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Cryobiological characteristics of red blood cells from human umbilical cord blood

by

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Abstract

There has been significant interest in the literature on the possibility of using red blood cells (RBCs) derived from umbilical cord blood (cord RBCs) for neonatal transfusions. Low stability of cord RBCs during hypothermic storage necessitates the development of a cryopreservation method to prevent fast deterioration of these cells and create a stable supply for clinical transfusions.

The goal of this work was to understand the physiology of cord RBCs from a cryobiological perspective. Investigations were conducted on several levels, from assessing the initial quality of cord RBCs after their isolation from cord blood, to the determining the cryobiological parameters of the cord RBC membrane.

Results indicated that whole cord blood can be stored at room temperature for up to 65 hours without significant decrease in cord RBC quality. Cord blood:anticoagulant volume ratio was found to be a major determinant of cord RBC quality. I developed a new method to measure rapid osmotically-induced changes in RBC volume, which is based on the direct relationship between RBC volume and the intensity of autofluorescence of intracellular hemoglobin. This method was used to determine osmotic parameters of cord RBCs, such as the osmotically inactive fraction of cell volume, osmotic permeability to water and the cryoprotectant glycerol, the Arrhenius activation energies for water and glycerol permeabilities, and osmotic tolerance limits were determined. The permeability of cord RBCs varied, in comparison to adult RBCs, depending on

temperature and osmolality. Cord RBCs appeared to be more osmotically resistant to swelling than adult RBCs. When cryopreserved using standard high glycerol – slow cooling method designed for adult RBCs, cord RBCs showed lower recovery compared to adult RBCs.

This thesis provides all pieces of a puzzle required to create the cryopreservation technique for cord RBCs in future, starting from their initial quality upon isolation from umbilical cord blood and ending with cryobiological parameters of cord RBC membrane. If a successful cryopreservation technique for cord RBCs is created, these cells can be turned from a waste product into a superior blood product for intrauterine and neonatal transfusions that will significantly improve prenatal and postnatal medical care.

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Abbreviations

RBC	red blood cell
Hb	hemoglobin
HbF	fetal hemoglobin
HbA	adult hemoglobin
p50	partial pressure of oxygen required for hemoglobin to be 50% saturated
2,3-DPG	2,3–diphosphoglycerate
ATP	adenosine triphosphate
PS	phosphatidylserine
QMP	Quality Monitoring Program
CPA	cryoprotective agent
Me_2SO	dimethyl sulfoxide
HES	hydroxyethyl starch
PVP	polyvinylpyrrolidone
CPD	citrate-phosphate-dextrose
SAGM	saline-adenine-glucose-mannitol
PEO-1500	polyethylene oxide with molecular weight 1500
CP2D	citrate-phosphate-double dextrose
ACD-A	anticoagulant citrate dextrose solution-formula A
AS-1	additive solution 1
AS-3	additive solution 3
MetHb	methemoglobin
K^+	potassium
Na^+	sodium
WBC	white blood cells
Hct	hematocrit
MCV	microcorpuscular volume
MCH	microcorpuscular hemoglobin

MCHC	microcorpuscular hemoglobin concentration
EI_{max}	maximum theoretical elongation index
K_{EI}	shear stress required to achieve half of the EI_{max}
MP	microparticle
CD47	cluster of differentiation 47
MFI	mean fluorescence intensity
NaCl	sodium chloride
CPDA-1	citrate-phosphate-dextrose-adenine-1
LD	leukodepleted
NLD	non-leukodepleted
HiCN	cyanmethemoglobin
HK	hexokinase
ADP	adenosine diphosphate
NAD^+	nicotinamide adenine dinucleotide
G6PD	glucose-6-phosphate dehydrogenase
NADH	nicotinamide adenine dinucleotide phosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
NEM	N-ethylmaleimide
CFDA	carboxyfluorescein diacetate
RFU	relative fluorescence unit
V	Volt
PBS	phosphate-buffered saline
ANOVA	analysis of variance
Na_2HPO4	sodium phosphate dibasic
HCl	hydrochloric acid
NaOH	sodium hydroxide
EDTA	ethylenediaminetetraacetic acid
dH_2O	distilled water

Symbols

L_p	hydraulic conductivity
P_s	osmotic permeability to solutes
V_b	osmotically inactive cell volume
E_a	Arrhenius activation energy
$P_{glycerol}$	osmotic permeability to glycerol
C	concentration
A	absorbance of the solution
M	molecular mass of hemoglobin monomer
F	dilution factor
ε	extinction coefficient
l	light path
Hb_S	supernatant hemoglobin concentration
Hb_T	total hemoglobin concentration
ΔA	(absorbance of the sample solution) – (absorbance of the blank solution)
V_{sample}	total volume of the sample
v	sample volume used in the assay
C_{met}	methemoglobin concentration
C_{oxy}	oxyhemoglobin concentration
C_{deoxy}	carboxyhemoglobin concentration
λex	excitation wavelength
λem	emission wavelength
V	equilibrium cell volume
V_0	isotonic cell volume
F	equilibrium cell autofluorescence
F_0	isotonic cell autofluorescence
m	slope
c	intercept
V_w	equilibrium volume of water in the cell
V_{w0}	isotonic volume of water in the cell

π	experimental osmolality
π_0	isotonic osmolality
b	osmotically inactive fraction of the cell
t	time
A	cell surface area
R	universal gas constant
T	absolute temperature
C_s^i	intracellular concentration of permeant solute
C_s^e	extracellular concentration of permeant solute
C_i^i	intracellular concentration of impermeant solute
C_i^e	extracellular concentration of impermeant solute
S	number of solute molecules
k	fitting constant
P_f	osmotic water permeability
SAV	vesicle surface area to volume ratio
MVW	molar volume of water
$C_{in(t=0)}$	initial intravesicular osmolality
C_{out}	extravesicular osmolality
V_{glyc}	volume of glycerol in the cell

Chapter 1

Introduction

1.1 Pathophysiology and treatment of fetal and neonatal anemias

Fetal and neonatal anemias are among the most serious complications of pregnancy and postnatal development. A common cause of fetal anemia is the immune hemolytic disease that results from blood group incompatibility between the mother and the fetus [1]. Other causes include defects in hemoglobin structure and synthesis, fetomaternal or twin-to-twin hemorrhages, and parvovirus B19 infections [2]. Intrauterine transfusion of red blood cells (RBCs) replaces RBCs of the fetus that have been destroyed or replaces fetal RBCs with RBCs negative for the antigen that causes immune response of the mother (exchange transfusion) [1]. Intrauterine transfusion is the most effective treatment currently available for fetal anemia, having both a high success rate (87.2%) and a low pregnancy loss rate (2.8%) [3]. Approximately 17 intrauterine transfusions were performed in Alberta in 2009 between the high risk obstetrical clinics in Edmonton and Calgary [4].

Neonatal anemia can either result from fetal anemia, or develop after birth as a result of hemorrhage due to obstetric complications, frequent drawing of blood for laboratory testing, or impaired RBC production by the bone marrow [2]. This type of anemia is magnified in extent and duration among premature infants and is termed the anemia of prematurity. As in the case of fetal anemia, RBC

transfusion is the most commonly used treatment of neonatal anemia [5-8]. In Edmonton, 1852 and 1462 neonatal transfusions were performed in 2008 and 2009 respectively [4].

1.2 Adult vs cord red blood cell transfusions to neonates

RBCs used in intrauterine and neonatal transfusions are derived from adult donors [1, 5-9]. However, adult RBCs are different from those present in the blood of a fetus or neonate [2, 10-14]. RBCs obtained from umbilical cord blood (cord RBCs), usually discarded during isolation of stem cells from cord blood [15-17], may be a superior alternative for intrauterine and neonatal transfusions [18, 19].

Cord RBCs are unique cells that differ from adult RBCs in membrane composition and biophysical properties [2, 11], hemoglobin (Hb) structure [2, 10-14, 20], metabolism, and enzymatic profile [10, 12]. One of the most physiologically important differences is the high concentration of fetal hemoglobin (HbF) in cord RBCs. HbF has a higher affinity to oxygen compared to adult hemoglobin (HbA) [20]. The position of hemoglobin oxygen dissociation curve as a function of age is demonstrated in Figure 1-1. Partial pressure of oxygen required for hemoglobin to be 50% saturated is called p50, and can be determined from an oxygen dissociation curve. Figure 1-1 clearly shows a gradual shift to the right of oxygen dissociation curve as an infant ages, accompanied by an increase in p50 and, therefore, a decrease in hemoglobin affinity to oxygen

[10]. The affinity of Hb to oxygen is regulated by 2,3–diphosphoglycerate (2,3-DPG). When 2,3-DPG binds to Hb chains, it causes the release of oxygen to tissues; when 2,3-DPG dissociates from Hb, it allows the binding of oxygen [10, 21]. 2,3-DPG molecules are not as strongly bound to gamma-chains in the nonoxygenated state of HbF, as they are to beta-chains of HbA, which allows HbF to bind oxygen more easily; however, release less oxygen to the tissues [20]. Additionally, the concentration of 2,3-DPG is lower in cord RBCs compared to adult RBCs [22].

HbF is ideally suited to the intrauterine environment. There are many problems associated with adult RBC transfusions to fetuses and neonates that can be resolved by replacing adult RBCs with cord RBCs. These include:

- **Improved Oxygen Delivery Dynamics**

  Due to the high concentration of HbF, fetal blood has a higher affinity to oxygen than mother's blood, which facilitates the uptake of oxygen from placenta by the fetus [13]. If RBCs of a fetus are replaced with adult RBCs during intrauterine transfusion, the latter will not be able to bind an adequate amount of oxygen from mother's blood due to their lower oxygen affinity.

  Adult RBC transfusions to premature infants have been associated with the increased incidence of retrolental fibroplasia – the abnormal growth of blood vessels in the retina that may lead to blindness [23-25]. In 1950,

Mallek and Spohn reviewed the cases of retrolental fibroplasia in three hospitals. They found that 13 of 15 cases occurred in the hospital where anaemic neonates were routinely given blood transfusions as a treatment, whereas only two cases occurred in the hospitals where blood transfusions to neonates were not a common practice [25]. In 1952, Hepner *et al.* reported the study of 20 neonates, eight of whom developed retrolental fibroplasia. Every neonate with retrolental fibroplasia received a blood transfusion; moreover, the number and volume of transfusions were significantly larger in cases of infants with retrolental fibroplasia than without one [24]. Clark *et al.* in 1981 showed that the combination of oxygen therapy and blood transfusions significantly increased the incidence of retrolental fibroplasia compared to the infants receiving oxygen therapy alone [23].

Even though the correlation between the risk of retrolental fibroplasia and blood transfusions is obvious from the aforementioned reports, the reason for this is not clearly understood. It was suggested that increased unloading of oxygen by adult RBCs upon transfusion may cause damage to immature retinal blood vessels due to oxygen toxicity [23]. This idea is strongly supported by the data of Fouron *et al.* who performed exchange transfusions in newborn lambs using adult lamb blood and observed the marked decrease in hemoglobin affinity for oxygen following transfusion [19]. Furthermore, Battaglia *et al.* reported that upon intrauterine exchange transfusions in sheep using adult sheep blood, the oxygen affinity of fetal

blood decreased and umbilical venous oxygen tension increased [18]. The risk of retrolental fibroplasia development after administration of the excessive amounts of supplemental oxygen to premature infants is currently being assessed in the Phase III clinical trial called “Canadian Oxygen Trial” [26].

Bronchopulmonary dysplasia, a chronic inflammatory lung disease that can lead to respiratory dysfunction [27], can be another possible complication of adult RBC transfusions to neonates. Bronchopulmonary dysplasia results from the damage of lung tissue caused by the oxygen toxicity and ventilation pressure during mechanical ventilation of premature newborns [27]. Recently, the direct correlation between the incidence of bronchopulmonary dysplasia and adult blood transfusions has been demonstrated [28-30]. Collard *et al.* have shown that top-up transfusions of adult blood increased the pulmonary oxidative damage in premature babies [28]; however, the mechanism for this relationship needs to be further investigated.

- **Improved RBC Production**

  Transfusions of adult RBCs to neonates may suppress the production of erythropoietin - the regulator of erythropoiesis. The level of erythropoietin is controlled by tissue oxygen tension; therefore, by increasing the oxygen level in neonatal tissues, adult RBC transfusions may suppress RBC

production and further contribute to neonatal anemia [31]. Transfusion as treatment for anemia of prematurity may actually prolong the duration of reticulocytopenia in this setting.

- **Reduction in Hemolytic Transfusion Reactions**

  Hemolytic transfusion reactions, caused by blood group incompatibility between the donor and the recipient, although very rare nowadays, are among the most severe complications of adult RBC transfusions [32]. ABO and RhD blood groups are the ones most commonly tested for; however, non-ABO/RhD blood group antigens also play a significant role in the development of hemolytic reactions [8]. RBCs from cord blood are characterized by a lower expression of blood group antigens. Particularly, cord RBCs do not express Le^a, Sd^a, Ch, Rg, or AnWj and weakly express A, B, H, I, Le^b, P_1, Lu^a, Yt^a, Vel, Do^a, Do^b, Gy^a, Hy, Jo^a, Xg^a, and Bg [8]. Therefore, *in utero* cord RBC transfusions and transfusions to neonates may reduce the risk and severity of hemolytic transfusion reactions, as well as decrease the chance of recipient immunization by minor blood group antigens.

- **Reduced Risk of Disease Transmission**

  Another adverse effect of adult RBC transfusions is the transmission of viral infections, such as human immunodeficiency virus, hepatitis B and

C, and cytomegalovirus [33-36]. As it was demonstrated that cord blood has a lower risk of viral contamination [37-40], cord RBCs blood may help to overcome this problem.

