oxygen delivery capacity of transfused blood (13-15). As a consequence, the active debate on the clinical impact of stored RBCs has given rise to large clinical trials about the use of "fresh" vs. "old" RBCs (16-18).

The effect of the HSL on RBC quality during storage has also rejuvenated research efforts into novel additive solutions for better preservation of RBC *ex vivo* quality and function (19). Animal models are often used as a translational tool to understand the mechanisms behind the ageing of blood cells and possible clinical effects related to the quality of stored blood (20). Therefore, understanding biological differences between RBCs from animal model species and humans, as well as the effect of blood component manufacturing processes on cell quality are the first step in animal translational studies.

Previous studies have addressed the quality of rat RBCs using CPDA-1 as the additive solution; however, CPDA-1 is not widely used today in blood component manufacturing. To my knowledge, no study has been done using the current additive solutions for pRBC storage: SAGM or AS3. The differences in quality parameters between human RBCs in SAGM and AS3 are well documented in the literature, but this information is not available for rat RBCs (21). Also, little is known about the effects of component manufacturing and leukoreduction methods on the parameters important for RBC hemorheology, including deformability, aggregation and microvesiculation in rat RBCs. Therefore, the aim of this study was to evaluate baseline differences between rat and human RBCs in SAGM, including *in vitro* quality assays to examine membrane-related and hemorheology parameters. In addition, I investigated the impact of the buffy coat component manufacturing method on rat RBCs and

the effects of different additive solutions. Ultimately, the goal was to establish a rat pRBC production method that closely mimics human pRBC techniques current in use that can be applied in rat models of transfusion studying the HSL and novel RBC biopreservation strategies.

2.2 Materials and Methods

2.2.1 RBC collection and manufacturing

Ethics approval for the study was granted by the Canadian Blood Services and the University of Alberta Research Ethics Boards. Rat whole blood (n=6) was obtained from Sciences Animal Support Services (SASS) under the Animal Care & Use Committee for Biosciences (University of Alberta). The Sprague-Dawley rats were all male, between 8 and 9 weeks old with an average weight of 300 g. The blood was collected after anesthesia into 10 mL citrate-phosphate-dextrose (CPD) anticoagulant vacuum tubes (Haematologic Technologies Inc., Essex Junction, VT) by cardiac puncture. Harvested blood was centrifuged (2,200 x *g*, 10 min at 4 °C), and the plasma and buffy coat were removed by aspiration. The pRBCs were divided into two aliquots. One aliquot was resuspended in SAGM (MacoPharma, Mouvaux, France) and the second aliquot in AS3 (Haemonetics Corporation, Braintree, MA). Both maintained a proportion of 1:2 AS:RBC (vol/vol) and were leukoreduced at room temperature using 10.0 µm Versapor® membrane syringe filters (Pall Corporation, Ann Arbor, MI). Before leukoreduction, percent hemolysis was measured in both CPD-SAGM RBCs and CPD-AS3 RBCs. Immediately after leukoreduction, *in vitro* quality of CPD-SAGM RBCs and CPD-AS3 RBCs was analyzed, including percent hemolysis, ATP, 2,3-DPG, hematological indices, deformability, aggregation and microvesiculation, as described below.

After informed consent, human whole blood from six healthy volunteers was collected by venipuncture into CPD anticoagulant tubes. The blood was processed following the same method described above for rat blood. *In vitro* quality was analyzed within 3 hours of collection and included percent hemolysis, osmotic fragility, ATP, 2,3-DPG, supernatant potassium concentrations, hematological indices, deformability, aggregation and microvesiculation, as described below.

2.2.2 RBC *in vitro* quality assessment

Percent hemolysis

RBC hemolysis, which represents RBC breakdown and release of Hb as a marker of membrane damage and rupture, was measured spectrophometricaly using the cyanmethemoglobin method of Drabkin (22). In this method, RBCs and supernatants were diluted 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). The reagent converts most types of Hb to cyanmethemoglobin (HiCN) in a two-step reaction. First, hemoglobin is oxidized to methemoglobin that subsequently reacts with cyanide to form HiCN. HiCN's absorbance was measured at 540 nm on a spectrophotometer SPECTRAmax PLUS 384 (Molecular Devices Corporation, Sunnyvale, CA, USA). HiCN's absorbance is directly proportional to hemoglobin concentration, hemoglobin concentration was calculated according to the equation below (23):

$$C = \frac{A540 \; x \; M \; x \; F}{\varepsilon 540 \; x \; l \; x \; 1000} \qquad \text{(Eq. 2.1)}$$

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

ε540 = extinction coefficient of HiCN at 540 nm (11.0 cm-1·mM-1)

l = light path (cm)

Once supernatant and total hemoglobin values have been determined, the percent hemolysis was calculated as a ratio of the supernatant hemoglobin to the total Hb, with the hematocrit (Hct), measured by microhematocrit from the following equation (24):

$$\%\ hemolysis\ = \frac{HbS}{HbT}\ x\ (1 - Hct) \qquad \text{(Eq. 2.2)}$$

Where: Hct = hematocrit

HbS = supernatant hemoglobin (g/L)

HbT = total hemoglobin (g/L)

Commercial tri-level hemoglobin controls (low, medium and high ranges) were used as controls for total hemoglobin (Stanbio Laboratory, Boerne, TX).

Osmotic fragility[*]

Osmotic fragility was determined using a series of saline solutions with concentrations ranging from 0.0 g/L to 9.0 g/L. When RBCs are exposed to hypotonic solutions the cells will begin to swell and will eventually burst releasing hemoglobin. As hemoglobin absorbs light at 540 nm, the amount of hemoglobin can be measured and percent hemolysis calculated.

[*] Osmotic fragility was performed by HYRS student Diana Yu under my supervision.

Briefly, 10 µL of each RBC sample was added to 1000 µL of each saline solution. After a 30-minute incubation period, samples were spun at 2,200 x g for 5 min and the percent hemolysis was measured at 540 nm on a spectrophotometer SPECTRAmax PLUS 384 (Molecular Devices Corporation). Percent hemolysis in each saline solution was plotted against the saline solution concentration to determine the concentration that produced 50% hemolysis; this parameter was reported as mean corpuscular fragility (MCF). If the cells are less fragile they can tolerate more dilute solutions and the curve will be shifted to the left relative to the control curve (25).

ATP

ATP concentrations were determined enzymatically using a commercially available kit and controls (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). RBC samples were added to 10% trichloroacetic acid, vortexed and placed on ice. After centrifugation, clear supernatants were combined with the substrates (glucose and NAD^+) and enzymes (hexokinase and glucose-6-phosphate dehydrogenase) required for the enzymatic reaction to occur. ATP from the sample is used in two chemical reactions, which ultimately convert glucose into 6-phosphogluconate and NADH. The amount of NADH produced, which is proportional to the amount of ATP in the sample, was measured at 340 nm on SPECTRAmax PLUS 384 spectrophotometer (Molecular Devices Corporation) using the following equation:

$$ATP\ \left(\frac{\mu mol}{dL}\right) = \frac{\Delta A\ x\ Vsample\ x\ F\ x\ 100}{\varepsilon 340\ x\ l\ x\ v} \qquad \text{(Eq. 2.3)}$$

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

Vsample = sample volume used in the assay (µL)

F = dilution factor for sample

ε340 = extinction coefficient of NADH at 340 nm (6.3 cm-1·mM-1)

l = light path (cm)

v = total reaction volume (µL)

The amount of ATP in the sample was calculated as µmol/dL; this was further normalized using the total Hb concentration (µmol/g Hb) (25).

2,3-DPG

2,3-DPG concentrations were determined using a commercially available kit according to manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). RBC suspension was mixed with 0.6M perchloric acid and centrifuged to obtain supernatant. Supernatant was then mixed with 2.5M potassium carbonate to neutralize the perchloric acid and centrifuged again to obtain supernatant, used subsequently for 2,3-DPG measurement. 2,3-DPG from RBCs is used in the series of six chemical reactions, that ultimately result in production of glycerol-3-phosphate and consumption of two NADH molecules per each 2,3-DPG molecule. NADH absorbance was measured at 340 nm on SPECTRAmax PLUS 384 (Molecular Devices Corporation). The decrease of absorbance at this wavelength is equivalent to the amount of NADH that has been consumed in the reaction. The concentration of 2,3-DPG is indirectly related to the amount of NADH remaining in the reaction mixture (26). 2,3-DPG concentration in test samples was calculated using SoftMax Pro software (Molecular Devices Corporation) according to the equation below:

$$2{,}3-\mathrm{DPG}\ \left(\frac{\mathrm{mmol}}{\mathrm{L}}\right)=\frac{\Delta A\ x\ Vsample\ x\ F}{2\ x\ \varepsilon 340\ x\ l\ x\ v} \qquad \text{(Eq. 2.4)}$$

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

Vsample = sample volume used in the assay (µL)

F = dilution factor for sample

ε340 = extinction coefficient of NADH at 340 nm (6.3 cm-1·mM-1)

l = light path (cm)

v = total reaction volume (µL)

Distilled water was used as a blank and an in-house made 2,3-DPG control (2.5 mmol/L) was prepared from 2,3-Diphospho-D-glyceric acid pentasodium salt (SIGMA, St. Louis, USA). Final 2,3-DPG concentrations were adjusted to the Hb content of each sample and reported as µmol/g Hb.

*Supernatant potassium**

RBC samples were centrifuged at 2,200 x *g* for 10 minutes at 4 °C to obtain supernatants. Supernatant potassium concentrations were measured by indirect potentiometry using ion-selective electrodes on a chemistry analyzer (Unicel® DxC 800 System and SYNCHRON® System, Beckman Coulter, Inc., Fullerton, CA) (27) by Laboratory Services at University of Alberta Hospital. The SYNCHRON System(s) determines potassium ion concentration by indirect potentiometry utilizing a potassium ion selective electrode in conjunction with a sodium reference electrode. To measure potassium concentrations, a volume of sample (40 µL) was mixed with a buffered solution. The high molar strength buffer is used to establish a constant activity coefficient for potassium ions, calibrating the electrode to concentration values.

* Potassium supernatants were analyzed by Laboratory Services at the University of Alberta Hospital.

Hematological indices

The RBC indices consist of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), which were determined using a Coulter automated cell counter (Coulter AcT, Beckman Coulter, New York, NY) (27). As RBCs in suspension pass through the Coulter aperture, they displace their own volume of electrolyte, shortly increasing impedance of the aperture, which results in a pulse that is directly proportional to the cell volume. In addition, a known volume of the RBC suspension is passed through the aperture, so a count of the number of pulses can yield the RBC count in a sample. The instrument computes hematocrit value by summing the electronic volume of RBCs, while HiCN method is used for determining Hb concentration. MCV is determined by measuring the average volume of individual RBCs and is expressed in fl. MCH is the average weight of Hb in the RBC, expressed in absolute units (pg) and computed by dividing Hb concentration (g/L) by RBC count (RBCs/L). Finally, MCHC is the average concentration of Hb in each individual RBC, expressed as a percentage and computed by dividing Hb concentration by the hematocrit.

Deformability

RBC deformability was measured using a laser-assisted optical rotational cell analyzer (LORCA; Mechatronics, Zwaag, The Netherlands), by ektacytometry as previously described (28). RBCs were diluted 1:100 in a polyvinylpyrrolidone solution, and subjected to increasing shear stress at 37 °C. The diffraction pattern produced by the scatter of a laser beam at each stress was collected, and subsequently plotted as a deformability curve. Deformability curves were linearized using the Eadie-Hofstee method as published by Stadnick, *et al*. (28). Two parameters were extrapolated using this linearized function: the maximum elongation index

(EI_{max}), as a measure of deformability, and the stress required to reach half of the maximum elongation (K_{EI}), as a measure of rigidity (28).

Aggregation

RBC aggregation was measured by syllectometry using LORCA (Mechatronics). RBCs were washed two times with PBS and 400 µL of packed washed cells were mixed with 1% dextran (100 kDa) solution to an optimal Hct of 42-46%. The controls were prepared the same way and mixed with PBS (negative) and 3% dextran (positive). Briefly, the cells were subjected to a constant shear rate with rotation, to cause complete disaggregation. The aggregation process was started by abruptly stopping the shear rate. The laser backscatter intensity over time was measured (syllectogram) and the following aggregation parameters were generated: aggregation index (AI) in percentage (%), amplitude (Amp) in arbitrary units (au) and aggregation half-life ($t_{1/2}$) in seconds (s) (29) (Figure 2.1).

Microvesiculation[*]

The flow cytometry procedure was adapted from Almizraq *et al.* (25). Two tagged antibodies were used to label RBCs and RBC microparticles (MPs) from human and rat samples. Fluorescein isothiocyanate (FITC) anti-human CD235a antibody (MHGLA01 or MHGLA01-4, Invitrogen Life Technologies, Burlington, ON) was used as a marker for RBCs and MPs from human samples, while peridinin chlorophyll protein complex-cyanine 5.5 (PerCP-Cy™ 5.5) anti-rat erythroid cells antibody (BD PharMingen, San Jose, CA) was used as a marker for RBCs and MPs from rat samples. APC annexin V (BD PharMingen, San Jose, CA) was used to label phosphatidylserine (PS) according to manufacturer's instructions in

[*] The author would like to acknowledge George (Jingzhou) Huang (Flow Cytometry Laboratory - Department of Experimental Oncology) for his help with the adaptation of the flow cytometry method for measuring microparticles in rat RBCs.

both human and rat samples. RBCs (5 mL) were diluted with annexin-binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, pH 7.4) and 5 µL of each of the fluorescently-labeled monoclonal antibodies (FITC, APC, PerCP-Cy™ 5.5) were added. After 15 minutes of incubation in the dark at room temperature, the prepared samples were run on a FACSCalibur (BD Biosciences, San Jose, CA) equipped with a 488 nm argon laser and computer software (CellQuest, BD Biosciences) (25). Commercial isotype controls directed against glycophorin A (FITC mouse IgG1, k isotype control, BD PharMingen) and rat erythroid cells (PerCP-Cy™ 5.5 mouse IgM, k isotype control, BD PharMingen) were used to account for any nonspecific binding of the antibodies. Frozen RBCs served as the positive control for PS expression and microvesiculation (25). TruCOUNT beads (BD PharMingen) were used to determine the absolute number of MPs/µL. Forward scatter and side scatter, measured on a logarithmic scale, was used to distinguish between RBC and MP populations. Absolute numbers of MPs/µL were calculated using the equation:

$$\frac{MPs}{\mu L} = \left[\left(\frac{no.of\ glycophorin\ A-positive\ MP\ gated\ events}{TruCOUNT\ beads-gated\ events}\right) x \left(\frac{no.\ of\ beads\ per\ TruCOUNT\ tube}{volume\ of\ buffer\ added\ to\ TruCOUNT\ (\mu L)}\right)\right] x\ dilution\ factor$$

(Eq. 2.5)

2.2.3 Statistical analyses

Statistical analysis was performed using SPSS 20.0 software (IBM, Armonk, NY). Mann-Whitney's U non-parametric test was used to assess differences between rat and human RBCs and Wilcoxon signed-rank test was used to compare samples in different additive solutions (SAGM vs. AS3). Data were expressed as mean ± standard deviation and $p < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Differences between human and rat RBCs in SAGM additive solution

Table 2.1 shows the differences in RBC *in vitro* quality parameters between the two species. In this study the samples were filtered using 10.0 μm syringe filters as an adaptation for the small sample volume, while trying to mimic the buffy coat blood component manufacturing process. The level of leukoreduction achieved was 90 ± 5% for platelets and 71 ± 9% for white blood cells. The buffy coat blood component manufacturing method caused alterations to rat but not to human RBCs, as demonstrated by increased hemolysis (rat pre-leukoreduction: 3.03% ± 0.64% vs. human pre-leukoreduction: 0.24% ± 0.07%, p = 0.002; rat post-leukoreduction: 4.70% ± 0.83% vs. human post-leukoreduction: 0.34% ± 0.07%, p = 0.002; Figure 2.2). Free Hb levels were also measured in CPD-plasma, where no difference was observed (human: 1.13 ± 0.15 g/L vs. rat: 0.98 ± 0.18 g/L, p = 0.157), and in the SAGM supernatant of pRBCs where the levels in rat were greater than in human (human: 1.47 ± 0.07 g/L vs. rat: 8.95 ± 0.51 g/L, p = 0.034) (Figure 2.3). Rat RBCs were also more sensitive to osmotic stress than human RBCs, as shown by higher MCF values (p = 0.002). Rat RBCs had significantly lower levels of ATP compared to human RBCs (p = 0.004) while the levels of 2,3-DPG were similar between species (p = 0.054). Potassium levels were significantly higher in the supernatant of rat RBCs compared to humans (p = 0.002). Rat RBCs had decreased deformability (EI_{max}; p = 0.002) and decreased membrane rigidity (K_{EI}; p = 0.002) compared to human RBCs (Figure 2.4). RBCs characteristics, including the hematological indices MCV and MCH were significantly different between species (p = 0.002). Aggregation index and amplitude were significantly higher in human RBCs compared to rats (p = 0.016 and p = 0.004, respectively) and the aggregation half-life was lower (p = 0.037), indicating that aggregation happens faster in human RBCs (Figure 2.5). The number of MPs/μL was lower in

rats compared to humans (p = 0.010). Further analysis showed that there were also differences between the species in terms of MPs expressing PS (p = 0.004; Figure 2.6 B) and the mean fluorescence intensity (MFI) for the expression of PS on both RBCs (p = 0.004) and MPs (p = 0.004).

2.3.2 Rat RBCs in different additive solutions: SAGM vs. AS3

Table 2.2 shows the effect of different additive solutions on rat RBC *in vitro* quality parameters. Both EI_{max} (deformability) and K_{EI} (rigidity) were significantly increased when the packed RBCs were resuspended in AS3 compared to SAGM (p = 0.028 and p = 0.046, respectively). In AS3, percent hemolysis was significantly lower pre and post leukoreduction (Figure 2.2). ATP concentration was higher (p = 0.026) and 2,3-DPG concentration was lower (p = 0.028) in AS3 vs. SAGM. MCH values were comparable for both additive solutions (p = 0.288), while MCV values were lower (p = 0.028) and MCHC values were higher (p = 0.027) with the use of AS3. No significant differences in the aggregation behavior were observed between the RBCs resuspended in the two additive solutions (Figure 2.5). The number of MPs/μL was slightly lower in rat RBCs resuspended in AS3 compared to SAGM (47,806 ± 3,029 vs. 67,556 ± 13,318, p = 0.046). The percentage of PS exposure in both RBCs and MPs were significantly lower in the packed cells resuspended in AS3 (p = 0.028; Figure 2.6 A, 2.6 B). MFI for the expression of PS in RBCs resuspended in AS3 was also lower (p = 0.046).

2.4 Discussion

Differences between human and rat RBCs in SAGM, particularly in terms of membrane parameters and hemorheology, which play a significant role in the human RBC HSL are not well documented. One of the objectives of this study was to fill in gaps in the

current literature by evaluating the baseline hemorheological differences between human and rat RBCs in SAGM. In addition to deformability and aggregation, MP analysis is an important tool to assess quality and the effects of new preservation strategies, as MPs have been linked to various biological activities including inflammation, vascular function and immune response (30).

The concentration of MPs was lower in rat samples compared to human pRBC samples, while the exposure of PS in RBCs from both species was comparable. RBC intracellular ionized calcium concentration in rats range from 83-105 nM (31) while in humans the physiological range is 20-60 nM (32). Rat RBCs have also been described as more sensitive to exogenous Ca^{++} than human RBCs (33). Considering that PS externalization and microvesicle formation are directly correlated with calcium influx (34-36), a higher number of MPs would be expected in rat samples. On the other hand, RBC microvesiculation has also been described as a protective mechanism to avoid erythroptosis (36, 37) or to regulate membrane stability and avoid lysis (34, 36). In this context, the increased MPs combined with the lower hemolysis values indicates that the regulatory mechanisms in human RBCs are more effective than rat RBCs, allowing the cells to adapt better to different environments. Willekens and colleagues (2005) have demonstrated that in rats, RBC-derived MPs have high PS exposure and are easily removed from the circulation by liver Kuppfer cells, and that the same clearance mechanism is likely to happen in humans (35). In this study, the PS exposure in MPs from rat RBCs was higher than in humans. However further studies are necessary to determine if this high PS exposure plays a role *in vivo*.

The results show that the deformability of rat RBCs significantly differs from human RBCs. Lower EI_{max} and K_{EI} values suggests that rat RBCs are less deformable and less rigid

than human RBCs. Similar results have been described using ektacytometry (38) and the resistive pulse shape analysis technique in whole blood samples collected in ethylenediaminetetraacetic acid (EDTA) (39). Baskurt also showed a direct correlation between deformability and MCV values of various species, suggesting that RBCs with a smaller volume do not have to deform as much to pass through the microcirculation compared to larger RBCs (39). This study agrees with this finding, demonstrating that rat RBCs have lower MCV and decreased deformability compared to humans. The aggregation pattern of human and rat RBCs observed in this study is in agreement with previous reports for blood collected in EDTA (40). The percentage of aggregation is higher in human RBCs, and the cells also aggregate faster and to a greater extent than rat RBCs. Rat RBCs have a higher surface charge compared to human, demonstrated by a higher partition coefficient (40) which is expected to influence the aggregation behaviour, thereby explaining the different aggregation patterns observed in this study.

Hemolysis is one of the key quality control parameters evaluated in pRBCs before transfusion. It is well established that the percent hemolysis values of human pRBCs in CPD-SAGM at the end of the 42-day storage period should remain under 0.8% (27). The hemolysis in rat RBCs was more than ten times higher than in human RBCs after the component production process. I investigated whether the hemolysis could have been caused by the CPD anticoagulant, but found that the free Hb concentration in CPD-plasma of rat and human samples was similar. The free Hb concentration was measured again in the SAGM supernatant of both species and while in humans the concentration was virtually the same, in rats it had increased eight times. Potassium levels were higher in the supernatant of rat RBCs, in accordance with previously published data for RBCs in CPDA-1 (20). This is possibly as a

consequence of the pronounced hemolysis, with more intracellular potassium leaking to the supernatant. Comparison of osmotic fragility in RBCs from different mammalian species has shown that rat RBCs are among the least fragile (41). In this comparison to human RBCs I observed that, rat RBCs are more fragile. Correlations with cell volume have been made, showing that smaller cells are more fragile in hypotonic media (41) and more resistant to hypertonic media (42). The diffusional permeability of rat RBC membrane to water is higher than that of human RBC membrane (43), which may explain the increased sensitivity observed in hypotonic media and the differences observed in the corpuscular fragility between species. Osmotic behaviour is also a key element for cell interaction with additive solutions, especially hypertonic ones like SAGM (44).

Previously published studies of rat RBCs have used RBCs resuspended in CPDA-1 or EDTA blood, rather than additive solutions that are currently used. With the development of new additive solutions, it is important to know how the RBCs of rats, a species that is often used as a model in storage studies, respond to the additive solutions that are already in use. Human pRBC component manufacturing process is highly regulated, with well-established standards (21). One limitation of this study is that leukoreduction levels achieved for rat RBCs (90 ± 5% platelets; 71 ± 9% leukocytes) are below the human blood banking standard of 99 % reduction. Data to relate those standards to animal models of transfusion is lacking. This is in part due to a lack of understanding of how slight differences in the final product can benefit or affect the recipient of the RBC transfusion. Studies have already identified how different additive solutions and processes affect human, but not rat, RBCs. Another limitation of this study was that a direct comparison of human RBCs stored in SAGM and AS3 was not performed; however, some quality parameters have been examined before (45). Acker and

colleagues (2014) have previously reported no difference in ATP levels but increased 2,3-DPG levels with the use of AS3 compared to SAGM in human RBCs (26). In rat RBCs a significant increase in ATP and a decrease in 2,3-DPG levels using AS3 compared to SAGM were observed. The metabolic aspects seem to follow the same tendency as previously described for RBCs resuspended in CPDA-1, with human RBCs having higher levels of ATP compared to rat RBCs, with no difference in 2,3-DPG levels observed between the two species (20). The raw values for ATP and 2,3-DPG in rat RBC using SAGM were comparable to the values described for rat RBCs in CPDA-1.

In terms of hemorheology, I observed that rat RBC deformability improved (EI_{max}), while rigidity increased (K_{EI}), with the use of AS3 compared to SAGM. However, more studies need to be conducted to elucidate the exact mechanism of these differences. It has been shown that increased cellular dehydration caused by increased intracellular Hb results in greater cell rigidity and increases in the Hb-spectrin complex, which contributes to rigidity (46, 47). MCHC was higher for rat RBCs in AS3 compared to SAGM. This increased Hb concentration in AS3 RBCs may be the cause of the observed increase in rigidity. The aggregation behaviour of rat RBCs in AS3 and SAGM remained the same, with no statistically significant differences observed in aggregation index, amplitude and kinetics of aggregation between the two additive solutions. Compared to RBCs in SAGM, the number of MPs/µL was lower in rat RBCs resuspended in AS3, as was the PS exposure in RBCs and MPs. *In vivo*, PS exposure is related to increased adherence to the endothelium, which leads to hypercoagulability and vascular occlusion (48). Hemolysis was higher in rat RBCs in SAGM compared to AS3, which might be due to differences in the formulations of the two additive solutions. For example, mannitol, a membrane stabilizer that helps reduce hemolysis in human

RBCs (49), is present in SAGM but absent in AS3. Little is known about mannitol's effect on rat RBCs. The second notable difference between the two additive solutions is the NaCl concentration which is two times higher in SAGM (21). The higher concentration of Cl^- in SAGM might generate an imbalance in ion distribution across the RBC membrane, which could lead to activation of K^+/Cl^- co-transport resulting in increased potassium efflux and subsequently hemolysis.

2.5 Conclusion

In conclusion, rat RBCs differ from human RBCs in metabolic and membrane-related aspects findings, which should be taken into account when performing storage studies using a rodent model. Additive solutions play an important role in RBC preservation; however, SAGM, which is commonly used for human RBC storage, is not compatible with rat RBCs, as it causes high hemolysis and increased MP production. The present study suggest AS3 is a better alternative for rat RBC storage when conducting studies examining the HSL or new preservation strategies for RBCs that might require the use of animal models.

In this chapter, a processing method that mimics current blood bank processing methods was developed and tailored to rat RBCs taking into account the small blood volume and the sensitivity of rat RBCs to certain additive solutions. This also allowed for the characterization of *in vitro* quality parameters of fresh rat RBCs in comparison to human RBCs. The next chapter will focus on defining a liposome formulation with the potential to mitigate HSL in stored rat RBCs and the effect of this formulation on the hemorheology of stored rat RBCs as well as *in vivo* effects of transfusing liposome-treated rat RBCs in a rat model.

Table 2.1: *In vitro* quality parameters of human and rat RBCs. Mean values ± SD for fresh human and rat RBCs resuspended in SAGM post-leukoreduction are shown. au = arbitrary units.

RBC quality parameters	**Human RBCs**	**Rat RBCs**
Hemolysis (%)	0.34 ± 0.07	4.70 ± 0.83*
Osmotic fragility (MCF)	4.9 ± 0.3	5.9 ± 0.3*
ATP (µmol/g Hb)	3.1 ± 0.1	2.5 ± 0.2*
2,3-DPG (µmol/g Hb)	11.3 ± 1.3	14.1 ± 2.4
Supernatant		
K^+ (mmol/l)	1.4 ± 0.4	3.5 ± 0.6*
Hematologic indices		
MCV (fL)	95.1 ± 1.6	69.5 ± 2.9*
MCH (pg)	30.2 ± 0.7	22.6 ± 0.7*
MCHC (g/l)	318 ± 7	327 ± 12
Deformability		
EI_{max}	0.57 ± 0.01	0.52 ± 0.02*
RBC rigidity (K_{EI})	2.32 ± 0.51	0.76 ± 0.13*
Aggregation parameters		
AI (%)	53.4 ± 2.1	49.2 ± 2.3*
Amp (au)	27.4 ± 2.3	13.6 ± 0.7*
$t_{1/2}$ (s)	3.3 ± 0.3	4.0 ± 0.6*
Microvesiculation		
MPs/µL	85,377 ± 4,656	67,556 ± 13,318*
RBC-PS (%)	0.4 ± 0.0	0.6 ± 0.2
MP-PS (%)	6.8 ± 1.4	42.0 ± 5.0*
RBC-MFI	26.0 ± 1.7	86.3 ± 13.1*
MP-MFI	41.2 ± 7.1	187.3 ± 21.9*

* $p < 0.05$ compared to human RBCs.

Table 2.2: *In vitro* quality parameters of rat RBCs in different additive solutions. Mean values ± SD for fresh rat RBCs resuspended in SAGM and AS3 post-leukoreduction are shown. au = arbitrary units.

	Additive solutions	
Rat RBC quality parameters	**SAGM**	**AS3**
Hemolysis (%)	2.21 ± 0.68	0.87 ± 0.39*
ATP (µmol/g Hb)	2.6 ± 0.3	3.0 ± 0.3*
2,3-DPG (µmol/g Hb)	17.6 ± 2.2	13.8 ± 2.7*
Hematological indices		
MCV (fL)	62.9 ± 2.7	60.5 ± 2.0*
MCH (pg)	21.3 ± 0.8	21.2 ± 0.7
MCHC (g/l)	339 ± 5	350 ± 2*
Deformability		
EI_{max}	0.51 ± 0.02	0.55 ± 0.01*
RBC rigidity (K_{EI})	0.80 ± 0.17	0.98 ± 0.04*
Aggregation parameters		
AI (%)	53.8 ± 3.2	49.0 ± 4.2
Amp (au)	16.6 ± 3.6	14.8 ± 2.6
$t_{1/2}$ (s)	3.2 ± 0.5	4.0 ± 0.9
Microvesiculation		
MPs/µL	67,556 ± 13,318	47,806 ± 3,029*
RBC-PS (%)	0.6 ± 0.2	0.2 ± 0.0*
MP-PS (%)	42.0 ± 5.0	34.4 ± 3.4*
RBC-MFI	86.3 ± 13.1	74.8 ± 8.3*
MP-MFI	187.3 ± 21.9	173.2 ± 32.5

* $p < 0.05$ compared to SAGM.

Figure 2.1: Schematic representation of the aggregation procedure and indices obtained by syllectometry.