For the above stated reasons, there have been a number of safety and efficacy studies examining the use of cord RBCs to treat fetal and neonatal anemia [41-51]. These studies examining the clinical utility of autologous cord blood have demonstrated that this source of RBCs is a safe and effective alternative to adult RBCs for the treatment of anemia of prematurity [41-50]. More recent work has examined the efficacy and safety of cord RBCs isolated from cord blood [43, 47, 49]. While issues of bacterial contamination, clot formation, RBC hemolysis and low yield of RBCs have been raised as a cautionary note to the use of hypothermically stored autologous cord RBCs [50, 51], these risks could be significantly mitigated using long-term storage techniques and conventional biobanking practices.

1.3 Blood banking in transfusion medicine

1.3.1 Hypothermic storage of red blood cells

The first approach to blood preservation was storage of blood at refrigerated temperatures - so called hypothermic storage [52]. In 1914, sodium citrate blood anticoagulant was discovered. This was followed by the discovery of citrate-glucose preservative solution in 1916, that allowed storing blood for several days

after collection [53, 54]. These advances in blood preservation led to the creation of the first blood bank during World War I by Oswald Robertson. Robertson was transfusing wounded soldiers with universal group O blood stored in glass bottles with citrate-glucose solution on ice for 26 days [55]. In 1940 blood was separated into RBC and plasma components, and blood product manufacturing was initiated. Since then, preservative solutions have advanced significantly, permitting longer blood storage, however, the main principles of hypothermic blood preservation remained the same.

During hypothermic storage, RBCs undergo a number of changes, called hypothermic storage lesions. The major trigger of the sequence of hypothermic storage lesions is adenosine triphosphate (ATP) depletion. During storage, the products of RBC metabolism accumulate in the RBC bag which leads to a decrease in pH. The low pH environment slows down glycolysis and, therefore, results in a rapid drop in ATP concentration. Another significant effect of the low rate of glycolysis is the loss of 2,3-DPG. 2,3-DPG is an important modifier of hemoglobin affinity to oxygen and plays an important role in the delivery of oxygen to tissues. A low 2,3-DPG level may render RBCs incapable of normal oxygen delivery after transfusion into the patient. A low ATP concentration leads to accumulation of intracellular calcium which, in turn, triggers the loss of phospholipid asymmetry and expression of phosphatidylserine (PS) on the RBC surface. PS is normally present on the inner side of the RBC membrane and, when externalized, becomes the marker of apoptosis. As a defense mechanism, externalized PS is shed from RBCs in the form of microvesicles, the process

called microvesiculation. During microvesiculation, RBCs lose cytoskeletal and membrane proteins. Another significant consequence of ATP depletion is impairment of the normal work of sodium-potassium pumps, which disturbs the electrolyte balance and causes the loss of potassium from RBCs. Together with microvesiculation, a disturbed electrolyte balance results in a decreased RBC surface area to volume ratio, alterations in normal RBC shape (echinocytosis, spheroechinocytosis), decreased deformability and increased osmotic fragility. Other effects include increased RBC adhesiveness and aggregability. Another major trigger of RBC hypothermic storage lesions is oxidative injury. Free radicals, generated during hypothermic storage, attack hemoglobin (an oxygen-binding protein in RBC), membrane lipids and membrane proteins, such as band 3 (mediates an exchange of bicarbonate and chloride across cell membrane, therefore, facilitating exchange of carbon dioxide) and other cytoskeletal proteins. The final stage of RBC deterioration during hypothermic storage is RBC lysis, or hemolysis [52, 56-65]. More detailed information on hypothermic storage lesions of adult RBCs can be found in Table 1-1 [66].

1.3.2 Cryopreservation of red blood cells

During cryopreservation, RBCs are frozen and stored at the subzero temperatures below which biochemical reactions do not occur [67], such as -80°C and below. To achieve successful cryopreservation of RBCs, efforts typically target

avoidance of the two causes of damage, termed freezing injury. The first cause occurs if RBCs are cooled slowly. In this case, the formation of ice crystals in the extracellular space causes the osmotic efflux of water from the cell. Osmotic efflux of water leads to the increased concentration of intracellular solutes, causing potential cell damage due to solute toxicity. The other general cause of freezing injury takes place if RBCs are cooled rapidly. During rapid cooling there is not enough time for water to leave the cell, which leads to the supercooling of cell cytoplasm and the formation of ice crystals inside the cell [68].

Cryoprotective agents (CPAs) are substances that protect biological systems from injury during freezing and, therefore, are commonly used in RBC cryopreservation. Based on the mechanism of their protective action, there are two main classes of CPAs: permeating and non-permeating. Permeating CPAs protect cells from slow freezing injury. They penetrate the cell and act as an additional intracellular solvent, reducing concentration of other intracellular solutes during freezing when intracellular water turns into ice. Examples of permeating CPAs are dimethyl sulfoxide (Me_2SO), glycerol, ethylene and propylene glycols. Non-permeating CPAs protect cells from rapid freezing injury. Not being able to penetrate through the cell membrane, they act by dehydrating cells and, therefore, reducing the amount of water available for intracellular ice formation. Among non-permeating CPAs are sugars, polymers, and starches (hydroxyethyl starch (HES), polyvinylpyrrolidone (PVP)) [52, 69, 70].

In 1940, Dr. Charles Drew in New York was the first person to show that human RBCs, isolated from whole blood, can be frozen and later reconstituted

[53, 54]. In 1950, soon after Polge and Smith discovered the protective effect of glycerol during freezing of spermatozoa [71], glycerol was used for cryopreservation of RBCs [72]. To enable the removal of CPA from RBCs after thawing, Huggins developed the "cytoagglomeration" method in 1963. This method is based on the reversible binding of γ-globulins in blood plasma to lipoproteins on RBC membranes at acidic pH. Lowering the ionic strength of the solution renders γ-globulins precipitated together with RBCs. The supernatant is discarded and the whole procedure is repeated several times to fully remove the CPA. RBCs can be resuspended by increasing the ionic strength or pH of the solution [73].

The two most common methods used nowadays for the clinical cryopreservation of RBCs are high glycerol – slow cooling (the standard method in US and Canada) and low glycerol – rapid cooling (the standard method in Europe) [52]. High glycerol – slow cooling method involves adding the CPA glycerol to RBCs to a final concentration of about 40% (w/v), cooling at 1ºC/min to -80ºC (storage temperature), rapid thawing in a 37ºC water bath, and removing glycerol by a series of washes with sodium chloride solutions of decreasing concentrations [74]. Deglycerolized RBCs are stored in isotonic saline with 0.2% glucose at 4ºC and must be transfused within 24 hours [75]. After the high glycerol-slow cooling method, the average RBC freeze-thaw-wash recovery (the percentage of RBCs left in the sample after deglycerolization) was 85.2 ± 5.9% (SD) and the average 24 hour post-transfusion survival (the percentage of transfused RBCs present in recipient's circulation 24 hours after transfusion) was

83%, as reported by Valeri [76]. Based on Quality Monitoring program (QMP) data, acquired by Dr. Jason Acker's laboratory, Canadian Blood Services, Edmonton Center, freeze-thaw-wash recovery of RBCs was 84 ± 5% (SD) [77]. With the introduction of ACP-215 Automated Cell Processor, an automated functionally closed instrument for addition and removal of glycerol from RBCs, it became possible to maintain the acceptable quality of RBCs for up to 15 days after deglycerolization when stored in Additive Solution 3 (AS-3) at 4ºC. After 15 days of storage, RBCs processed in ACP-215 showed the mean 24 hour post-transfusion survival of 77 ± 9% (SD) and hemolysis (the release of free hemoglobin from RBCs as a result of membrane damage) of 0.6 ± 0.2% (SD) [76].

For the low glycerol – rapid cooling method, RBCs are mixed with an equal volume of the solution, containing 28% glycerol, 3% mannitol and 0.65% NaCl, to achieve the final glycerol concentration of 14% (v/v) [78]. The RBC-glycerol mixture is then frozen by immersion into liquid nitrogen (-196ºC), where RBCs are stored. Thawing is performed in a warm water bath (42-45ºC) and glycerol is removed by one wash with 16% mannitol in 0.9% NaCl and two washes with isotonic saline. After the low glycerol-rapid cooling method, the average RBC freeze-thaw-wash recovery was 95.9% and the average 24 hour post-transfusion survival was 96 ± 1.5% (SD) [78].

An alternative method for RBC cryopreservation is the one using non-permeating CPA HES. In this method, RBCs are mixed with HES (molecular weight 200,000 Da, degree of substitution 0.5) to a final HES concentration of

11.5% (w/w), frozen by immersion into liquid nitrogen, and stored at a temperature less than -130ºC. After thawing in a warm water bath, HES is either left in RBC suspensions, or removed by a single wash with isotonic saline [79]. Thomas reported the average recovery of RBCs cryopreserved with HES to be 98.8 ± 0.3% (SD) [80]. The average 30 minute stability of RBCs in isotonic saline, that closely imitates 24 hour post-transfusion survival, ranged from 86.3% to 93% [80-83].

Cryopreservation of RBCs allows storage for at least 10 years [84]. However, being a technically demanding and labor-intensive process [52], RBC cryopreservation is limited to rare blood groups, people with adverse antibody problems, as well as civil emergency and military applications [75, 85].

1.4 Cryopreservation as the method of choice for cord red blood cell storage

1.4.1 Why cryopreserve cord red blood cells?

Adult RBCs can be successfully stored at 1-6ºC in an anticoagulant/preservative solution (e.g. citrate-phosphate-dextrose/saline-adenine-glucose-mannitol (CPD/SAGM)) for 42 days [52]. In contrast, cord RBCs have low hypothermic storage stability [41, 86-88]. It was reported that hemolysis of cord RBCs reached 0.9% after only 14 days of storage [41] in CPD/SAGM, and was also significantly higher compared to adult RBCs after 35 days of storage [87, 88]. Such a high level of hemolysis exceeds the current acceptance criteria for RBCs used in

transfusion (hemolysis of less than 0.8%) [89]. Compared with adult RBCs, cord RBCs also have a higher level of extracellular potassium [41], lower adenosine triphosphate [87] and lower pH [86-88] after 35 days of hypothermic storage. Hypothermic storage lesions of cord RBCs reported in these studies, as well as conditions of cord RBC storage, are described in more details in Table 1-2.

Reports describing the effect of cryopreservation on cord RBCs are currently very limited. In her doctoral thesis, Bobrova investigated the effect of 1,2-propanediol, glycerol, and Me_2SO on phase transitions in placenta, fetal erythrocytes and extracellular space at subzero temperatures, with particular focus on vitrification and ice recrystallization upon thawing [90]. In this study, fetal RBCs were frozen with different concentrations of CPAs by immersion in liquid nitrogen (cooling rate approximately 200 °C/min), and subsequently thawed by immersion into a 40°C water bath. The best preservation of erythrocytes was achieved with 20-30% (w/v) 1,2-propanediol or glycerol, and 10-20% (w/v) Me_2SO. Bobrova also reported that high concentrations of CPAs cause changes in cord RBC morphology. In particular, 30-40% (w/v) glycerol cause swelling of erythrocytes and formation of stomatocytes, whereas 1,2-propanediol (30-40% (w/v)) and Me_2SO (any concentration) cause crenation of cord RBCs. Optimum equilibration temperatures were 20°C for glycerol and 0°C for 1,2-propanediol and Me_2SO. Finally, high concentrations of CPAs (glycerol and 1,2-propanediol > 40% (w/v) and Me_2SO > 30% (w/v)) resulted in damage of barrier functions of cord RBC membrane.

Another thesis by Zubov describes the effects of cryopreservation with extracellular CPA PEO-1500 (polyethylene oxide with molecular weight 1500 Da) on cytoskeleton and lipid asymmetry of cord RBC membranes [91]. Erythrocytes from cord blood were equilibrated with 15% PEO-1500 at 0-4°C or 37°C, frozen by immersion into liquid nitrogen, and subsequently thawed in a 40-42°C water bath. It was found that incubation with PEO-1500 strengthens interactions between cytoskeleton and cytoplasmic membranes through ankyrin, band 4.1 and band 4.2, as well as interactions between actin filaments within the cytoskeleton through band 4.9. Cryopreservation caused changes in the content of spectrin, ankyrin, band 3, bands 4.1, 4.2, and 4.9, however these changes were reversible upon return of RBCs to physiological conditions. Cryopreservation did not alter the expression of Glycophorin A on RBC membranes. Incubation with PEO-1500 at 0-4°C resulted in better preservation of the cytoskeletal proteins upon thawing, as well as lower percentage of phosphatidylserine expression on cord RBC membranes, which correlated with decreased hemolysis of cord RBCs after thawing.

Although these studies provide valuable information on the effect of four CPAs on cord RBC membrane, more detailed investigation and further optimization of freezing and thawing parameters (cooling and warming rate, final storage temperature, etc.) is required. Therefore, an effective cryopreservation method for cord RBCs still needs to be developed. Cryopreservation may prevent the deterioration of cord RBCs seen during liquid storage and maintain a high quality of cord RBC units for use in intrauterine transfusions. Also, for the small

volume top up transfusions in preterm and neonatal patients [92], it would be possible to cryopreserve cord RBCs from a single donor in small aliquots and, by thawing one at a time, perform multiple transfusions from a single source. This would limit the exposure of the neonate to multiple donors and the concomitant adverse effects.

1.4.2 Cell membrane as an important mediator of cell response to low temperatures

The cell membrane is an important mediator of cellular response to cryopreservation [68, 93-95], however the involvement of different membrane components in this process is poorly understood. The cell membrane regulates osmotically induced changes of the cell volume during freezing by governing the transport of water and solutes between the cytoplasm and the extracellular milieu [93]. Osmosis is the process of solvent transfer from solution of lower concentration of solute to solution of higher concentration of solute, when two solutions are separated by a semi-permeable membrane which allows for the transport of solvent, but blocks the transport of solute [96]. As an example, when ice forms outside the cell, the concentration of extracellular solutes increases. For cells with low membrane permeability to water osmotic efflux of water from the cell following extracellular ice formation is slow and, hence, intracellular water

content is high. High water content increases the probability of intracellular freezing during subsequent cooling, resulting in low post-thaw survival [97].

A number of osmotic parameters regulate the movement of water and solutes through the cell membrane and determine osmotic changes of the cell during cryopreservation. Hydraulic conductivity (L_p) and osmotic permeability to solutes (P_s) describe the rate at which these substances cross the cell membrane and are typically determined by measuring the rate of cell volume changes when placed in an anisotonic environment. The osmotically-inactive cell volume (V_b) is the volume of the cell that does not participate in osmotic behaviour of the cell. Arrhenius activation energy (E_a) describes the temperature dependence of the membrane permeability to water and solutes [98].

The osmotic parameters listed above can be used in mathematical modelling to predict optimized cryopreservation protocol. *CryoSim6* software was developed by Dr. Locksley McGann at the University of Alberta, Canada, and uses the phase diagrams of extracellular and intracellular solutions, osmotic properties of the cell membrane and their temperature dependencies to estimate changes in cell volume during cryopreservation. This information, in turn, is used to predict the probability of cell injury due to increased concentration of intra- and extracellular solutes and intracellular ice formation [99-101]. Using the approach of Karlsson et al., it is possible to mathematically predict the procedure for addition and removal of CPA, that will prevent excessive cell swelling or shrinking, as well as reduce CPA toxicity by minimizing the time of exposure to CPA [102]. Toxicity during CPA addition and removal can also be

mathematically modelled and minimized by using the toxicity cost function, the approach recently reported by Benson et al. [103]. Using these mathematical predictions, it is possible to derive the optimized cryopreservation parameters for a specific type of cells, such as type and concentration of CPA, CPA addition and removal procedure, cooling and warming rates, etc., which can later be empirically verified. Such simulations will greatly reduce the amount of time and expenses compared to empirical determination of cryopreservation parameters.

Cord RBCs differ from adult RBCs in their membrane composition and properties. Particularly relevant is the difference in membrane permeability to water and solutes. It has been reported, that, compared to adult RBCs, cord RBCs are approximately 1.3-1.4 times less permeable to water [104], have higher activation energy for water permeability [104], and are less permeable to some solutes, in particular glycerol [105]. However, literature values of cord RBC permeability are both limited and, being obtained by different experimental methods, controversial. Based on these observations, I propose the following hypothesis.

1.5 Hypothesis

Since the osmotic properties of RBCs, such as membrane permeability to water and cryoprotectant and osmotic tolerance limits, determine cell recovery after cryopreservation, differences in osmotic properties of cord and adult RBCs will

result in poorer recovery of cord RBCs after the conventional cryopreservation procedure for adult RBCs.

1.6 Objectives and approach

The research objectives of proposed project are the following:

1. **Examine the effect of pre-freeze storage conditions on the quality of cord RBCs**

   It is known that the temperature [106] and duration of pre-freeze storage [107] affect the quality of RBCs. Since cord RBCs are currently treated as a waste product post processing of collected cord blood for stem cell extraction, there is no incentive to monitor or preserve their quality. After cord blood is collected, it can be stored ideally at room temperature for up to 48 hours before being processed for stem cell extraction [108] and in some cases even longer. Presently the effects of pre-processing storage on the quality of cord RBCs is unknown. If cord RBCs are to be cryopreserved for clinical use, it is critical to ensure that a high quality of cord RBC product remains, following isolation from cord blood. Using conventional and novel methods for assessing RBC quality, I will examine if and how the quality of cord RBCs is affected by the duration of cord blood storage at room temperature and other cord blood characteristics. This information will allow to better control and possibly improve the

hypothermic storage conditions of cord blood prior to cord RBC isolation, and provide a more consistent product for studies on cord RBC cryopreservation.

2. Develop a new method to measure permeability of RBCs to water and solutes based on hemoglobin autofluorescence

To design effective cryopreservation procedures for cells and tissues and avoid freezing injury, it is critical to know cell osmotic parameters, such as permeability of cell membrane to water and solutes [109-112]. To determine cell membrane permeability, one needs to measure the rate of cell volume changes when placed in an anisotonic environment [113]. RBCs respond very quickly to changes in extracellular solute concentrations [104]. Therefore, it is difficult to measure the rate of RBC volume change with traditional methods, such as using an electronic particle counter [114]. Stopped-flow spectroscopy can quantify rapid changes of RBC volume and is commonly used to measure RBC osmotic permeability [104, 115]. Two stopped-flow approaches are used in this regard (light scattering [104] and self-quenching of fluorescent dye [115]), but both have limitations. Therefore, development of an improved method to determine cord RBC permeability is warranted.

I will develop a method to capture rapid changes in RBC volume in response to exposure of cells to an anisotonic environment by measuring changes in intrinsic hemoglobin fluorescence intensity. This

method will not require addition of any fluorescent dye and will potentially overcome limitations of the traditionally used methods.

3. Determine the osmotic parameters for cord RBCs and adult RBCs

Knowledge of osmotic parameters of cord RBCs is required to design effective cryopreservation methods for these cells. However, literature data on cord RBC permeability to water and glycerol are both limited and controversial [104, 116]. This necessitates experimental verification of these data in order to establish the difference between cord RBC and adult RBC membranes and a baseline for the cord RBC membrane characteristics. The following osmotic parameters will be determined for cord RBCs and adult RBCs:

- hydraulic conductivity (L_p) and osmotic permeability to glycerol ($P_{glycerol}$)

- activation energy to describe the temperature dependence of water and glycerol permeabilities (E_a)

- osmotically inactive cell volume (V_b)

- osmotic tolerance limits

I will use the method developed in Research Aim # 2 to measure L_p, $P_{glycerol}$, and E_a, for cord RBCs and adult RBCs. In addition, I will

determine osmotic tolerance limits for fresh and frozen RBCs in the absence and in the presence of glycerol.

4. **Examine the recovery of cord RBCs cryopreserved using the standard high glycerol – slow cooling method**

   Osmotic parameters of the cell membrane are major determinants of cell response to freezing and thawing, as well as addition and removal of cryoprotectant. If these differences are confirmed in Research Objective 3, it is reasonable to assume that recoveries of cord RBCs and adult RBCs after conventional high glycerol – slow cooling cryopreservation method will be different. Comparison of post-thaw recoveries of cord RBCs and adult RBCs is required to test my original hypothesis.

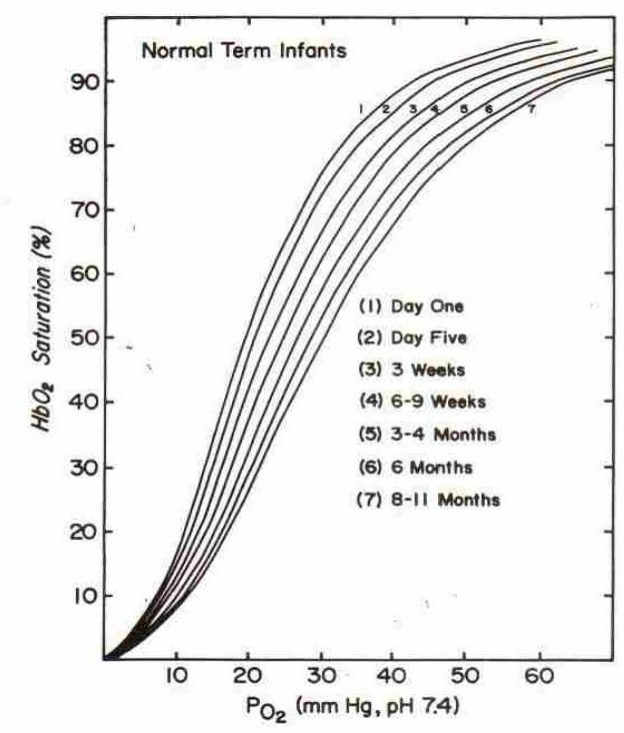

Figure 1-1. Oxygen dissociation curves of human hemoglobin from term infants of different age groups. Figure is adopted from *Oski FA. The unique fetal red cell and its function. E. Mead Johnson Award Address. Pediatrics. 1973 Mar;51(3):494-500* [10].

Table 1-1. Canadian Blood Services Quality Monitoring Program data on quality measures of adult RBCs during 42 days of hypothermic storage.

Quality parameter	**Fresh (5 days)**	**Expiry (42 days)**
ATP, µmol/g Hb	3.83 ± 0.607	1.66 ± 0.430
2,3-DPG, µmol/g Hb	5.31 ± 3.19	0.558 ± 1.02
MetHb, %	3.47 ± 1.51	3.71 ± 1.53
Hemolysis (Drabkin's), %	0.12 ± 0.05	0.33 ± 0.17
Supernatant K^+, mmol/L	12 ± 3	47 ± 5
Supernatant Na^+, mmol/L	142 ± 5	119 ± 5
p50, mmHg	23.15 ± 2.310	17.87 ± 5.000
WBC count, 10^9/L	0.4 ± 1.7	0.3 ± 1.0
RBC count, 10^{12}/L	6.33 ± 0.43	6.25 ± 0.45
Hemoglobin (Coulter), g/L	189 ± 14.0	183 ± 12.8
Hct (Coulter), %	58.6 ± 3.31	60.1 ± 4.03
MCV, fL	92.8 ± 4.90	96.3 ± 5.30
MCH, pg	29.9 ± 2.10	29.3 ± 1.90
MCHC, g/L	322 ± 14.9	305 ± 9.60
Platelet count, 10^{12}/L	11 ± 53	7 ± 25
RBC morphology index	70.5 ± 10.2	42.8 ± 4.8
EI_{max}	0.599 ± 0.034	0.573 ± 0.043
K_{EI}	2.65 ± 0.79	2.54 ± 0.77
#MPs/µL	65820 ± 35476	188206 ± 72815
PS^+ MPs, %	39.7 ± 13.5	38.2 ± 9.2
MFI PS on MPs	67 ± 22	54 ± 14
$CD47^+$ MPs, %	40.2 ± 13.8	50.1 ± 9.9
MFI CD47 on MPs	207 ± 22	199 ± 14
PS^+ RBCs, %	2.5 ± 0.6	3.2 ± 1.2
MFI PS on RBCs	34 ± 6	39 ± 8
$CD47^+$ RBCs, %	98.1 ± 2.8	99.2 ± 1.1
MFI CD47 on RBCs	184 ± 29	209 ± 34

Data from QMP testing 2012, performed in Canadian Blood Services, Edmonton Center, Dr. Jason Acker's laboratory [66]. RBC units were stored at 1-6°C for 42 days. CPD, CP2D, or ACD-A was used as an anticoagulant. SAGM, AS-1, or AS-3 was used as a preservative. Data is reported as mean ± SD.

Table 1-1 (continued)

CPD – citrate-phosphate-dextrose , CP2D – citrate-phosphate-double dextrose, ACD-A – anticoagulant citrate dextrose solution-formula A, SAGM – saline-adenine-glucose-mannitol, AS-1 – Additive Solution 1, AS-3 – Additive Solution 3, ATP – adenosine triphosphate, 2,3-DPG – 2,3–diphosphoglycerate, MetHb – methemoglobin, K^+ – potassium, Na^+ – sodium, p50 – partial pressure of oxygen required for hemoglobin to be 50% saturated, WBC – white blood cells, RBC – red blood cell, Hct – hematocrit, MCV – microcorpuscular volume, MCH – microcorpuscular hemoglobin, MCHC – microcorpuscular hemoglobin concentration, EI_{max} – maximum theoretical elongation index, K_{EI} – the shear stress required to achieve half of the EI_{max}, MP – microparticle, PS – phosphatidylserine, CD47 – cluster of differentiation 47, "marker of self" on RBCs, MFI – mean fluorescence intensity.

Table 1-2. Literature data on hypothermic storage lesions of fetal and adult RBCs.

Measured parameters	Duration of storage (days)								Source
	Fresh		14		21		35		
	Fetal RBC	Adult RBC	Fetal RBC	Adult RBC	Fetal RBC	Adult RBC	Fetal RBC	Adult RBC	
RBCs (10^6/µL)	4.6±0.9* 5.2±0.6*	6.6±0.3			 5.4±0.48 5.6±0.49		4.7±0.9* 5.1±0.5* 5.5±0.5* 5.6±0.45*	 6.7±0.3 6.5±0.51	*Garritsen 2003, Brune 2007 (NLD)* *Brune 2007 (LD)* *Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)*
MCV (fL)	114.3±6.5 106.3±2.8*	85.6±4.8			 117.9±6.67 113.8±6.39		117±8.6 111.1±3.3* 122.1±7.6* 116.1±6.9*	 96.4±7.1 90.5±3.87	*Brune 2007 (NLD)* *Brune 2007 (LD)* *Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)*
Hct (%)	52.6±10.0 56.0±5.6	56.5±1.3			 0.64±0.05 0.64±0.06		55.2±11.1 57.1±4.8* 0.67±0.05* 0.65±0.06*	 63.5±1.4 0.58±0.03	*Garritsen 2003, Brune 2007 (NLD)* *Brune 2007 (LD)* *Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)*
WBC count (per µL)	6300±2600* 0.5±0.4*	2.1±0.9			 4500±2270 4500±1740		4200±200* 0.0±0.0 3900±2160* 3700±1510*	 0.0±0.0 <0.001	*Garritsen 2003, Brune 2007(NLD)* *Brune 2007 (LD)* *Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)*
Thrombocyte count (per µL)	62685±24100* 958±101*	3568±1193			 94000±40700 96000±44600		54666±11785* 0.0±0.0 83300±37700* 96000±43900*	 0.0±0.0 <0.015	*Garritsen 2003, Brune 2007(NLD)* *Brune 2007 (LD)* *Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)*
pH	6.4±0.1* 6.7±0.02*	7.6±0.2			 6.44±0.13 6.50±0.96		6.1±0.1* 6.4±0.0* 6.23±0.16* 6.32±006*	 6.8±0.1 6.56±0.14	*Garritsen 2003, Brune 2007 (NLD)* *Brune 2007 (LD)* *Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)*
ATP (µmol/gHb)	3.7±0.9	3.3±0.4					1.2±0.5*	2.3±0.4	*Garritsen 2003*

Table 1-2 (continued)

Hemolysis (%)	0.0±0.0 0.05±0.03 0.01±0.05	0.0±0.0 0.004±0.002	0.9	0.1	 1.35±0.64 1.43±0.91		1.1±0.8* 1.1±0.7 1.0±0.7 2.2±0.67* 1.8±1*	0.2±0.1 0.2±0.1 0.7±0.14	*Eichler 2000* *Garritsen 2003* *Brune 2007 (NLD)* *Brune 2007 (LD)* *Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)*
K^+ (mmol/L)			22.7	15.8	 52.4±10.1 53.7±10.8		 69.3±11.3* 72.2±12.4*	 45.2±3.0	*Eichler 2000* *Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)*
Na^+ (mmol/L)					120.7±12.9 140.6±11.9		106.6±12.8 122.1±12.1	113.8±18	*Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)*
Total Hb (g/dL) Total Hb (mmol/L)	17.1±3.5 10.4±2.1 11.8±1.1	18.9±0.4 11.5±0.2					17.1±3.3 10.4±0.2 11.8±1.1	19.3±0.5 11.7±0.4	*Garritsen 2003* *Brune 2007 (NLD)* *Brune 2007 (LD)*
Free Hb (mg/dL) Free Hb (mmol/L)	19.4±19.2 0.012±0.012 0.030±0.010*	14.5±5.4 0.001±0.000			 630±310 820±690		416.9±254.5* 1050±400* 1320±1200* 0.256±0.152 0.280±0.017*	82.8±42.4 130±30 0.051±0.025	*Garritsen 2003* *Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)* *Brune 2007 (NLD)* *Brune 2007 (LD)*
Osmotic resistance (% NaCl for 50% lysis)					0.51 0.49		0.53 0.49	0.52	*Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)*
Glucose (mmol/L)					19.3±6.3 19.9±10.5		16.7±10.7 20.1±10.9	22.1±6.3	*Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)*
Lactate (mmol/L)					3.67±0.71 3.76±0.77		4.2±0.8* 4.2±1.1	3.45±0.31	*Khodabux 2011 (SAGM)* *Khodabux 2011 (AS-3)*

*p<0.05 vs adult

Table 1-2 (continued)

Details of the studies:

Eichler 2000 [41]: fetal RBCs were collected from premature and mature neonates, non-leukodepleted, stored in CPD/SAGM.