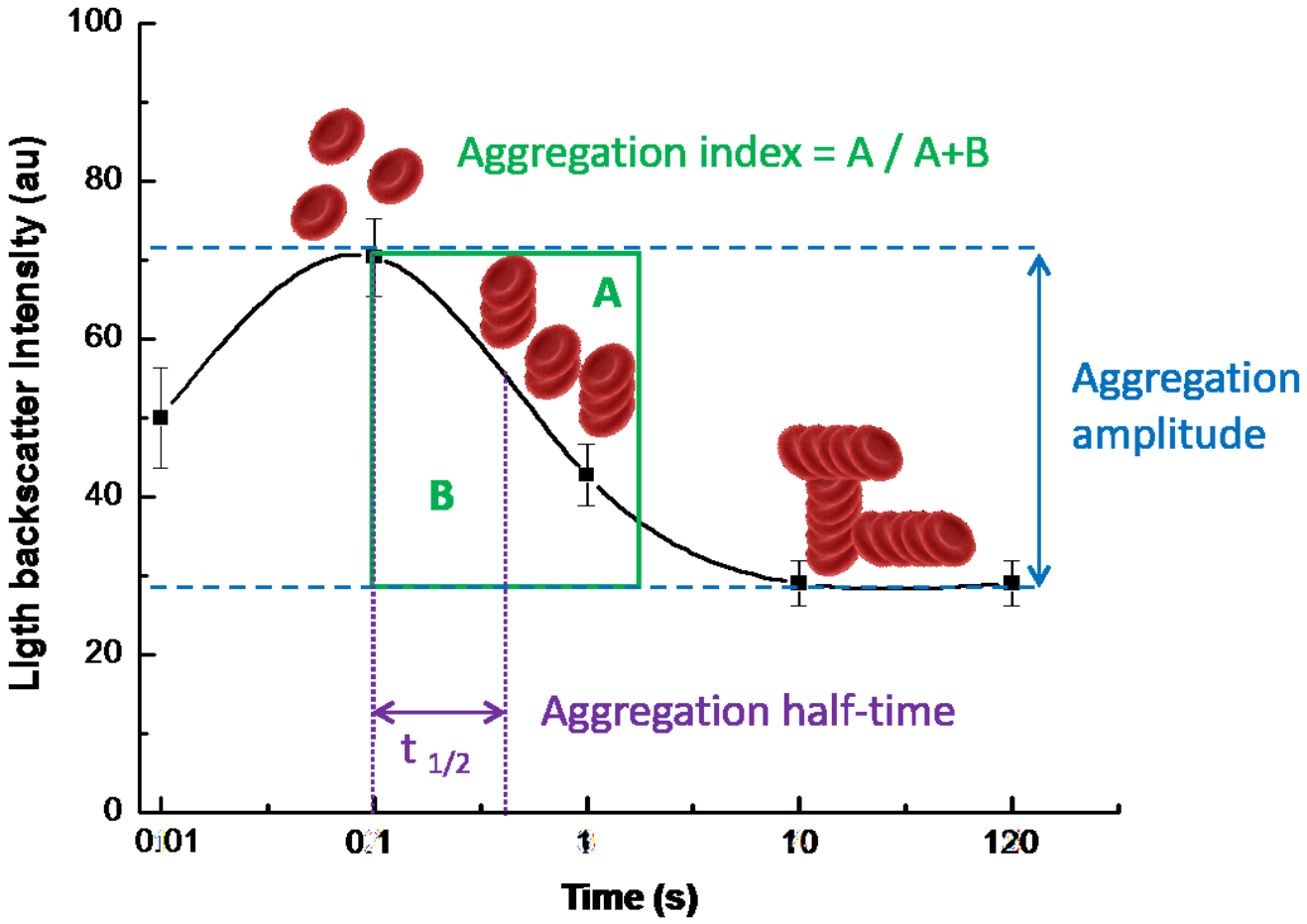


Figure 2.2: Percent hemolysis in fresh human RBCs resuspended in SAGM and in rat RBCs resuspended in SAGM and AS3, pre and post leukoreduction. Shown is the mean ± SD (n= 6). * Significant ($p < 0.05$) compared to human RBCs resuspended in SAGM and ** significant ($p < 0.05$) compared to rat RBCs resuspended in AS3.

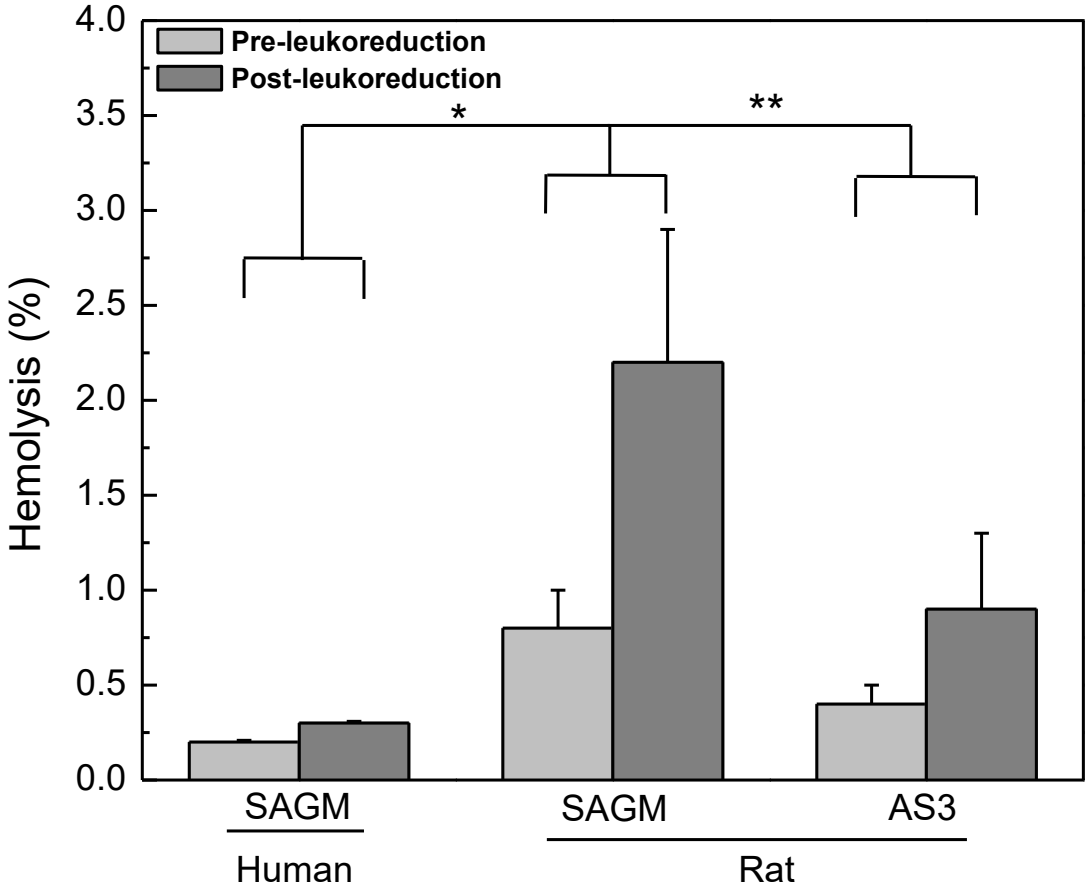


Figure 2.3: Free hemoglobin in CPD plasma and SAGM supernatants pre and post leukoreduction in human and rat. Shown is the mean ± SD (n= 4). * Significant ($p < 0.05$) compared to human.

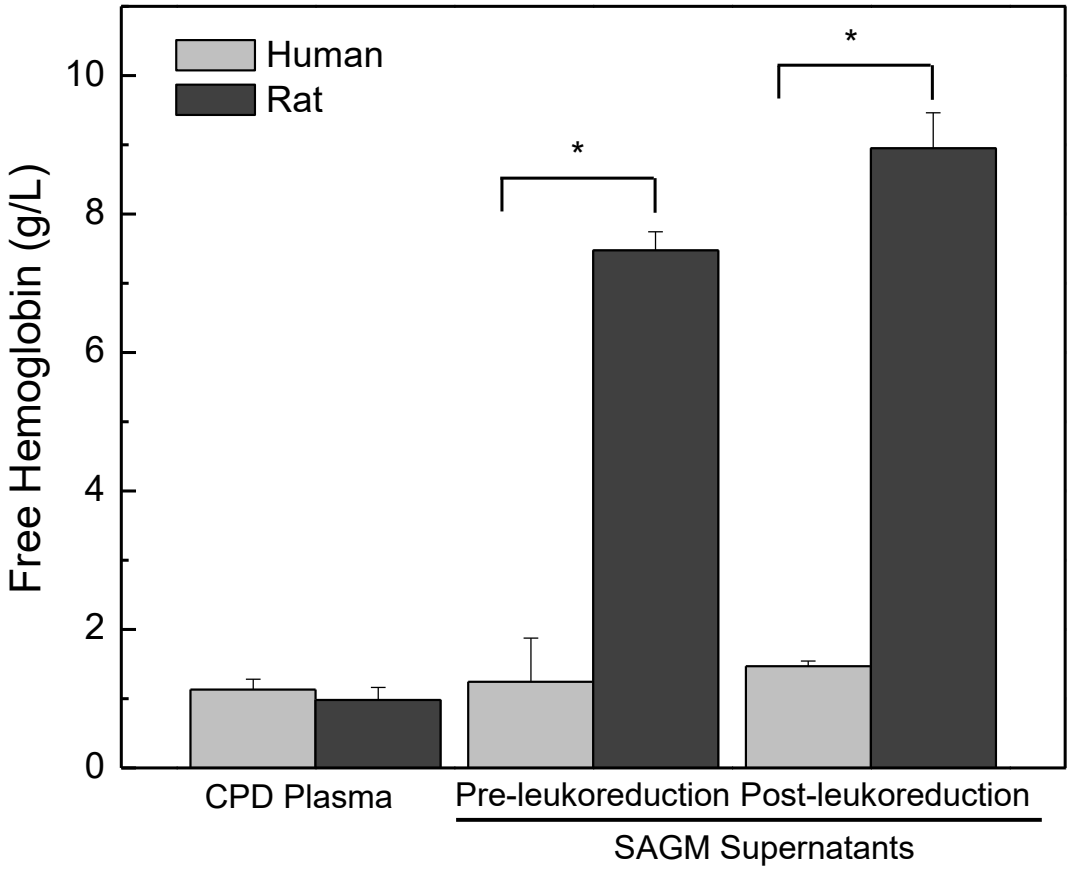


Figure 2.4: Deformability of human and rat fresh RBCs resuspended in SAGM. Shown is the mean ± SE (n= 6) of the elongation indices at different shear stresses after Eadie-Hofstee linearization. The two variables shown (EI_{max} and K_{EI}) are extrapolated from the lines: EI_{max} = y-intercept (deformability) and K_{EI} = slope (rigidity).

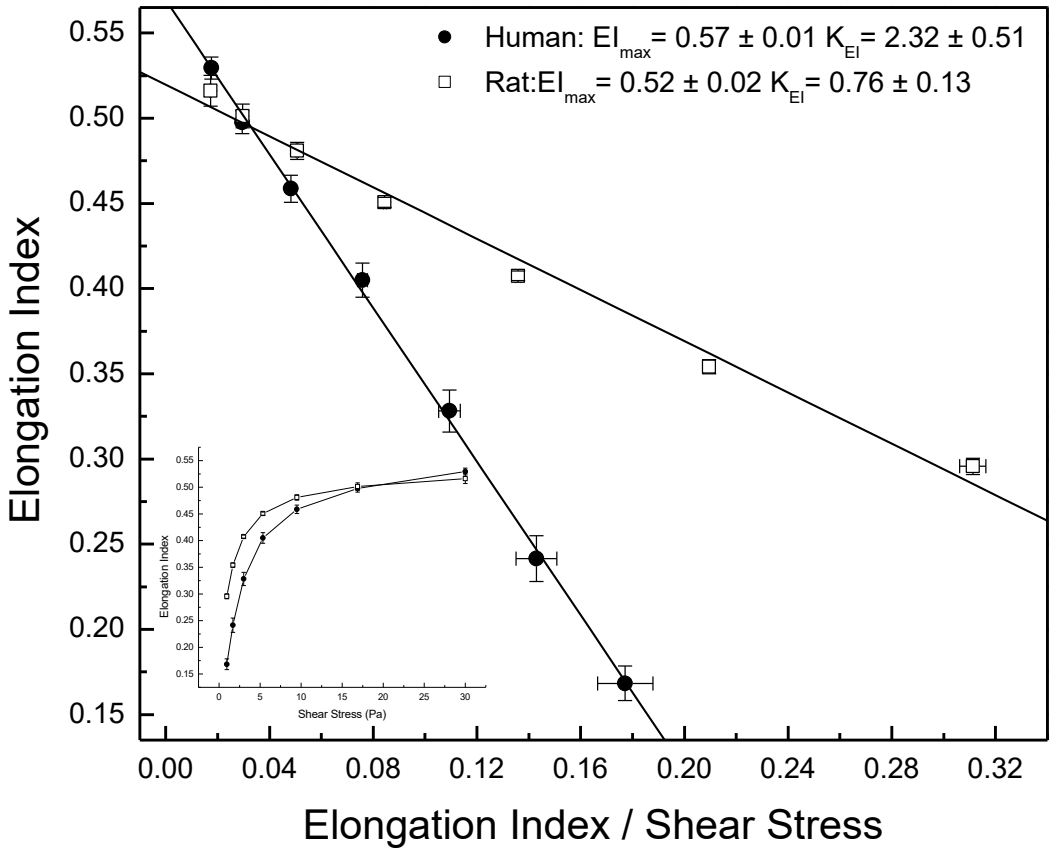


Figure 2.5: RBC aggregation in fresh human RBCs resuspended in SAGM and in rat RBCs resuspended in SAGM and AS3. Syllectograms (light scatter x time) showing RBC aggregation patterns (n=6). Negative control (PBS) = no aggregation; Positive control (3% dextran) = high aggregation.

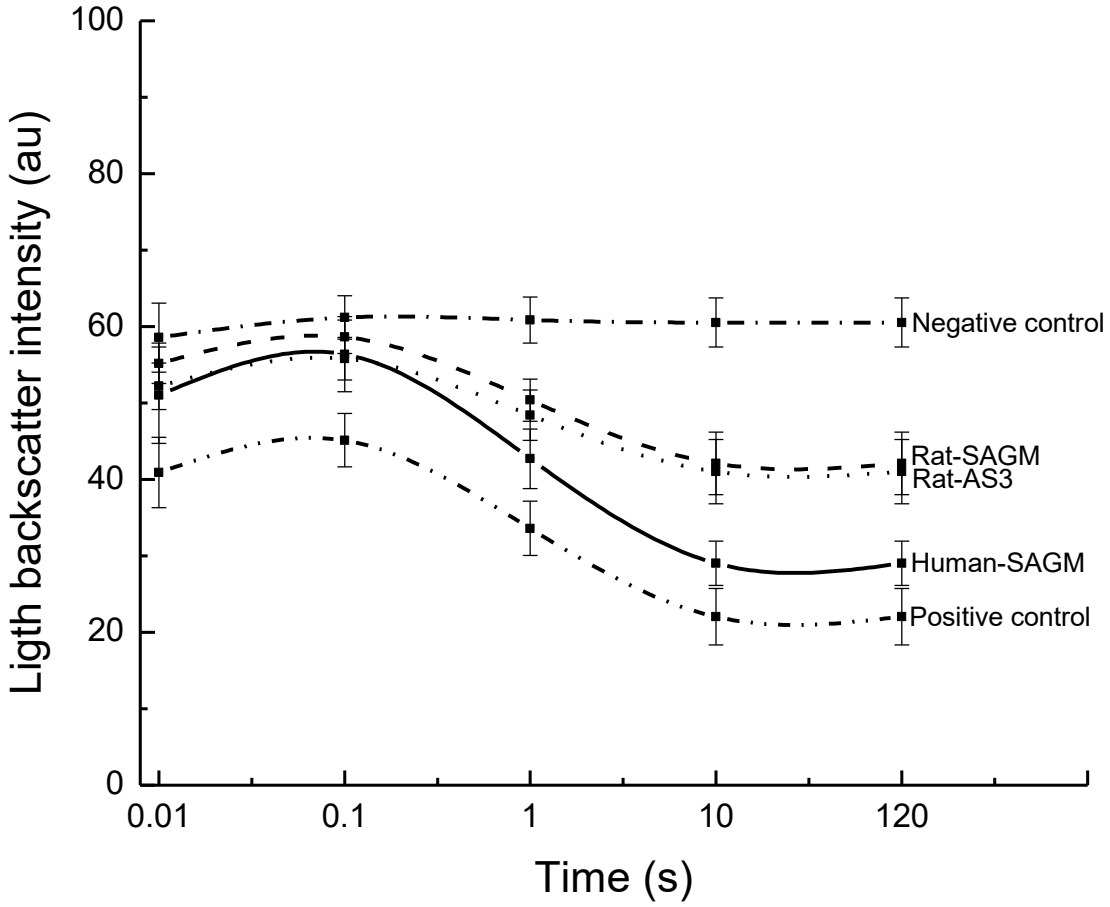
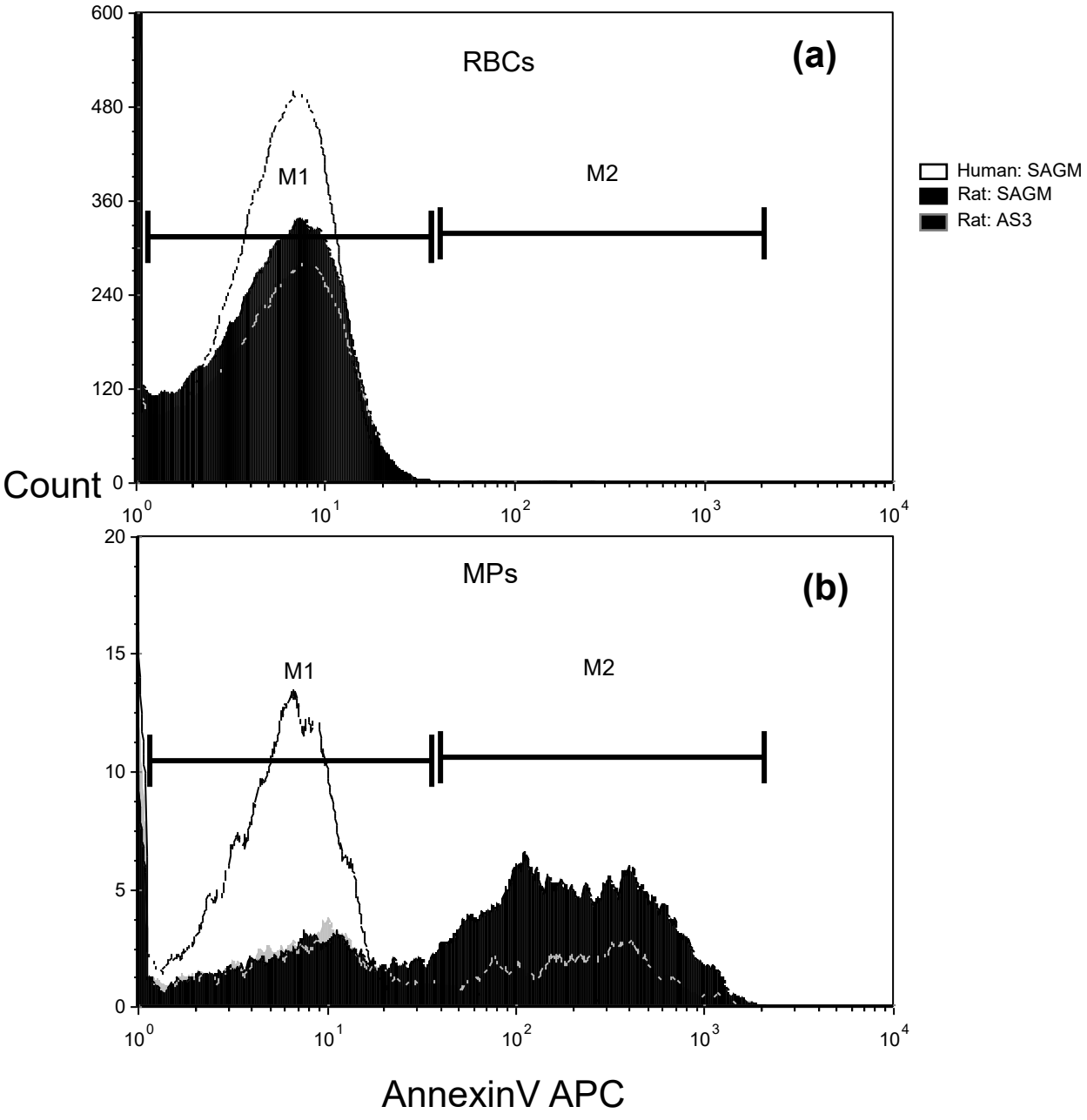


Figure 2.6: Percentage of phosphatidylserine (PS) exposure by RBCs (A) and MPs (B) in fresh human and rat RBCs resuspended in SAGM and in fresh rat RBCs resuspended in AS3. Histogram: region M1 is negative for PS exposure while, region M2 is positive.



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Chapter 3*

Effect of liposome treated red blood cells in an anemic rat model

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3.1 Introduction

The clinical benefits of blood transfusion were only made possible through the development of techniques to preserve *ex vivo* RBC viability, which allowed the blood donor and transfusion recipient to be separated in time and space (1). Additive solutions are commonly used in blood banks to extend RBC *in vitro* storage for up to six weeks prior to transfusion (2, 3).

Although use of additive solutions extends storage length of RBC units, the quality of stored RBCs progressively decreases during hypothermic storage (1-6 °C). RBCs undergo a series of biochemical and biomechanical changes, collectively known as "hypothermic storage lesion" (4). Characteristics of the HSL include RBC membrane remodeling, decreased metabolites, loss of intracellular potassium, oxidative injury of proteins and lipids, PS exposure, membrane loss, MP release and ultimately hemolysis (5-7). Recent analyses of RBC membrane lipids during refrigerated storage in SAGM revealed that the cholesterol content is more stable, while major changes occur to phospholipids, with a general decrease in lipids of the major phospholipid classes (8, 9). There are concerns that the accumulation of metabolic and cellular byproducts during storage might be related to post-transfusion adverse effects and decreased RBC viability, although there is still no accurate evidence to prove causality (10-13).

Apart from the use of additive solutions, other approaches to decrease RBC membrane injury during storage have been investigated such as rejuvenating solutions (14), anaerobic storage (15) and the use of plasticizers (16) without much success. Since the cell membrane plays an important role in post-transfusion survival and oxygen delivery capacity of RBCs (17-19), recent research has focused on the use of liposomes - synthetic lipid vesicles - to mitigate RBC membrane injury during hypothermic storage (20-23). The mechanism of

interaction between liposomes and human RBCs involves adsorption of the vesicles to the RBC surface, as well as liposome content incorporation through membrane fusion (20). RBCs are not able to synthesize phospholipids, so the main pathway for membrane phospholipid renewal *in vivo* is through exchange with plasma lipoproteins (24).The motivation for the use of liposomes is based on the transfer of lipids between liposome and RBC membrane (21, 22), as it mimics what happens *in vivo*, and has been previously shown to change the membrane composition of human RBCs, ultimately improving cell deformability, membrane fluidity and reducing hemolysis upon storage (21, 23). It is unknown whether liposomes can be used to mitigate elements of RBC membrane storage lesion unaddressed by current additive solutions, including MP shedding, PS exposure and impaired hemorheology.

Small animal models are necessary to provide evidence of the safety of liposome treatment and guide future pre-clinical translational and clinical studies (25, 26). Rat models have been widely used to study effects of transfusion, different blood products and blood substitutes on the cardiovascular system (25, 27-29). Like human RBCs, rat RBCs are anucleate flexible binconcave discs, with similar structure and biochemical pathways. Similarities also exist in the asymmetry of the RBC membranes, and their phopholipid and protein composition (30-32). This animal model of anemia/myocardial ischemia provides a clinically relevant and objective means to measure the effects of transfusion of RBCs stored in novel conditions. Since the myocardium has a basal oxygen extraction ratio of 55-70% (28), it has little capacity to increase oxygen extraction, making it particularly vulnerable to the effects of anemia and an ideal model system to measure injury response to transfusion.

While previous research on application of liposomes for mitigating the HSL largely focused on the *in vitro* mechanics of RBC and liposome interaction, as well as the quality of rat RBCs in current additive solutions for pRBC, this study investigated the effects of

liposome treatment on RBC rheologic properties, as well as *in vivo* assesment of efficacy and safety of transfused liposome treated RBCs in a rat model. Considering that chemical characteristics of liposomes (charge, saturation) are important determinants of liposome-RBC interaction (33-35), the aim of this study was to assess the effect of different liposome formulations on rat RBC HSL and hemorheology. Finally, the *in vivo* outcomes of transfusing liposome treated RBCs in an anemic rat model of myocardial ischemia and reperfusion was examined.

3.2 Materials and Methods

3.2.1 RBC collection and manufacturing

Rat whole blood was obtained from Sciences Animal Support Services (University of Alberta). The Sprague-Dawley rats were all male, between 8 and 9 weeks old with an average weight of 300 g. The blood was collected and processed as previously described in Chapter 2.

3.2.2 Liposome synthesis

Phospholipids for liposome synthesis were obtained from Avanti Polar Lipids (Alabaster, AL) and cholesterol was obtained from Sigma-Aldrich (St. Louis, MO). Multilamellar vesicles (MLVs) were prepared by the technique of Bangham & Horne (1964) (36). Briefly, chloroform solutions of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, 16:0), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, 18:1), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS, 18:1), and cholesterol were thoroughly mixed at a molar ratio of 7:3, resulting in a 25 mM final lipid solution. Four different formulations were obtained: saturated uncharged (DPPC:cholesterol, 7:3 mol%), unsaturated uncharged (DOPC:cholesterol, 7:3 mol%), saturated charged (DPPC:cholesterol:DOPS, 6:3:1 mol%) and unsaturated charged (DOPC:cholesterol:DOPS, 6:3:1 mol%). Chloroform solvent was

evaporated from the lipid mixture using a dry nitrogen stream, and subsequently lyophilized for at least 12 h (Virtis, AdVantage,Wizard -2.0, Gardiner, NY) to create a thin lipid film. Dry lipids were hydrated in HEPES-NaCl buffer containing 135 mM NaCl and 20 mM HEPES (pH 7.4, 276 mOsm). Small unilamellar vesicles were obtained by an extrusion technique (37) using a Lipex™ extruder (Northern Lipids Inc, Vancouver, BC) with 200 nm pore size Nuclepore® polycarbonate membranes (Whatman, Clifton, NJ).

3.2.3 Liposome characterization

Size

The mean Z-average size (nm) of the liposomes used in this study was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Briefly, 50 µL of liposomes were diluted in 0.9% NaCl solution, vortexed and transferred to a cuvette for DLS analysis. When a monochromatic light (i.e. a laser) shines through a solution containing spherical particles in Brownian motion (random motion of particles suspended in a fluid) and the light hits a moving particles it causes a change in the frequency of a wave (Doppler Shift), changing the wavelength of incoming light. This change is related to the size of the particle and can be calculated using Stokes-Einstein equation.

$$Dh = \frac{kB\ x\ T}{3\pi\ x\ \eta\ x\ Dt} \qquad \text{(Eq. 3.1)}$$

Where: Dh = hydrodynamic diameter

kB = Boltzmann's constant

T = temperature

η = dynamic viscosity

Dt = translational diffusion coefficient

Phosphate content

Liposome phosphate content was measured using a modification of the Fiske & Subbarow method for determining phosphorus (38, 39). Organic phosphorus in the sample is oxidized (digested) with sulfuric acid. Ammonium molybdate reacts with phosphate in the sample forming a phosphomolybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid. The color is proportional to the phosphorus concentration and its maximum absorption was measured at 820 nm on a spectrophotometer SPECTRA max PLUS 384 (Molecular Devices Corporation, Sunnyvale, CA, USA). A 3mM NaH_2PO_4 stock solution was used to generate a standard curve. A phosphate assay standard curve was obtained by plotting the absorbance against the phosphate concentration of standards (0, 75, 225, 300 and 600 nmol PO_4), and further used to calculate the phospholipid content of liposome preparations, according to the following equation:

$$Total\ lipid\ conncetration\ (mM) = \frac{C_{PO4}\ x\ Vs}{\%\ PO_4}\ X\ 100 \qquad \text{(Eq. 3.2)}$$

Where: C_{PO4} = phosphate concentration (nmol) from the absorbance standard curve

Vs = volume of liposomes (µL)

% PO_4 = percent of phosphate in the sample (70 for liposome preparations)

3.2.4 Liposome treatment

Previous studies have shown no added benefit of increasing the liposome treatment concentration beyond 2 mM (40). Therefore, this concentration was applied for all the liposome studies included in this thesis.

The effects of liposome composition on rat RBC in vitro quality

The packed rat RBCs were incubated at 37 °C with either HEPES-NaCl solution (non-treated control) or 2 mM lipid from four different liposome formulations. The RBCs were gently rotated during incubation. The *in vitro* quality of DPPC/DOPC/ DPPC-PS/ DOPC-PS RBCs (n=3 each) and control RBCs (n=3) was analyzed immediately after the liposome treatment and at weekly intervals up to 5 weeks of hypothermic storage and included percent hemolysis, hematological indices, deformability, and cholesterol and phospholipid concentrations, as described below.

The effects of DOPC liposome treatment on rat RBC hemorheology

The packed rat RBCs were incubated for 30 min at 37 °C with either HEPES-NaCl solution (non-treated control) or 2 mM lipid from DOPC liposomes. The RBCs were gently rotated during incubation. The *in vitro* quality of DOPC RBCs (n=7) and control RBCs (n=7) was analyzed immediately after the liposome treatment and upon 1 week and 6 weeks of hypothermic storage and included percent hemolysis, hematological indices, deformability, aggregation and microvesiculation, as described below.

3.2.5 RBC *in vitro* quality assessment

Rat RBC quality was assessed by percent hemolysis (Drabkin's), hematological indices (Coulter), deformability and aggregation (LORCA), microvesiculation (flow cytometry) all as described in Chapter 2, Section 2.2.2 and cholesterol/phospholipid concentrations.

Density gradient separation using Ficoll-Paque (GE Healthcare Biosciences, Uppsala, Sweden) was performed to separate RBCs from liposomes adsorbed to the RBC membrane, as well as free liposomes in the incubation mixture (20). Lipid extraction was performed according to Rose & Oklander (1965) (41). Briefly, RBCs were washed 3x in 0.9% NaCl. After the last wash, RBCs were hemolysed with distilled water, allowed to stand for 15 min and then centrifuged. The pellet was resuspended in distilled water and transferred to a glass tube, isopropanol was added and after 1 h of incubation, chloroform was added to the mixture. After another hour, the extraction tubes were centrifuged at 500 x g for 30 min at 4 °C. The top layer containing the lipid extract was transferred to 3 glass tubes, dried using a N_2 stream and frozen at -20 °C for further cholesterol and phospholipid analysis.

The EnzyChrom™ AF Cholesterol Assay Kit (E2CH-100; BioAssay Systems, Hayward, CA) was used to determine the amount of cholesterol in the extracts according to manufacturer's instructions (21). The kit consists of a single reagent that combines cholesterol ester hydrolysis, oxidation and color reaction in one step. In addition to a cholesterol standard (300 mg/dL). The color intensity of the reaction product at 570nm is directly proportional to total cholesterol concentration in the sample. A standard curve was obtained by plotting the absorbance against the cholesterol concentration of standards (0 – 100 mg/dL cholesterol, 10 mg/dL increments), and further used to calculate the cholesterol content of the samples, according to the following equation:

$$cholesterol\ (mg/dL) = \frac{A_S - A_b}{slope} \qquad \text{(Eq. 3.3)}$$

Where: A_s = absorbance of the sample

A_b = absorbance of the blank

Slope = slope of the linear regression fit

Phospholipids in the extracts were estimated by spectrophotometric phosphorus determination after an acid digestion as previously described in Section 3.2.3 of this chapter.