Garritsen 2003 [87]: fetal RBCs were collected from premature and mature neonates, non-leukodepleted, stored in CPD/SAGM at 2-6 °C. Adult RBCs were leukodepleted and stored under the same conditions as fetal RBCs. N=390 fetal RBC units, n=12 adult RBC units.

Brune 2007 [86]: fetal RBCs were collected from mature neonates, either leukodepleted or non-leukodepleted, stored in CPD/SAGM at 4 °C. Adult RBCs were leukodepleted and stored under the same conditions as fetal RBCs. N=390 fetal RBC units, n=12 adult RBC units.

Khodabux 2011 [88]: fetal RBCs were collected from premature neonates, non-leukodepleted, stored in CPDA-1/SAGM or CPDA-1/AS-3 at 2-6 °C. Adult RBCs were leukodepleted and stored in CPDA-1/SAGM at 2-6 °C. Data is presented as mean ± SD. N=34 fetal RBC units in SAGM, n=13 fetal RBC units in AS-3, n=10 adult RBC units.

Abbreviations used: RBC – red blood cell, MCV – microcorpuscular volume, Hct – hematocrit, WBC – white blood cell, ATP – adenosine triphosphate, K^+ – potassium, Na^+ – sodium, Hb – hemoglobin, NaCl – sodium chloride, CPD – citrate-phosphate-dextrose, CPDA-1 – citrate-phosphate-dextrose-adenine-1, SAGM – saline-adenine-glucose-mannitol, LD – leukodepleted, NLD – non-leukodepleted, AS-3 – additive solution-3.

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Chapter 2

Quality of Red Blood Cells Isolated from Room Temperature – Stored Umbilical Cord Blood[*].

2.1 Introduction

Fetal and neonatal anemias are among the most serious complications of pregnancy and postnatal development. The causes of fetal anemia include immune haemolytic disease [1], defects in haemoglobin structure and synthesis, fetomaternal or twin-to-twin hemorrhages, and parvovirus B19 infections [2]. Neonatal anemia, on the other hand, can either result from fetal anemia, or develop after birth as a result of hemorrhage due to obstetric accidents, frequent drawing of blood for laboratory testing, or impaired red blood cell (RBC) production by bone marrow [2]. The most commonly used treatments for fetal and neonatal anemia are transfusions of red blood cells (RBCs), either intrauterine [1] or intravenous [3-6], to help replace the lost RBCs of the fetus or neonate.

RBCs used in intrauterine and neonatal (intravenous) transfusions are derived from adult donors [1, 3-7]. Adult RBCs are different from those present in the blood of a fetus or neonate [2, 8-12]. Neonatal RBCs obtained from umbilical cord blood (cord RBCs) are usually discarded during isolation of stem cells from

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cord blood [13-15]. This waste product may offer a superior alternative for intrauterine and neonatal transfusions [16, 17].

Cord RBCs are unique cells that differ from adult RBCs in membrane composition and biophysical properties [2, 9], haemoglobin (Hb) structure [2, 8-12, 18], metabolism, and enzymatic profile [8, 10]. One of the most important physiological differences is the high concentration of fetal hemoglobin (HbF) in cord RBCs. This is practically absent in adult RBCs (normal adult HbF is <1%) [18]. HbF has a higher affinity for oxygen compared to adult hemoglobin (HbA). This allows HbF to bind oxygen more easily, with a left shift of the oxygen dissociation curve and the release of less oxygen to the tissues [18].

There are many problems associated with adult RBC transfusions to fetuses and neonates that can be resolved by replacing adult RBCs with cord RBCs. Due to the high concentration of HbF, fetal blood has higher affinity for oxygen than the mother's blood, which facilitates the uptake of oxygen from the placenta by the fetus [11]. The practice of administering adult RBC transfusions to premature infants has been associated with the increased incidence of retrolental fibroplasia – the abnormal growth of blood vessels in the retina that may lead to blindness [19-21]. Another possible complication of adult RBC transfusions to neonates is bronchopulmonary dysplasia, a chronic inflammatory lung disease that can lead to respiratory dysfunction [22]. Several researchers have demonstrated a direct correlation between the incidence of bronchopulmonary dysplasia and adult blood transfusions [23-25].

The practice of transfusions of RBCs derived from umbilical cord blood to treat neonatal anemia has recently gained a lot of interest [26-35]. A number of studies have demonstrated that transfusions of autologous cord RBCs are both safe and effective in the treatment of anemic neonates [26-32]. Some, however, have expressed concerns with cord blood transfusions, including the potential high risk of bacterial contamination, low hypothermic storage stability, and small volume of umbilical cord blood collections [28, 33]. These concerns, however, can be overcome through use of novel or superior long-term storage techniques for cord RBCs.

Adult RBCs can be successfully stored at 1-6ºC in an anticoagulant/preservative solution (e.g. citrate-phosphate-dextrose/saline-adenine-glucose-mannitol (CPD/SAGM)) for 42 days [36]. In contrast, cord RBCs deteriorate much faster under the same conditions and cannot be stored for more than 14 days without significant decrease in quality [28, 37]. Cryopreservation and subsequent storage at ultra-low temperatures may preserve cord RBCs and maintain a high quality of cord RBCs for use in intrauterine and neonatal transfusions. Despite many studies having documented the successful cryopreservation of adult RBCs, no protocol for cryopreservation of cord RBCs has been developed.

Since cord RBCs are currently treated as a waste product post processing of collected cord blood for stem cell extraction, there is no incentive to monitor or preserve their quality. After cord blood is collected, it can be stored ideally at room temperature for up to 48 hours before being processed for stem cell

extraction [38]. Longer pre-processing room temperature storage for stem cell extraction is permissible when there is strict monitoring of cell viability, CD 34+ cell content and viability and colony forming assay potential. Presently the effects of pre-processing storage on the quality of cord RBCs are unknown.

A number of conventional methods exist for assessing RBC quality. RBC hemolysis is used as an indicator of RBC membrane damage that results in the release of free Hb into extracellular space. A decrease in adenosine triphosphate (ATP), the energy substrate of RBCs, has been observed as one of the markers of RBC aging during hypothermic storage [39]. The concentration of 2,3–diphosphoglycerate (2,3-DPG) in RBCs is another important quality parameter, since 2,3-DPG regulates oxygen exchange between Hb and tissues by mediating the binding of oxygen to Hb, as well as the release of oxygen into tissues [8, 40]. Finally, the concentration of methemoglobin (metHb) acts as an indicator of RBC oxidative injury.

In addition, novel predictors of RBC *in vitro* quality, such as RBC deformability, microvesiculation, and extracellular expression of phosphatidylserine (PS) and cluster of differentiation 47 (CD47), have been recently incorporated into RBC quality assessments [41, 42]. The deformability of RBCs enables their passage through small blood capillaries and is therefore a critical property for maintaining normal blood flow [43]. Microvesiculation is the process of generating microparticles - phospholipid vesicles 0.04 - 1.5 μm in diameter - by eukaryotic cells as a result of different types of cell stimulation [44]. Notably, microvesiculation has been shown to

increase during hypothermic storage of adult RBCs [45]. Glycophorin A (CD235a) is a glycoprotein abundantly present on the RBC membrane [46], and its expression on microparticles is used as a marker of RBC origin [44]. PS is a membrane phospholipid that is normally found within the inner leaflet of the plasma membrane, but during apoptosis, it is translocated to the outer leaflet. Annexin V is a phospholipid-binding protein with a high affinity for PS and is used to determine the percentage of cells within a population that are actively undergoing apoptosis. It has been shown that PS is exposed on the surface of RBCs during hypothermic storage [47]. CD47 is an erythrocyte surface antigen that has been shown to be a "marker of self". RBCs lacking this antigen on their surface are rapidly cleared from the circulation by macrophages in the spleen [48]. It has been demonstrated that the expression of CD47 on RBCs decreases during storage and may be an important determinant of post-transfusion in-vivo survival [49].

If cord RBCs are to be cryopreserved for clinical use, it is critical to ensure that a high quality of cord RBC product remains after isolation from cord blood. The objective of the present study was to examine if and how the quality of cord RBCs is affected by the duration of cord blood storage at room temperature and other cord blood characteristics.

2.2 Methods

Cord RBC collection

Cord RBCs were obtained from the Alberta Cord Blood Bank (Suite 408 College Plaza, 8215-112 Street, Edmonton, AB T6G 2C8, Canada), as a waste product, after stem cell isolation from umbilical cord blood. Women with healthy, full-term pregnancies who met the Alberta Cord Blood Bank criteria for inclusion and gave informed consent were eligible to donate cord blood. Cord blood collections were performed by a trained physician or midwife attending the delivery, from either the undelivered or delivered placenta. The umbilical vein was punctured and cord blood was collected by gravity into a blood collection bag (Fenwal, Inc., Lake Zurich, IL, USA) containing 35 mL of CPD anticoagulant. After collection, whole umbilical cord blood was stored at room temperature and processed at the Alberta Cord Blood Bank facility according to a previously described double collection procedure [50]. Ethics approval for the study was obtained from the University of Alberta Health Research Ethics Board (Biomedical Panel).

Cord blood characteristics

Upon receipt of a packed cord RBC unit, a number of cord blood characteristics were documented from the Alberta Cord Blood Bank Collection Form. The time

between cord blood collection and the start of processing that cord blood spent at room temperature was calculated (age of cord blood). The baby's gender and weight, the cord blood collection method (*in utero* or delivered placenta), and cord blood hematocrit were recorded. The ratio of cord blood volume to anticoagulant volume (cord blood/CPD) was calculated by dividing the reported volume of cord blood in mL (w/o anticoagulant) by 35 mL (volume of CPD in blood bag). Additionally, the percentage of HbF in cord blood was determined using the standard Kleihauer-Betke kit (Sure Tech Diagnostic, Associates, Inc., St. Louis, MO, USA).

Assessment of cord RBC quality

Standard indicators of RBC quality

Cord RBCs were tested immediately after their isolation from cord blood for conventional indicators of RBC quality.

Percent hemolysis

RBC hemolysis, which is the release of free hemoglobin from RBCs as a result of membrane damage, was determined by spectrophotometric measurement of cyanmethemoglobin according to Drabkin's method [51]. Drabkin's method is considered to be a reference method for hemoglobin determination. In this

method, packed RBC sample and supernatant, obtained after spinning down packed RBC sample in Eppendorf 5415 R microcentrifuge, were mixed with Drabkin's reagent to determine total and supernatant hemoglobin concentrations, respectively. Drabkin's reagent converts most forms of hemoglobin into cyanmethemoglobin (HiCN). During this reaction, hemoglobin iron is oxidized from Fe^{II} to Fe^{III} (methemoglobin), and methemoglobin then reacts with cyanide, forming HiCN. HiCN's absorbance was measured on spectrophotometer SPECTRA max PLUS 384 microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) at 540 nm. Since HiCN's absorbance is directly proportional to hemoglobin concentration, hemoglobin concentration was calculated using SoftMax Pro software (Molecular Devices Corporation, Sunnyvale, CA, USA) according to the equation below:

$$C = \frac{A_{540} \times M \times F}{\varepsilon_{540} \times l \times 1000} \qquad \text{(Eq. 2-1)}$$

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

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

l = light path (cm) [52]

Hematocrit of RBC sample was measured using the microhematocrit centrifuge (Hettich, Tuttlingen, Germany) as the ratio of the volume occupied by packed RBCs to the volume of a whole RBC sample [46]. After total and supernatant hemoglobin concentrations were determined, percent hemolysis was calculated as follows:

$$Hemolysis\ (\%) = \frac{(100 - Hct) \times Hb_S}{Hb_T} \quad \text{(Eq. 2-2)}$$

where Hct = hematocrit (%)

Hb_S = supernatant hemoglobin concentration (g/L)

Hb_T = total hemoglobin concentration (g/L)

Controls for total hemoglobin were prepared from Stanbio Tri-Level Hemoglobin controls (Stanbio Laboratory, Boerne, TX, USA).

ATP concentration

ATP concentration of RBCs was determined using the commercial ATP Hexokinase FS kit (DiaSys Diagnostic Systems GmbH). 10% trichloroacetic acid was added to RBC suspension to cause protein precipitation. After that,

suspension was centrifuged to obtain supernatant, used subsequently for ATP measurement. ATP from RBCs is used in the following two chemical reactions:

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

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

where *HK*-hexokinase

ADP - adenosine diphosphate

NAD^{+} - nicotinamide adenine dinucleotide

G6PD - glucose-6-phosphate dehydrogenase

NADH - nicotinamide adenine dinucleotide phosphate

All reactants and enzymes are present in the reagents from the kit, except for ATP, which is provided by RBCs. NADH absorbance was measured on SPECTRA max PLUS 384 microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) at 340 nm. Since the amount of NADH produced is directly proportional to the amount of ATP in RBC sample, ATP concentration was calculated using SoftMax Pro software (Molecular Devices Corporation, Sunnyvale, CA, USA) according to the equation below:

$$ATP\ \left(\frac{\mu mol}{dL}\right) = \frac{\Delta A \times V_{sample} \times F \times 100}{\varepsilon_{340} \times v \times l} \quad \text{(Eq. 2-3)}$$

where ΔA = (absorbance of the sample solution) – (absorbance of the blank solution)

V_{sample} = total volume of the sample

F = dilution factor of sample preparation

ε_{340} = extinction coefficient of NADH at 340 nm (6.3)

v = sample volume used in the assay

l = light path (cm)

ATP concentration can be converted from µmol/dL to µmol/g Hb using the equation below [53]:

$$C\ \left(\frac{\mu mol}{g}\ Hb\right) = \frac{C\ (\frac{\mu mol}{dL}) \times 10}{Hb\ (\frac{g}{L})} \quad \text{(Eq. 2-4)}$$

Blank sample was prepared with distilled water and control sample was prepared with ATP standard (DiaSys Diagnostic Systems GmbH, Holzheim, Germany).

2,3-DPG concentration

RBC 2,3-DPG concentration was determined using the commercially available 2,3-DPG assay kit (Roche, 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. All reactants and enzymes are present in the reagents from the kit, except for 2,3-DPG, which is provided by RBCs. NADH absorbance was measured on SPECTRA max PLUS 384 microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) at 340 nm. The amount of NADH left in the reaction mixture is indirectly proportional to the concentration of 2,3-DPG in RBC sample. 2,3-DPG concentration in test samples was calculated using SoftMax Pro software (Molecular Devices Corporation, Sunnyvale, CA, USA) according to the equation below:

$$2{,}3-DPG\left(\frac{mmol}{L}\right)=\frac{\Delta A\times V_{sample}\times F}{2\times\varepsilon_{340}\times l\times v} \qquad \text{(Eq. 2-5)}$$

where ΔA = (absorbance of the sample solution) – (absorbance of the blank solution)

V_{sample} = total reaction volume

F = dilution factor for blood

ε_{340}= extinction coefficient of NADH at 340 nm (6.3)

l = path length (cm)

v = sample volume used in the assay

Blank sample was prepared with distilled water and control sample was prepared with in-house made 2,3-DPG controls. 2,3-DPG controls were prepared from 2,3-Diphospho-D-glyceric acid pentasodium salt (SIGMA, St. Louis, USA) to a 2.5 mmol/L concentration.

2,3-DPG concentration in control samples was calculated using SoftMax Pro software according to the equation below:

$$2,3-DPG\left(\frac{mmol}{L}\right)=\frac{\Delta A\times 2\times V_{sample}}{2\times\varepsilon_{340}\times l\times v} \qquad \text{(Eq. 2-6)}$$

where all variables are defined the same as in above equation [54].

Methemoglobin concentration

Concentration of metHb was measured spectrophotometrically on the SPECTRA max PLUS 384 microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA). RBCs were diluted 1:500 in distilled water and the concentration of each type of haemoglobin (oxyhemoglobin (C_{oxy}), metHb (C_{met}), carboxyhemoglobin (C_{deoxy})) was determined by measuring the absorption of lysed RBCs at four different wavelengths (560 nm, 576 nm, 630 nm, 700 nm), since each type of haemoglobin has a unique absorption peak. Concentrations of different types of Hb were calculated as the amount of heme monomer in mol/L using the following formulas [55]:

$$C_{met} = 501 \times \left[\left(-0.361416 \times (A_{560} - A_{700})\right) + \left(0.174064 \times (A_{576} - A_{700})\right) + \left(2.68255 \times (A_{630} - A_{700})\right)\right] \times 10^{-4}$$

(Eq. 2-7)

$$C_{oxy} = 501 \times \left[\left(-0.741711 \times (A_{560} - A_{700})\right) + \left(1.01587 \times (A_{576} - A_{700})\right) - \left(0.279425 \times (A_{630} - A_{700})\right)\right] \times 10^{-4}$$

(Eq. 2-8)

$$C_{deoxy} = 501 \times \left[\left(1.35699 \times (A_{560} - A_{700})\right) + \left(0.739456 \times (A_{576} - A_{700})\right) - \left(0.671847 \times (A_{630} - A_{700})\right)\right] \times 10^{-4}$$

(Eq. 2-9)

where *C* = concentration of each type of Hb (mol/L),

A = absorbance at each wavelength.

These concentrations were also converted to g/L of tetrameric haemoglobin:

$$C\left(\frac{g}{L}\right) = \frac{C(\frac{mol}{L})\times 64000}{4} \qquad \text{(Eq. 2-10)}$$

Note, that extinction coefficients change with pH, therefore these equations only work for solutions at a pH of 7.0 [56].

Novel indicators of RBC quality

RBC deformability was analyzed via ektacytometry using the Laser-assisted Optical Rotational Cell Analyzer (LORCA, Mechatronics, Zwaag, The Netherlands). In ektacytometry, the RBC suspension is subjected to different levels of shear stress during rotation at different speeds, which causes the RBCs to elongate to different extents. A laser beam, shone through the RBC suspension, is refracted by RBCs, and the shape of the diffraction pattern is used to determine EI_{max}, the maximum theoretical elongation index, and K_{EI}, the shear stress required to achieve half of the EI_{max} [43]. A high EI max suggests RBCs are highly deformable, whereas a high KEI means that RBCs are very rigid and, hence, more force needs to be applied for RBCs to elongate. For ektacytometry

experiments, RBCs were diluted 1:100 in polyvinylpyrrolidone (PVP, Mechatronics, Zwaag, The Netherlands). All measurements were performed at 37°C. Deformability data was analyzed using Eadie-Hofstee linearization as previously described by Stadnick et al. [57].

Flow cytometry was used to assess RBC microvesiculation, as well as expression of PS and CD47 by both RBCs and RBC microparticles according to the protocol developed in our laboratory [58, 59]. To prepare RBC samples, RBC concentrates were diluted 1:5650 in Annexin V binding buffer (prepared in-house and contained 140 mM sodium chloride, 2.5 mM calcium chloride, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) in two steps. To eliminate any small particles originally present in the buffer, the buffer was sterile filtered through 0.2 μm Supor Membrane VacuCap 60 Bottle-top filters (Pall Life Scienes, Ann Arbor, MI, USA) before being used for any cell dilutions. Nine hundred eighty five microliters of RBC suspension was then labelled with 5 μL each of: FITC-conjugated anti-glycophorin A (Invitrogen Corporation, Camarillo, CA, USA), APC-conjugated Annexin V (BD Pharmingen, Franklin Lakes, NJ, USA) and PE-conjugated anti-CD47 (BD Pharmingen, Franklin Lakes, NJ, USA), and incubated for at least 15 minutes in the dark at room temperature.

A number of controls were used in this assay. Unstained RBCs served as a negative control. To determine the degree of non-specific binding of antibodies, isotype controls were prepared by labeling 990 μL of RBCs with 5 μL each of: PE-conjugated mouse IgG1, κ (BD Pharmingen, Franklin Lakes, NJ, USA) and

FITC-conjugated mouse IgG1, κ (Invitrogen Corporation, Camarillo, CA,USA). The positive control for RBC microparticles and PS externalization was prepared by treating fresh RBCs with N-ethylmaleimide (NEM) (Sigma-Aldrich, St. Louis, MO, USA), as previously described by Stewart et al. [60], and subsequently labeling NEM-treated RBCs with fluorescent antibodies in the same way as a test sample. Annexin V binding buffer was run alone to check for the purity of the buffer and absence of microparticle artifacts and the Annexin V binding buffer with added fluorescent antibodies was run as a blank control.

Prepared samples were then analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) with a low flow rate. Uniform Polystyrene Microspheres 1.01 μm in diameter (Bangs Laboratories, Inc., Fishers, IN, USA) were used as a size reference to set a gate around the desired population of microparticles and only microparticles less than 1.01 μm in diameter were included in the analysis. Only microparticles positive for Glycophorin A were considered to be of RBC origin and, therefore, further quantified and analyzed for PS and CD47 expression. Data analysis was performed using CellQuest Pro software, Version 6.0 (BD Biosciences, San Jose, CA, USA). After analysis, flow cytometry output data was used to calculate the percentage of microparticle events in each RBC sample, the percentage of RBCs and RBC microparticles expressing PS and CD47, as well as the mean fluorescence intensity (MFI) of PS and CD47 on RBCs and RBC microparticles.

Statistical analysis

Three types of statistical analysis were used in the present study: correlation, regression, and Student-t test. Statistical analysis was conducted using Statistical Analysis System (SAS) software, Version 9.1 (SAS institute Inc., Cary, NC, USA). To investigate the strength of the relationship between cord blood characteristics and cord RBC quality parameters, correlation analysis was performed. Spearman rank coefficients were calculated for the baby's gender and cord blood collection site, which are discrete variables, while Pearson correlation coefficients were calculated for other cord blood characteristics, which are continuous variables. The use of regression analysis permitted the effects of multiple cord blood characteristics on RBC quality measures to be examined in a model simultaneously. In regression analysis, the backward selection approach was used to select the variable(s) for the final statistical model. First, all of the characteristic variables were entered into the model. Then variables possessing the highest p-values (indicating no effect) were removed one by one until all of the remaining variables comprising the model had a significance of a p-value <0.1. Student-t test was used for comparison of cord blood characteristics and cord RBC quality parameters between groups of cord blood units based on baby's gender and a method of cord blood collection. The significance level was set to 0.05.

Contributions of others

Personnel of Alberta Cord Blood Bank provided cord RBC samples for this study. Dr. John Akabutu (Alberta Cord Blood Bank) helped with designing experiments and interpreting the data. Dr. Qi-long Yi (Canadian Blood Services) performed statistical analysis for the present study. Ruqayyah Almizraq (Department of Laboratory Medicine and Pathology, University of Alberta), Jayme Tchir (Canadian Blood Services), and Ann Berg participated in developing flow cytometry method.

2.3 Results

A total of 30 cord RBC samples were tested in this study. Most of the samples tested were between 17 and 48 hours old (Figure 1). The average age of cord blood samples was 30.6±10.3 hours (mean±SD). The ratio of cord blood volume to the volume of CPD anticoagulant in cord blood collections ranged from 0.77 to 3.83 (Figure 2), the average ratio being 1.84±0.80 (mean±SD)

Relationship between cord blood characteristics and cord RBC quality measures

Table 1 shows correlations between cord blood characteristics and RBC quality measures. There was a fair, negative correlation between cord blood storage time and 2,3-DPG content of RBCs ($r=-0.431$, $p=0.017$). A fair, positive correlation between cord blood hematocrit and deformability of RBCs was observed ($r=0.392$, $p=0.035$). Also, there was a moderately strong, positive relationship between the percentage of HbF in RBCs and their deformability ($r=0.668$, $p=0.002$). Other cord blood characteristics, such as baby's gender, baby's weight, cord blood volume, the ratio of cord blood volume to anticoagulant volume, and cord blood collection method, were not significantly correlated with any of the cord RBC quality measures.

Regression analysis revealed many significant effects of cord blood characteristics (predictor variables) on cord RBC quality measures (outcome variables). Regression coefficients show the nature of relationship between the predictor and the outcome. Absolute values of regression coefficients show how much the outcome changes when the predictor changes. A + / -sign before the coefficient show the direction in which the change takes place (increase or decrease). There was a significant relationship between the age of cord blood and the 2,3-DPG concentration of RBCs, with a regression coefficient of -0.214 ($p=0.012$). There was a significant relationship between the cord blood volume and the ATP content of RBCs, with a regression coefficient of 0.010 ($p=0.030$).

There was a significant negative relationship between the baby's weight and hemolysis, with a regression coefficient of -0.001 (p=0.018). There were significant relationships between HbF content of RBCs and a number of cord RBC quality measures, such as hemolysis, with regression coefficient of -0.030 (p=0.009), 2,3-DPG content, with a regression coefficient of -0.082 (p=0.007), and deformability, with a regression coefficient of 0.004 (p=0.002). The effect of HbF content on RBC rigidity was modified by baby's gender, so that no significant effect was observed for males; however, for females RBC rigidity decreased with the increase in HbF content (regression coefficient -0.068, p=0.002). A positive relationship was observed between the ratio of cord blood volume to anticoagulant volume and PS expression on RBCs with a regression coefficient of 3.836 (p=0.028). Females had an increase in CD47 expression on RBCs of 0.742% compared to males (p=0.037), which was modified by cord blood hematocrit. Cord blood RBCs collected from *in utero* placenta had an increase in PS expression on their surface of 4.106% compared to RBCs collected from delivered placenta (p=0.013).

A number of interactions between predictor variables were observed, wherein the effect of one predictor on cord RBC quality measure was further enhanced by a similar direction change of another predictor. Interactions between the following parameters were found: baby's weight and HbF, age of cord blood and HbF, baby's gender and HbF, the ratio of cord blood volume to the volume of anticoagulant and the method of cord blood collection, and baby's gender and cord blood hematocrit. To illustrate some of the above interactions, there was an

interaction of the effect of the age of cord blood and HbF content on 2,3-DPG concentration. Since both the age of cord blood and HbF content were negatively associated with 2,3-DPG, when the age of cord blood increased, for samples with higher HbF content, the decrease in 2,3-DPG content was stronger than for samples with lower HbF content. The effect of HbF on hemolysis was modified by the change in baby's weight; in particular, cord blood samples with lower HbF content taken from smaller babies had higher hemolysis than those taken from larger babies. The effect of the ratio of cord blood volume to anticoagulant volume on RBC PS expression was modified by the method of cord blood collection, so that PS expression on RBCs collected from *in utero* placenta decreased with increase in cord blood/anticoagulant ratio.