3.2.6 Effects of transfusing DOPC-treated rat RBCs in an anemic rat model of myocardial ischemia and reperfusion*

Male Sprague-Dawley rats (200-300 g) were used to experimentally evaluate *in vivo* effect of transfusing liposome-treated RBCs. All animals were provided water and food ad libitum and housed in a temperature- and humidity-controlled facility with 12-hour light and dark cycles. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Experimental protocols were approved by the Animal Use Committee at the University of Western Ontario. Anemia was induced in rats by a combination of iron-deficient diet (10-20 ppm; TestDiet 5859, Richmond, IN) and phlebotomy (2-3 mL) twice weekly to reach target hemoglobin (Hb) levels of 80-90 g/L. Immediately after phlebotomy, rats were infused with an equal volume of 10% pentastarch (Bristol-Myers Squibb Canada, Montreal, Quebec, Canada) (28). Myocardial ischemia and reperfusion (I/R) was induced by occlusion of the left descending coronary artery for 45 minutes, followed by 24 hours of reperfusion according to previous reports (25, 28). Anemic rats were transfused immediately after coronary artery ligation with either non-treated RBCs or DOPC-treated RBCs stored for one week. After transfusion, Hb levels, infarct size and 24 h-survival were measured. Infarct size was determined according to previous reports (42, 43). Briefly, 3 mL of Evans blue dye was

* *In vivo* experiments were performed in collaboration with Dr. Qingping Feng and Dr. Ian Chin-Yee from the University of Western Ontario, London, ON.

injected into the left ventricle to distinguish between perfused and nonperfused areas of the heart. Hearts were then excised and cut into four transverse slices from the apex to base. Sections were stained with 1.5% triphenyltetrazolium chloride (TTC) for 30 min at 37°C, and then weighed. The nonischemic area (blue), area at risk (red), and infarct area (pale) were quantitated using an image analysis system (Sigma ScanPro, Ashburn, VA). Percent weight of nonischemic, risk, and infarct area were calculated. Infarct size was expressed as a percentage of the weight of the infarct area to the area at risk.

3.2.7 Statistical analyses

Statistical analysis was performed using SPSS 22.0 software (IBM, Armonk, NY). Mann-Whitney's U non-parametric test was used to assess differences between liposome-treated rat RBCs and controls, and between anemic rats transfused with liposome-treated RBCs vs. non-treated RBCs. Wilcoxon signed-rank test was used to assess differences between liposome-treated rat RBCs and controls over storage time. Data were expressed as mean ± standard deviation and $p < 0.05$ was considered statistically significant.

3.3 Results

The characteristics of the liposome formulations used for all studies comprised in this chapter are shown in Table 3.1.

3.3.1 The effects of liposome composition on rat RBC in vitro quality

Immediately after incubation, all liposome treatments resulted in significant decrease in percent hemolysis, with the effect most prominent with DOPC-treated RBCs (1.6 ± 0.1% vs 3.1 ± 0.2 %, $p = 0.050$; Figure 3.1A). Liposome treatment did not significantly alter rat RBC membrane deformability (EI_{max}: Control 0.58 ± 0.00; DOPC-PS 0.59 ± 0.01; DOPC 0.57 ±

0.00; DPPC-PS 0.57 ± 0.01; DPPC 0.58 ± 0.00, p > 0.050) or hematological indices (MCV, MCH and MCHC). DOPC liposome treatment resulted in significant increase in rat RBC phospholipid concentration, while DPPC liposome treatment seemed to induce loss of phospholipids from the rat RBC membranes (1.56 ± 0.20 mg/ g Hb vs 0.76 ± 0.07 mg/ g Hb, p = 0.003). The cholesterol: phospholipid ratio in DOPC-treated rat RBCs was comparable to control (0.49 ± 0.06 vs 0.51 ± 0.08, p = 0.800; Figure 3.2). All liposome treated RBCs continued to exhibit decreased hemolysis after 5 weeks of storage compared to the control (p = 0.050), with uncharged formulations resulting in lower hemolysis than charged liposomes (3.4 ± 0.2% vs 3.9 ± 0.4%, p = 0.010) (Figure 3.1A). In terms of rigidity (K_{EI}), DOPC-treated RBCs remained significantly lower compared to control RBCs even after 5 weeks of storage (Figure 3.1B). Based on these findings, the DOPC formulation was selected for further studies on hemorheology.

3.3.2 The effects of DOPC liposome treatment on rat RBC hemorheology

Table 3.2 describes the hemorheology parameters of liposome-treated and control RBCs in additive solution 3 (AS3), both immediately after treatment (fresh) and after 6 weeks of hypothermic storage. DOPC liposome treatment resulted in significant changes in fresh liposome-treated RBCs compared to control RBCs, such as a decrease in percent hemolysis (1.7 ± 0.2% vs. 2.3 ± 0.3%, p = 0.018), MCH (22.0 ± 1.0 vs. 22.3 ± 0.9, p = 0.018) and MCHC (355 ± 11 vs. 375 ± 7, p = 0.046) an increase in rigidity (K_{EI}: 0.84 ± 0.10 vs. 0.77 ± 0.07, p = 0.043) and Hct (51.7 ± 2.3 % vs. 49.8 ± 1.3 %, p = 0.041). The intensity of PS exposure was the same in control and liposome-treated MPs (MP-MFI/MP-PS%, p >0.050). PS-MFI of microparticles decreased over time in both liposome-treated and control MPs (Table 3.2). After six weeks of hypothermic storage, liposome-treated RBCs continued to

differ from control RBCs by exhibiting an increased Hct (47.1 ± 1.7 % vs. 45.0 ± 1.5 %, p = 0.028) and lower rigidity (K_{EI}: 1.03 ± 0.18 vs. 1.16 ± 0.13, p = 0.043). Flow cytometry analysis also showed increased PS exposure in liposome-treated RBCs (1.1 ± 0.2 vs. 0.8 ± 0.2, p = 0.018) while PS exposure in MPs was significantly lower (55.8 ± 6.5 vs. 34.8 ± 8.5, p = 0.031; Figure 3.3). The intensity of PS exposure was lower in liposome-treated MPs (= less PS/MP) than in control MPs as shown by MP-MFI/MP-PS% values (Figure 3.4), with a more prominent decrease observed in liposome treated MPs (63.1% vs. 39.2%, p = 0.009). In terms of aggregation parameters, there was no significant difference between control and liposome-treated RBCs. However, control RBCs showed a significant decrease in aggregation index (AI: 41.9 ± 7.2 % vs. 29.9 ± 4.1 %, p = 0.018) and an increase in aggregation half time ($t_{1/2}$: 6.5 ± 2.9 s vs. 12.5 ± 2.8 s, p = 0.018) after six weeks of storage that was not observed in liposome-treated RBCs (Figure 3.5). The only change observed in liposome-treated RBCs was a decrease in aggregation amplitude (Amp: 12.9 ± 1.7 au vs. 10.9 ± 1.4 au, p = 0.018).

3.3.3 Effects of transfusing DOPC-treated rat RBCs in an anemic rat model of myocardial ischemia and reperfusion

The Hb levels prior to transfusion were similar in both control and liposome-treated groups (84.2 ± 7.1 vs. 83.5 ± 5.3 g/L, p = 1.000). The *in vivo* study showed that the 24 h-survival after myocardial I/R was the same in both liposome-treated (7 out of 7) and control groups (7 out of 7). No significant difference was observed in the Hb levels (control: 99.8 ± 3.0 vs. lipo: 101.9 ± 2.5 g/L, p = 0.197; Figure 3.6) and infarct size between the two groups (control: 53.3 ± 13.1 vs. lipo: 45.3 ± 8.4%, p = 0.223; Figure 3.7). Note that two rats from the liposome-treated group had to be excluded from the statistical analysis of infarct size due to very small infarct sizes attributed to the surgical procedure.

3.4 Discussion

Several elements of RBC membrane storage lesion remain unaddressed by current additive solutions (4). A membrane-based approach to preserve RBC quality, such as the use of liposomes, may mitigate MP shedding, PS exposure and impaired hemorheology. This study investigated the effects of liposome treatment on rat RBC HSL and rheologic properties as well as the outcomes of transfusing liposome treated RBCs in a rat model.

All the liposome formulations tested succesfully decreased hemolysis in rat RBCs compared to non-treated controls. The same effect has also been described for human RBCs (23). RBCs treated with liposomes exhibited higher Hct and lower supernatant Hb concentrations, showing that the treatment stabilizes the cell membrane, preventing Hb leakage. The exact mechanism by which liposomes reduce hemolysis has not been completely elucidated, but based on the *in vitro* mechanisms of liposome-cell interactions previously described, the possible interactions include lipid transfer, adsorption, fusion or a combination of these (21, 44). The DOPC liposomes used in this study, which were small unilamellar vesicles (SUV), neutral (not charged) and fluid (always kept above the Tc of the lipid) had the most prominent effect in reducing hemolysis of rat RBCs. Based on those characteristics, the most probable RBC-liposome interaction types are membrane fusion and lipid transfer (22, 44). Renooij & Van Golde (1976) (45) have reported that lipid exchange between rat RBCs and lipid vesicles is a temperature dependent process that only becomes significant at temperatures higher than 20 °C, which explains why a difference was observed in hemolysis immeadiately after incubation with liposomes at 37 °C, but was no longer detectable after the storage period at 4 °C.

The concentration of MPs was comparable between the two treatment groups when fresh and after the 6-week storage period. However, some slight differences in the PS profile of those MPs, lead me to argue whether they would have the same biological activity. The percentage of PS (+) MPs decreased in control samples and remained stable in liposome-treated samples over time, with overall values being comparable between the groups for both time points evaluated (fresh and 6 weeks). An interesting observation is that even though the percentage of PS (+) MPs is comparable in both groups after 6 weeks, MPs from liposome-treated group exposed less PS per MP compared to control (as shown by MP-MFI/MP-PS% values; Table 3.2; Figure 3.4). Percentage of PS (+) RBCs increased over time in both groups, with a more prominent increase in the liposome-treated group. The increase in PS (+) RBCs seems to be a natural process that occurs to rat RBCs during storage time. The slightly higher increase observed in liposome-treated samples might be due to the cholesterol/phospholipid ratio of the liposome formulation used (0.4:1), and therefore can be easily modified. According to Van Meer and colleagues (1980) (46) liposomes with cholesterol/phospholipid ratio lower than 1:1 can extract cholesterol from RBCs resulting in altered stability that will directly impact membrane lipid asymmetry. PS exposure is often associated with increased inflammation, coagulation and endothelial injury (47, 48) so it would be fair to assume that less PS exposure on MPs could result in less adverse events. On the other hand, more PS exposure is also present in senescent cells and correlates with increased *in vivo* uptake by the reticuloendothelial system (49) which would potentially result in faster removal of those MPs from the circulation. Further investigation is needed to establish the clinical relevance of these findings and understand whether the two types of MPs are equivalent or whether one type would produce more deleterious effects than the other.

RBC membrane characteristics such as surface charge, phospholipid composition and membrane mechanical behavior are the most commonly considered biophysical properties that influence aggregation behavior (50). Increased aggregation results in an increase in blood viscosity, affects microcirculatory blood flow and organ perfusion (51). In terms of aggregation parameters, there was no significant difference between the two groups. However, fewer changes were observed in the liposome-treated group over time suggesting a stabilization effect of liposomes on RBCs that minimizes the effects of HSL. The only change observed was a decrease in aggregation amplitude, related to the quantity of 3D aggregates formed. Therefore, the differences in aggregation behavior within control and liposome-treated RBCs over time could be attributed to changes resulting from the liposome treatment.

Deformability measurements can be described by two distinct parameters EI_{max} and K_{EI}. The EI_{max} represents how much elongation the cells can tolerate and directly relates to the elasticity of the RBC membrane, while the K_{EI} is a measurement of cell rigidity that relates to the cytoskeleton/membrane assembly rather than the membrane alone. In this study, both treatment groups presented an equivalent decrease in EI_{max} over the 6 weeks of storage. The decrease in deformability might be explained by membrane loss in the form of MPs that was not overcome by the formulation used. The K_{EI} from liposome-treated RBCs increased significantly immediately after treatment and remained stable during storage, while in control RBCs it significantly increased over time and to a greater extent. Lipid transfer or fusion of liposomes with RBCs in the treatment group could alter the association of the membrane with the cytoskeleton, changing the rigidity at first but then maintaining it at a constant rigidity throughout storage. It has been reported that phosphorylation of major proteins such as Ankyrin, band 4.1 and band 4.9 can weaken the rigidity of the cytoskeleton by reducing the binding affinity (52).

Rat RBCs, despite having similar characteristics to human RBCs, which make them a suitable model for transfusion studies, still differ from human RBCs in many metabolic and membrane-related aspects (53). The liposome formulation used in the *in vivo* study did not cause deleterious effects in terms of the clinical outcomes of ischemia/reperfusion in anemic rats, and therefore appears to be safe. Hu and colleagues (2010, 2012) using anemic Sprague-Dawley rats and the same I/R procedure, reported that baseline infarct size for anemic rats that did not received transfusion was about 70% (25, 28). Comparing to the data presented here this value is 17% larger compared to the group that received control RBCs and 25% larger compared to the group that received DOPC-treated RBCs. However, the safety observed in rats cannot be directly translated to human safety, since previous drugs that were shown to be safe in animal models later failed safety tests in humans (54). Therefore, as in many studies involving animal models to mimic clinical settings in humans, the results presented need to be interpreted with caution due to limitations such as species differences, group sizes and a possible underestimation of the adaptive response in animals, as summarized by Hartung (2008) (55). The present study was limited to examining the acute effects of transfusing liposome-treated RBCs in a relatively small number of animals per group. Hypersensitivity reactions mediated by complement activation could lead to cardiovascular and hemodynamic alterations (56), as well as coagulation disturbances and transient thrombocytopenia caused by liposome-platelets interactions (57-59) were some of the acute outcomes of concern and that could have resulted in decreased survival of the animals after transfusion of liposome-treated RBCs. In terms of chronic outcomes, the main concern would be immune system modulation that could lead to inflammation, immune suppression or autoimmune responses.

The elements of rat RBC HSL are similar to human, except that the rat RBC storage lesion of seven days is equivalent to the human RBC storage lesion of 29 days (60). This

animal model has been used in the past to evaluate effects of hypothermic storage lesion and leukoreduction (25, 28, 29). By using sensitive measures of specific organ injury and myocardial infarct size, is possible to quantitate the effects of anemia, as well as differentiate between fresh and stored blood transfusion. The liposome formulation used in the *in vivo* study did not show superior effects in terms of the outcomes of I/R in anemic rats.

3.5 Conclusion

From the four formulations tested unsaturated-uncharged liposomes (DOPC) were the most beneficial for rat RBCs and were therefore used to further evaluate effects on hemorheology and *in vivo* study. DOPC liposome treatment resulted in an overall improvement in rat RBC hemorheology upon storage. However, the changes observed with liposome treatment *in vitro* were not sufficient to improve the outcomes of myocardial I/R in anemic rats transfused with liposome-treated RBCs.

Even so, because the *in vivo* study did not prompt any immediate safety concerns, the beneficial effects of liposome treatment should continue to be investigated on stored human RBCs, by exploring new formulations and combinations of treatments. In addition to further studies confirming these safety results and exploring the long-term effects, more studies need to be done to address the *in vivo* viability and oxygen delivery efficacy of this transfusion product.

Next chapter will investigate the effect of DOPC liposomes on stored human RBCs, while determining the ideal treatment time and its effect on hemorheology.

Table 3.1: Formulations, size and lipid content of liposomes used in the studies. Mean values ± SD for experiments that required more than one batch of liposomes.

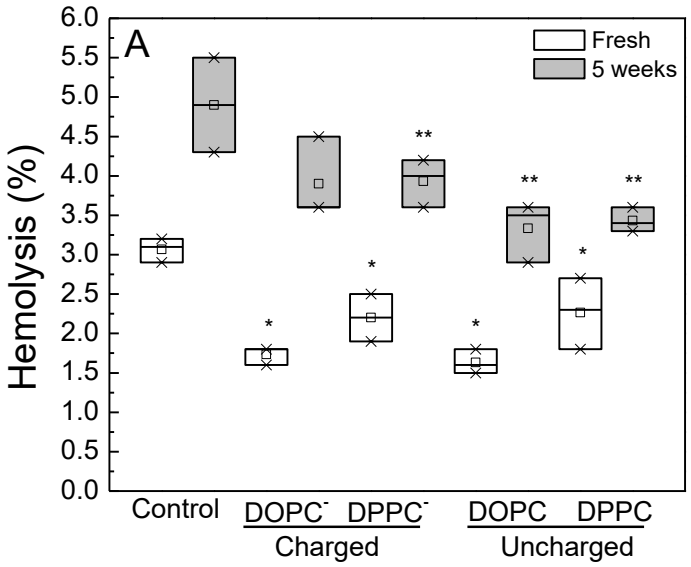
Study	Formulation	Size (nm)	Lipid content (mM)
Effect of formulation	DOPC:Chol (70:30)	127.3	16.8
	DOPC:PS:Chol (60:10:30)	130.5	17.0
	DPPC:Chol (70:30)	177.8	16.1
	DPPC:PS:Chol (60:10:30)	147.9	21.5
Hemorheology	DOPC:Chol (70:30)	129.6 ± 1.8	19.4 ± 0.1
In vivo	DOPC:Chol (70:30)	130.7 ± 5.0	19.4 ± 1.5

Table 3.2: Hemorheology parameters of DOPC-treated RBCs and control rat RBCs. Mean values ± SD for DOPC-treated and control rat RBCs immediately after treatment and after 6 weeks of hypothermic storage are shown. au = arbitrary units.

	Control RBCs		***DOPC-treated RBCs***	
RBC hemorheology	**Fresh**	**6 weeks**	**Fresh**	**6 weeks**
Hemolysis (%)	2.3 ± 0.3	4.7 ± 0.7‡	1.7 ± 0.2*	5.0 ± 0.6‡
Hematologic indices				
MCV (fL)	62.1 ± 2.2	64.1 ± 2.5‡	61.9 ± 2.5	64.4 ± 3.0‡
MCH (pg)	22.3 ± 0.9	24.1 ± 1.1‡	22.0 ± 1.0*	24.2 ± 1.2‡
MCHC (g/l)	360 ± 10	375 ± 7	355 ± 11*	376 ± 6‡
Deformability				
EI_{max}	0.56 ± 0.01	0.40 ± 0.02‡	0.56 ± 0.02	0.39 ± 0.02‡
K_{EI}	0.77 ± 0.07	1.16 ± 0.13‡	0.84 ± 0.10*	1.03 ± 0.18*
Aggregation parameters				
AI (%)	41.9 ± 7.2	29.9 ± 4.1‡	34.7 ± 9.2	30.7 ± 6.1
Amp (au)	11.7 ± 1.8	10.6 ± 2.0	12.9 ± 1.7	10.9 ± 1.4‡
$t_{1/2}$ (s)	6.5 ± 2.9	12.5 ± 2.8‡	9.1 ± 4.4	11.4 ± 4.5
Microvesiculation				
MPs/μL	24,663 ± 1,529	444,084 ± 103,366‡	26,339 ± 2,897	569,258 ± 115,981‡
RBC-PS (%)	0.3 ± 0.0	0.8 ± 0.2‡	0.3 ± 0.0	1.1 ± 0.2*‡
MP-PS (%)	35.1 ± 4.7	24.8 ± 7.8‡	34.3 ± 4.0	28.5 ± 9.3
RBC-MFI	68.3 ± 6.1	59.3 ± 9.2	74.0 ± 17.0	53.3 ± 5.0‡
MP-MFI	146.3 ± 32.3	49.1 ± 8.5‡	131.6 ± 32.5	44.9 ± 2.0‡
MP-MFI/MP-PS%	3.9 ± 0.8	1.8 ± 0.2‡	3.7 ± 0.9	1.5 ± 0.3*‡

* $p < 0.05$ compared to control RBCs of corresponding age, ‡ $p < 0.05$ compared to fresh RBCs of the same group.

Figure 3.1: Percent hemolysis (A) and rigidity (B) in rat RBCs treated with charged and uncharged liposome formulations. Shown in the boxplots (n= 3) are: 99% percentile (higher x), 1% percentile (lower x) and mean (□). * Significant ($p < 0.05$) compared to fresh control RBCs; ** significant ($p < 0.05$) compared to control RBCs stored for 5 weeks.



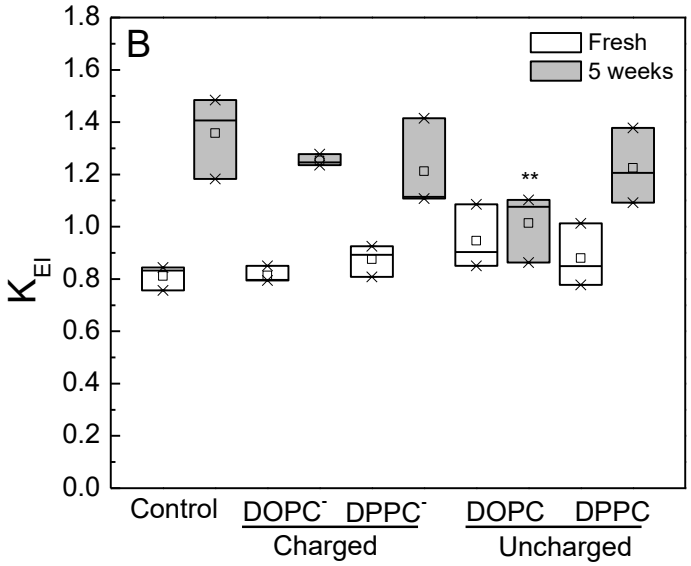


Figure 3.2: Cholesterol/Phospholipid ratio in rat RBCs treated with saturated and unsaturated liposome formulations. Shown in the boxplots (n= 3) are: 99% percentile (higher x), 1% percentile (lower x) and mean (□). * Significant ($p < 0.05$) compared to control RBCs.

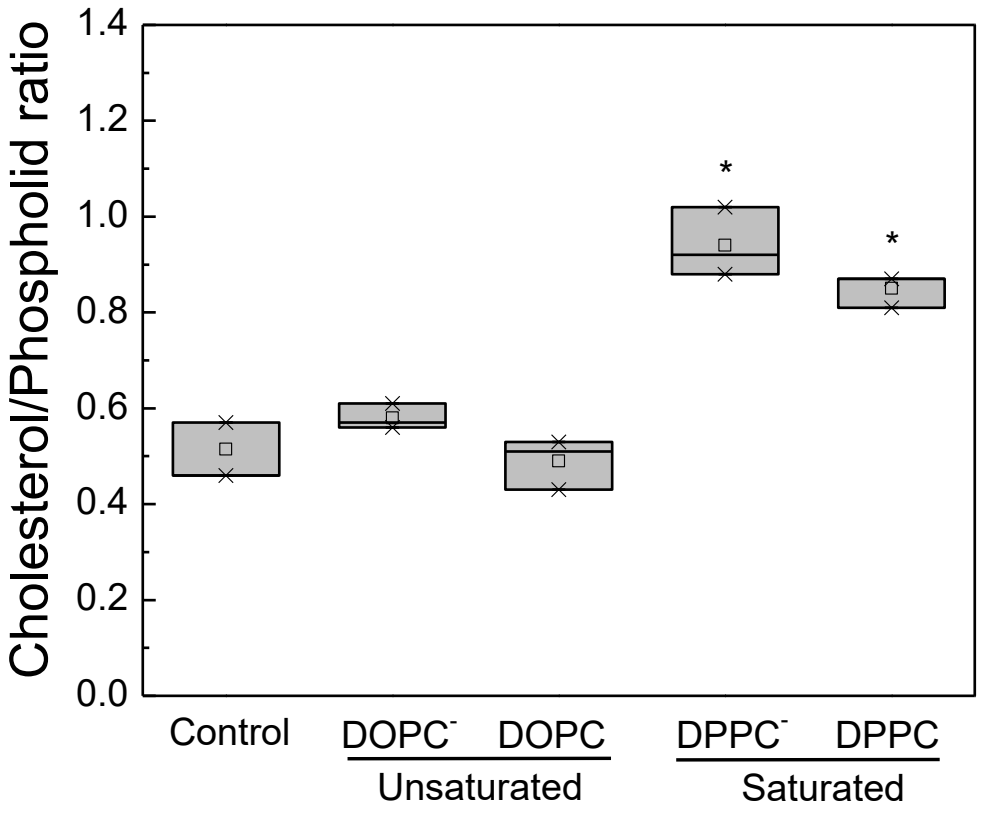


Figure 3.3: Percentage of phosphatidylserine (PS) exposure by MPs in fresh control and liposome-treated rat RBCs and after 6 weeks of storage. Histogram: region M1 is negative for PS exposure while, region M2 is positive.

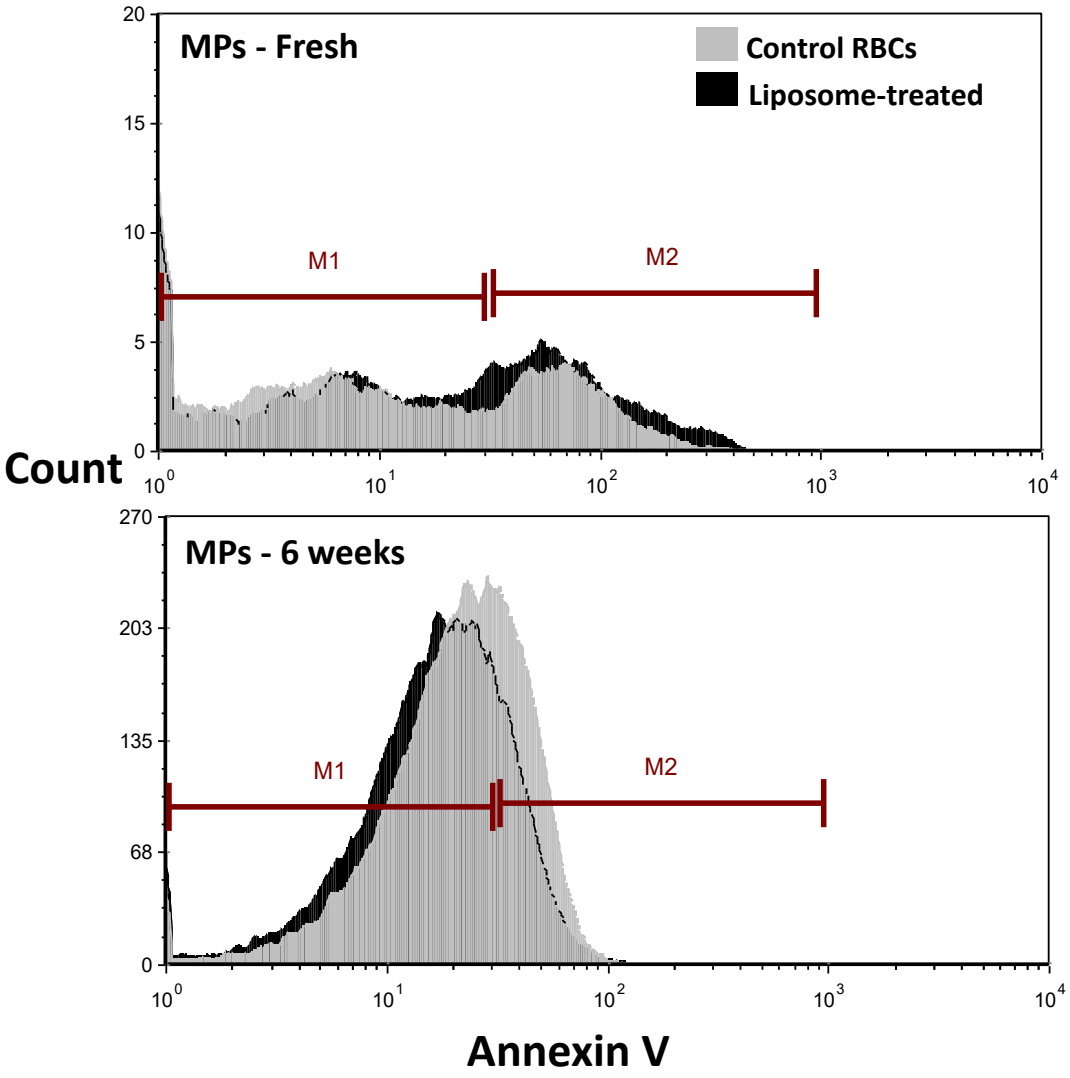


Figure 3.4: Intensity of phosphatidylserine (PS) exposure (MFI) divided by percentage of PS (+) MPs in fresh control and liposome-treated rat MPs and after 6 weeks of storage. * Significant ($p < 0.05$) compared to control MPs of same age.

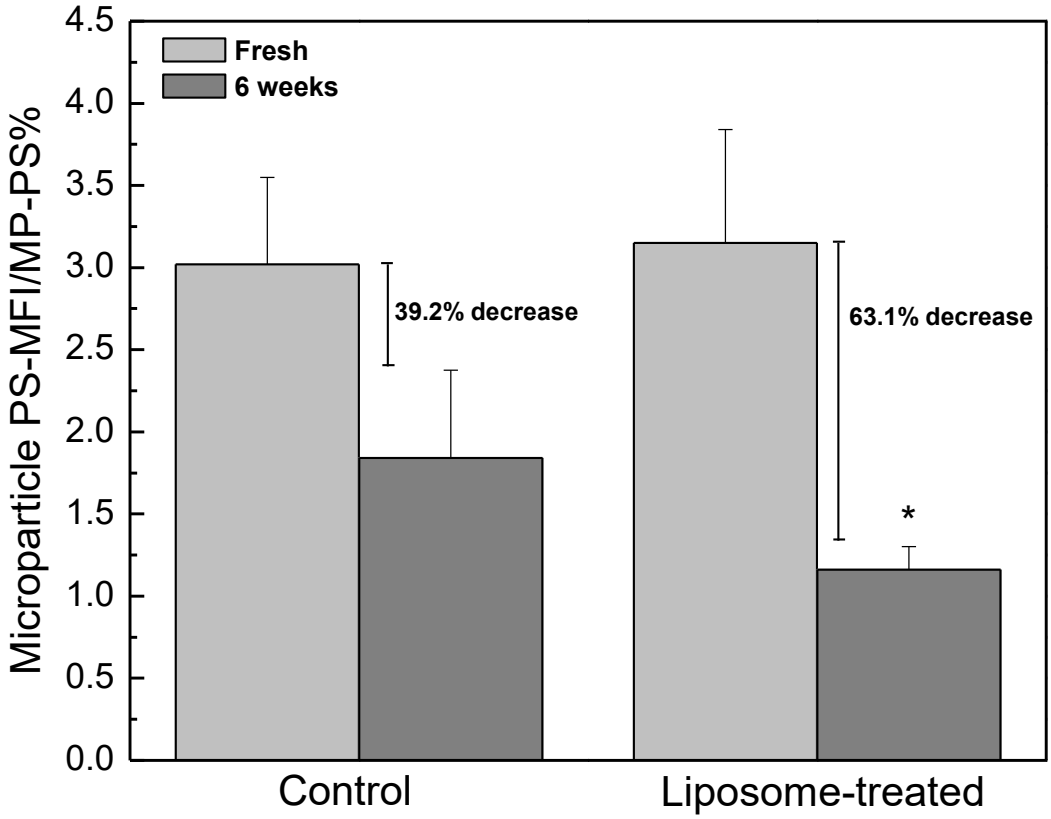


Figure 3.5: RBC aggregation in control and liposome-treated rat RBCs (fresh and after 6 weeks of storage). Syllectograms (light scatter x time) showing RBC aggregation patterns (n=7). Negative control (PBS) = no aggregation; Positive control (3% dextran) = high aggregation.