Effect of baby's gender and the method of cord blood collection on cord RBC quality

The results of comparison of cord blood characteristics and cord RBC quality measures between cord blood groups based on baby's gender and a method of cord blood collection are presented in Table 2. There were no significant differences noted in the cord blood characteristics and cord RBC quality parameters between males and females, or between cord blood collected from delivered or *in utero* placentas (Table 2).

2.4 Discussion

The goal of this study was to examine if and how the quality of RBCs isolated from whole cord blood is affected during cord blood storage at room temperature, and secondly, whether or not any of several cord blood characteristics impact the quality of cord RBCs. The primary purpose of the cord blood units used in this study was for stem cell extraction according to well established protocols. After collection, whole cord blood is stored at room temperature for a variable amount of time dictated by the time of birth, transportation to the processing facility and laboratory processing hours. It is then processed for stem cell isolation. Little is known about the effect of such storage conditions on the quality of RBCs present in cord blood. If cord RBCs are potentially superior for intrauterine and neonatal transfusions, and are to be cryopreserved for clinical use, there is an incentive to ensure their best quality upon isolation from umbilical cord blood.

Effect of cord blood storage time on cord RBC quality

During the first 65 hours of cord blood storage at room temperature, the only observed change was a decrease in the 2,3-DPG concentration of RBCs (Table 1). Other RBC quality measures were not affected. It has been reported that 2,3-DPG disappears very quickly from adult RBCs during hypothermic storage and is usually no longer detectable by end of the first week [61], though it is easily

replenished after RBCs are transfused into the patient [62]. Therefore, if cord RBCs are to be cryopreserved for clinical use within 65 hours after cord blood collection, the drop in 2,3-DPG may not pose a serious risk for maintaining an acceptable quality of cord RBCs after cryopreservation.

Effect of cord blood:anticoagulant ratio on cord RBC quality

Evidence in the literature suggests that the ratio of adult blood volume to the volume of anticoagulant/preservative solution is important and affects RBC quality [63, 64]. For adult whole blood collections, the standard ratio of blood volume to CPD anticoagulant volume is 7:1 [65]. In this study, blood:CPD ratio for cord blood units ranged from 0.77:1 to 3.83:1, due to the variable volumes obtained at the time of collection. These volumes are not predictable and hence an optimal cord blood/ anticoagulant ratio cannot be specified. A number of scientific reports indicate that the quality of adult RBCs from under- [63, 64] or over collected blood units is suboptimal as compared with defined standard collections [64]. Evidence from this study suggests that the same may be true for cord blood collections. Particularly, lower cord blood hematocrit was correlated with lower RBC deformability (Table 1). Since cord blood hematocrit was measured after cord blood was mixed with CPD, cord blood hematocrit was partly determined by the ratio of cord blood volume to the volume of anticoagulant. Anticoagulant is acidic, therefore, when this ratio is too low (in the case of small

cord blood volumes), the pH of cord blood will be reduced. This eventually results in RBC damage early in storage. A positive association between cord blood volume and ATP content, observed in the present study, also supports the argument about the importance of ratio of volumes. Higher cord blood volumes result in higher blood:CPD ratio, and, therefore, higher ATP concentration. Finally, my results show that a higher baby's weight was correlated with lower RBC hemolysis. This can be explained by the fact that in this study bigger babies on average had higher cord blood volumes, which in turn resulted in higher blood:CPD ratio. On the other hand, regression analysis showed that a lower ratio of cord blood to anticoagulant was associated with lower expression of the apoptotic marker PS on RBCs. Combined, these observations imply that cord RBC quality is governed by an optimal ratio of cord blood to anticoagulant that will need to be maintained during cord blood collections for RBC transfusion *in utero* or for neonates. Since it is very difficult to predict the volume of cord blood that can be collected from the placenta, an alternative solution is to select only cord blood units with a certain specified cord blood:anticoagulant ratio for cord RBC transfusion purposes.

Hemoglobin F content and RBC deformability

I have observed the positive correlation between the content of HbF in RBCs and RBC deformability (Table 1). It is known that HbF decreases gradually during the

last trimester of fetal development and premature babies have higher percentage of HbF than full-term neonates [2]. Although the literature contains contradictory data on deformability of cord RBCs, Lindercamp et al. showed that RBCs of pre-term babies have higher cellular deformability than RBCs of full-term babies [66]. Together, these two pieces of evidence are in agreement with my observation.

In conclusion, there was no significant decrease in quality measures of cord RBCs during the first 65 hours of whole cord blood storage at room temperature. The ratio of cord blood volume to anticoagulant volume in cord blood collection bag is important and an optimal ratio needs to be maintained in order to ensure that a good quality cord RBCs are preserved. Knowledge of cord RBC quality upon isolation from cord blood is important to design procedures for cord RBC preservation. This, in turn, may result in the development of a novel blood product from a currently discarded byproduct of cord blood cell processing that may offer a superior alternative for treatment of fetal and neonatal anemias.

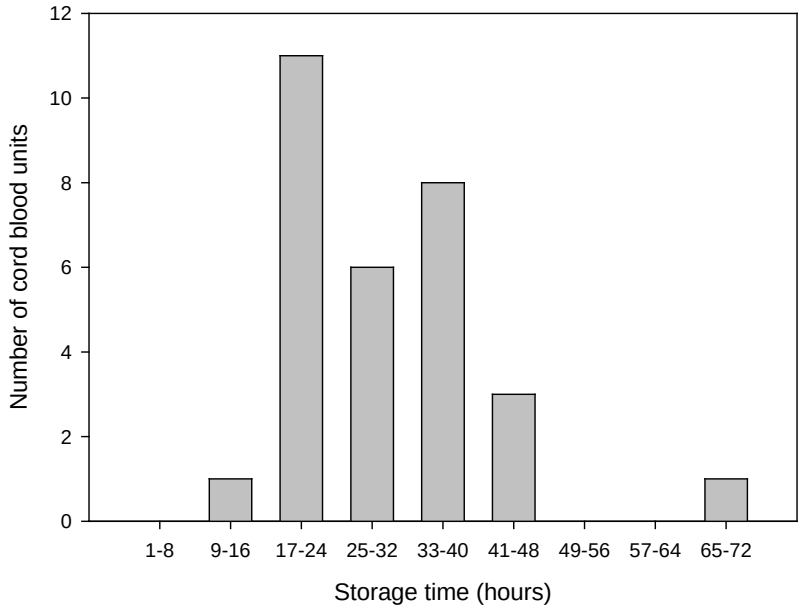

Figure 2-1. The duration cord blood samples spent at room temperature prior to testing.

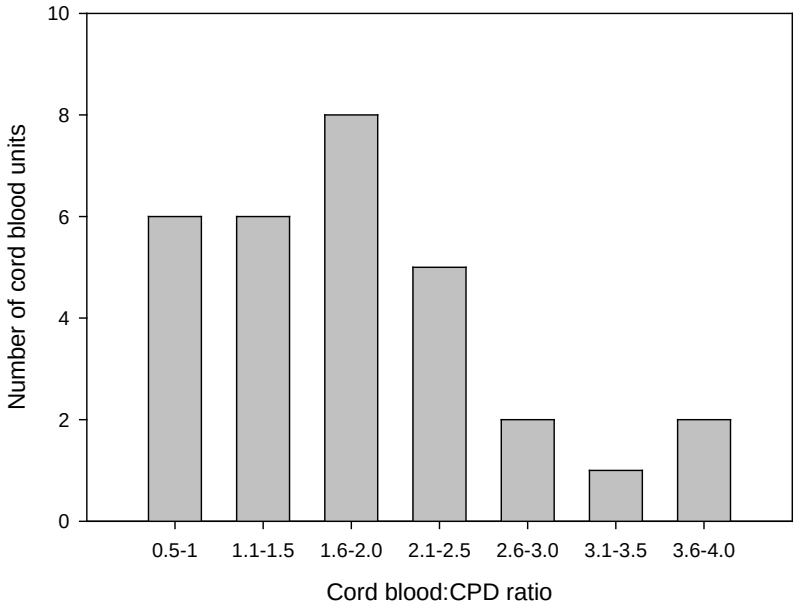

Figure 2-2. The distribution of the ratio of cord blood volume to CPD anticoagulant volume in collected cord blood samples.

Table 2-1. Correlation between cord blood characteristics and cord RBC quality measures.

Cord blood characteristic Cord RBC quality measure	Age of cord blood (hours) **30.6± 10.3**	Cord blood volume (mL) **64±28**	Cord blood Hct (L/L) **0.30± 0.04**	Baby's gender	Baby's weight (g) **3468± 490**	Cord blood collection site	HbF (%) **90.1±5.8**	Cord blood/ CPD **1.84± 0.80**
ATP (μmol/g Hb) **2.78±0.69**	-0.229 (0.241)	0.337 (0.080)	0.316 (0.109)	-0.241 (0.216)	0.042 (0.841)	0.021 (0.914)	0.177 (0.497)	0.337 (0.080)
Hemolysis (%) **0.12±0.04**	-0.127 (0.505)	-0.144 (0.448)	-0.104 (0.590)	0.055 (0.773)	-0.085 (0.672)	-0.013 (0.946)	-0.358 (0.145)	-0.144 (0.448)
MetHb (%) **4.60±1.80**	0.098 (0.606)	-0.143 (0.451)	-0.236 (0.217)	0.019 (0.919)	-0.001 (0.995)	0.032 (0.866)	0.321 (0.194)	-0.143 (0.451)
2,3-DPG (mmol/L) **0.11±0.20**	-0.431* (0.017)	-0.133 (0.484)	-0.181 (0.346)	-0.231 (0.220)	-0.072 (0.720)	0.019 (0.919)	-0.342 (0.165)	-0.133 (0.484)
Deformability **0.446±0.030**	-0.119 (0.531)	0.323 (0.081)	0.392* (0.035)	0.012 (0.951)	0.254 (0.201)	0.160 (0.397)	0.668** (0.002)	0.323 (0.081)
Rigidity **1.47±0.33**	-0.277 (0.138)	0.010 (0.960)	-0.093 (0.630)	0.144 (0.448)	-0.127 (0.529)	0.225 (0.233)	-0.375 (0.126)	0.010 (0.960)
MP events (%) **0.3±0.2**	-0.126 (0.540)	0.031 (0.882)	-0.240 (0.249)	-0.200 (0.327)	0.305 (0.156)	-0.088 (0.668)	0.145 (0.592)	0.031 (0.882)
PS^+ RBCs (%) **5.2±0.9**	0.008 (0.969)	0.015 (0.938)	-0.023 (0.910)	-0.170 (0.388)	-0.080 (0.705)	0.193 (0.325)	-0.084 (0.741)	0.015 (0.938)
CD47+ RBCs (%) **99.9±0.1**	-0.144 (0.465)	-0.215 (0.271)	-0.371 (0.057)	-0.322 (0.095)	0.014 (0.947)	-0.236 (0.227)	-0.309 (0.212)	-0.215 (0.271)
PS^+ MP (%) **61.3±18.2**	-0.151 (0.461)	-0.171 (0.404)	-0.292 (0.157)	0.084 (0.682)	-0.125 (0.569)	0.104 (0.612)	-0.056 (0.836)	-0.171 (0.404)
$CD47^+$ MP (%) **88.2±24.2**	0.121 (0.557)	-0.076 (0.713)	-0.218 (0.295)	0.105 (0.608)	-0.209 (0.338)	0.265 (0.191)	0.137 (0.612)	-0.076 (0.713)
MFI of PS on RBCs **45±10**	-0.086 (0.663)	0.102 (0.606)	-0.202 (0.313)	-0.152 (0.440)	0.093 (0.660)	-0.222 (0.257)	0.344 (0.163)	0.102 (0.606)
MFI of CD47 on RBCs **274±83**	-0.129 (0.513)	-0.033 (0.869)	-0.105 (0.601)	-0.071 (0.718)	-0.070 (0.739)	-0.107 (0.587)	-0.070 (0.781)	-0.033 (0.869)
MFI of PS on MPs **324±55**	0.135 (0.494)	0.007 (0.972)	0.155 (0.439)	0.027 (0.892)	0.035 (0.868)	-0.164 (0.403)	-0.137 (0.588)	0.007 (0.972)
MFI of CD47 on MPs **776±370**	-0.168 (0.394)	-0.095 (0.632)	0.097 (0.629)	-0.080 (0.684)	-0.118 (0.574)	-0.136 (0.491)	-0.344 (0.163)	-0.095 (0.632)

The numbers in the table are correlation coefficients (p value in brackets), that show the strength of a relationship between two parameters. Coefficients between 0.3 and 0.5 represent a fair linear relationship and coefficients between 0.6 and 0.8 represent moderately strong linear relationship. A + / - sign before the coefficient indicates the direction of the correlation (positive or negative). The numbers in bold are mean±SD for each tested parameter. * correlation is significant at 0.05 level, ** correlation is significant at 0.01 level. Hct – hematocrit, HbF – fetal hemoglobin, CPD – citrate-phosphate-dextrose (anticoagulant), ATP – adenosine triphosphate, MetHb – methemoglobin, 2,3-DPG – 2,3–diphosphoglycerate, PS – phosphatidylserine, MP – microparticle, MFI – mean fluorescence intensity.

Table 2-2. Comparison of cord blood characteristics and cord RBC quality measures between cord blood groups based on baby's gender and a method of cord blood collection.

Cord blood characteristics and cord RBC quality parameters	Baby's Gender			Method of cord blood collection		
	Male (n=17)	Female (n=13)	p-value	Delivered placenta	Placenta *in utero*	p-value
Age of cord blood	30.2 (11.8)	31.2 (8.3)	0.7908	24.5 (0.4)	31.3 (10.6)	0.0028*
Cord blood volume	60 (23)	70 (34)	0.3310	61 (25)	65 (29)	0.8113
Cord blood Hct	0.30 (0.05)	0.31 (0.04)	0.7943	0.31 (0.03)	0.30 (0.05)	0.8676
Baby's weight	3546 (557)	3336 (333)	0.2906	3230 (407)	3498 (499)	0.3821
HbF (%)	88.5 (5.6)	91.5 (6.0)	0.2891	. (.)	90.1 (5.8)	.
Cord blood/CPD	1.71 (0.66)	2.01 (0.96)	0.3310	1.73 (0.70)	1.85 (0.82)	0.8113
ATP (μmol/g Hb)	2.87 (0.58)	2.68 (0.83)	0.4811	2.68 (0.46)	2.80 (0.72)	0.7936
Hemolysis (%)	0.12 (0.05)	0.12 (0.05)	0.7987	0.11 (0.02)	0.12 (0.05)	0.6607
MetHb (%)	4.54 (1.62)	4.67 (2.07)	0.8531	4.46 (1.68)	4.61 (1.84)	0.8941
2,3-DPG (mmol/L)	0.11 (0.15)	0.11 (0.26)	0.9933	0.05 (0.03)	0.12 (0.21)	0.5923
Deformability	0.445 (0.029)	0.447 (0.032)	0.8972	0.437 (0.014)	0.447 (0.031)	0.5834
Rigidity	1.41 (0.21)	1.56 (0.43)	0.2025	1.27 (0.23)	1.49 (0.33)	0.2747
MP events (%)	0.3 (0.2)	0.2 (0.1)	0.2483	0.9 (0.1)	0.3 (0.2)	0.8861
PS^+ RBCs (%)	5.4 (1.0)	5.1(0.7)	0.3842	4.7 (1.4)	5.3 (0.8)	0.2113
$CD47^+$ RBCs (%)	99.9 (0.1)	99.8 (0.2)	0.0706	99.9 (0.1)	99.9 (0.1)	0.4089
PS^+ MPs (%)	61.2 (21.8)	61.5 (11.2)	0.9770	57.2 (2.4)	61.9 (19.3)	0.6848
$CD47^+$ MPs (%)	87.6 (30.1)	89.2 (10.2)	0.8674	76.3 (16.6)	89.8 (24.8)	0.3736
MFI of PS on RBCs	47 (7)	44(13)	0.3924	51 (10)	45 (10)	0.2715
MFI of CD47 on RBCs	275 (73)	271 (99)	0.8983	299 (114)	271 (82)	0.5814
MFI of PS on MPs	324 (62)	324 (46)	0.9946	366 (74)	319 (52)	0.1730
MFI of CD47 on MPs	837 (434)	694 (258)	0.3209	899 (336)	761 (378)	0.5528

The numbers in the table are absolute values for each parameter (standard deviation in brackets). * $p<0.05$ (Student-t test). Hct – hematocrit, HbF – fetal hemoglobin, CPD – citrate-phosphate-dextrose (anticoagulant), ATP – adenosine triphosphate, MetHb – methemoglobin, 2,3-DPG – 2,3–diphosphoglycerate, PS – phosphatidylserine, MP – microparticle, MFI – mean fluorescence intensity.

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Chapter 3

A method to measure permeability of red blood cell membrane to water and solutes using intrinsic fluorescence

3.1 Introduction

Cryopreservation – preservation of cells and tissue at subzero temperatures below which biochemical reactions do not occur at appreciable rates – is an effective means to preserve the quality and functionality of biological material for clinical transplantation and research purposes [1]. To prevent damage to cells during exposure to such low temperatures, special chemical compounds termed cryoprotectants are used. Glycerol is a common cryoprotectant used in cryopreservation of red blood cells [2]. Cell membrane plays a very important role in cryopreservation, governing the transport of water and solutes between the cytoplasm and the extracellular milieu and regulating osmotic changes of the cell during freezing [3]. To design effective cryopreservation procedures for cells and tissues and avoid freezing injury, it is critical to know cell osmotic parameters, such as permeability of cell membrane to water and solutes [4-7]. To determine cell membrane permeability, one needs to measure the rate of cell volume changes when placed in an anisotonic environment [8]. Red blood cells (RBCs) respond very quickly to changes in extracellular solute concentrations [9]. Therefore, it is difficult to measure the rate of RBC volume change with

traditional methods, such as using an electronic particle counter [10]. Stopped-flow spectroscopy can quantify rapid changes of RBC volume and is a common method used to measure RBC osmotic permeability. Two stopped-flow approaches are used in this regard. The first measures RBC volume based on changes in the intensity of light scattered by RBCs [9, 11-13]. However, the limitation of this method is that several factors besides cell volume can influence the intensity of scattered light. Among these factors are the dependence of cell refractive index on the intracellular permeable solute concentration [14], discoid shape of RBCs [13], or membrane aggregation [15]. The second approach monitors changes in cell volume based on the self-quenching of a fluorescent dye, such as carboxyfluorescein diacetate (CFDA), entrapped inside the cell. This method was first introduced by Chen et al. [15], and was later used by others for RBCs [16, 17]. However, a significant portion of light emitted by fluorescent dye is absorbed by hemoglobin (Hb), which complicates this technique [18].

Evidence suggests that RBCs obtained from umbilical cord blood (cord RBCs) contain the same type of Hb as blood of newborns [19] and may be a superior alternative to adult RBCs presently transfused to neonates with anemia [20-22]. Although cord RBCs are usually discarded during hematopoietic stem cells isolation from cord blood, a number of studies have demonstrated that transfusions of autologous cord RBCs are both safe and effective in the treatment of anemic neonates [23-29]. Unlike RBCs from adult blood, cord RBCs deteriorate very quickly during traditional storage at 1-6°C [25, 30] and would benefit from low temperature preservation. Despite many studies having

documented the successful cryopreservation of adult RBCs, no protocol for cryopreservation of cord RBCs has been developed.

Knowledge of osmotic permeability of cord RBCs to water and solutes is required to design effective cryopreservation methods for these cells. However, literature data on cord RBC permeability are both limited and controversial [9, 31]. Therefore, development of the improved methods to determine cord RBC permeability is warranted. Literature reports indicate that RBC Hb has autofluorescent properties [32-35]. Hirsch et al. showed that adult hemoglobin (HbA) and fetal hemoglobin (HbF), when excited at 280 nm, emit light with a maximum intensity at ~325 nm [32]. Alpert et al. demonstrated maximum emission of human HbA at 334 nm [33]. I hypothesized that RBC permeability to water and solutes can be measured using intrinsic fluorescence (autofluorescence) of intracellular Hb. Rapid changes in RBC volume in response to exposure of cells to am anisotonic environment can be captured by measuring changes in intrinsic Hb fluorescence intensity, since measured fluorescence is reduced at increased Hb concentrations resulting from reduced cell volume.

3.2 Materials and Methods

Experimental samples

Two sources of RBCs were used for this study: RBCs from peripheral blood of adult donors (adult RBCs), and RBCs from umbilical cord blood (cord RBCs). The Canadian Blood Services Network Center for Applied Development in Vancouver provided adult RBCs for my experiments. RBCs were leukocyte-reduced, stored in CPD (citrate-phosphate-dextrose) anticoagulant and SAGM (saline-adenine-glucose-mannitol) preservative at 1-6°C, and were used in experiments within 10 days of collection. The Alberta Cord Blood Bank supplied cord RBCs, as a waste product, after stem cell isolation from umbilical cord blood. Cord blood collected from a placenta was stored at room temperature for up to 67 hours prior to stem cell isolation [36]. Leftover cord RBCs were stored in our laboratory at 1-6°C and used in experiments within 24 hours of isolation from cord blood. Prior to experimentation, cord RBCs were washed with saline 1-3 times by centrifugation at 2200 g, 4°C for 5 minutes. The hematocrit of cord RBCs was then adjusted to approximately 60% by the addition of saline to the RBC pellet. Ethics approval for the study was obtained from the University of Alberta Health Research Ethics Board (Biomedical Panel) and Canadian Blood Services Research Ethics Board.

Experimental solutions

RBC autofluorescence was monitored under rapid kinetic and equilibrium conditions, as a function of extracellular osmolality in the presence of various concentrations of sodium chloride (NaCl). NaCl solutions were prepared by diluting 12% (w/v) NaCl stock solution (Baxter, Deerfield, IL, USA) with distilled water to yield final concentrations of 0.46, 0.68, 0.8, 0.9, 1.25, 1.6, 2.3, 2.55, 3.5, and 6.1% (w/v). Glycerol (3.5% (w/v)) – NaCl (0.9% (w/v)) solution was prepared by diluting 35 g glycerol (99.5+%, Sigma Aldrich, Inc., St. Louis, MO, USA) and 9 g NaCl (>=99.0%, Sigma Aldrich, Inc., St. Louis, MO, USA) with distilled water to 1 L.

Experimental system

A SpectraMax GEMINI EM dual-scanning fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used to measure equilibrium autofluorescence of RBCs and Hb in various osmolalities. Data were acquired and analyzed using SOFTmax PRO software (Version 5.3, Molecular Devices, Sunnyvale, CA, USA). All measurements were made at ambient temperature (mean = 24.6°C, range 22.1 – 27.8°C).

An SX20 stopped-flow reaction analyser (Applied Photophysics, Ltd., Leatherhead, UK) was used to measure rapid kinetic changes of RBC autofluorescence upon mixing with solutions of various osmolalities. Excitation and emission slit widths were set to 3 mm (equivalent to a wavelength bandwidth of 13.95 nm). The 20 μL optical cell had a 10 mm pathlength and 1 ms dead time (during which mixing occured). Data were acquired using ProData SX software (Applied Photophysics, Ltd., Leatherhead, UK). All measurements were made at ambient temperature (mean = 20.2°C, range 19.3 – 21.2°C).

Integrity of RBCs in experimental solutions

RBC hemolysis (membrane damage) was measured in all experimental NaCl solutions (NaCl concentration ranging from 0.68% (w/v) to 3.5% (w/v)). Fifty μL RBCs were pipetted into 1 mL NaCl solution and were allowed to equilibrate at room temperature for approximately 5 minutes. RBC hemolysis was determined by spectrophotometric measurement of total and supernatant cyanmetHb according to Drabkin's method [37]. Controls for total Hb were prepared from Stanbio Tri-Level Hb controls (Stanbio Laboratory, Boerne, TX, USA) [38]. The hematocrit of the RBC sample was measured, using a microhematocrit centrifuge (Hettich, Tuttlingen, Germany), as the ratio of the volume occupied by packed RBCs to the volume of a whole RBC sample [39].

Preparation of lysed RBCs (negative control)

Adult RBCs were lysed by three consecutive freeze-thaw cycles; cells were immersed in liquid nitrogen, and were then thawed at 37°C. Lysed RBCs were run in parallel with intact RBCs in equilibrium and kinetics experiments. Specifically, control samples were prepared by adding 5 μL (in equilibrium experiments) or 20 μL (in kinetics experiments) lysed RBCs into 1 mL NaCl solution (NaCl concentration ranging from 0.68% (w/v) to 3.5% (w/v)).

Fluorescence spectrum of adult and cord RBCs

I chose 280 nm as an excitation wavelength for both cord and adult RBCs based on literature values [32]. The maximum emission wavelength was determined in four NaCl solutions; 0.68%, 0.9%, 1.6%, and 3.5% (w/v). Emission spectra of adult and cord RBCs were scanned in a fluorescence microplate reader (range 330 - 750 nm, 325 nm cut-off filter, step 10 nm) and in a stopped-flow analyzer (range 280 – 650 nm, step 2 nm).

Measurement of RBC autofluorescence

Equilibrium

I measured autofluorescence of RBCs equilibrated in NaCl solutions at concentrations of 0.68%, 0.8%, 0.9%, 1.25%, 1.6%, 2.55%, and 3.5% (w/v) NaCl. 5 μL RBCs were pipetted into 1 mL NaCl solution and equilibrated at room temperature for approximately 5 minutes to allow the RBC volume to stabilize. 200 μL of each sample were pipetted in triplicate into 96-well Microfluor 1 black flat bottom microtiter plates (ThermoScientific, Rochester, NY, USA). After mixing, samples were excited at 280 nm, and emission was measured at the optimized wavelength in top-read mode. Background autofluorescence of NaCl solutions (without RBCs) was also measured in triplicate and later subtracted from autofluorescence of RBC samples. Lysed RBCs served as a control. Fluorescence was expressed in Relative Fluorescence Units (RFUs).

Rapid kinetics

In a SX20 stopped-flow reaction analyser, osmotically-driven changes in RBC volume were induced by mixing RBCs with equal volume of experimental solutions of varying osmolality. RBC autofluorescence was recorded simultaneously as a function of time after mixing. The RBC suspension for

stopped-flow experiments was prepared by adding 20 μL RBCs to 1 mL of NaCl solution. The sample cell was illuminated at 280 nm, and emission was measured at the optimized wavelength. Fluorescence intentity was measured as a voltage (V, volts).

NaCl experiments

To achieve final extracellular NaCl concentrations of 0.68%, 0.9%, 1.6%, and 3.5% (w/v), the RBC suspension (in 0.9% (w/v) NaCl) was rapidly mixed in a 1:1 ratio with 0.46%, 0.9%, 2.3%, and 6.1% (w/v) NaCl, respectively. Total stopped-flow drive volume was set to approximately 120 μL. 1000 fluorescence data points were collected during the 10 s period immediately following mixing. Measurements were made in triplicate at each NaCl concentration. Autofluorescence of NaCl solutions (without RBCs) was also measured and subtracted from RBC autofluorescence (background). Lysed RBCs served as a control.