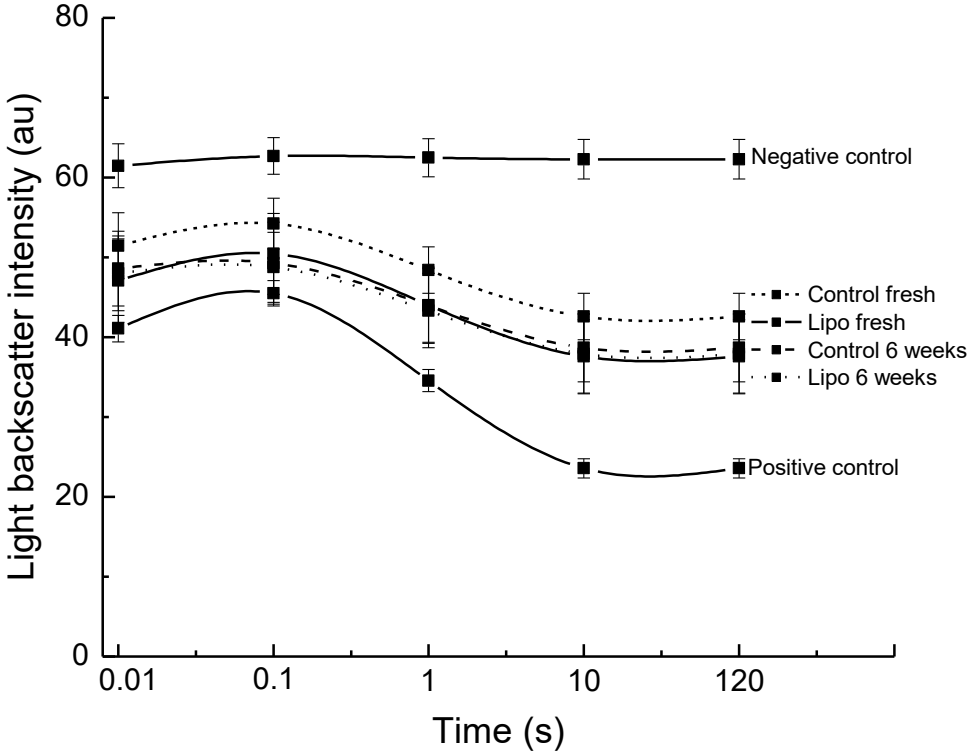


Figure 3.6: Hemoglobin levels in rats from liposome and control groups before and after transfusion. Shown in the boxplots (n= 7) are: 99% percentile (higher x), 1% percentile (lower x) and mean (□).

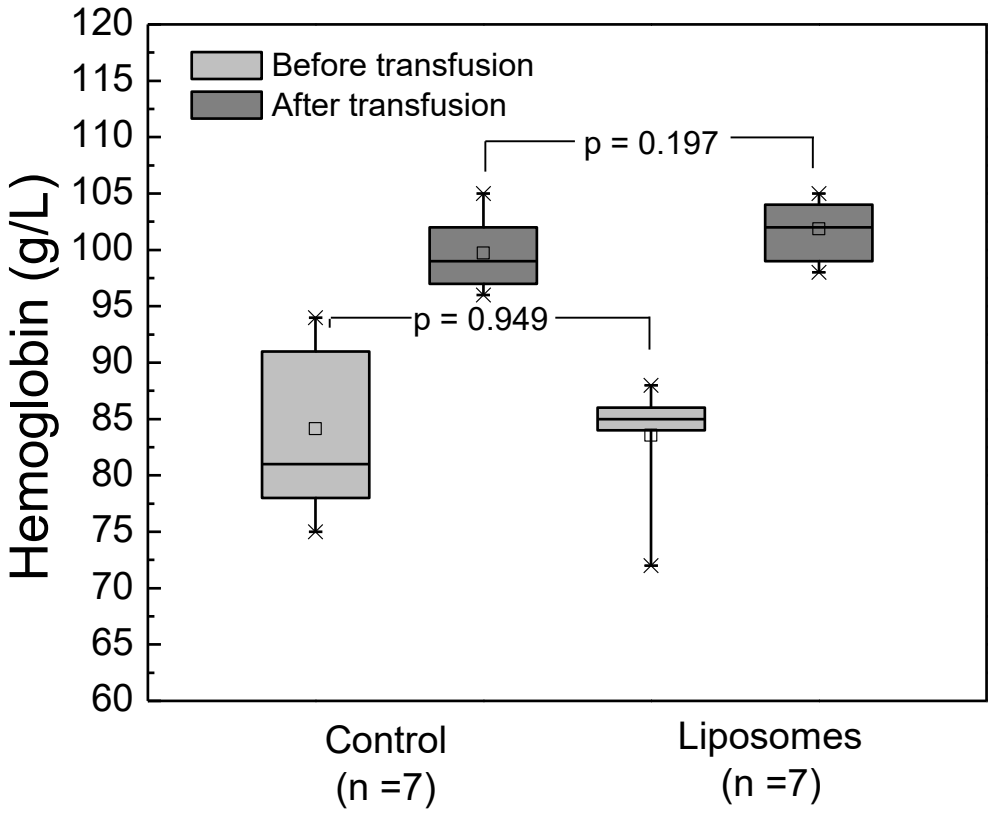
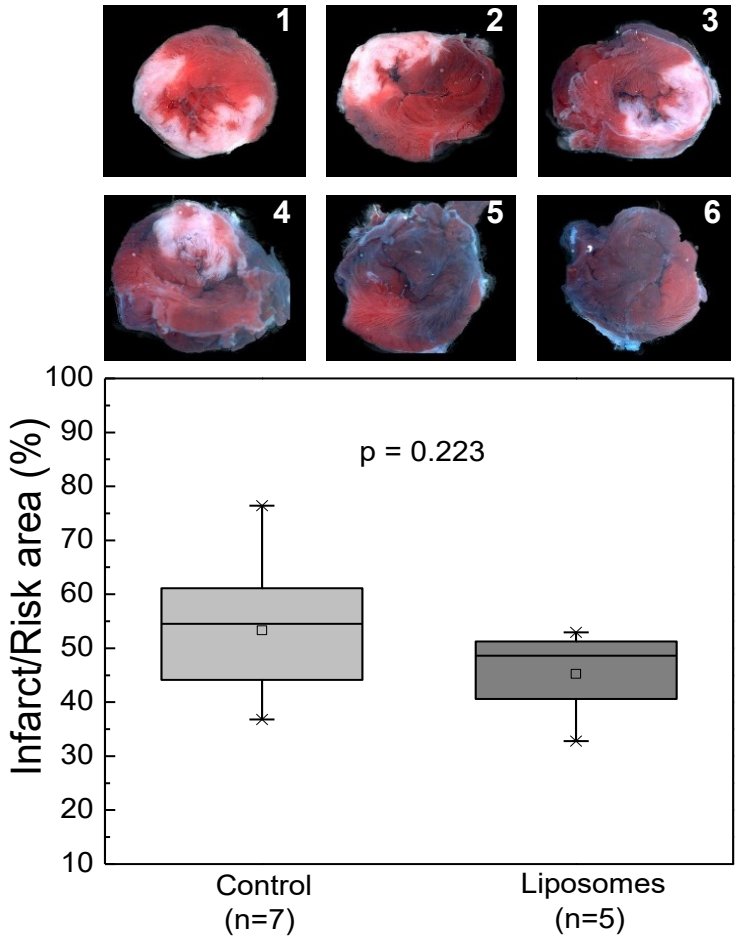


Figure 3.7: Effect of liposome treated rat red blood cells on infarct size after ischemia and reperfusion in anemic rats. Pictures 1-6 represent transverse sections of the same heart from apex to base stained with 2% triphenyltetrazolium chloride (TTC) where infarct size was measured. Blue area: non-ischemic, red area: at risk area, white area: infarct area. Shown in the boxplots are: 99% percentile (higher x), 1% percentile (lower x) and mean (□). Baseline Infarct/risk area in anemic rats after I/R without transfusion is ~ 70% (Source: Hu *et al.*, 2010; Hu *et al.*, 2012).



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Chapter 4

The effect of liposome treatment on hemorheology and metabolic profile of human RBCs

4.1 Introduction

Ex vivo cold storage of RBCs for transfusion has long been associated with a set of changes to cell characteristics that results in reduced *in vitro* quality, including cell shape change, loss of membrane, decreased deformability, altered aggregation behavior, metabolic depletion and, finally, hemolysis. These RBC changes are now all known collectively as HSL (1). Cold storage slows down metabolism but does not completely supress it, so the cells will continue to age, along with a confined and fixed environment that will accumulate cell metabolism by-products and cell waste that will contribute to changes in pH, that in turn will affect cellular responses (2).

Studies of HSL have characterized that the most significant changes start to happen between 2 and 3 weeks of storage (~ 21 days), period after which, some changes become irreversible. For example, RBC changes shape after loss of membrane (discoid shape to spherocytes)(3, 4) and 2,3-DPG is also no longer detectable after two weeks, although studies have shown that normal levels are restored within 72 h of transfusion (5, 6). Additive solutions provide RBCs with an excess of nutrients, substrates for metabolism and membrane stabilizers that help maintain cellular structure and metabolism for longer periods (up to six weeks) during *ex vivo* storage (7).

One important topic of HSL not addressed by additive solutions and metabolic rejuvenation is the loss of phospholipid asymmetry that culminates in membrane loss. The loss of RBC membrane phospholipid asymmetry ultimately leads to PS externalization, which culminates in MP formation and release (8, 9). Several mechanisms can be attributed to MP shedding from RBCs including protein oxidation (10), ATP depletion (11, 12) and increased intracellular Ca2+ concentrations (13). Current strategies to preserve *in vitro* RBC quality prior to transfusion do not adequately address the loss and remodeling of RBC membrane.

Numerous studies using RBCs as membrane models have utilized liposomes as means for modifying the composition of membranes and have documented the effect of these manipulations (14, 15). Liposomes have been shown to influence membrane fluidity and thermal phase behavior (16, 17) of human RBCs, but no studies have evaluated the effect of liposomes as a strategy for decreasing MP concentration or changing its composition during hypothermic storage. The intent of the liposome treatment is to provide a source of phospholipids for membrane renewal and conservation of phospholipid asymmetry; since RBCs are not able to synthesize phospholipids and their *in vivo* source (lipoproteins) do not remain in the bag after whole blood is processed into packed RBCs (18). Considering the aforementioned facts, it is important to evaluate when the liposome treatment should be performed in human RBCs so the cells would be most benefited by the liposomal supply of phospholipids.

In chapter 3, I described an assessment of the impact of liposome treatment on hemorheology of rat RBCs (19) with an observation that DOPC liposome treatment resulted in an overall improvement in rat RBC hemorheology upon storage. Therefore, it is important to evaluate if those effects remain true for human RBCs. In addition, there are no reports on the effect of membrane manipulations using liposomes on the metabolic profile of cells. Although it is not expected that the treatment will induce significant changes in the metabolic profile, it is essential to make sure the treatment will not further deplete metabolic markers (i.e. ATP, 2,3-DPG). With those observations in mind, the aim of this study was to evaluate the effect of liposome treatment time on human RBCs and to assess the effect of liposome treatment on hemorheology and metabolic profile of human RBCs using the same liposome formulation that rendered significant changes to rat RBCs.

4.2 Materials and Methods

4.2.1 RBC collection and manufacturing

Ethical approval for the study was granted by both the Canadian Blood Services (CBS) and the University of Alberta Research Ethics Boards. Ten leukoreduced packed RBC units in saline-adenine-glucose-mannitol buffy coat processed were obtained from the CBS Network Centre for Applied Development (netCAD) and processed from whole blood as previously described (20).

4.2.2 Liposome treatment

Unilamellar (DOPC:cholesterol, 7:3 mol%) liposomes were synthesized as described in Chapter 3, Section 3.2.2.

***The effect of liposome treatment time on human RBC in vitro quality**[*]*

Four leukoreduced packed RBC units in CPD-SAGM obtained from the CBS netCAD were pooled and split. One pool containing four Rh and ABO-matched RBC units was generated and subsequently split to produce four equivalent RBC products. RBCs were divided into three sets of tubes of control and liposome-treated samples (n= 4 each) and treated at day 2, day 21 and day 42 of hypothermic storage with a final assessment at day 45. The packed RBCs were incubated at 37 °C with either HEPES-NaCl solution (non-treated control) or 2 mM lipid DOPC liposomes (147.2 nm). The *in vitro* quality of DOPC-treated RBCs (n=4) and control RBCs (n=4) was analyzed immediately after the liposome treatment (for each treatment day) and at days 42 and 45 of hypothermic storage and included percent

[*] The author would like to acknowledge Angela Hill (Acker Lab research technician) for her help with the pool and split procedure.

hemolysis, hematological indices, deformability, aggregation and microvesiculation, as described in Chapter 2, Section 2.2.2.

The effects of DOPC liposome treatment on human RBC hemorheology and metabolic content*

The packed human RBCs were incubated for 1 h at 37 °C with either HEPES-NaCl solution (non-treated control) or 2 mM lipid from DOPC liposomes (123.1 nm). The *in vitro* quality of DOPC RBCs (n=6) and control RBCs (n=6) was analyzed immediately after the liposome treatment and upon 3 week and 6 weeks of hypothermic storage and included percent hemolysis, hematological indices, deformability, aggregation, supernatant potassium, ATP and 2,3-DPG as described in Chapter 2, Section 2.2.2.

4.2.3 Statistical analyses

Statistical analysis was performed using SPSS 23.0 software (IBM, Armonk, NY). Two-way ANOVA was used to assess the effect of treatment (control vs. liposome) and treatment time (d2, d21, d42) on *in vitro* quality parameters. When a significant interaction between treatment and treatment time was found, simple main effects were analyzed using Bonferroni post hoc test. When no significant interaction was found, main effects were reported followed by Tukey's Post Hoc Test when needed. Paired Student's T test or Wilcoxon test was used to assess differences between liposome-treated RBCs and controls over storage time. Data were expressed as mean ± standard deviation (or mean ± standard error, where indicated) and $p < 0.05$ was considered statistically significant.

* Potassium supernatants were analyzed by Laboratory Services at the University of Alberta Hospital. ATP and 2,3-DPG assays were performed by MLS honors student Melissa Shyian under my supervision.

4.3 Results

4.3.1 The effect of liposome treatment time on human RBC in vitro quality

Table 4.1 summarizes the test between-subjects effects of two-way ANOVA. No significant differences between treatment type or treatment time were detected overall for aggregation parameters AI (model $p = 0.356$) and aggregation half time (model $p = 0.321$), therefore no further analysis was conducted. There was no significant interaction between treatment type and treatment time for ektacytometry parameters EI_{max} $F(2,18) = 0.22$, $p = 0.803$ and K_{EI} $F(2,18) = 0.35$, $p = 0.709$; aggregation amplitude $F(2,18) = 1.25$, $p = 0.310$; MCH $F(2,18) = 0.01$, $p = 0.990$; MCHC $F(2,18) = 3.05$, $p = 0.072$ and MP-MFI $F(2,18) = 0.59$, $p = 0.563$, therefore main effects were reported after post hoc analysis. There was a significant interaction between treatment and treatment time for hemolysis $F(2,18) = 26.14$, $p < 0.001$, MCV $F(2,18) = 20.09$, $p < 0.001$, MP concentration $F(2,18) = 18.67$, $p < 0.001$, MP-PS% $F(2,18) = 8.22$, $p = 0.003$ and MP-MFI/MP-PS% $F(2,18) = 5.21$, $p = 0.016$. Therefore, results were interpreted in the context of the interaction and simple effects reported.

Table 4.2 summarizes RBC quality parameters affected by treatment time. Deformability of samples treated on day 2 and day 21 was slightly lower compared to samples treated on day 42 (day 2: 0.54 ± 0.00 vs. 0.55 ± 0.00, $p < 0.001$; day 21: 0.54 ± 0.00 vs. 0.55 ± 0.00, $p < 0.001$). Rigidity of samples treated on day 2 and day 21 was lower compared to samples treated on day 42 (day 2: 2.12 ± 0.02 vs. 2.32 ± 0.02, $p < 0.001$; day 21: 2.16 ± 0.02 vs. 2.32 ± 0.02, $p < 0.001$). Aggregation amplitude was significantly lower on samples treated on day 2 compared to treated on day 42 (16.3 ± 0.3 vs. 17.3 ± 0.3, $p = 0.037$). MCH of samples treated on day 2 was lower compared to samples treated on day 21 (29.1 ± 0.1 vs. 29.5 ± 0.1, $p = 0.006$). MCHC of samples treated on day 2 was lower compared to samples

treated on day 21 (297 ± 1 vs. 302 ± 1, p = 0.001) and on day 42 (297 ± 1 vs. 302 ± 1, p = 0.001). MP-MFI was significantly lower in samples treated at day 2 compared to day 21 (p = 0.010) and 42 (p < 0.001); and on day 21 treatment compared to day 42 (p = 0.042).

K_{EI} was also affected by treatment type with DOPC-treated RBCs having higher rigidity than control RBCs (2.25 ± 0.02 vs. 2.16 ± 0.02, p = 0.002) (Figure 4.1 A). Aggregation amplitude was affected by treatment type with DOPC-treated RBCs having higher amplitude compared to control (17.2 ± 0.2 vs. 16.5 ± 0.2, p = 0.040) (Figure 4.1 B).

Table 4.3 summarizes simple effects analysis for RBC quality parameters where the interaction between treatment type and treatment time was significant. DOPC-treated RBCs had significantly lower hemolysis than control RBCs when treated at day 2 (p < 0.001) and day 21 (p <0.001), but there was no difference in hemolysis between treatments when RBCs were treated at day 42 (p = 0.093). DOPC-treated RBCs had significantly higher MCV than control RBCs when treated at day 2 (p < 0.001) and day 21 (p <0.001), but there was no difference in MCV between treatments when RBCs were treated at day 42 (p = 0.501). DOPC-treated RBCs had significantly higher MP concentration than control RBCs when treated at day 2 (p < 0.001) and day 21 (p <0.001), but there was no difference in MP concentration between treatments when RBCs were treated at day 42 (p = 0.074). DOPC-treated RBCs had significantly higher MP-PS% than control RBCs when treated at day 2 (p < 0.001) and day 21 (p = 0.001), but there was no difference in MP-PS% between treatments when RBCs were treated at day 42 (p = 0.715). DOPC-treated RBCs had significantly lower MP-MFI/MP-PS% than control RBCs when treated at day 2 (p = 0.001) and day 21 (p = 0.005), but there was no difference in MFI/MP-PS% between treatments when RBCs were treated at day 42 (p = 0.771) (Figure 4.2).

4.3.2 The effects of DOPC liposome treatment on human RBC hemorheology and metabolic content

Table 4.4 describes the hemorheology and metabolic parameters of liposome-treated and control RBCs, both immediately after treatment (fresh) and after 3 and 6 weeks of hypothermic storage. Ektacytometry analysis showed no significant effect of liposome treatment on maximum elongation (EI_{max}) in any of the time points evaluated. DOPC-treated RBCs showed increased rigidity immediately after treatment (K_{EI}: 2.08 ± 0.27 vs. 1.84 ± 0.27, p = 0.040), which stabilized after 6 weeks of storage resulting in significantly lower values compared to the control group (K_{EI}: 2.27 ± 0.33 vs. 2.39 ± 0.23, p = 0.048) (Figure 4.3). Syllectometry analysis showed a decrease in aggregation half time of liposome-treated RBCs (3.4 ± 0.4 s vs. 3.8 ± 0.4 s, p = 0.046) immediately after treatment. After 6 weeks of storage, aggregation index (AI) and aggregation amplitude (Amp) were significantly increased in liposome-treated RBCs (AI: 45.38 ± 1.92% vs. 41.54 ± 4.10%, p = 0.020) (Amp: 16.38 ± 2.17 au vs. 12.22 ± 3.29 au, p = 0.019) while aggregation half time was lower when compared to control (4.93 ± 0.46 s vs. 6.05 ± 1.21 s, p = 0.035) (Figure 4.4). Although hemolysis was significantly lower immediately after treatment in the liposome group (0.15 ± 0.13% vs. 0.18 ± 0.14%, p = 0.042), a significant difference was not detected after 3 and 6 weeks of storage. Despite comparable hemolysis levels at 3 and 6 weeks, DOPC-treated RBCs showed significantly increased potassium levels for the aforementioned time-points (3 weeks: 31.2 ± 2.7 mmol/l vs. 30.8 ± 2.7 mmol/l, p = 0.007; 6 weeks: 45.0 ± 3.0 mmol/l vs. 43.8 ± 3.4 mmol/l, p = 0.013). MCH was comparable between treatments throughout storage, while MCHC was slightly increased in DOPC-treated RBCs after 6 weeks of storage (306 ± 5 g/l vs. 302 ± 7 g/l, p = 0.003) and MCV was slightly decreased (96.5 ± 6.2 fL vs. 97.3 ± 6.4 fL, p = 0.028). 2,3-DPG levels were comparable throughout storage and no longer detectable after 6

weeks. ATP levels were initially comparable in both groups but was slightly decreased in DOPC-treated RBCs compared to control after 6 weeks of storage (1.3 ± 0.5 μmol/g Hb vs. 1.7 ± 0.6 μmol/g Hb, $p = 0.008$).

4.4 Discussion

Several elements of RBC membrane storage lesion remain unaddressed by current RBC preservation approaches. A liposome-based approach has been previously applied to rat RBCs, resulting in hemorheological improvements. This study evaluated the effect of liposome treatment time on human RBCs; and investigated the effects of liposome treatment on human RBC HSL and rheological properties as well as the metabolic profile.

The parameter least affected by liposome treatment was aggregation, with treatment time having no detectable effect on aggregation index and aggregation kinetics ($t_{1/2}$). Aggregation amplitude was the only parameter affected by treatment time, with results showing that samples treated at the beginning of storage (day 2) had lower amplitude at expiry, compared to samples that were treated at expiry (day 42). High aggregation amplitude could lead to impaired microcirculatory flow, and this hemorheologic alteration has been thought to contribute to the pathophysiology of slow coronary flow (21). RBC deformability and rigidity (K_{EI}) were both affected by liposome treatment time. Maximum elongation was comparable until day 21 and slightly increased when RBCs were treated at day 42, although is not likely that a difference of 0.01 in deformability would have a significant clinical impact. RBC membrane rigidity was also increased when the treatment was performed at expiry (day 42) compared to day 2 and 21. Treatment type also had an impact on rigidity, with liposome-treated RBCs exhibiting slightly higher rigidity compared to control. Rigidity can be increased by the cholesterol content of RBC membrane, which is why patients with high cholesterol

levels also present an increase in RBC rigidity (22). Liposome formulation used in this study had a 0.4 C/P ratio, therefore is not likely that transfer of cholesterol from liposomes to RBCs had taken place (23), consequently the increase in rigidity cannot be explained by cholesterol enrichment. On a separate note, studies have shown the transfer of cholesterol from RBCs to liposomes (17). Therefore the increase in ridigity could be linked to membrane loss in the form of MPs or a change in the normal C/P ratio of RBC membrane.

Hematological indices MCH and MCHC were slightly affected by treatment time, with lower MCH for samples treated on day 2 compared to day 21 and lower MCHC for samples treated on day 2 compared to day 21 and 42. Although a statistical significance was observed, the differences were too small to be considered clinically relevant. While MCH remained whithin the reference range (29 ± 2 pg) at expiry, MCHC decreased to similar values in both treatment groups (300 ± 1 g/l vs. 301 ± 1 g/l, $p = 0.184$) remaining below reference range (340 ± 20 g/l) (24). This means that there was less hemoglobin in each RBC, regardless of their volume. Hemoglobin can be lost through membrane leak or bound to MPs (25, 26). MCV was increased in DOPC-RBCs treated on day 2 and 21, but not day 42, while hemolysis reversely decreased in DOPC-treated RBCs when treated at day 2 and 21, but at day 42 values were comparable to control. Liposomes might have fused with the membrane or transferred enough phospholipids that caused the change in volume while decreasing hemolysis (27). The type of liposomes used in this study would interact with RBCs by fusion, phospholid transfer or a combination of both (27). This uptake of lipids could help stabilize cell membrane and compensate for lipid loss that would help decrease hemolysis.

Microparticle profile revealed that MP concentration and percentage of PS(+) MPs were higher in DOPC-RBCs treated at day 2 and 21 compared to control, but not day 42.

While MPs from DOPC-treated RBCs treated at day 2 and 21 exposed less PS per MP compared to control (as shown by MP-MFI/MP-PS% values; Table 4.3; Figure 4.2). Mean fluorescence intensity (MFI) of MPs increased as treatment time increased (d42 > d21 > d2). The time taken between PS exposure and MP release it not well known for RBCs, but in other cell types entering apoptosis it occurs within hours (28). Considering that, MP release is a time-dependent process it is not surprising that samples treated at day 42 had lower MP concentration (i.e. there was not enough time to shed compared to samples treated earlier). MP concentration in DOPC-treated RBCs was significantly higher while inversely presenting higher MCV for the same treatment times. This might suggest that the lipid enrichment provided by the liposome treatment is compensating the membrane loss, which would explain why even after shedding membrane pieces through MPs, their cell volume was not reduced. It could also provide an explanation to the different MP characteristics, with DOPC-treated MPs exposing less PS per MP (as per MP-MFI/MP-PS% values).

The DOPC liposomes used in this study, which were small unilamellar vesicles (SUV), neutral (not charged) and fluid (always kept above the Tc of the lipid) had the same characteristics as the ones used for previous rat studies and were also able to reduce hemolysis of human RBCs immediately after treatment, although during 3 and 6 weeks assessments this effect was no longer observed. Inversely, supernatant postassium levels for 3 and 6 weeks of storage was slightly increased for DOPC-treated samples. Acker and colleagues (2014) have reported supernatant potassium values at expiry of 46.2 ± 4.3 and 47.4 ± 4.0 mmol/l, using current blood manufacturing methods (29), therefore the values observed for DOPC-treated samples (45.0 ± 3.0 mmol/l) would be still clinically acceptable.

DOPC treatment increased the percent and extent of RBC aggregation while reducing aggregation time. Aggregation behavior that usually decreases with prolonged storage time seemed to be better preserved in DOPC-treated RBCs resembling values observed in fresh control RBCs. While the increase in the extent of aggregation might not seem of advantage, it is important to point that although the amplitude was increased by liposome treatment it remained within what has been reported for healthy individuals (21, 30, 31).

Metabolic profile was not significantly altered by liposome treatment, no differences were observed for 2,3-DPG and only a slight decrease in ATP levels were detected after 6 weeks of storage in DOPC-treated samples. Intracellular ATP levels decrease progressively throughout storage and correlates to post-transfusion RBC viability towards the end of storage (32). ATP is also necessary for the function of many pumps and transporters that maintain RBC shape and to repair cellular damage (33). Considering that transmembrane lipid transporters are ATP-dependent, this could explain lower values observed for the liposome-treated samples.

4.5 Conclusion

Overall liposome treatment seemed to be more beneficial when performed at the beginning of storage up to day 21. Treatment at expiry (day 42) resulted in a lack of effect of liposome treatment on *in vitro* quality parameters. These results support the practice of treating RBCs while they are still fresh (up to 7 days of storage), as it was done in the previous chapter for rat RBCs.

This chapter characterized for the first time the effect of DOPC liposomes on *in vitro* quality of human RBCs. DOPC liposome treatment resulted in modest improvements in

human RBC hemorheology upon storage, with no significant impact on metabolic profile. Liposome treatment resulted in more significant changes immediately after treatment or at the end of storage, with the 3 week assessment revealing no significant changes between treatment groups. This indicates that liposome-treated RBCs in the middle of the storage duration do not differ from control RBCs, and therefore there would be no added clinical benefit of the treatment if they were to be transfused at that time point.

Next studies will focus on the immune profile of liposome-treated RBCs in order to evaluate and predict possible undesirable interactions with cells of the immune system.

Table 4.1: Two-way ANOVA for RBC quality parameters. Treatment type (control vs. liposomes) and treatment time (d2, d21, d42) were fixed factors. Control and liposome-treated RBCs (n = 4 each) assessed on day 42 of hypothermic storage.

Quality parameters		***Effect of treatment type***		***Effect of treatment time***		***Interaction***	
	Model P	**F**	**P**	**F**	**P**	**F**	**P**
Hemolysis (%)	< 0.001	132.64	< 0.001	2.33	0.126	26.14	< 0.001
Deformability							
EI_{max}	0.001	1.58	0.224	18.25	< 0.001	0.22	0.803
K_{EI}	< 0.001	13.54	0.002	27.16	< 0.001	0.35	0.709
Hematologic indices							
MCV (fL)	< 0.001	55.69	< 0.001	19.65	< 0.001	20.09	< 0.001
MCH (pg)	0.040	1.87	0.188	6.49	0.008	0.01	0.990
MCHC (g/L)	0.001	1.91	0.184	11.93	0.001	3.05	0.072
Aggregation							
AI (%)	0.356	1.60	0.222	1.04	0.373	1.12	0.348
Amp (au)	0.037	4.90	0.040	3.87	0.040	1.25	0.310
t ½ (s)	0.321	2.36	0.142	1.04	0.374	0.95	0.406
Microvesiculation							
MPs/μL	< 0.001	119.23	< 0.001	16.50	< 0.001	18.67	< 0.001
MP-PS (%)	< 0.001	25.45	< 0.001	0.70	0.510	8.22	0.003
MP-MFI	0.001	1.40	0.253	17.97	< 0.001	0.59	0.563
MP-MFI/MP-PS%	0.001	15.84	0.001	3.57	0.050	5.21	0.016

Table 4.2: Two-way ANOVA for RBC quality parameters affected by treatment time. Mean values ± SE for samples treated on day 2, day 21 and day 42. Main effect of treatment time followed by post hoc analysis (Tukey's test).

Treatment time	**EI_{max}**	**F**	**P**
Day 2	0.54 ± 0.00^{a}	18.25	< 0.001
Day 21	0.54 ± 0.00^{a}		
Day 42	0.55 ± 0.00^{b}		
Treatment time	**K_{EI}**		
Day 2	2.12 ± 0.02^{a}	27.16	< 0.001
Day 21	2.16 ± 0.02^{a}		
Day 42	2.32 ± 0.02^{b}		
Treatment time	**MCH (pg)**		
Day 2	29.1 ± 0.1^{a}	6.49	0.008
Day 21	29.5 ± 0.1^{b}		
Day 42	29.3 ± 0.1^{ab}		
Treatment time	**MCHC (g/L)**		
Day 2	297 ± 1^{a}	11.93	0.001
Day 21	302 ± 1^{b}		
Day 42	302 ± 1^{b}		
Treatment time	**Amp (au)**		
Day 2	16.3 ± 0.3^{a}	3.87	0.040
Day 21	17.0 ± 0.3^{ab}		
Day 42	17.3 ± 0.3^{b}		
Treatment time	**MP-MFI**		
Day 2	251.8 ± 9.2^{a}	17.97	< 0.001
Day 21	295.6 ± 9.2^{b}		
Day 42	330.1 ± 9.2^{c}		

Means followed by different letters are significantly different ($P < 0.050$ Tukey's test).

Table 4.3: Two-way ANOVA for RBC quality parameters with significant interaction between treatment type and treatment time. Mean values ± SE for control and DOPC-treated RBCs treated on day 2, day 21 and day 42. Simple effects of treatment time x treatment type after post hoc analysis (Bonferroni's test).

Hemolysis (%)	**Control**	**DOPC**	**P**
Day 2	0.91 ± 0.02	0.57 ± 0.02	< 0.001
Day 21	0.82 ± 0.02	0.65 ± 0.02	< 0.001
Day 42	0.80 ± 0.02	0.75 ± 0.02	0.093
MCV (fL)			
Day 2	97.1 ± 0.1	98.5 ± 0.1	< 0.001
Day 21	97.2 ± 0.1	98.2 ± 0.1	< 0.001
Day 42	97.1 ± 0.1	97.0 ± 0.1	0.501
MPs/µL			
Day 2	78275 ± 3642	132513 ± 3642	< 0.001
Day 21	78549 ± 3642	111971 ± 3642	< 0.001
Day 42	79595 ± 3642	89351 ± 3642	0.074
MP-PS (%)			
Day 2	32.3 ± 1.9	45.5 ± 1.9	< 0.001
Day 21	33.5 ± 1.9	44.7 ± 1.9	0.001
Day 42	41.4 ± 1.9	40.4 ± 1.9	0.715
MP-MFI/MP-PS%			
Day 2	8.29 ± 0.54	5.24 ± 0.54	0.001
Day 21	9.05 ± 0.54	6.61 ± 0.54	0.005
Day 42	8.02 ± 0.54	8.25 ± 0.54	0.771

Table 4.4: Hemorheology and metabolic parameters of DOPC-treated RBCs and control human RBCs. Mean values ± SD for DOPC-treated and control RBCs immediately after treatment and after 3 and 6 weeks of hypothermic storage are shown. au = arbitrary units.

	Control RBCs			***DOPC-treated RBCs***		
Hemorheology	**Fresh**	**3 weeks**	**6 weeks**	**Fresh**	**3 weeks**	**6 weeks**
Hemolysis (%)	0.18 ± 0.14	0.32 ± 0.14	1.14 ± 0.36	0.15 ± 0.13*	0.30 ± 0.15	1.14 ± 0.47
Supernatant K^+ (mmol/l)	3.5 ± 0.4	30.8 ± 2.7	43.8 ± 3.4	3.4 ± 0.4	31.2 ± 2.7*	45.0 ± 3.0*
Hematologic indices						
MCV (fL)	91.9 ± 5.5	93.6 ± 5.8	97.3 ± 6.4	92.3 ± 5.0	93.9 ± 5.6	96.5 ± 6.2*
MCH (pg)	32.0 ± 2.4	29.9 ± 2.1	29.3 ± 2.2	32.0 ± 2.4	29.9 ± 2.1	29.6 ± 2.2
MCHC (g/l)	348 ± 8	319 ± 6	302 ± 7	346 ± 8	318 ± 6	306 ± 5*
Deformability						
EI_{max}	0.60 ± 0.02	0.58 ± 0.01	0.53 ± 0.01	0.60 ± 0.01	0.58 ± 0.02	0.53 ± 0.02
K_{EI}	1.84 ± 0.27	2.03 ± 0.35	2.39 ± 0.23	2.08 ± 0.27*	2.13 ± 0.45	2.27 ± 0.33*
Aggregation parameters						
AI (%)	50.1 ± 2.5	46.9 ± 2.6	41.5 ± 4.1	53.7 ± 3.8	47.3 ± 2.6	45.4 ± 1.9*
Amp (au)	21.4 ± 2.8	17.6 ± 3.2	12.2 ± 3.3	21.1 ± 3.0	18.0 ± 2.5	16.4 ± 2.2*
$t_{1/2}$ (s)	3.8 ± 0.4	4.5 ± 0.6	6.1 ± 1.2	3.4 ± 0.4*	4.5 ± 0.5	4.9 ± 0.5*
Metabolism						
ATP (µmol/g Hb)	3.8 ± 0.4	2.7 ± 0.5	1.7 ± 0.6	3.6 ± 0.6	3.0 ± 0.8	1.3 ± 0.5*
2,3-DPG (µmol/g Hb)	5.4 ± 2.3	0.9 ± 1.3	0 ± 0	5.5 ± 2.3	0.5 ± 0.7	0 ± 0

* $p < 0.05$ compared to control RBCs of corresponding age.

Figure 4.1: Estimated marginal means for K_{EI} (A) and aggregation amplitude (B) of DOPC-treated and control human RBCs.

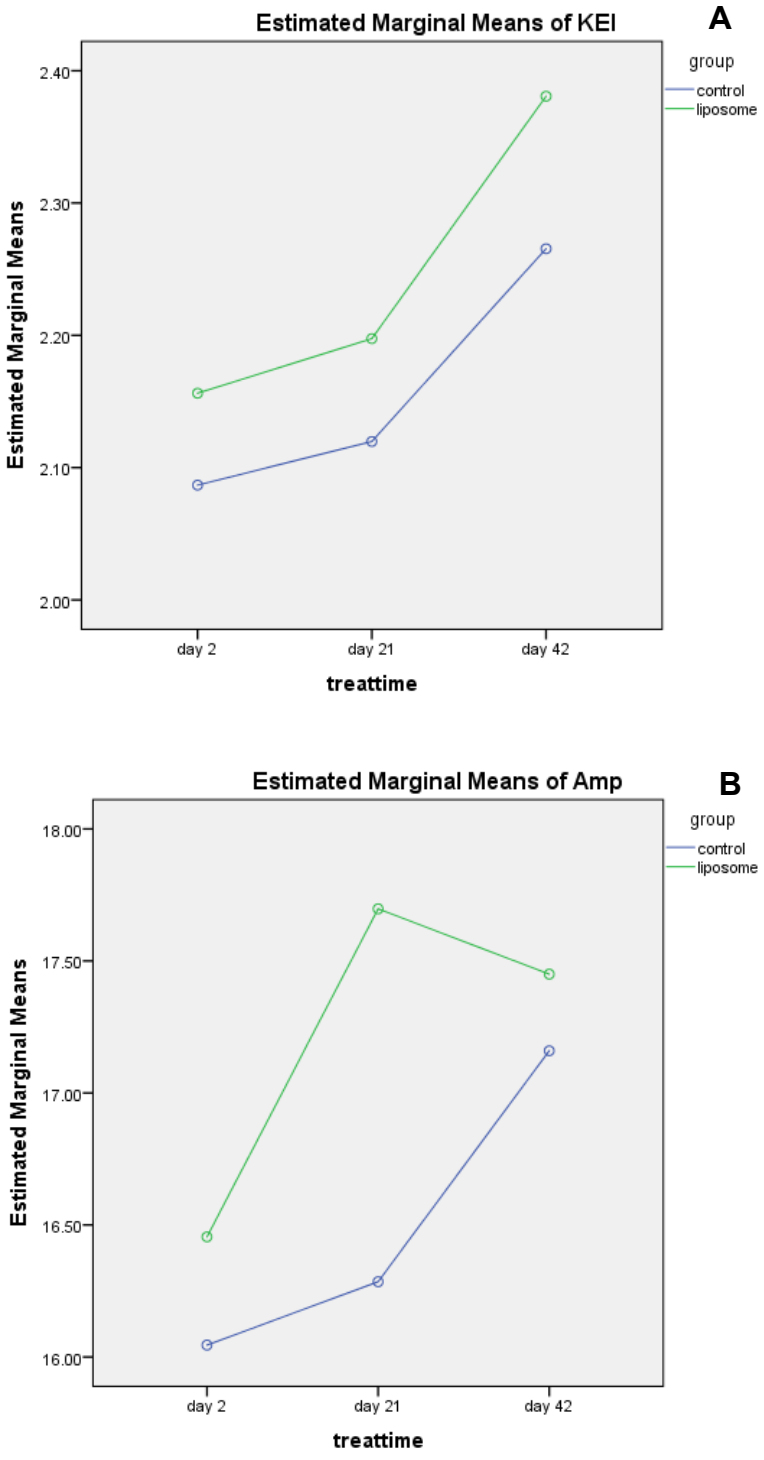


Figure 4.2: Intensity of phosphatidylserine (PS) exposure (MFI) divided by percentage of PS (+) MPs in control and DOPC-treated MPs. Mean values ± SD for control and DOPC-treated MPs treated at day 2, day 21 and day 42. * Significant ($p < 0.05$) compared to control MPs treated on the same.

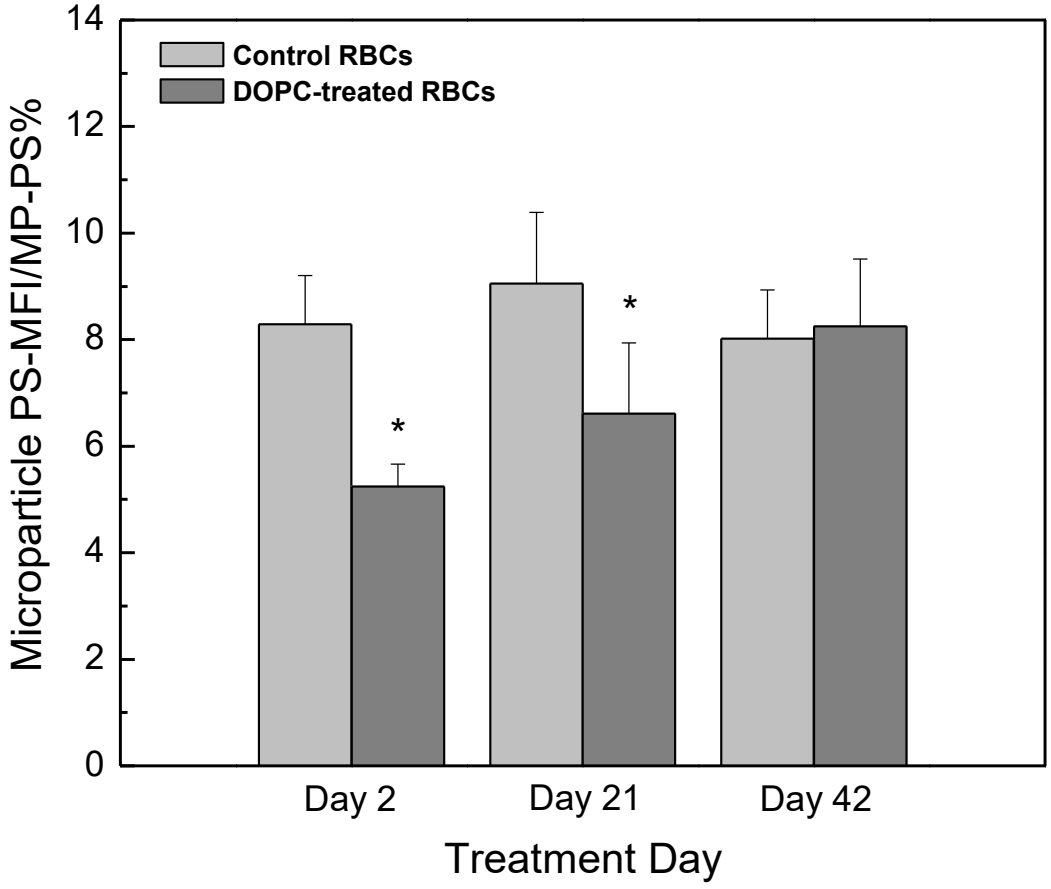


Figure 4.3: Deformability curves of fresh and stored DOPC-treated and control RBCs. Maximum elongation index (EI_{max}) and rigidity (K_{EI}) are shown (n= 6). * Significant ($p < 0.05$) compared to fresh control RBCs.

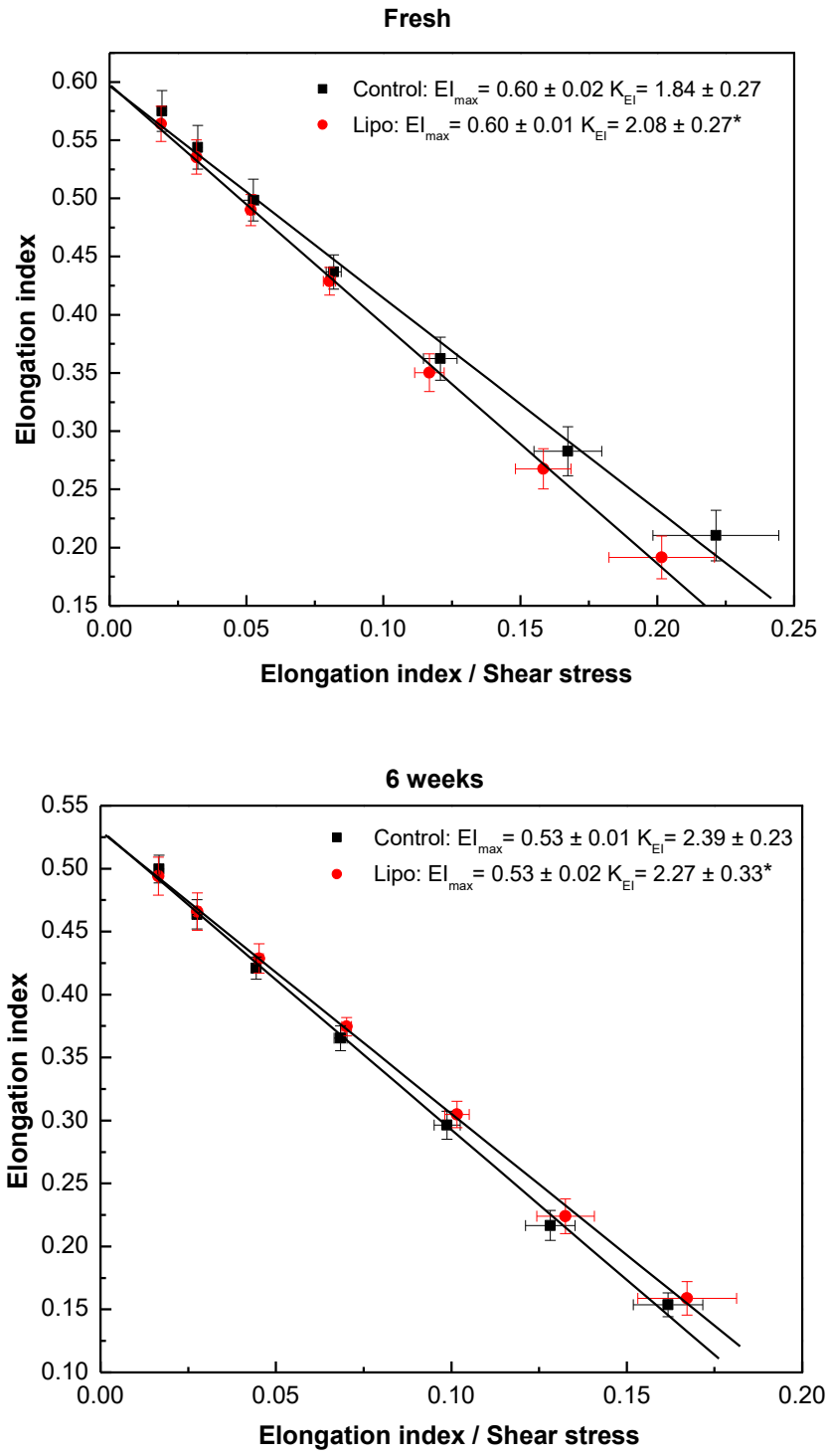
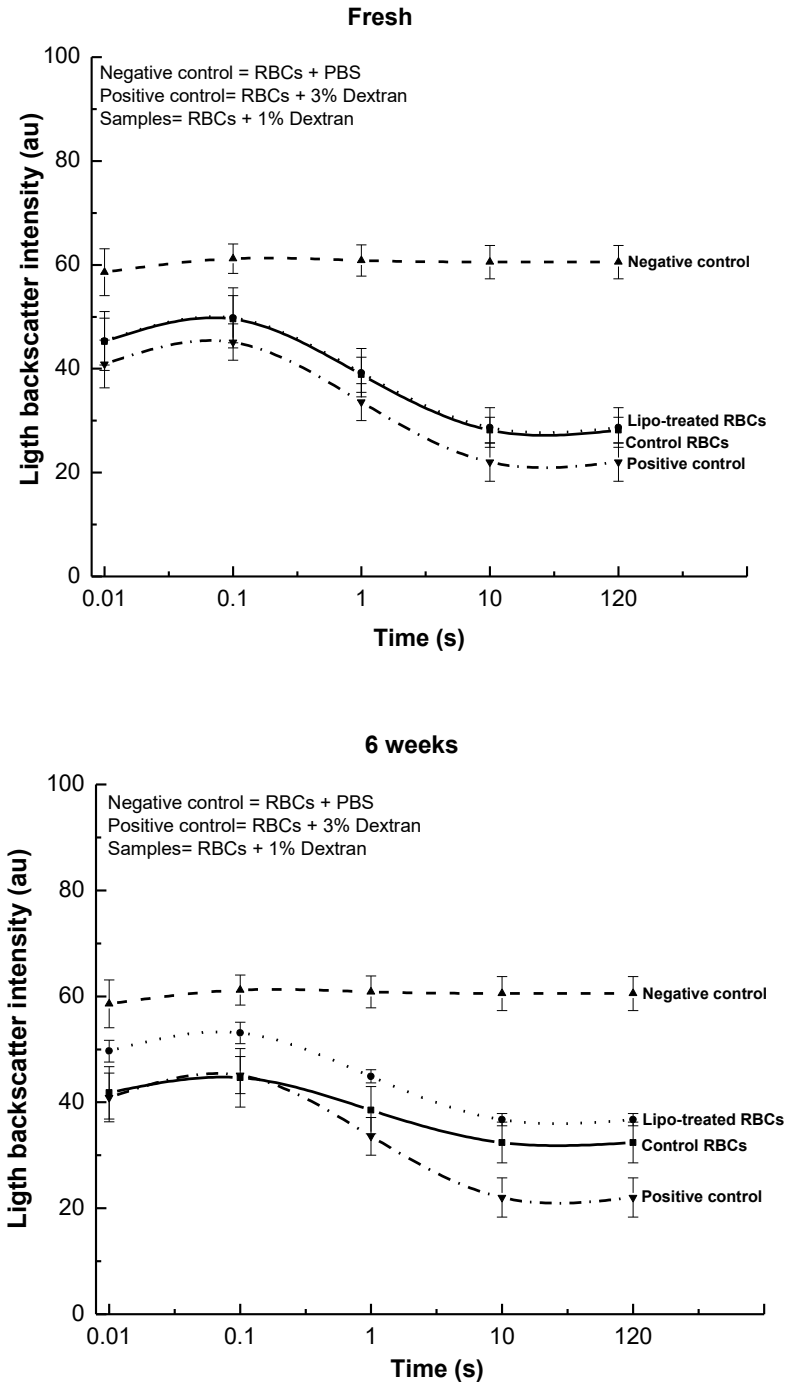


Figure 4.4: RBC aggregation in control and DOPC-treated RBCs (fresh and after 6 weeks of storage). Syllectograms (light scatter x time) showing RBC aggregation patterns (n=6). Negative control (PBS) = no aggregation; Positive control (3% dextran) = high aggregation.



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Chapter 5*

The effect of liposome treatment on the immune profile of red blood cells and supernatants

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5.1 Introduction

Red blood cells (RBCs) for transfusion are stored for a maximum of 42 days under hypothermic conditions, during which *in vitro* storage results in cells undergoing several biochemical and biomechanical changes, known as the "hypothermic storage lesion" (HSL). Some of the membrane-related consequences of HSL include RBC membrane remodeling with phosphatidylserine (PS) exposure, loss of intracellular potassium, oxidative injury of proteins and lipids, membrane loss, microparticle (MP) release and ultimately hemolysis (1, 2). Since membrane damage is a major consequence of HSL and current strategies to preserve *in vitro* RBC quality prior to transfusion do not adequately address the loss and remodeling of RBC membrane, this thesis's approach has focused on liposome treatment of RBCs to mitigate HSL. Previous studies have shown that liposomes improve human RBC *in vitro* quality by minimizing membrane damage occurring during 42-day hypothermic storage (3, 4). *In vivo* transfusion of liposome-treated RBCs in a rat model has not prompted safety concerns (5).

The use of older blood units (≥ 21 days) has been associated with adverse effects in transfused patients, although no direct causality has been proven (6). The adverse effects described include transfusion related acute lung injury, systemic inflammatory response syndrome, modulatory effects on hemostasis, inflammation and coagulation (7, 8). Three components of HSL are of particular interest in relation to immune modulation: PS exposure, MP shedding and the release of hemoglobin derived from RBC breakdown. These components have been related to vascular and immunoregulatory impairment that could lead to thrombosis, hypertension and inflammation (9-11). Clinical studies on post-transfusion inflammatory markers have only focused on a few cytokines (12, 13), resulting in a lack of a comprehensive panel to evaluate cytokine release and other aspects of immune modulation, like endothelial cell activation and adhesion molecules expression, and immune response by phagocytic cells.

Although the previous *in vivo* study suggested that the changes observed with liposome treatment *in vitro* were not sufficient to improve the outcomes of myocardial ischemia/reperfusion in anemic rats (5), differences in the PS profile suggested that there may be potential immune modulation, which could affect the safety and efficacy of liposome-treated RBCs.

Microparticle shedding is a natural process that occurs in all cells as part of activation or apoptosis (14), and in case of RBCs as part of calcium influx and metabolic depletion during *ex vivo* storage (15, 16). Depending on the process by which they are formed, composition of MPs may be affected, in ageing RBCs they are characterized by larger size and lower PS exposure compared to the ones from younger cells (17). MPs derived from blood cells have been generally shown to exhibit both procoagulant and proinflammatory activities (17, 18) making them a concern in transfusion medicine.

There are currently several liposome-based drugs on the market and several others being used in clinical trials (19). In spite of that, there is no current regulatory guideline specifically designed to test the immunotoxicity of nanoparticles, with most guidelines and toxicity studies suggested by regulatory bodies being based on guidelines used for conventional drugs (20). Reflection papers from the European Medicines Agency (EMA) to guide the development of liposome formulations similar to the ones already in use, suggests *in vitro* and *in vivo* immune reactogenicity assays such as macrophage/basophil activation assays and testing for complement activation-related pseudoallergy (CARPA) in sensitive animal models to evaluate potential adverse events. While under non-clinical pharmacodynamics studies it suggests: “where possible the development of *in vitro* tests capable of characterising any interaction between liposomes and target cells or other cells where the interaction is toxicologically relevant is encouraged” (21). Because the types of phospholipids used in this

project have already been used in clinical formulations (19), instead of focusing on standard assays it is important to evaluate other cell-based assays that are relevant to this novel liposome application.

Although cholesterol and phospholipids are common substances of cell membrane in any living organism, some of those lipids are also involved in recognition by the reticuloendothelial system. Therefore, their exposure can function as a signal for several biological processes from apoptosis and removal from circulation, to initiation of the clotting cascade and inflammatory response, as is it the case for phosphatidylserine in red blood cells and platelets (22-24). The cell types chosen for the *in vitro* assays were monocytes/macrophages and endothelial cells, not only because they are important for immune response but also for their role in inflammatory and clotting response (25).

The Monocyte Monolayer Assay (MMA) has been used since early 80's in transfusion to predict clinical significance of red blood cell alloantibodies (26) and later to assess phagocytosis of RBCs affected by sickle cell disease and infected by *Plasmodium falciparum* (27, 28). Since monocytes/macrophages represent the first line of defense of the immune system against foreign materials (29) this assay is useful in predicting the immune response to membrane changes caused by liposomes that might be undesired (i.e. early removal from circulation).

Human Umbilical Vein Endothelial Cells (HUVECs) are widely used to model endothelial biology and vascular pathology and more recently to evaluate biomaterials, biopharmaceuticals, and nanoparticles as part of preclinical tests (30, 31). HUVECs are primary, non-immortalized cells and possess several advantages for being well characterized, easily available, generating pure isolates, availability of pooled preparations and relatively simplicity of culture and maintenance, while generating great amount of cells by rapid

expansion (32). Cytokines are signalling molecules secreted from a wide variety of cells to stimulate immune response in inflammation, infection and trauma (33). While adhesion molecules are cytokine-induced proteins, expressed on the surface of endothelial cells that binds to leukocytes resulting in an inflammatory response (34).

As current progress points towards the use of liposomes in gene therapy, vaccines and biopreservation (35, 36), the interaction of liposomes with the immune system both *in vitro* and *in vivo* is of interest as part of preclinical studies to determine the immunotoxicity of liposomes and guide future translational studies. Considering that, most adverse effects in transfused patients are related to inflammation, as well as, the importance of trying to predict the *in vivo* immune response to avoid immunotoxicity and the changes reported in Chapter 4 after liposome treatment. This study aimed to assess the impact of liposome-induced membrane changes on the immune profile of liposome-treated RBCs by evaluating their interaction with endothelial cells and monocytes; and the resulting immune response derived from this interaction, in the form of cytokine release, adhesion molecules expression and phagocytosis. In addition, the impact of liposome treatment on MP-rich supernatants was also assessed, by evaluating interactions with endothelial cells (cytokines and adhesion molecules) and coagulation profile.

5.2 Materials and Methods

5.2.1 RBC collection

Ethical approval for the study was granted by both the Canadian Blood Services (CBS) and the University of Alberta Research Ethics Boards. Six leukoreduced packed RBC units in saline-adenine-glucose-mannitol buffy coat processed were obtained from the CBS Network

Centre for Applied Development (netCAD) and processed from whole blood as previously described (37).

5.2.2 Liposome Treatment

Unilamellar (DOPC:cholesterol, 7:3 mol%) liposomes were synthesized as described in Chapter 3, Section 3.2.2. Each packed RBC unit was divided into two aliquots and incubated at 37 °C for 1 h with either HEPES-NaCl solution (non-treated control) or 2 mM lipid from DOPC/Chol liposomes (131 nm). The RBCs were gently rotated during incubation. DOPC-treated and control RBCs were used in the assays immediately after treatment (day 2 of hypothermic storage) and after 42 days of hypothermic storage.

5.2.3 Human Umbilical Vein Endothelial Cells (HUVECs) Culture*

HUVECs (C2519A, Lonza Group Ltd., Walkersville, MD, USA) were purchased as pooled primary cells and cultured as previously described (38, 39). The cells were cultured as a monolayer in tissue culture flasks until confluent and then transferred to 12-well flat-bottomed culture plates and used for experiments at passage 2-3. Growth medium was replaced with basal media with 1% fetal bovine serum (FBS) 24 h prior to the incubation with RBCs and supernatants.

5.2.4 Incubation with RBCs and MPs

HUVECs were incubated with 25% (v/v) control and DOPC-treated RBCs as well as control and DOPC-treated supernatants containing MPs in endothelial basal medium -2 (EBM-2) to a final volume of 1 mL, for 24 h at 37 °C and 5% CO_2. After the 24-h incubation

* The author would like to acknowledge Dr. Leah Marquez-Curtis (research associate LMP/CME) for the training in HUVECs culture.

period, supernatants were collected and centrifuged at 10,000 x g for 5 min. The cell-free supernatants were then stored at -80 °C for further cytokine analysis. HUVECs cells were washed and harvested using StemPro® Accutase® (Gibco® by Life Technologies, Grand Island, NY, USA) for adhesion molecules analysis. Lipopolysaccharide (LPS) from Escherichia coli, serotype 055:B5 (Sigma Aldrich, St. Louis, USA) in a concentration of 20 µg/mL EBM-2/1% FBS was used as a positive control while phosphate-buffered saline (PBS) in EBM-2/1% FBS was used as a negative control.

5.2.5 Adhesion Molecules Expression by HUVECs

HUVECS were washed with staining media 0.1% bovine serum albumin (BSA) in PBS (pH = 7.4) and stained with phycoerythrin (PE) anti-human CD106 antibody as a marker of vascular cell adhesion molecule [VCAM]-1, allophycocyanin (APC) anti-human CD-62E as a marker of E-selectin and fluorescein isothiocyanate (FITC) anti-human CD31 as an endothelial cell marker (BD Pharmingen, San Diego, CA, USA). Commercial isotype controls (PE mouse IgG1, κ isotype control; APC mouse IgG1, κ isotype control; FITC mouse IgG1, κ isotype control, BD Pharmingen) were used to account for any nonspecific binding of the antibodies. After 15 minutes of incubation in the dark at room temperature, the prepared samples were fixed with a 4% (v/v) formaldehyde solution (Sigma-Aldrich, St. Louis, USA) and run the next day on a FACSCanto II flow cytometer (BD Biosciences, Erembodegem, Belgium) using FACSDiva (BD Biosciences) computer software. Lipopolysaccharide (LPS) from Escherichia coli, serotype 055:B5 (Sigma Aldrich, St. Louis, USA) in a concentration of 20 µg/mL EBM-2/1% FBS was used as a positive control while phosphate-buffered saline (PBS) in EBM-2/1% FBS was used as a negative control.

5.2.6 Peripheral Blood Mononuclear Cells (PBMCs) Isolation

Three buffy coat residual products produced as a by-product from the separation of plasma and red blood cells from whole blood were obtained from netCAD (Vancouver, Canada) and centrifuged to obtain a pooled, concentrated buffy coat layer, which was then diluted with two volumes of RPMI-1640 media (Sigma-Aldrich, St. Louis, USA). This suspension (10 mL) was layered over 30 mL of Ficoll-Paque PLUS (GE Healthcare UK Ltd., England) and centrifuged (400 x g, 40 minutes, 20°C, acceleration=1, brakes=0). The resulting layer of PBMCs and platelets was removed and washed three times to remove platelets before being resuspended in 37 °C RPMI-1640 media. The viable cell count was determined by the trypan blue (Sigma-Aldrich, Oakville, ON, Canada) exclusion method and the cell suspension adjusted to 2.0 x 10^6/mL.

5.2.7 Monocyte Monolayer Assay (MMA)*

The MMA was adapted from Branch and colleagues (26, 40). The cell suspension (1 mL) was placed on glass coverslips coated with poly-L-lysine (Sigma-Aldrich, Oakville, ON, Canada) and incubated at 37 °C with 5% CO_2 for one hour. They were then washed three times with 1 mL of PBS at 37 °C to remove non-adherent cells and to prepare the resulting monocyte monolayer for subsequent testing. Each sample (0.5 mL of 10% RBCs in PBS) was added to 0.5 mL culture media for each coverslip. Positive controls were prepared from O positive RBCs that were washed in PBS and incubated with IgG anti-D. RBC suspension (1 mL) was placed on each coverslip monolayer and incubated (2 hours, 37 °C, 5% CO_2). A negative control coverslip was covered with 1 mL culture media. The supernatants were then

* MMA assay was performed by MLS honors student Melissa Shyian under my supervision.

collected, spun at 10 000 x g to obtain a cell-free supernatant, which was frozen at -80 °C for subsequent cytokine analysis. The coverslips were washed three times with 1 mL 37 °C PBS to remove non-phagocytosed RBCs, stained with Hema 3 stain (Fisher Scientific, Kalamazoo, MI, USA), and mounted to a microscope slide. Monocytes (100 cells) were counted and the RBC phagocytic index was calculated.

5.2.8 Multiplex Cytokine Analysis*

Luminex multi-analyte profiling (xMAP) technology from Luminex employ proprietary bead sets which are measurable under flow cytometry-based instruments. After the bead captures an analyte from a test sample, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. Each individual microsphere is identified and the result is quantified based on fluorescent reporter signals. The capability of adding multiple conjugated beads to a biological sample results in the ability to measure multiple cytokines from that sample (41).

Luminex technology was used to measure 40 cytokines with MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panels (EMD Millipore Toronto, Canada).The 38-plex human cytokine/chemokine panel (38plex: HCYTMAG-60K-PX38) consists of (interleukin [IL]-1RA, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, interferon [IFN]-γ, IFN-α2, eotaxin, epidermal growth factor [EGF], vascular endothelial growth factor [VEGF], granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor [GM-CSF], monocyte chemoattractant protein [MCP]-1, MCP-3, macrophage inflammatory protein

* Multiplex Cytokine analysis was performed by Dr. Trang Duong from The Hospital for Sick Children Research Institute in Toronto.

[MIP]-1α, MIP-1β, tumor necrosis factor [TNF]-α and TNF-β, fibroblast growth factor [FGF]-2, transforming growth factor [TGF]-α, macrophage-derived chemokine [MDC], fractalkine, sCD40L, Flt-3L, GRO [CXCL1], IP-10 [CXCL10]) in addition to IL-11 (IL-11: HCYP3MAG-63K) and RANTES (RANTES: HCYTOMAG-60K). The assay was performed in accordance with the manufacturer's instructions as previously described (42).

5.2.9 Coagulation (PT and aPTT) in MP-rich supernatants

Prothrombin time (PT)

Clotting time by the PT method was assessed using the KC1 analyzer (Trinity Biotech plc, Bray, Co. Wicklow, Ireland), which uses a mechanical endpoint detection method. Supernatants (25 µL) from control and DOPC-treated samples were combined with 25 µL normal fresh frozen plasma. This mixture was incubated at 37°C for 60 seconds in a test well containing a metal ball, and then 100 µL warmed thromboplastin reagent (Werfen Group IVD, Lexington, MA, USA) was added. The time to clotting was measured based on the time taken for the clot to stop the metal ball from oscillating. Samples were tested in duplicate and the mean clotting time was used for statistical analysis.

Activated partial thromboplastin time (aPTT)

Clotting time by the aPTT method was also analyzed using the KC1 analyzer (Trinity Biotech plc, Bray, Co.). Supernatants (50 µL) from control and DOPC-treated samples were combined with 50 µL normal fresh frozen plasma and 100 µL aPTT reagent (Werfen Group IVD) containing kaolin and negatively charged phospholipids. This mixture was incubated at 37°C for 180 seconds in a test well containing a metal ball, and then 100 µL warmed 0.025 M calcium chloride reagent (Werfen Group IVD) was added. The time to clotting was detected

by mechanical endpoint. Samples were tested in duplicate and the mean clotting time was used for statistical analysis.

5.2.10 Statistical Analyses

Statistical analysis was performed using SPSS 23.0 software (IBM, Armonk, NY). Paired T test or Wilcoxon signed-rank test were used to assess differences between liposome-treated RBCs and MPs, and control RBCs and MPs. Spearman's correlation was used to evaluate cytokine release and adhesion molecules expression by HUVECs. Data were expressed as mean ± standard deviation and $p < 0.05$ was considered statistically significant.

5.3 Results

Cytokine release from HUVECs after Incubation with Control and DOPC-treated RBCs at Day 2 of Hypothermic Storage

From the 40 cytokines/chemokines measured, six (IL-1α, IL-1β, IL-2, IL-3, IL-9 and TNFβ) were below the detection limit and thirty-four were not significantly altered by the DOPC treatment compared to control at day 2 of hypothermic storage (Table 5.1). HUVECs release of interferons IFNα2 (DOPC-treated: 11.1 ± 2.5 pg/mL vs. control: 17.5 ± 3.4 pg/mL, $p = 0.009$) and IFNγ (DOPC-treated: 2.4 ± 0.4 pg/mL vs. control: 3.3 ± 0.7 pg/mL, $p = 0.030$) was significantly decreased after incubation with DOPC-treated RBCs compared to control RBCs. The release of cytokines IL-15 and IL-17 was also decreased in DOPC-treated RBCs at day 2 of storage (IL-15: 2.0 ± 0.4 pg/mL vs. 2.8 ± 0.5 pg/mL, $p = 0.024$; IL-17: 1.1 ± 0.3 pg/mL vs. 1.6 ± 0.1 pg/mL, $p = 0.006$) as well as hematopoietin Flt-3L (8.5 ± 2.4 pg/mL vs. 11.6 ± 2.3 pg/mL, $p = 0.010$). The growth factor FGF-2 was significantly increased after

incubation with DOPC-treated RBCs compared to control RBCs (127.6 ± 16.0 pg/mL vs. 53.5 ± 19.5 pg/mL, p = 0.002).

Cytokine release from HUVECs after Incubation with Control and DOPC-treated RBCs at Day 42 of Hypothermic Storage

From the 40 cytokines/chemokines measured, fourteen (IL-1α, IL-1β, IL-2, IL-3, IL-5, IL-9, IL-10, TNFα, TNFβ, MDC, IL-12p70, sCD40L, IL-17 and TGFα) were below the detection limit and thirty-seven were not significantly altered by the DOPC treatment compared to control at day 42 of hypothermic storage (Table 5.1). The cytokines IL-1α, IL-1β, IL-2, IL-3, IL-9 and TNFβ remained below detection limit throughout storage. HUVECs release of cytokine IL-11 was significantly increased after incubation with DOPC-treated RBCs compared to control RBCs (4.2 ± 0.4 pg/mL vs. 3.5 ± 0.6 pg/mL, p = 0.005) as well as growth factor FGF-2 (DOPC: 46.4 ± 21.2 pg/mL vs. Control: 15.3 ± 5.7 pg/mL, p = 0.015) at day 42 of storage. The growth factor FGF-2 was the only cytokine that remained increased after incubation with DOPC-treated RBCs compared to control RBCs throughout storage. The release of chemokine IP-10 was significantly decreased after incubation with DOPC-treated RBCs compared to control RBCs (4.1 ± 3.3 pg/mL vs. 8.6 ± 2.1 pg/mL, p = 0.016).

Cytokine release from HUVECs after Incubation with Control and DOPC-treated MP-rich supernatants at Day 2 of Hypothermic Storage

From the 40 cytokines/chemokines measured, three (IL-1α, IL-2, IL-3) were below the detection limit and thirty were not significantly altered by the DOPC treatment compared to control at day 2 of hypothermic storage (Table 5.2). The colony-stimulating factor GM-CSF was significantly increased in DOPC supernatants compared to control (24.0 ± 10.2 pg/mL vs. 14.6 ± 7.5 pg/mL, p = 0.007). Chemokines GRO (6337.6 ± 4125.2 pg/mL vs. 3678.5 ± 3820.2 pg/mL, p = 0.002), MCP-3 (164.3 ± 71.9 pg/mL vs. 107.5 ± 53.5 pg/mL, p = 0.027) and IL-8

(6306.7 ± 1362.1 pg/mL vs. 3462.2 ± 2842.0 pg/mL, p = 0.029) were also significantly increased in DOPC supernatants. DOPC supernatants also had slightly higher concentrations of sCD40L (9.0 ± 1.8 pg/mL vs. 7.8 ± 2.1 pg/mL, p = 0.020), IL-17 (2.7 ± 0.4 pg/mL vs. 2.2 ± 0.5 pg/mL, p = 0.006) and IL-6 (305.2 ± 181.0 pg/mL vs. 211.0 ± 163.6 pg/mL, p = 0.031).

Cytokine release from HUVECs after Incubation with Control and DOPC-treated MP-rich supernatants at Day 42 of Hypothermic Storage

From the 40 cytokines/chemokines measured, six (TGF- α, IL-1α, IL-9, IL-1β, IL-3, IL-5) were below the detection limit and thirty-three were not significantly altered by the DOPC treatment compared to control at day 42 of hypothermic storage (Table 5.2). Growth factor FGF-2 was increased in DOPC supernatants compared to control (45.8 ± 12.3 pg/mL vs. 25.5 ± 10.7 pg/mL, p = 0.028).

HUVECs Adhesion Molecules Expression: Effect of Liposome Treatment

The expression of VCAM-1 in the negative control was 3.5 ± 3.2% and in the positive control was 16.7 ± 1.4%. The expression of E-selectin in the negative control was 4.1 ± 2.2% and in the positive control was 12.7 ± 1.6%. The expression of both VCAM-1(15.3 ± 5.6% vs. 6.3 ± 0.9%, p = 0.008) and E-selectin (18.0 ± 6.3% vs. 6.6 ± 0.7%, p = 0.004) by HUVECs was significantly increased after incubation with liposome-treated RBCs compared to control, at day 2 of storage (Figure 5.1A). At day 42 of hypothermic storage, the expression of VCAM-1 by HUVECs was higher when incubated with liposome-treated RBCs (16.8 ± 4.9% vs. 10.9 ± 2.8%, p = 0.028), while the expression of E-selectin increased to a similar extent for incubation with both control and liposome-treated RBCs (37.2 ± 8.4% vs. 41.0 ± 5.5%, p = 0.249) (Figure 5.1B). Control and liposome-treated supernatants induced similar expression of VCAM-1 (7.1 ± 3.6% vs. 5.2 ± 1.2%, p = 0.237) and E-selectin (9.2 ± 4.9% vs. 7.3 ± 1.0%, p = 0.402) by HUVECs at day 2 of storage. At day 42 of storage, control and liposome-treated

supernatants also induced similar expression of VCAM-1 (12.6 ± 4.7% vs. 11.1 ± 2.6%, p = 0.526) and E-selectin (29.8 ± 6.5% vs. 26.9 ± 2.7%, p = 0.397) by HUVECs.

HUVECs Adhesion Molecules Expression: Effect of Storage

The expression of both VCAM-1(6.3 ± 0.9% vs. 10.9 ± 2.8%, p = 0.011) and E-selectin (6.6 ± 0.7% vs. 37.2 ± 8.4%, p = 0.031) by HUVECs was significantly increased after incubation with control RBCs at day 42 of storage compared to day 2. The expression of E-selectin by HUVECs was higher at day 42 compared to day 2 of storage (18.0 ± 6.3% vs. 41.0 ± 5.5%, p = 0.001), while the expression of VCAM-1 did not increase significantly with storage after incubation with DOPC-treated RBCs (15.3 ± 5.6% vs. 16.8 ± 4.9%, p = 0.617). The expression of both VCAM-1 (7.1 ± 3.6% vs. 11.7 ± 4.6%, p = 0.045) and E-selectin (9.2 ± 4.9% vs. 29.8 ± 6.5%, p < 0.001) by HUVECs was significantly increased after incubation with control supernatants at day 42 of storage compared to day 2. The expression of both VCAM-1 (5.2 ± 1.2% vs. 11.6 ± 2.6%, p = 0.002) and E-selectin (7.3 ± 1.0% vs. 26.9 ± 2.7%, p < 0.001) by HUVECs was significantly increased after incubation with DOPC supernatants at day 42 of storage compared to day 2.

Correlation of Cytokine release and Adhesion Molecule Expression by HUVECs

Table 5.3 shows Spearman's correlation coefficients and p-values for cytokine release by HUVECs when incubated with control and DOPC-treated RBCs at day 2 (IL-15, IL-17, IFNγ, IFNα2, FGF-2, Flt-3L) and 42 (IL-11, FGF-2, IP-10) of hypothermic storage and the expression of adhesion molecules. No correlation was found between the release of cytokines/chemokines and the expression of adhesion molecules (VCAM-1 and E-selectin) when HUVECs was incubated with control RBCs, regardless of storage time. When DOPC-treated RBCs were incubated with HUVECs at day 2 of storage there was a strong negative correlation between the release of IL-15 (r_s = -0.943, p = 0.017) and IL-17 (r_s = -0.853, p =

0.033) and the expression of VCAM-1, as well as the release of IFNα2 (r_s = -0.899, p = 0.017) and the expression of E-selectin. When DOPC-treated RBCs were incubated with HUVECs at day 42 of storage there was a strong positive correlation between the release of IL-11 (r_s = 0.943, p = 0.017) and the expression of VCAM-1.

Cytokine release from Monocytes after Incubation with Control and DOPC-treated RBCs at Day 2 of Hypothermic Storage

From the 40 cytokines/chemokines measured, twenty-one were below the detection limit and thirty-eight were not significantly altered by the DOPC treatment compared to control at day 2 of hypothermic storage (Table 5.4). Monocytes release of IL-12p70 was slightly decreased after incubation with DOPC-treated RBCs compared to control RBCs (1.4 ± 0.2 pg/mL vs 1.6 ± 0.2 pg/mL, p = 0.043) while IL-5 was significantly increased in DOPC-treated RBCs compared to control RBCs (1.0 ± 0.2 pg/mL vs 0.7 ± 0.1 pg/mL, p = 0.042).

Cytokine release from Monocytes after Incubation with Control and DOPC-treated RBCs at Day 42 of Hypothermic Storage

From the 40 cytokines/chemokines measured, twenty-eight were below the detection limit and thirty-nine were not significantly altered by the DOPC treatment compared to control at day 42 of hypothermic storage (Table 5.4). Monocytes release of fractalkine was significantly decreased after incubation with DOPC-treated RBCs compared to control RBCs (0.6 ± 1.6 pg/mL vs 7.1 ± 3.9 pg/mL, p = 0.039).

Phagocytosis of Control and DOPC-treated RBCs by Monocytes

The MMA resulted in phagocytic indexes findings of zero for both control and DOPC-treated RBCs at day 2 and day 42 of hypothermic storage (positive controls: 61 ± 22% at day 2; 72 ± 11% at day 42).

Coagulation (PT and aPTT) in control and DOPC-treated MP-rich supernatants

PT results were comparable at day 2 (19.2 ± 0.8s vs. 19.8 ± 1.9s, p = 0.471) but significantly decreased in stored supernatants from liposome-treated RBCs compared to control (16.7 ± 0.6s vs. 18.7 ± 0.6s, p = 0.003) (Figure 5.2A). The opposite occurred for aPTT which was slightly decreased at day 2 in supernatants from liposome-treated RBCs compared to control (41.1 ± 1.2s vs. 43.0 ± 0.8s, p = 0.043) but at day 42, a significant difference was no longer observed (43.9 ± 1.1s vs. 45.3 ± 1.0s, p = 0.106) (Figure 5.2B).

5.4 Discussion

Liposomes have been shown to improve human RBC *in vitro* quality by minimizing membrane damage occurring during hypothermic storage (3). The emerging use of nanoparticles in therapeutics (i.e. drug delivery) raises safety questions about their interaction with the immune and coagulation systems that could ultimately lead to severe consequences in patients. This study evaluated a comprehensive panel of cytokines and other aspects of immune modulation, like endothelial cell adhesion molecules expression, and immune response by phagocytic cells as part of the investigation on the effects of liposome-treated RBCs and supernatants on immune response, as well as the effect of liposome-treated supernatants on coagulation profile.

Recent studies on transfusion-related reactions are pointing to manufacturing methods and donor factors as the main cause of adverse events, even showing that fresh RBCs can be potentially more immunogenic than old RBCs as the results observed in this study indicates (43-45). A possible mechanism involves the presence of soluble HLA antigens from donor's plasma and the presence of residual leukocytes that can become activated or undergo apoptosis consequently releasing cytokines (46). Cytokines are signalling molecules secreted

from a wide variety of cells to stimulate immune response in inflammation, infection and trauma (33). Cytokine release was more pronounced after incubation with fresh RBCs (day 2 of hypothermic storage) regardless of treatment for both HUVECs and PBMCs compared to RBCs at day 42 of hypothermic storage, as shown by the higher number of cytokines that were below the detection limit at day 42.

Interferons (IFNγ and IFNα2) were significantly decreased when HUVECs were incubated with DOPC-treated RBCs at day 2 of storage; at day 42 a significant difference was no longer observed. Endothelial cells do not secrete type II interferons like IFNγ, but they respond to IFNγ by secreting chemokine IP-10 (interferon-γ-inducible protein 10) which attracts T-cells to inflammation sites (47). Therefore, the IFNγ detected on the samples might have been produced by residual leukocytes. While IP-10 secretion was comparable at day 2 of storage, at day 42 HUVECs release of IP-10 was decreased after incubation with DOPC-treated RBCs compared to control. At day 2 of storage, IL-6 secretion by HUVECs showed a trend towards lower levels ($p = 0.058$) after incubation with DOPC-treated RBCs, which might be linked to the significantly lower secretion of IL-15 and IL-17 observed after incubation with DOPC-treated RBCs. Endothelial activation with release of IL-6 supports the expansion of T helper (Th)17 under inflammatory conditions (48), Th17 cells will in turn produce inflammatory cytokine IL-17, while IL-15 has also been shown to trigger IL-17 production *in vitro* (49).

Although no significant difference was observed in the release of VEGF by HUVECs when incubated with the two RBC groups (control and liposome-treated), both samples experienced a significant decrease in the release of this growth factor with storage time (51.2% for DOPC-treated RBCs versus 43.7% for control RBCs). VEGF has been implicated

as a potential mediator of transfusion-related acute lung injury (TRALI) (50) which is one of the most serious complications of transfusion, characterized by acute respiratory distress and lung edema, presenting high indices of morbidity and mortality (51). Lower concentrations of Flt-3L were released by HUVECs when incubated with DOPC-treated RBCs at day 2, compared to control RBCs, while at day 42 of storage the levels of Flt-3L were comparable regardless of the treatment. Flt-3L is a hematopoietin produced in great amounts by endothelial cells (52), but its main role in immunity is related to the generation and differentiation of dendritic cells, which in turn control T regulatory cells. Considering that anti-inflammatory responses tend to be higher in response to inflammation (53), the increased levels of Flt-3L might be an effort to counteract the increased levels of pro-inflammatory cytokines released during incubation with control RBCs. The Fibroblast Growth Factor-2 (FGF-2) has been associated with many functions like angiogenesis, endothelial cell proliferation and wound healing (54). FGF-2 was the only cytokine released by HUVECs that remained significantly increased after incubation with DOPC-treated RBCs compared to control RBCs throughout storage. This is particularly concerning because FGF-2 is up-regulated in inflammatory conditions like atherosclerosis (55) and following blood vessel injury (56).

A great number of cytokines were not detected after incubation with RBCs at day 42 of storage, and most of the significant differences observed between treatments at day 2 were no longer detectable (with the exception of FGF-2). At day 42, besides the aforementioned difference in IP-10 release, the only detectable difference in cytokine release was in IL-11 that was significantly increased in HUVECs incubated with DOPC-treated RBCs. IL-11 has been

shown to have protective effects on immune-mediated injury in HUVECs without inhibition of pro-inflammatory response (57).

Supernatants were able to induce a greater cytokine response from HUVECs than the cell fraction. This observation is not surprising considering supernatants will contain all the metabolic and cellular by-products, including biologically active MPs of all sizes, compositions and different cell origins (i.e. leukocytes, platelet and RBC-derived) (58). A similar pattern of upregulation of cytokines has also been reported for fresh vesicles incubated with PBMCs compared to older vesicles (42 days) (59), that was similar to what was observed for HUVECs (more stimulation with day 2 supernatants compared to day 42). On day 2 DOPC-treated supernatants showed a more pro-inflammatory profile with upregulation of pro-inflammatory cytokines (GM-CSF, IL1-7, IL6), chemoattractants (GRO, IL-8, MCP-3) and anti-inflammatory sCD40L. On day 42 DOPC-treated and control supernatants had a similar profile, with only a small increase in growth factor (FGF-2) secretion in DOPC supernatants. Considering that a control sample containing liposomes only was not able to induce the same effects on cytokine release by HUVECs (data not shown), the observed changes could be a result of liposome interaction with other cells (leukocytes, platelets) or changes to RBC MPs.

Overall, the monocytes response to the incubation with DOPC-treated and control RBCs in the form of cytokine release was lower when compared to HUVECs. This fact is probably due to the shorter duration of incubation (2 h for monocytes compared to 24 h for HUVECs). One of the few changes observed between treatments was a decrease in the release of IL-12p70 by monocytes when incubated with DOPC-treated RBCs at day 2 of storage compared to control RBCs. IL-12 is required to induce the production of IFN-γ by Th1 cells (60), but no similar decrease was observed in IFN-γ from the same sample. The levels of IL-5

released by monocytes were higher when incubated with DOPC-treated RBCs. Th2 cells produce IL-5 and is implicated in allergy-related inflammation by activation of eosinophils (61). After incubation with RBCs at day 42 of storage, the only significant difference observed was an increase in the release of the chemokine Fractalkine by monocytes when incubated with control RBCs compared to DOPC-treated RBCs. Fractalkine is a critical mediator in the host inflammatory response leading to vascular injury by promoting adhesion and extravasation of leucocytes to inflammatory infiltrates, and is usually up-regulated in endothelial cells in inflammatory disease states (62). Previous reports have shown that a 2 h incubation is enough to produce detectable levels of acute phase cytokines by monocytes (63) but since this might not be true for all cytokines/chemokines the short incubation time constitutes a limitation of the study regarding cytokine release by monocytes.

Adhesion molecules are cytokine-induced proteins, expressed on the surface of endothelial cells that binds to leukocytes resulting in an inflammatory response (34). Liposome-treated RBCs increased the expression of VCAM-1 by HUVECs at day 2 and at day 42 of storage compared to control RBCs. The α4β1 integrin on RBC membrane binds to VCAM-1 on endothelial cells (64) and has been associated with increased adherence of sickle RBCs (65). The level of expression of α4β1 in mature RBCs is not likely to be changed by liposome treatment but signaling cascades may result in activation of this integrin and could regulate RBC adhesion to endothelium (66). An increase in E-selectin expression by HUVECs was observed at day 2 of storage for liposome-treated RBCs compared to control, but at day 42 of storage no significant difference was observed. Sialic acid serves as the binding site for E-selectin on RBCs (64). E-selectin also increased significantly with storage in both groups, which contrasts with the fact that old RBCs tend to have less sialic acid content on their

membranes (67) and therefore increased expression of E-selectin at day 42 of storage must have been stimulated by other factors. The cytokines TNFα and IL-1β that are known to induce the expression of E-selectin (68) were almost undetectable, requiring further investigation into the exact mechanism of adhesion molecules expression that was observed. Control and DOPC-treated supernatants induced similar expression of VCAM-1 and E-selectin regardless of treatment time. While the expression of both adhesion molecules increased significantly over time in both treatment groups. One limitation of these results is that RBCs were incubated with HUVECs under static conditions rather than flow conditions (as it happens *in vivo*), therefore the adhesion molecules expression as well as cytokine release response might be exacerbated by the extended contact between cells. Because of these factors, the likelihood that the observed changes might be biologically significant are minimal, but cell assays closely mimicking *in vivo* conditions should be designed to provide a definite answer. The effect of liposome-treated supernatants on the expression of VCAM-1 and E-selectin was comparable to control throughout storage.

Since monocytes/macrophages represent the first line of defense of the immune system against foreign materials (29) this assay is useful in predicting the immune response to membrane changes caused by liposomes. In this study, phagocytosis for both control and liposome-treated RBCs was found to be zero at day 2 and day 42 of hypothermic storage. A study by Veale and colleagues (2014) using the human monocytic cell line THP-1 has rendered different results showing significantly increased *in vitro* phagocytosis of RBCs at day 1 and at day 42 of storage (69). THP-1 cells are a transformed monocyte cell line, therefore results obtained using this cell line may not necessarily be generalized to normal monocytes-macrophages. However, a study evaluating *in vivo* and *in vitro* phagocytosis in a mouse model

showed the absence of *in vitro* phagocytosis of senescent RBCs by bone marrow and spleen-derived macrophages (70). This may suggest that changes observed in senescent RBCs as well as in stored RBCs (i.e. PS exposure) were not sufficient to induce phagocytosis *in vitro*, and the membrane changes promoted by the liposome treatment did not induce further recognition by phagocytic cells *in vitro* nevertheless, the possibility that they might be recognized *in vivo* cannot be excluded.

Coagulation markers were slightly altered in DOPC-treated supernatants compared to control, showing a procoagulant tendency. Studies evaluating coagulation activity in stored blood have attributed these effects early in storage to the presence of PS, tissue factor and factor XIa, but later in storage other factors might contribute to procoagulant activity (71). The procoagulant activity seen early in storage might be due to factors affecting the intrinsic pathway (since there is an increase in aPTT over storage time), while coagulation activity at the end of storage might be due to factors affecting PT and the extrinsic pathway (since there is a decrease in PT over storage time).

5.5 Conclusion

Liposome treatment did not result in significant changes to the immune profile of stored RBCs. There were significant changes in adhesion molecules expression by HUVECs, as well as FGF-2 release, that were exclusively observed on incubations with DOPC-treated RBCs, and therefore require further investigations about its possible clinical implications. However, liposome treatment did not induce additional immune response from endothelial cells or monocytes *in vitro*, suggesting that such RBCs are not likely to be removed prematurely from the circulation upon transfusion.

Liposome treatment resulted in a small change in the immune profile of fresh supernatants only, indicated by higher cytokine release. In addition, supernatants showed slightly enhanced clotting response, warranting further investigations. These results are less concerning because a simple wash procedure prior to transfusion would likely eliminate any deleterious effects associated with the supernatant portion.

These findings combined with previous *in vivo* results from Chapter 3 showing the ability of DOPC-treated RBCs to raise hemoglobin levels in transfused anemic rats without increasing mortality in addition to results from Chapter 4 showing improvement in hemorheology without affecting the metabolic profile of human RBCs, make liposome treatment a potential candidate for application in red blood cell preservation for transfusion purposes as well as open the possibility for clinical use with other cell types.

The next chapter will evaluate the combination of liposome treatment with rejuvenating solution, a strategy currently used in blood banks to achieve metabolic restoration.

Table 5.1: Cytokine/Chemokine released by HUVECs after incubation with DOPC-treated and control RBCs at day 2 and day 42 of hypothermic storage. Mean values ± SD (n = 6) are shown.

Hypothermic storage	***Day 2***		***Day 42***	
Cytokines/ Chemokines (pg/mL)	**Control RBCs**	**DOPC-treated RBCs**	**Control RBCs**	**DOPC-treated RBCs**
IL-1RA	8.4 ± 1.4	6.2 ± 1.6	6.8 ± 3.6	5.0 ± 3.1
FGF-2	53.5 ± 19.5	127.6 ± 16.0*	15.3 ± 5.7	46.4 ± 21.2*
Eotaxin	123.2 ± 65.3	112.2 ± 67.8	76.7 ± 54.0	81.6 ± 34.4
TGFα	0.9 ± 0.5	0.1 ± 0.3	< OOR	< OOR
Flt-3L	11.6 ± 2.3	8.5 ± 2.4*	8.1 ± 2.7	8.2 ± 1.8
GM-CSF	14.3 ± 4.2	10.2 ± 3.0	20.9 ± 11.0	14.7 ± 7.4
Fractalkine	81.8 ± 9.4	70.9 ± 6.4	35.8 ± 16.0	26.8 ± 11.2
IFNα2	17.5 ± 3.4	11.1 ± 2.5*	19.2 ± 9.8	17.2 ± 6.1
IFNγ	3.3 ± 0.7	2.4 ± 0.4*	2.3 ± 0.9	2.0 ± 0.7
GRO	104.1 ± 30.7	75.7 ± 22.1	90.0 ± 44.7	75.2 ± 22.7
IL-10	2.8 ± 0.8	2.6 ± 0.4	< OOR	< OOR
MCP-3	15.3 ± 2.5	12.0 ± 3.5	11.5 ± 3.0	9.0 ± 2.1
IL-12p40	5.7 ± 1.7	3.6 ± 1.2	5.1 ± 2.9	2.4 ± 2.8
MDC	9.3 ± 2.3	7.4 ± 1.0	< OOR	< OOR
IL-12p70	4.0 ± 1.0	3.1 ± 0.4	< OOR	< OOR
IL-13	2.2 ± 0.3	2.0 ± 0.4	6.4 ± 3.3	3.0 ± 2.1
IL-15	2.8 ± 0.5	2.0 ± 0.4*	2.0 ± 1.6	1.6 ± 1.4
sCD40L	4.5 ± 1.4	2.8 ± 2.2	< OOR	< OOR
IL-17	1.6 ± 0.1	1.1 ± 0.3*	< OOR	< OOR
IL-1α	< OOR	< OOR	< OOR	< OOR
IL-9	< OOR	< OOR	< OOR	< OOR
IL-1β	< OOR	< OOR	< OOR	< OOR
IL-2	< OOR	< OOR	< OOR	< OOR
IL-3	< OOR	< OOR	< OOR	< OOR
IL-4	5.6 ± 1.4	4.2 ± 1.2	6.7 ± 4.0	5.0 ± 3.3
IL-5	1.3 ± 0.3	1.2 ± 0.1	< OOR	< OOR
IL-6	131.9 ± 40.5	74.4 ± 34.9	181.3 ± 107.7	136.2 ± 85.5
IL-7	7.6 ± 1.8	6.5 ± 2.0	21.6 ± 12.6	22.4 ± 5.4
IL-8	406.5 ± 154.0	270.3 ± 78.7	437.3 ± 256.8	313.1 ± 138.4
IP-10	14.4 ± 3.3	11.7 ± 4.4	8.6 ± 2.1	4.1 ± 3.3*
MCP-1	11672.7 ± 841.1	8980.2 ± 3317.0	9028.9 ± 5375.8	6053.8 ± 3968.4
MIP-1α	6.2 ± 1.9	5.6 ± 2.8	5.9 ± 3.1	4.6 ± 2.9
MIP-1β	3.3 ± 1.8	1.9 ± 2.1	5.7 ± 1.5	4.4 ± 1.1
TNFa	1.3 ± 0.3	0.9 ± 0.3	< OOR	< OOR
TNFb	< OOR	< OOR	< OOR	< OOR
VEGF	80.6 ± 15.8	81.9 ± 32.7	45.3 ± 22.8	40.0 ± 18.4
EGF	4.6 ± 0.8	4.0 ± 0.6	1.1 ± 1.7	1.5 ± 1.6
G-CSF	22.2 ± 6.0	18.9 ± 5.1	24.1 ± 13.5	16.8 ± 6.7
IL-11	3.3 ± 1.0	3.4 ± 0.4	3.5 ± 0.6	4.2 ± 0.4*
RANTES	423.3 ± 228.6	360.5 ± 183.9	318.9 ± 187.5	313.6 ± 141.5

* $p < 0.05$ compared to control RBCs of correspondent age, < OOR: out (below) of detection range.

Table 5.2: Cytokine/Chemokine released by HUVECs after incubation with DOPC-treated and control supernatants at day 2 and day 42 of hypothermic storage. Mean values ± SD (n = 6) are shown.

Hypothermic storage	***Day 2***		***Day 42***	
Cytokines/ Chemokines (pg/mL)	**Control Supernatants**	**DOPC-treated Supernatants**	**Control Supernatants**	**DOPC-treated Supernatants**
IL-1RA	12.8 ± 3.0	12.0 ± 1.9	11.5 ± 3.2	8.7 ± 3.1
FGF-2	53.8 ± 9.1	56.6 ± 6.5	25.5 ± 10.7	45.8 ± 12.3*
Eotaxin	19.1 ± 5.4	21.9 ± 4.3	30.2 ± 10.5	32.5 ± 16.9
TGFα	1.7 ± 0.6	1.8 ± 0.4	OOR <	OOR <
Flt-3L	16.1 ± 2.7	17.2 ± 1.8	12.1 ± 1.6	11.2 ± 3.7
GM-CSF	14.6 ± 7.5	24.0 ± 10.2*	17.8 ± 5.9	17.9 ± 15.5
Fractalkine	130.7 ± 39.1	134.3 ± 32.7	52.6 ± 7.5	42.4 ± 14.6
IFNα2	20.9 ± 7.1	23.8 ± 4.9	29.3 ± 4.7	30.3 ± 10.7
IFNγ	4.1 ± 0.8	4.8 ± 1.2	4.0 ± 1.2	3.3 ± 1.9
GRO	3678.5 ± 3820.2	6337.6 ± 4125.2*	1504.8 ± 1200.3	2262.9 ± 4284.5
IL-10	3.6 ± 0.5	3.6 ± 0.8	1.6 ± 1.9	0.7 ± 1.1
MCP-3	107.5 ± 53.5	164.3 ± 71.9*	26.9 ± 10.5	19.5 ± 9.4
IL-12p40	9.2 ± 1.5	9.6 ± 1.9	8.7 ± 3.2	6.4 ± 3.1
MDC	10.5 ± 1.1	12.0 ± 2.7	3.6 ± 1.9	2.4 ± 2.9
IL-12p70	5.8 ± 1.4	6.0 ± 1.3	2.8 ± 0.6	2.0 ± 2.1
IL-13	2.5 ± 0.5	2.7 ± 0.4	9.6 ± 2.0	9.0 ± 3.3
IL-15	4.0 ± 0.9	4.5 ± 1.2	5.4 ± 1.8	4.0 ± 2.7
sCD40L	7.8 ± 2.1	9.0 ± 1.8*	4.7 ± 2.5	3.5 ± 3.2
IL-17	2.2 ± 0.5	2.7 ± 0.4*	1.2 ± 1.0	0.9 ± 1.2
IL-1α	OOR <	OOR <	OOR <	OOR <
IL-9	1.3 ± 0.2	1.4 ± 0.2	OOR <	OOR <
IL-1β	0.5 ± 0.6	0.5 ± 0.5	OOR <	OOR <
IL-2	OOR <	OOR <	1.69 ± 1.03	0.42 ± 1.03
IL-3	OOR <	OOR <	OOR <	OOR <
IL-4	10.7 ± 2.5	11.4 ± 3.0	15.9 ± 1.9	13.2 ± 4.3
IL-5	1.9 ± 0.4	1.9 ± 0.3	OOR <	OOR <
IL-6	211.0 ± 163.6	305.2 ± 181.0*	130.3 ± 57.0	148.7 ± 198.4
IL-7	14.3 ± 4.3	15.6 ± 2.2	43.7 ± 9.8	43.5 ± 10.9
IL-8	3462.2 ± 2842.0	6306.7 ± 1362.1*	1757.9 ± 1099.2	1831.0 ± 974.0
IP-10	26.4 ± 11.2	32.5 ± 13.7	14.4 ± 5.1	10.8 ± 4.9
MCP-1	11606.1 ± 441.7	11786.6 ± 247.2	14608.6 ± 555.5	12118.2 ± 3446.8
MIP-1α	10.1 ± 2.0	11.9 ± 1.5	10.4 ± 2.6	9.0 ± 2.8
MIP-1β	6.6 ± 1.6	6.6 ± 1.9	7.1 ± 1.2	5.9 ± 1.6
TNFa	2.0 ± 0.6	1.9 ± 0.5	0.7 ± 0.6	0.6 ± 0.7
TNFb	2.3 ± 0.6	2.4 ± 0.5	0.3 ± 0.7	0.7 ± 1.1
VEGF	142.2 ± 38.2	164.0 ± 33.3	91.0 ± 21.5	68.8 ± 28.4
EGF	4.9 ± 0.6	5.5 ± 1.3	4.1 ± 1.0	3.1 ± 1.7
G-CSF	35.2 ± 13.4	36.8 ± 9.9	40.7 ± 14.6	42.4 ± 18.3
IL-11	3.6 ± 0.8	3.5 ± 0.3	4.1 ± 0.2	4.5 ± 0.8
RANTES	85.5 ± 59.1	101.9 ± 32.7	134.4 ± 76.3	171.0 ± 125.7

* $p < 0.05$ compared to control RBCs of correspondent age, < OOR: out (below) of detection range.

Table 5.3: Correlation of cytokine release with the expression of adhesion molecules by HUVECs when incubated with control and DOPC-treated RBCs at day 2 and 42 of hypothermic storage.

Day 2	***Control RBCs***				***DOPC-RBCs***			
Adhesion molecules	**VCAM-1**		**E-selectine**		**VCAM-1**		**E-selectine**	
Cytokine/Chemokine	(r_s)	P	(r_s)	P	(r_s)	P	(r_s)	P
IL-15	0.029	1.00	0.314	0.56	-0.943	0.02*	-0.829	0.06
IL-17	0.794	0.06	0.353	0.50	-0.853	0.03*	-0.765	0.10
IFNγ	0.486	0.36	0.371	0.50	-0.200	0.72	-0.257	0.66
IFNα2	0.667	0.14	0.116	0.80	-0.812	0.06	-0.899	0.02*
FGF-2	0.257	0.66	0.771	0.10	0.143	0.80	0.029	1.00
Flt-3L	0.371	0.50	0.771	0.10	0.377	0.42	0.232	0.66
Day 42	(r_s)	P	(r_s)	P	(r_s)	P	(r_s)	P
IL-11	0.698	0.14	0.698	0.14	0.943	0.02*	0.771	0.10
FGF-2	0.029	0.92	0.029	0.92	0.771	0.10	0.714	0.14
IP-10	-0.177	0.71	-0.177	0.71	0.235	0.66	0.000	1.00

r_s = Spearman's correlation coefficient, * $p < 0.05$

Table 5.4: Cytokine/Chemokine released by monocytes after incubation with DOPC-treated and control RBCs at day 2 and day 42 of hypothermic storage. Mean values ± SD (n = 6) are shown.

Hypothermic storage	***Day 2***		***Day 42***	
Cytokines/ Chemokines (pg/mL)	**Control RBCs**	**DOPC-treated RBCs**	**Control RBCs**	**DOPC-treated RBCs**
IL-1RA	OOR <	OOR <	OOR <	OOR <
FGF-2	2.8 ± 3.6	4.2 ± 5.3	1.4 ± 3.4	2.8 ± 4.3
Eotaxin	12.1 ± 5.9	12.6 ± 4.8	13.0 ± 5.9	12.6 ± 7.1
TGFα	OOR <	OOR <	OOR <	OOR <
Flt-3L	OOR <	OOR <	3.4 ± 2.7	3.6 ± 3.0
GM-CSF	OOR <	OOR <	OOR <	OOR <
Fractalkine	4.2 ± 7.0	9.8 ± 11.3	7.1 ± 3.9	0.6 ± 1.6*
IFNα2	OOR <	OOR <	OOR <	OOR <
IFNγ	1.2 ± 0.4	1.3 ± 0.3	OOR <	OOR <
GRO	12.1 ± 4.5	10.5 ± 5.3	7.8 ± 3.1	6.2 ± 1.3
IL-10	1.7 ± 0.2	1.7 ± 0.3	OOR <	OOR <
MCP-3	4.0 ± 4.9	5.1 ± 4.4	5.3 ± 1.0	5.9 ± 1.8
IL-12p40	OOR <	OOR <	OOR <	OOR <
MDC	3.0 ± 1.7	1.7 ± 1.9	OOR <	OOR <
IL-12p70	1.6 ± 0.2	1.4 ± 0.2*	OOR <	OOR <
IL-13	OOR <	OOR <	OOR <	OOR <
IL-15	OOR <	OOR <	OOR <	OOR <
sCD40L	OOR <	OOR <	OOR <	OOR <
IL-17	OOR <	OOR <	OOR <	OOR <
IL-1α	OOR <	OOR <	OOR <	OOR <
IL-9	OOR <	OOR <	OOR <	OOR <
IL-1β	2.0 ± 1.2	2.3 ± 1.0	OOR <	OOR <
IL-2	OOR <	OOR <	OOR <	OOR <
IL-3	OOR <	OOR <	OOR <	OOR <
IL-4	OOR <	OOR <	OOR <	OOR <
IL-5	0.7 ± 0.1	1.0 ± 0.2*	OOR <	OOR <
IL-6	OOR <	OOR <	OOR <	OOR <
IL-7	OOR <	OOR <	OOR <	OOR <
IL-8	91.9 ± 52.1	86.3 ± 38.1	35.6 ± 17.8	23.4 ± 8.1
IP-10	3.3 ± 2.6	3.0 ± 2.6	OOR <	OOR <
MCP-1	28.7 ± 12.8	32.2 ± 19.0	32.5 ± 12.5	30.5 ± 13.6
MIP-1α	14.9 ± 7.5	14.4 ± 3.9	1.2 ± 2.9	0.6 ± 1.5
MIP-1β	OOR <	OOR <	OOR <	OOR <
TNFa	4.6 ± 2.0	4.4 ± 1.5	1.6 ± 1.3	0.9 ± 0.8
TNFb	OOR <	OOR <	OOR <	OOR <
VEGF	OOR <	OOR <	OOR <	OOR <
EGF	OOR <	OOR <	OOR <	OOR <
G-CSF	1.8 ± 2.1	1.1 ± 1.2	OOR <	OOR <
IL-11	3.2 ± 0.5	2.8 ± 0.5	4.4 ± 0.7	4.1 ± 0.7
RANTES	98.5 ± 44.2	93.3 ± 36.9	93.9 ± 41.5	89.3 ± 30.0

* $p < 0.05$ compared to control RBCs of correspondent age, < OOR: out (below) of detection range.

Figure 5.1: Adhesion molecules expression at day 2 (A) and day 42 (B) of storage by HUVECs incubated with control and DOPC-treated RBCs. Shown is the mean ± SD (n= 6). Negative control (PBS) and positive control (LPS 20 µg/mL). * Significant ($p < 0.05$) compared to control RBCs.

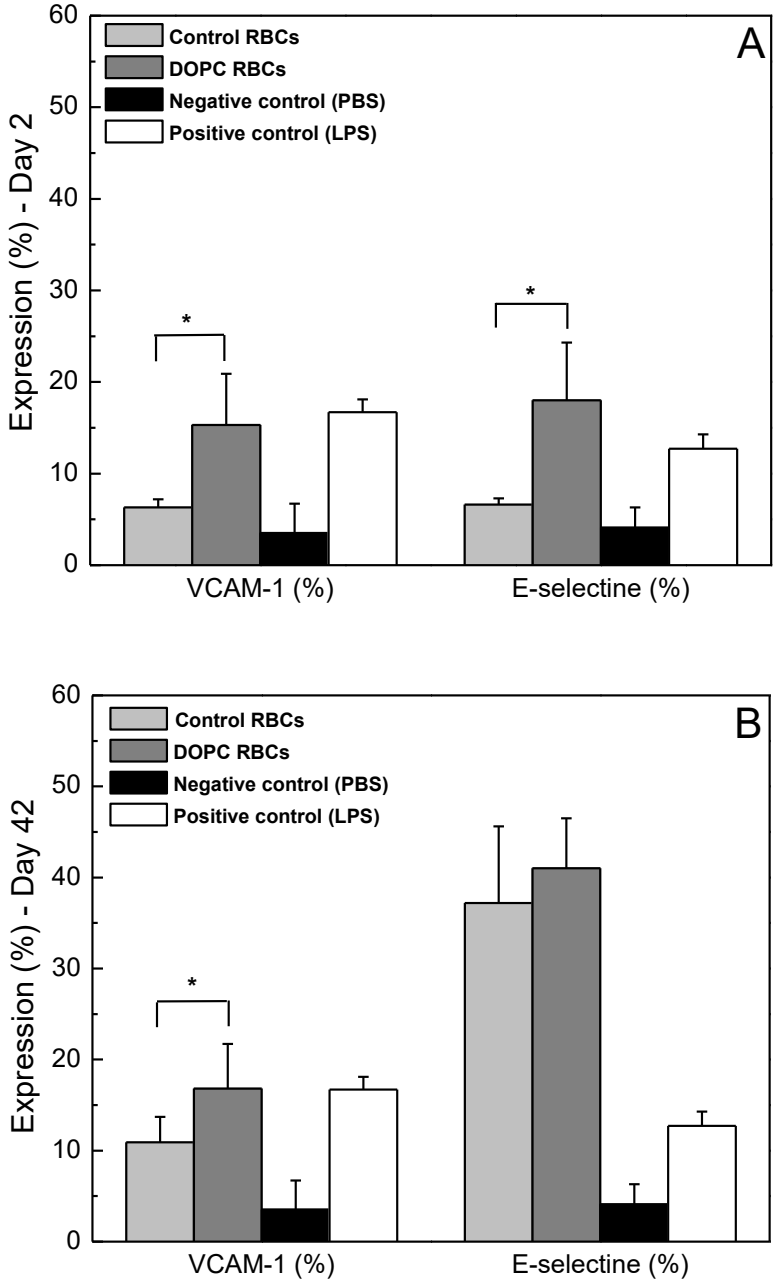
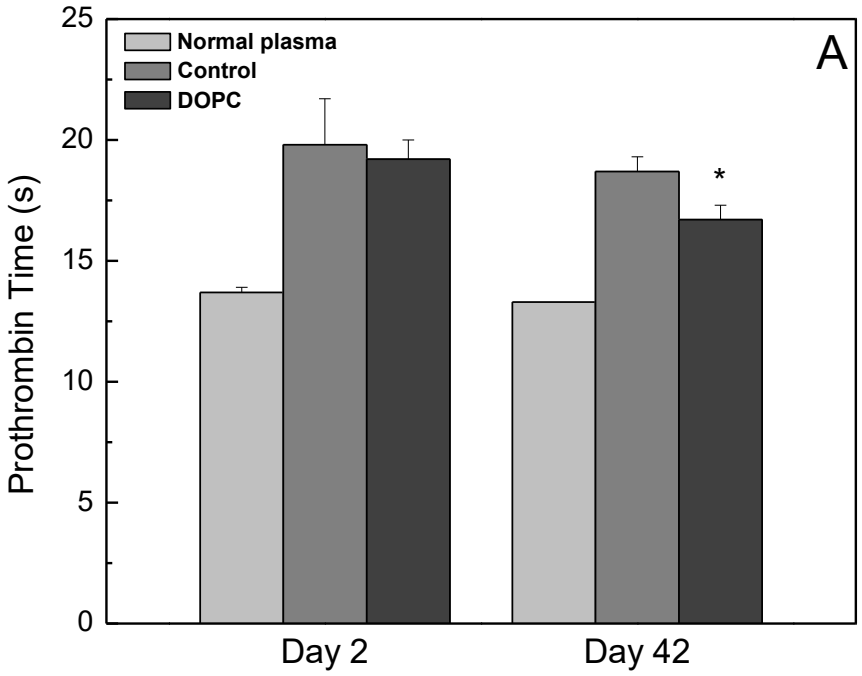
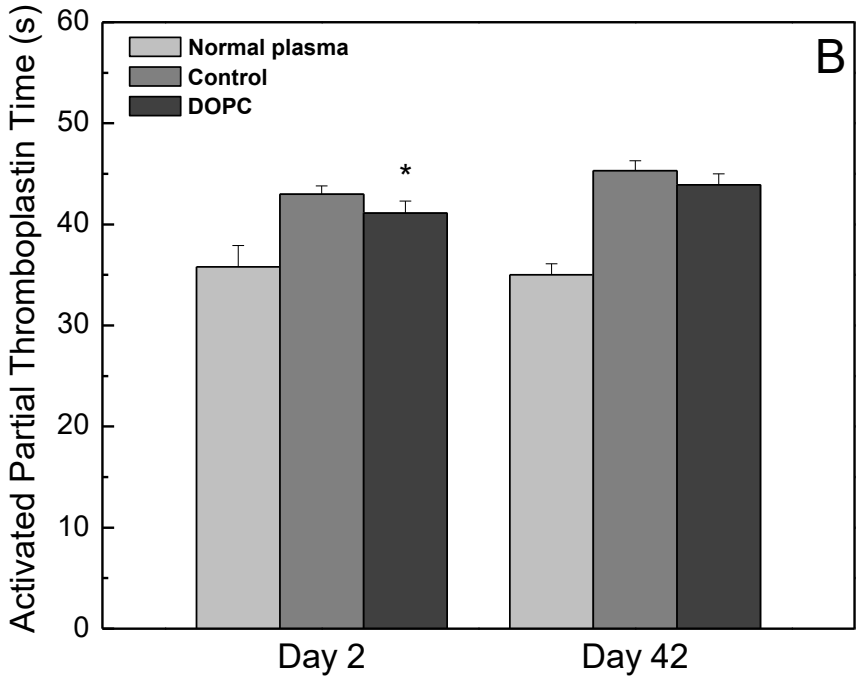


Figure 5.2: Prothrombin time (A) and Activated partial thromboplastin time (B) of control and DOPC-treated supernatants. Shown is the mean ± SD (n= 6). * Significant ($p < 0.05$) compared to control.





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Chapter 6

Combination of liposomes and rejuvenation treatment for minimizing hypothermic storage lesion of red blood cells

6.1 Introduction

As described in Chapter 1, HSL remains an unsolved problem for red cell preservation. Therefore, a number of alternate approaches have been investigated in order to decrease cell injury during storage and perhaps increase RBC shelf life. The techniques vary from new generation additive solutions (1, 2) to anaerobic storage (3), the use of plasticizers (4) and rejuvenating solutions (5).

Rejuvenating solutions have been described since the early 70's, originally for freezing of outdated stored RBCs (6, 7); they are composed of substrates to boost metabolism and contain pyruvate, inosine, phosphate and adenine, sometimes also referred to as the acronym PIPA (8). Pyruvate, inosine and phosphate support 2,3-DPG synthesis, while adenine supports ATP synthesis (Figure 6.1). Inosine and phosphate will originate substrates used in the pentose shunt that will ultimately lead to glyceraldehyde-3-phosphate (G3P), pyruvate to lactate conversion generates a NAD molecule needed for further conversion of G3P to 1,3-DPG (9).

The rejuvenation process involves incubating packed RBCs with the rejuvenating solution for 1 h at 4 °C (cold rejuvenation) or at 37 °C (warm rejuvenation). Cold rejuvenation is sometimes preferred so that RBCs are not exposed to varying temperatures during storage that could potentially affect their quality, in addition that are concerns that warm temperatures could promote contamination and bacterial growth in RBC units (10, 11). The incubation is followed by washing to remove the contents of rejuvenating solutions that are potentially toxic (nephrotoxicity) and the excess of lactate produced by the conversation of pyruvate, that could decrease blood pH and cause metabolic acidosis after transfusion (6, 10).

A rejuvenating solution named Rejuvesol is the only commercially available solution with FDA-approval (9). There are many reports in the literature using both Rejuvesol (5, 9,

12) and in house prepared solutions (13) showing an increase in ATP and 2,3-DPG levels following rejuvenation.

Rejuvenating solutions, while useful in restoration of metabolites, might not mitigate the membrane part of storage lesion. Cell membrane plays an important role in post-transfusion survival and oxygen delivery capacity of RBCs (14, 15). The use of liposomes in transfusion is relatively safe, as shown in Chapter 3. While improving RBC *in vitro* quality by minimizing membrane damage occurring during 42-day storage, as shown in Chapter 4, liposomes do not result in significant changes to the immune profile of stored RBCs, as shown in Chapter 5. Considering the effects of liposomes on membrane quality and the effect of rejuvenating solutions on restoring metabolism, the objective of this study was to evaluate the effect of combining liposome treatment and rejuvenation on the quality of stored RBCs.

6.2 Materials and Methods

6.2.1 RBC collection and manufacturing

Ethical approval for the study was granted by both the Canadian Blood Services (CBS) and the University of Alberta Research Ethics Boards. Five leukoreduced packed RBC units in saline-adenine-glucose-mannitol buffy coat processed were obtained from the CBS Network Centre for Applied Development (netCAD) and processed from whole blood as previously described (16).

6.2.2 Liposome and rejuvenation treatments *

Unilamellar (DOPC:cholesterol, 7:3 mol%) liposomes were synthesized as described in Chapter 3, Section 3.2.2. Five leukoreduced packed RBC units in CPD-SAGM obtained from

* Hematological indices and deformability were performed with the help of MSc student Betty Kipkeu and Doctoral student Ruqayyah Alzmiraq (Lab Medicine and Pathology).

the CBS netCAD were pooled and split. One pool containing five Rh and ABO-matched RBC units was generated and subsequently split to produce five equivalent RBC products. The units produced were segregated into four experimental groups sham control (S), liposome-treated (L), rejuvenation-treated (R) and liposome + rejuvenation-treated (L+R) with treatment at various periods during hypothermic storage (day 7, 21, 42). The FDA-approved rejuvenation solution, Rejuvesol (Citra Labs, Zimmer Biomet, Braintree, MA, USA) was used for rejuvenation treatment. RBCs were divided into four sets of 150 mL bags of S, L, R and L+R and treated at day 7, day 21 and day 42 of hypothermic storage with a final assessment at day 45 (Figure 6.2). The leukoreduced pRBCs were incubated for 1 h at 37 °C with HEPES-NaCl (sham), unilamellar liposomes (DOPC:CHOL, 7:3 mol%, 2 mM lipid, 138.6 nm), rejuvenation-treated (Rejuvesol) and unilamellar liposomes plus rejuvenation (DOPC:CHOL, 7:3 mol%, ≈ 1 mM lipid, 138.6 nm + Rejuvesol) at different storage periods and the *in vitro* quality was accessed. The RBCs were gently rotated during incubation.

The *in vitro* quality of RBCs (n=1 per group/per treatment day) was tested in duplicates and analyzed immediately after treatment (for each treatment day) and at days 42 and 45 of hypothermic storage. Parameters analyzed included percent hemolysis using Drabkin's method, hematological indices performed on a Coulter Counter, deformability and aggregation performed on LORCA, ATP and 2,3-DPG using spectrophotometric methods, all as described in Chapter 2, Section 2.2.2.

6.2.3 Combining liposomes and Rejuvesol*

For the L+R treatment, liposomes were spun at 15,000 x g at 4 °C for 40 min and the pellet ressuspended with Rejuvesol. During this procedure, it was noted that the centrifugation

* Rejuvesol was provided by Zimmer Biomet (IN, USA).

step was not successful in pelleting all the liposome content and part of it remained in the supernatant. Supernatants were then collected and stored at 4 °C, along with an aliquot of the original liposome suspension for further particle analysis to determine the amount of liposomes lost in this step.

Tunable resistive pulse sensing (TRPS)

TRPS technique, also referred to as scanning ion occlusion sensing (SIOS), was used to determine total particle concentration. This method of particle analysis is similar to the Coulter method, but allows for the individual analysis of particles in the nanoscale range (50 - 10,000 nm). When a single nanoparticle passes through a tunable nanopore, it displaces a volume of electrolyte, causing a temporary increase in the electrical resistance of the circuit and corresponding decrease in the measured current. The magnitude of the current reduction and the frequency of the pulses are respectively related to the particle size and concentration (17).

Liposome suspension and supernatant samples were diluted 1:1000 in Solution A of the Izon reagent kit (Izon Science Ltd., Cambridge, MA, USA) and measured on a NP200 nanopore (85-500 nm) using CPC200 calibration particles (Izon Science Ltd.) as controls.

6.2.4 Statistical analyses

Statistical analysis was performed using SPSS 23.0 software (IBM, Armonk, NY). Non-parametric One-way ANOVA (Kruskall-Wallis test) was used to assess the effect of treatment on *in vitro* quality parameters at day 42 HS regardless of treatment time. When a significant difference was found, Mann-Whitney test was used for paired comparisons. Data were expressed as mean ± standard deviation and $p < 0.05$ was considered statistically significant.

6.3 Results

Particle analysis revealed a concentration of 5.3 x 10^{16} particles/mL in the original liposome suspension, while in the supernatants the concentration was 2.6 x 10^{16} particles/mL, accounting for a loss of 2.7 x 10^{16} particles/mL (50.9 %).

Table 6.1 summarizes RBC quality parameters after non-parametric one-way ANOVA. Percent hemolysis was significantly decreased in all treatments compared to sham control (0.60 ± 0.06%): L (0.53 ± 0.01%, p = 0.042), R (0.43 ± 0.02%, p = 0.004), L+R (0.48 ± 0.06%, p = 0.020) (Figure 6.3). Hemolysis levels were comparable in the combined treatment (L+R) compared to individual treatments L (p = 0.293) and R (p = 0.107). No differences in treatments were observed for hematological indices MCV (p = 0.684), MCH (p = 0.649) and MCHC (p = 0.791).

Ektacytometry analysis showed an increase in maximum elongation (EI_{max}) in R (0.55 ± 0.01, p = 0.010) and L+R (0.55 ± 0.01, p = 0.010) treatments compared to S (0.53 ± 0.01) but not L (0.53 ± 0.01, p = 0.936) (Figure 6.4). The combined treatment was comparable to R (p = 0.872) and resulted in a greater EI_{max} than L (p = 0.004). RBC rigidity (K_{EI}) increased in all treatments compared to sham (1.19 ± 0.07): L (1.28 ± 0.06, p = 0.025), R (1.44 ± 0.17, p = 0.010) and R+L (1.44 ± 0.06, p = 0.004) (Figure 6.4). The combined treatment was comparable to R (p = 0.749) but increased to a greater extent when compared to L (p = 0.004). Analysis of variance did not show significant differences among treatments for aggregation index (AI, p = 0.081) and aggregation half-time ($t_{1/2}$, p = 0.092). However, aggregation amplitude was significantly increased by R treatment only (24.1 ± 1.7 au vs. 19.1 ± 1.4 au, p = 0.004). The combined treatment was comparable to L (p = 0.337) and R (p = 0.262).

ATP levels were significantly higher in all treatments compared to sham (1.64 ± 0.14 µmol/g Hb): L (2.00 ± 0.21 µmol/g Hb, p = 0.010), R (4.70 ± 1.20 µmol/g Hb, p = 0.004),

L+R (5.00 ± 1.56 μmol/g Hb, p = 0.004). ATP levels in the combined treatment was comparable to R (p = 0.749) and significantly higher compared to L (p = 0.004). The levels of 2,3-DPG were no longer detectable in S and L treatments at day 42. The combined treatment was comparable to R (2.38 ± 3.26 μmol/g Hb vs. 2.62 ± 2.20 μmol/g Hb, p = 0.868).

6.4 Discussion

Liposomes have been shown to minimize RBC membrane damage occurring during 42-day hypothermic storage, while rejuvenation solutions have been shown to restore RBC metabolism. The objective of this study was to evaluate a potential synergistic effect of combining liposome and rejuvenation treatment on RBC *in vitro* quality.

Rejuvenation treatment increased metabolites ATP and 2,3-DPG in accordance with reports by previous studies (7, 9, 13, 18). The increase in ATP has been associated with improvements in morphology (13, 19) and decreased endothelial adhesion (5, 20), that might result from the restoration of ion and lipid transport across the membrane which are ATP-dependent processes (21, 22), while the restoration of 2,3-DPG is associated with an increase in oxygen release capacity (23).

Percent hemolysis levels were decreased in all treatments compared to sham control. The reduction in hemolysis is consistent with results presented in Chapter 4 (Tables 4.3 and 4.4) for liposome-treated RBCs. The lower levels of hemolysis for rejuvenation treatment alone are also in agreeement with a study from Tchir and colleagues (2013), showing that rejuvenated samples had significantly lower hemolysis levels throughout storage compared to sham treated samples (13). The combined treatment however failed to show a synergistic effect on percent hemolyis reduction. This might have been due to liposome loss (≈51%)

during the centrifugation procedure to combine both treatments, that resulted in lower liposome concentration in the combined treatment and constitutes a limitation of this study.

While liposome treatment has caused changes to RBC rigidity, no changes to elongation index have been observed in previous chapters. Rejuvenation and combined treatment both had increased elongation index, which, considering previous results of liposome effect on hemorheology in Chapter 4, is probably due to rejuvenation rather the liposome treatment, since liposome treatment alone has never been shown to affect elongation index. This increase in elongation index is the opposite of what was observed during a study using cold rejuvenation (13), but agrees with a warm rejuvenation study showing partial recovery of mechanical damage in RBCs following rejuvenation (24). The same cold rejuvenation study showed no changes to rigidity (K_{EI}) (13) while the results of this study showed an increase in rigidity for all treatments. The changes observed could be due to cytoskeletal rearrangement or changes in the interaction between the lipid bilayer and cytoskeleton that might require biochemical activation and therefore would be favored by physiological temperatures and inhibited by cold temperatures (25, 26). Because no significant changes were observed in MCV and MCH for the same treatments, the increase in rigidity cannot be explained by a reduction in surface area-to-volume ratio and increased internal viscosity, that is usually related to changes in rigidity (27, 28). The choice for warm rejuvenation was based on the fact that liposome treatment is also performed at 37 °C. The difference between this result and previous results using cold rejuvenation might suggest that, while both types of rejuvenation are able to raise metabolites, the same effect might not be true regarding aspects of storage lesion related to the cell membrane. Membrane fluidity depends on temperature and composition, with cholesterol being a major determinant of fluidity (29). In order to maintain cell function, membrane lipid bilayers are normally fluid at

physiological temperatures (30). Cell membrane is less fluid at low temperatures, which in turn affects membrane permeability (31). Since there is an accumulation of calcium in stored and aged RBCs that is directly related to RBC deformability (32, 33), cold rejuvenation might not allow for calcium efflux of RBCs due to decreased membrane fluidity and permeability at 4 °C resulting in inferior effects on deformability compared to warm rejuvenation.

There are no reports in the literature evaluating the effects of rejuvenation on aggregation parameters. While there was no significant effect of treatments regarding aggregation index and aggregation half-time, a significant increase in the extent of aggregation (amplitude) compared to sham control was observed for rejuvenation treatment only. An increase in aggregation amplitude is often seen in abnormal RBCs (i.e. sickle cell disease) (34) and some disease states (i.e. unstable angina, bacterial infection) (35) as a consequence of changes in cellular properties (i.e. surface charge, glycocalyx) that favor the formation of larger 3D aggregates. Cellular factors like shape, surface charge (related to the membrane sialic acid content) and glycocalyx thickness are intrinsic cell properties known to influence aggregation behavior (36). RBCs tend to have less sialic acid as they age (37, 38) as well as a decrease in glycocalyx thickness (39), which would facilitate aggregation by reducing surface charge and increasing cell affinity respectively, coupled to restoration of morphology attributed to rejuvenation (40) could account for the increase in aggregation amplitude observed. Although statistical significance does not always translate to clinical significance, the amplitude results observed here for the rejuvenation treated RBCs are comparable to values reported for patients with sickle cell hemoglobin C disease (34) and therefore require further investigation.

Rejuvesol is slightly hypertonic (416 mOsm/kg H_2O). Liposomes behave like cell membranes when subjected to osmotic changes (41), therefore the exposure to a hypertonic

environment most likely caused them to shrink (42). The shrinkage could affect the integrity of the lipid bilayer and consequently their interaction with RBC membranes. Neutral liposomes are also prone to aggregation, which is usually reversible by agitation, but since pH and ionic strength also play a role in aggregation (43, 44) more information on the effects Rejuvesol (pH = 6.7-7.4) might have on liposome aggregation are needed.

This study was a limited study of the effects of combining liposomes and rejuvenation on the quality of RBCs. Lipid concentration on the combined treatment was a limiting factor of the study, as well as the small sample size used. In order to make the combined treatment less time consuming, DOPC liposomes and Rejuvesol were pooled and used as a one-step treatment, which might have affected liposome integrity and consequently their interactions with RBCs, therefore characterizing another limitation. Further studies need to be done to characterize liposome properties when subjected to a medium like Rejuvesol.

The investigation of the effects of the combined treatment on RBC quality still has merit, and future studies to re-test this hypothesis should be designed, while addressing the limitations presented here. If liposomes prove to be incompatible with Rejuvesol, one viable option would be to divide the treatments into two sequential steps.

6.5 Conclusion

Both rejuvenation and liposome treatments improved the quality of stored RBCs compared to sham control. The combined treatment (L+R) did not have a greater impact in improving *in vitro* quality of stored RBCs compared to rejuvenation alone. Further investigation should address whether rejuvenation solutions affect the ability of liposomes to interact with RBC membranes. Another alternative would be combining the treatments as a

sequential two-step treatment (i.e. rejuvenation followed by liposome treatment) to help avoid incompatibilities that might result in less efficacy.

Table 6.1: Non-parametric One-way ANOVA for RBC quality parameters. Mean ± SD are shown (n=3) for sham control (S), liposome (L), rejuvenation (R) and liposome + rejuvenation (L+R) treated RBCs assessed on day 42 of hypothermic storage.

Quality parameters	**S**	**L**	**R**	**L+R**	***P***
Hemolysis (%)	0.60 ± 0.06	0.53 ± 0.01*	0.43 ± 0.02*	0.48 ± 0.06*	0.002
Deformability					
EI_{max}	0.53 ± 0.01	0.53 ± 0.01‡	0.55 ± 0.01*	0.55 ± 0.01*	0.001
K_{EI}	1.19 ± 0.07	1.28 ± 0.06*‡	1.44 ± 0.17*	1.44 ± 0.06*	0.001
Hematological indices					
MCV (fL)	99.5 ± 0.4	99.8 ± 0.3	99.4 ± 0.8	99.7 ± 0.9	0.684
MCH (pg)	30.7 ± 0.2	30.8 ± 0.2	30.6 ± 0.1	30.6 ± 0.2	0.649
MCHC (g/L)	309 ± 3	308 ± 2	308 ± 1	307 ± 3	0.791
Aggregation					
AI (%)	45.6 ± 2.6	48.8 ± 2.0	48.5 ± 1.4	47.9 ± 0.7	0.081
Amp (au)	19.1 ± 1.4	20.4 ± 0.9	24.1 ± 1.7*	20.4 ± 5.9	0.020
$t_{1/2}$ (s)	4.8 ± 0.6	4.1 ± 0.3	4.2 ± 0.3	4.3 ± 0.2	0.092
Metabolites					
ATP (µmol/g Hb)	1.64 ± 0.14	2.00 ± 0.21*‡	4.70 ± 1.20*	5.00 ± 1.56*	< 0.001
2,3-DPG (µmol/g Hb)	0 ± 0	0 ± 0	2.62 ± 2.20	2.38 ± 3.26	0.026

*$p < 0.05$ compared to sham control, ‡ $p < 0.05$ compared to combined treatment

Figure 6.1: Red blood cell energy metabolism. Overview of metabolic pathways and contribution of PIPA to the generation of ATP and 2,3-DPG.

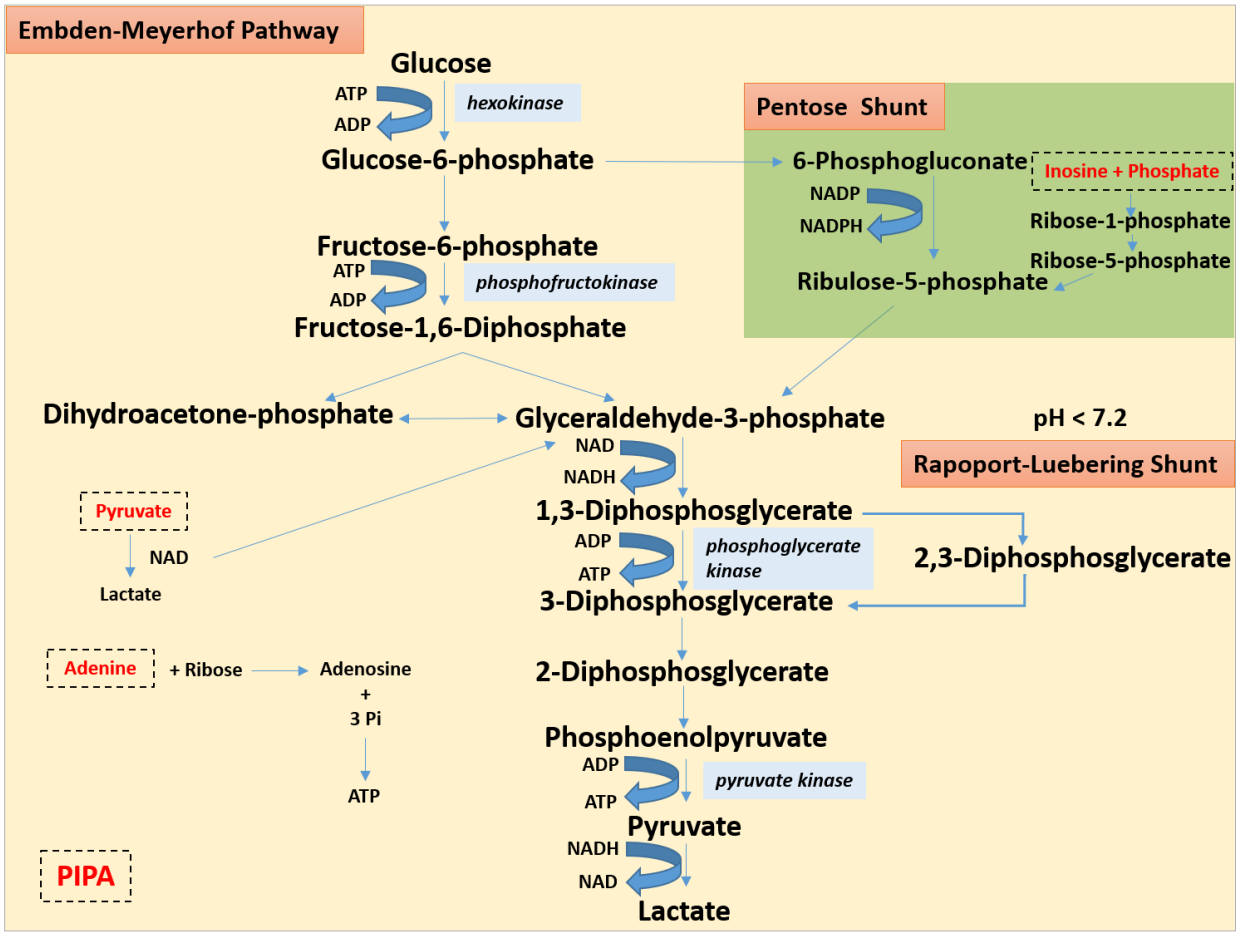
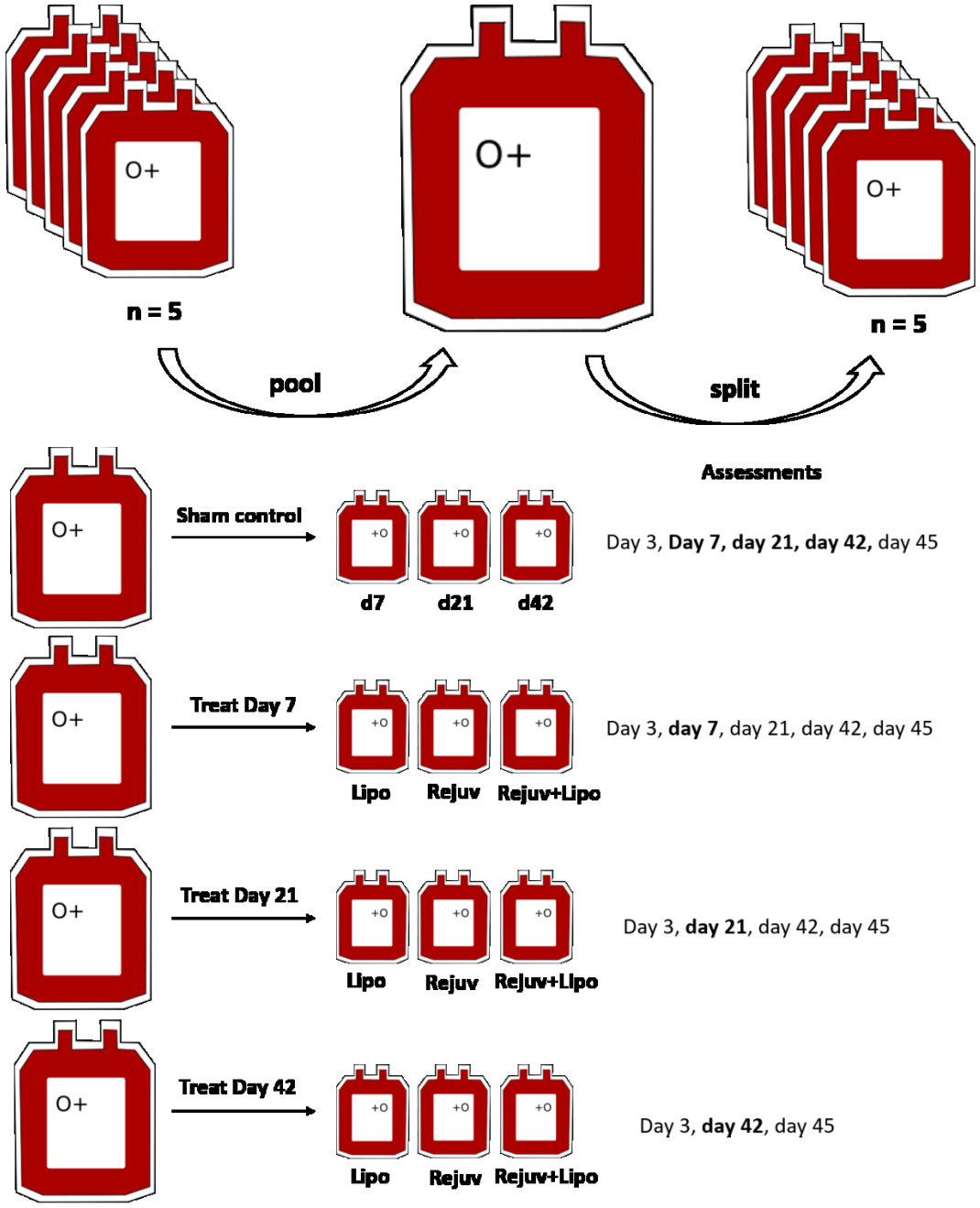


Figure 6.2: Experimental design of the study.



***In vitro* quality assessment**

Membrane:	***Metabolism:***
- Percent hemolysis	- ATP
- Hematological indices	- 2,3-DPG
- Deformability	
- Aggregation	

Figure 6.3: Percent hemolysis in human RBCs at day 42 of HS. Shown is the mean ± SD (n= 3). * Significant ($p < 0.05$) compared to sham control. Dotted line showing the cut off value for blood banked stored RBCs at day 42 of HS in Canada.

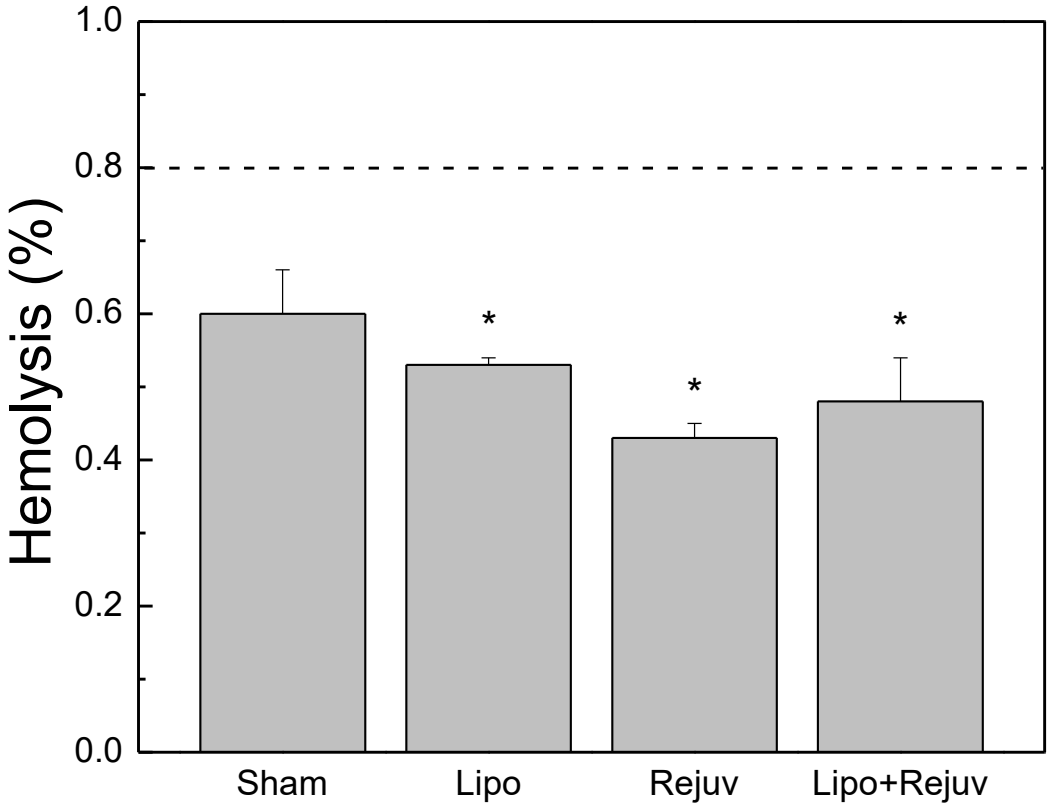
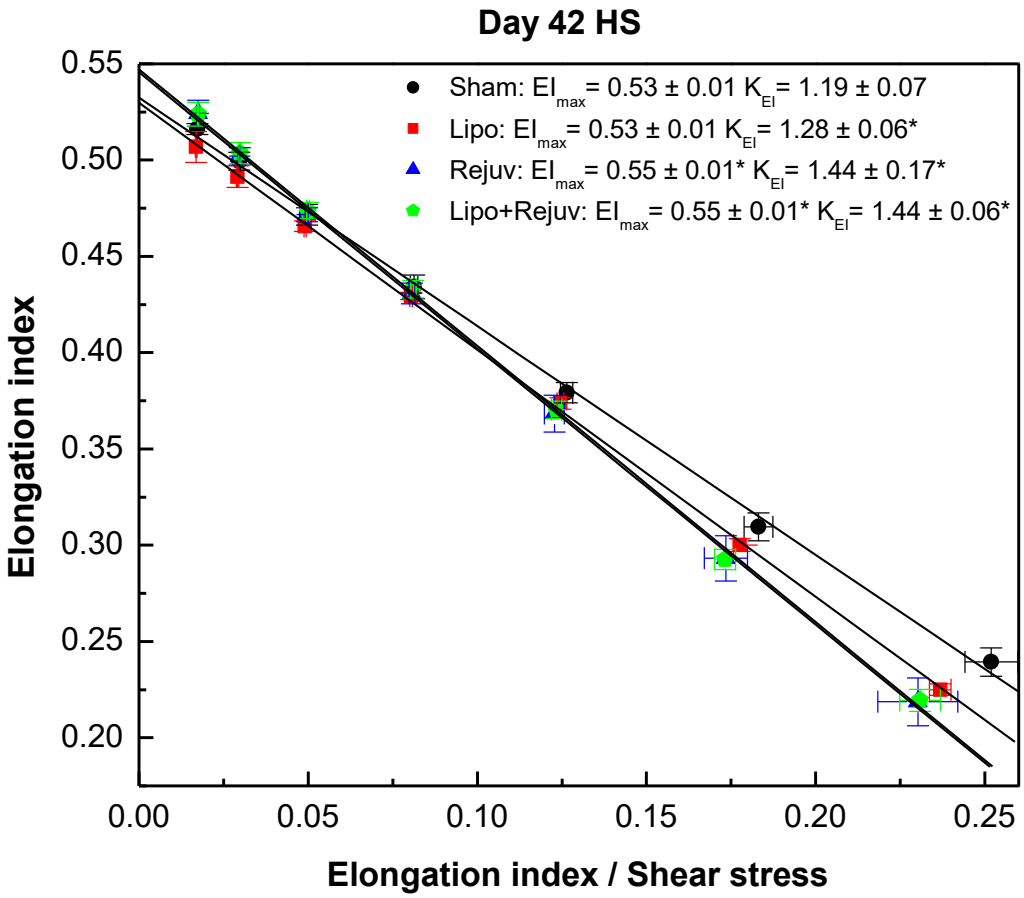


Figure 6.4: Deformability curves of sham, liposome-treated, rejuvenation-treated and liposome + rejuvenation-treated RBCs at day 42 of HS. Maximum elongation index (EI_{max}) and rigidity (K_{EI}) are shown (n= 3). * Significant ($p < 0.05$) compared to sham control RBCs.



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Chapter 7

General discussion and conclusions

7.1 Review of thesis objectives and summary of results

Many advances have been made in transfusion medicine since the first solution to preserve blood *ex vivo* was discovered by Rous and Turner in 1915 (1). Over the last 102 years, new knowledge and innovations in the field have allowed the establishment and expansion of modern blood banking. However, despite many successful strategies that allowed blood to be stored for longer periods, the decrease in quality of long stored blood caused by HSL is still a problem that has not been overcome by current strategies. Therefore, new research efforts are necessary to guarantee a better quality and safe supply for those in need of this life saving treatment.

The work presented here had the objective to advance the field of transfusion medicine and biopreservation by increasing our understanding of RBC hypothermic storage lesion and by investigating a novel approach to mitigate membrane injury occurring during hypothermic storage, involving the use of liposomes. Investigations were first conducted on an animal model, and developed further to evaluate several aspects of liposome treatment on human RBCs that would characterize it as a promising tool for RBC preservation.

Although the differences in quality parameters between human RBCs in SAGM and AS3 are well documented in the literature, this information is not available for rat RBCs (2). Also, little is known about the effects of component manufacturing and leukoreduction methods on the parameters important for RBC hemorheology, including deformability, aggregation and microvesiculation in rat RBCs. Considering that, the key of this research was to evaluate a new approach to HSL that directly influenced membrane characteristics, it is important that membrane-related parameters have been well characterized in the animal model species before any intervention. The objective of the first experimental study, described in Chapter 2, was to evaluate baseline differences between rat and human RBCs following a

novel adaptation of the buffy coat component production method (rat and human RBCs in SAGM). The purpose was to examine metabolic, membrane-related and hemorheology parameters in addition to investigate the impact of the buffy coat component manufacturing method on rat RBCs and the effects of different additive solutions (rat RBCs in AS3 and SAGM). Eventually, the goal was to establish a rat pRBC production method that closely mimics current human pRBC techniques to be used consistently in the next studies. This study provided confirmation of significant differences between RBC species in metabolic and membrane-related aspects, which should be taken into account when performing preclinical transfusion studies using rat models. It also showed that SAGM, which is commonly used for human RBC storage, is not compatible with rat RBCs, as it causes high hemolysis and increased MP production, a fact that was unknown until now. The recommendation originated from this study is the use of a buffy coat production method for rat RBCs (plasma and buffy coat removal, addition of compatible AS and leukoreduction) as well as the use of AS3 as a better alternative for rat RBC storage when conducting studies examining the HSL or new preservation strategies for RBCs that might require the use of rat models. Since the quality of stored RBCs vary with different production methods (3, 4), this study recommends that standard processing protocols mimicking blood bank production and storage be developed for specific species used as animal models in transfusion medicine studies evaluating the age of blood.

After establishing a rat pRBC production method and a baseline for rat RBC *in vitro* quality parameters, my next study aimed to evaluate the effect of different liposome formulations on rat RBC HSL and the *in vivo* outcomes of transfusing liposome-treated RBCs in an anemic rat model, as described in Chapter 3. This study investigated the effects of liposome treatment on RBC rheologic properties, as well as *in vivo* assesment of efficacy and

short-term safety of transfusing liposome treated RBCs in a rat model. Four liposome formulations were evaluated with differences in saturation (saturated and unsaturated) and surface charge (neutral and negatively charged), considering that chemical characteristics are important determinants of liposome-RBC interaction (5-10). This study showed that all formulations had an effect in reducing hemolysis and the beneficial effect of liposome treatment on rat RBC membrane was related to the saturation level of liposome phospholipids, rather than liposome charge. Unsaturated-uncharged liposomes (DOPC) were the most beneficial for rat RBCs and therefore were used to further evaluate effects on hemorheology and in the *in vivo* study. It was further demonstrated that DOPC liposome treatment overall improved rat RBC hemorheology upon storage. However, in terms of efficacy the animals that received liposome-treated RBCs did not show significant reduction in infarct size compared to the control group, suggesting that the changes observed with liposome treatment *in vitro* were not sufficient to improve the outcomes of myocardial I/R in anemic rats. Despite that, because the *in vivo* study did not prompt any immediate safety concerns, the effects of liposome treatment continued to be investigated on stored human RBCs in the next chapters. This was the first report of banked RBC treatment with liposomes followed by storage and *in vivo* transfusion of liposome-treated RBCs. This study recommended further investigation to confirm these safety results and explore the long-term effects of transfusing liposome-treated RBCs, as well as to address the *in vivo* viability and oxygen delivery efficacy of liposome-treated RBCs.

Stemming from questions posed in previous chapters, Chapter 4 experiments aimed to answer whether treatment of human RBCs with DOPC liposomes (as used for all previous rat studies) would have the same beneficial effects on hemorheology as observed in rat RBCs. In addition to evaluating the time point during RBC storage where liposome treatment would

have the most beneficial effects, considering that most significant changes attributed to HSL start to happen around 21 days of storage (11-13). This study showed that DOPC liposome treatment resulted in modest improvements in human RBC hemorheology upon storage, with no significant impact on metabolic profile. Half-way through storage the changes promoted by the liposome treatment dissipate resulting in similar quality to control samples, only to resurface again at the end of storage (6 weeks). This observation is of clinical importance because it shows that during this period when the quality of liposome-treated RBCs reaches a plateau, there would be no added clinical benefit of the treatment if they were to be transfused at that time point. This study also showed that liposome treatment should be performed at the beginning of storage preferably (≤ 7 days) up to 21 days.

Since studies associate old blood with adverse events in transfused patients involving modulatory effects on hemostasis, inflammation and coagulation (14-16), the next study had the objective to assess the impact of liposome-induced membrane changes on the immune profile of liposome-treated RBCs as well as the impact of liposome treatment on MP-rich supernatants as described in Chapter 5. The interaction of liposomes with the immune system both *in vitro* and *in vivo* is of interest as part of preclinical studies to determine the immunotoxicity of liposomes and guide future translational studies. The purpose of this study was to assess the interactions of liposome-treated RBCs with cells of the immune system using cell-based assays (HUVECs and monocytes) and the resulting immune response derived from this interaction, in the form of cytokine release, adhesion molecules expression and phagocytosis. In addition, the impact of liposome treatment on MP-rich supernatants was also assessed, by evaluating interactions with endothelial cells (cytokines and adhesion molecules) and the coagulation profile. This is the first report of immunomodulatory effects using a comprehensive panel of cell-based assays relevant to blood bank and this intended liposome

application. This study showed that liposome treatment did not result in significant changes to the immune profile of stored RBCs as per cytokine profiles of HUVECs and monocytes. However, further investigation on the clinical implications of adhesion molecules expression by HUVECs, as well as FGF-2 release that were exclusively increased on incubations with DOPC-treated RBCs are recommended. Liposome treatment did not induce additional immune response from endothelial cells or monocytes *in vitro*, suggesting that such RBCs are not likely to be removed prematurely from the circulation upon transfusion. It was also demonstrated that liposome treatment resulted in a small change in the immune profile of fresh supernatants only, indicated by higher cytokine release as well as slightly enhanced clotting response. Considering these effects would probably be mitigated by a washing procedure, it does not invalidate the potential for liposomes in RBC preservation. Finally, this study revealed that the membrane changes observed with liposome treatment in previous chapters do not necessarily translate into amelioration of immune and coagulation profile of stored RBCs (lower pro-inflammatory and pro-coagulant activity); however, the fact that it did not result in immunotoxicity is encouraging and still make liposome treatment a possible candidate for RBC preservation.

Providing stored RBCs with a source of phospholipids is important for membrane renewal, but so is the metabolic machinery in assuring appropriate function of enzymes, ion channels, transporters and pumps that will contribute to membrane improvements and long-term conservation. The final study of this thesis aimed to evaluate the effect of combining liposome treatment and rejuvenation on the quality of stored RBCs, as described in Chapter 6. Since rejuvenating solutions are useful in restoration of metabolites (17-20) and liposomes are useful in membrane restoration, the hypothesis that combining these treatments would result in better preservation of RBC quality after 42-day storage was tested. This is the first study to

address membrane and cell metabolism for improved biopreservation. This study demonstrated that individual liposome and rejuvenation treatments improved the quality of stored RBCs compared to sham treated. However, the combined treatment did not have a greater impact in improving *in vitro* quality of stored RBCs compared to rejuvenation alone. Additional investigation on the effect of rejuvenating solutions on the ability of liposomes to interact with RBC membranes were proposed. Due to the study limitations, it was not possible to exclude the possibility that combining liposome and rejuvenation treatments would improve the quality of stored RBCs and therefore this hypothesis should be re-tested using a sequential two-step treatment rather than the one-step treatment used.

My original hypothesis stated: "Liposome treatment of stored RBCs will improve *in vitro* membrane quality resulting in reduced *in vitro* production of proinflammatory and procoagulant markers and a safe transfusion product in an anemic rat model." The information obtained from this work provided new and important information on how liposome treatment does improve *in vitro* RBC quality, which does not translate into reduction of *in vitro* production of proinflammatory and procoagulant markers, although the final transfusion product showed relative safety in the rat model.

7.2 Contributions to science and future directions

This thesis has made a number of contributions to the fields of transfusion medicine and biopreservation, as demonstrated by four published papers in top journals in the fields of transfusion medicine, liposomes research and biopreservation.

1) It has produced new knowledge about the impact of blood processing manufacturing on rat RBCs using current additive solutions and a baseline of the *in vitro* membrane and metabolic quality markers, as well as established a processing method more suitable for use in

animal models of transfusion evaluating HSL. This knowledge is important, as it will help reduce one cause of variability in animal studies of transfusion that might add to the lack of translation to human studies.

2) It has demonstrated the effect of different liposome formulations on rat RBC hemorheology and determined for the first time the short-term safety of transfusing liposome-treated RBCs in an animal model. This will advance the knowledge base of biopreservation strategies using liposomes, while increasing the data on biocompatibility of liposomes that make them such promising tools for diverse applications in medical research.

3) It has established the ideal liposome treatment time in human RBCs and fully characterized for the first time the effects of DOPC liposome treatment on membrane and metabolic *in vitro* quality parameters in human RBCs at three different time points of hypothermic storage (fresh, 3 and 6 weeks). This was also the first attempt to perform the liposome treatment on packed human RBCs produced according to blood bank standards and characteristics (i.e. CPD-SAGM, leukoreduced, 55-65 % Hct) as previous attempts used small volume whole blood collections and different processing steps (i.e. different anticoagulants, no leukoreduction) and only evaluated 5% Hct suspensions. This work provides a foundation to guide future research using liposomes to preserve banked RBCs and other cell types (i.e. platelets, cord blood).

4) It has produced novel information about the potential effects of DOPC-treated RBCs and supernatants on the immune response using different cell types, a comprehensive cytokine panel and endothelial activation markers, relevant to current understanding of *in vivo* inflammatory effects. Furthermore, it has produced novel information on the coagulation profile of the supernatant portion, which has been implicated in adverse reactions related to thrombotic events in transfused patients.

5) Finally, a first attempt to a novel idea of combining liposome and rejuvenation treatments was demonstrated, and by the lack of synergistic effect of the combined treatment, it was concluded that treatments may be performed in two consecutive steps to avoid possible incompatibilities and loss of efficacy of liposomes.

Future research in this area should focus on evaluating this DOPC liposome formulation with varying cholesterol content, as a way to maximize the beneficial effects on the RBC membrane reported here (i.e. decreased hemolysis, aggregation behavior) while minimizing some of the undesired effects (i.e. increase MP release). Another aspect of the liposome approach that deserves further investigation as part of the efforts to improve RBC hypothermic storage, is the loading of liposomes with antioxidants (i.e. ascorbic acid, glutathione), cryoprotectants and rejuvenating solutions or its individual components (i.e. inosine, pyruvate) for delivery during storage. Because the hypothermic lesion results from a combination of different mechanisms (membrane, oxidative and metabolic), the association of liposomes with metabolic restoration or antioxidant protection might have a synergistic effect in protecting RBCs during hypothermic storage. Antioxidants loaded in the bilayer or aqueous core of liposomes have been shown to protect DPPC vesicles from radiation damage (21). In a similar way, antioxidants added to an RBC unit have been shown to protect against oxidative damage of gamma irradiation (22). Since evidence suggests that liposome-encapsulated antioxidants can be superior to the corresponding free antioxidants against oxidative damage (23), I believe that the combined action of liposomes and antioxidants could provide a new alternative for mitigating RBC storage lesion. Further investigation into different ways for combining liposomes with rejuvenation treatment is also recommended.

I believe that the use of liposomes to mitigate HSL is a strategy worth pursuing further. Considering the vast number of possible liposome formulations, it is imaginable that an ideal

one would be capable of improving the quality of stored RBCs to an even greater extent than the results reported here for this particular formulation. I also think that this would open new possibilities of preservation systems for other clinically relevant cell types and I hope this work helps guide future research and developments in biopreservation that will help advance cellular therapies and improve patient's lives.

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