Glycerol experiments

Experiment 1 – addition of glycerol to RBCs. In this experiment, RBCs were exposed to hypertonic concentration of glycerol. RBCs suspended in saline were mixed 1:1 with 3.5% (w/v) glycerol, which resulted in a final extracellular glycerol concentration of 1.75% (w/v). As a control, RBCs were mixed 1:1 with

saline (osmotic equilibrium conditions) and fluorescence measured as a function of time. Thereafter, this equilibrium curve was subtracted from the curve obtained with RBCs + 1.75% (w/v) glycerol.

Experiment 2 – removal of glycerol from RBCs. In this experiment, RBCs were first loaded with 3.5% (w/v) glycerol, then mixed 1:1 with saline to cause efflux of glycerol from the cell down the concentration gradient. As a control, RBCs loaded with 3.5% (w/v) glycerol were mixed 1:1 with 3.5% (w/v) glycerol in saline (osmotic equilibrium conditions) and fluorescence measured as a function of time. Thereafter, this equilibrium curve was subtracted from the curve obtained with RBCs in 3.5% (w/v) glycerol + saline.

In both experiments, 1000 fluorescence data points were collected during 60 s of acquisition.

Equilibrium RBC volume measurement

Equilibrium volumes of RBCs in NaCl solutions were determined on a Coulter Electronic Particle Counter (ZB1, Coulter Electronics, Inc., Hialeah, FL, USA) equipped with a pulse-height analyzer (The Great Canadian Computer Company, Spruce Grove, AB, Canada) [40]. RBCs were diluted in 10 mL of 0.68%, 0.9%, 1.6%, and 3.5% (w/v) NaCl to approximately 20000 cells/mL and were allowed

to equilibrate at room temperature for at least 5 min. Current pulses, proportional to cell volumes, were measured as RBCs passed through a 50 μm aperture. Measurements were made in triplicate in each experimental solution. At each NaCl concentration, 5 μm latex beads (Beckman Coulter, Inc., Fullerton, CA, USA) with known bead volume were used for calibration. Thereafter, calculated calibration factor was used to convert instrument output data to RBC volumes [41].

Testing correlation between RBC autofluorescence and volume

Relative RBC volumes were calculated by dividing the RBC volume measured in each experimental NaCl solution by the RBC volume measured in isotonic 0.9% (w/v) NaCl. Relative RBC autofluorescence under equilibrium conditions was calculated in two steps. First, the mean value of autofluorescence during the last 3 s of stopped-flow autofluorescence data acquisition (at the autofluorescence plateau) was calculated. Then, the relative RBC autofluorescence was calculated by dividing RBC autofluorescence in each experimental NaCl solution by RBC autofluorescence in isotonic 0.9% NaCl.

Fluorescence as a function of hemoglobin concentration

A concentrated Hb stock solution was prepared by shrinking RBCs in hypertonic salt solution to increase intracellular Hb concentration, then lysing RBCs. Adult RBCs were centrifuged at 2200g, 4°C for 10 min, the supernatant was discarded, and the RBC pellet diluted in 3.5% (w/v) NaCl. After repeating this centrifugation step, the RBC pellet was immersed in liquid nitrogen and thawed at 37°C until no ice crystals were visible. This freeze-thaw cycle was repeated two further times in order to lyse the RBCs. The concentration of Hb in the stock solution was measured using Drabkin's method [37]. A reverse pipetting technique was used to measure the extremely viscous stock solution of Hb. Several concentrations of Hb were then prepared by diluting this stock solution with saline. The Hb concentration in each of these dilutions was also measured by Drabkin's method. Thereafter, the fluorescence intensity of Hb solutions was measured on a fluorescence microplate reader using the optimized settings (see "Optimized experimental settings" section in Results).

Statistical analysis

One to three samples were used per experiment; most samples were tested in triplicate. The strength of the relationship between relative RBC volume and

relative RBC autofluorescence was assessed through Pearson's correlation analysis (SPSS Statistics software, version 19, IBM, Armonk, NY, United States). Correlations were considered significant at the 0.05 level.

Contributions of others

Personnel of Alberta Cord Blood Bank provided cord RBC samples for this study. Study hypothesis (stating that RBC permeability to water and solutes can be measured using intrinsic fluorescence (autofluorescence) of intracellular hemoglobin) was generated with assistance of Dr. Jason Acker. Dr. Aldo Olivieri and Dr. Andy Holt (Department of Pharmacology, University of Alberta) granted me the access to the stopped-flow system for the duration of this study, helped with setting up the stopped-flow system, and participated in developing the method to measure permeability of red blood cell membrane to water and solutes using intrinsic fluorescence. Dr. Locksley McGann participated in helpful discussion about hemoglobin self-quenching phenomenon.

3.3 Results

Upon equilibration in 0.68% - 3.5% (w/v) NaCl aqueous solutions, the range for hemolysis was 0.47-0.78% for adult RBCs and 0.70-1.0% for cord RBCs. In all

experimental solutions, except for 0.8 and 3.5% NaCl, hemolysis of cord RBCs was higher compared to adult RBCs ($p \leq 0.05$).

Optimized experimental settings

All samples were excited at 280 nm [32]. During fluorescence emission scanning on a stopped-flow analyzer, the maximum emission peak shifted towards a shorter wavelength as the concentration of NaCl was increased. Therefore, for stopped-flow analyses, the median maximum emission wavelengths for adult and cord RBCs were used with all experimental solutions. Shifting of the emission peak was not observed when measurements were in the fluorescence microplate reader, perhaps due to the larger scanning step used, compared with that used with the stopped-flow analyzer (10 nm versus 2 nm). Based on the results of fluorescence emission scans, the following optimized emission wavelengths for RBC autofluorescence were used: A. Adult RBCs: λex 280 nm, λem 340 nm, cut-off filter 325 nm (spectrofluorometer); λex 280 nm, λem 314 nm (stopped-flow analyzer) (Figure 3-1A); B. Cord RBCs: λex 280 nm, λem 340 nm, cut-off filter 325 nm (spectrofluorometer); λex 280 nm, λem 315 nm (stopped-flow analyzer) (Figure 3-1B).

Volume-dependent changes in RBC autofluorescence

The intensity of RBC autofluorescence and RBC volume were directly related. Specifically, the equilibrium autofluorescence of RBCs was inversely related to the concentration of NaCl solution in which cells were suspended. Over the range of experimental NaCl concentrations (0.68% to 3.5% w/v), equilibrium RBC autofluorescence was highest in 0.68% NaCl and lowest in 3.5% NaCl (Figure 3-2, for adult and cord RBCs). In contrast, when intact RBCs were replaced with lysed RBCs, autofluorescence was similar regardless of the solution tonicity (Figure 3-3).

Rapid mixing of RBCs with hypotonic NaCl solution to cause cell swelling caused an increase in autofluorescence, measured in a stopped-flow analyser; cell shrinking upon mixing with hypertonic solution resulted in a decrease in autofluorescence (Figure 3-4). Mixing of RBCs with isotonic (0.9% w/v) NaCl did not change autofluorescence intensity. In all solutions, autofluorescence reached equilibrium within a few seconds of mixing. With increase in tonicity of the solution, there was an increase in the rate of change in RBC autofluorescence. In particular, after mixing with 0.68% (w/v) NaCl, autofluorescence of adult RBCs reached a plateau after ~3 s, whereas upon mixing with 3.5% (w/v) NaCl, autofluorescence plateaued after ~0.5 s. RBCs had similar intensity of autofluorescence at time 0, immediately after mixing with NaCl solution, regardless of the tonicity of the solution they were suspended in.

After autofluorescence stabilized, the increase in autofluorescence intensity (plateau minus baseline) was inversely related to the concentration of NaCl solution RBCs were suspended in, the greatest increase observed with 0.68% (w/v) NaCl and the smallest in 3.5% (w/v) NaCl. When lysed RBCs were rapidly mixed with experimental NaCl solutions, no change in autofluorescence occurred regardless of the solution tonicity (Figure 3-5).

There were notable differences in autofluorescence kinetics between cord RBCs and adult RBCs (Figure 3-6). Upon mixing with anisotonic NaCl solutions, autofluorescence of cord RBCs took longer to stabilize compared with adult RBCs. For example, after mixing with 0.68% (w/v) NaCl, fluorescence of adult RBCs reached a plateau after approximately 3 s, whereas it took approximately 5 s for cord RBC fluorescence to stabilize.

When adult RBCs were suspended in 1.75% (w/v) glycerol solution, autofluorescence initially dropped, then gradually increased, and finally reached a plateau within 60 s. Furthermore, when RBCs loaded with 3.5% (w/v) glycerol were mixed rapidly with isotonic saline, autofluorescence initially increased, then gradually decreased, and finally reached a plateau within 60 s (Figure 3-7).

Correlation between autofluorescence and volume of RBCs

There was a strong positive correlation between equilibrium relative cell volume (V/V_0) and equilibrium relative cell autofluorescence intensity (F/F_0) for both

adult RBCs (correlation coefficient = 0.967, P = 0.033) and cord RBCs (correlation coefficient = 0.969, P = 0.031) (Figure 3-8).

Effect of RBC concentration on autofluorescence intensity

Absolute value of autofluorescence of the test sample changed as a function of the amount of RBCs added to it. When 1 to 10 μL packed RBCs (55 – 65% hematocrit) were added to 1 mL NaCl solution, fluorescence increased proportionally to the amount of RBCs added. When 10 to 50 μL packed RBCs (55 – 65% hematocrit) were added to 1 mL NaCl solution, fluorescence of the test sample gradually leveled out as the amount of RBCs in it increased (Figure 3-9). Note that the first four datapoints appear to have a negative value of fluorescence. This is due to the fact that in very dilute RBC suspensions, background autofluorescence of NaCl solutions (without RBCs) + the microplate well was higher that autofluorescence of RBC samples themselves. Therefore, subtraction of background autofluorescence from RBC autofluorescence rendered a negative value.

Self-quenching of intracellular hemoglobin

Results showed that quenching of fluorescence occurs at high Hb concentrations (Figure 3-10). On measuring autofluorescence of Hb solutions, ranging from 1 g/L to 448 g/L, it was found that from 1 to 10 g/L fluorescence increases with increasing Hb concentration, from 10 to 30 g/L fluorescence changes little with increasing concentration, and upwards of 30 g/L fluorescence decreases with increasing Hb concentration.

3.4 Discussion

The aim of this work was to develop a new method for measuring the fast kinetics of RBC volume changes in an anisotonic environment using intrinsic fluorescence of intracellular Hb. Knowledge of the kinetics of RBC volume changes as a function of extracellular osmolality is critical for the determination of the RBC osmotic permeability to water and solutes. Knowledge of osmotic permeability of cord RBCs is required for the development of an effective cryopreservation protocol for these cells. However, at present, available data are limited and controversial.

In one of the traditional methods for the determination of osmotic permeability, cells are loaded with the self-quenching dye CFDA [16,

17]. Since CFDA is membrane-impermeable, then when the cell volume decreases, the concentration of the intracellular dye will increase, leading to quenching and a decrease in observed fluorescence. Following the same principle, when the cell volume increases, fluorescence intensity also increases. In the present study I hypothesized that fluorescent Hb, already present inside RBCs, can act as an "intrinsic dye" and be used to measure changes in cell volume.

Integrity of RBCs in experimental solutions

A RBC hemolysis assay demonstrated that ~99% RBCs were intact in experimental NaCl solutions and were thus able to respond to changes in extracellular osmotic gradient by changing cell volume. Hemolysis of cord RBCs in most experimental solutions was increased compared with adult RBCs. This can be explained by the decreased stability of cord RBCs during hypothermic storage compared with adult RBCs, which results in higher levels of hemolysis [25, 30], especially in the absence of any preservatives.

Fluorescence spectrum of cord and adult RBCs

The fluorescence spectrum measured for RBCs was similar to that reported in the literature. Hirsch et al. (1980) showed that HbA and HbF, when excited at 280

nm, emit maximum light at ~325 nm [32]. Alpert et al. (1980) reported that maximum emission of human Hb A_0 takes place at 334 nm [33]. My results showed maximum emission at 314 nm and 315 nm for adult and cord RBCs, respectively, measured on the stopped-flow apparatus, and at 340 nm measured on the fluorescence microplate reader. The difference in emission peaks obtained on these two instruments may be due to the fact that 325 nm was the shortest wavelength available for the cut-off filter on fluorescence microplate reader and, considering 10 nm step, the reading of emission started only at 330 nm. The RBC emission peak obtained on the stopped-flow apparatus was quite broad, from about 300 nm to about 400 nm, and the maximum emission of 340 nm obtained on the fluorescence microplate reader corresponds well to that broader peak. Modest differences between these values and previously reported data may be explained by differences in instrumentation used by multiple research groups. Hirsch et al. used front-face optics [32], whereas the stopped-flow system I used had right angle optics. In contrast with values reported by Hirsch et al. (1980) [32], I also showed that there are slight differences in emission maxima between cord and adult RBCs, cord RBC peak emission being shifted 1 nm towards longer wavelengths.

Change in RBC autofluorescence is a volumetric response

My results indicated that there is a strong positive correlation between autofluorescence intensity and volume of RBCs. Equilibrium and kinetic experiments in NaCl solutions showed that a decrease in RBC volume in hypertonic NaCl solutions was associated with a decrease in autofluorescence intensity, and that an increase in RBC volume in hypotonic salt solutions led to an increase in autofluorescence (Figures 3-2 and 3-4). These observations were supported by a strong correlation between relative RBC fluorescence and relative RBC volume in equilibrium confirmed on a Coulter Electronic Particle Counter (Figure 3-8).

Similarly, the addition or removal of glycerol in RBC samples resulted in changes in RBC autofluorescence that were consistent with established patterns of cell volume changes upon addition or removal of permeable cryoprotectant [6]. When RBCs are suspended in hypertonic glycerol solution, cells initially shrink but then swell as cryoprotectant slowly permeates the cell, with the volume stabilizing after glycerol movement reaches equilibrium. Similarly, when RBCs loaded with glycerol are suspended in isotonic saline, cells first swell as water enters the cell as a result of osmotic pressure, followed by a reduction in cell volume as glycerol slowly diffuses from the cell into the extracellular space, until equilibrium is reached.

Mechanism – hemoglobin self-quenching

My data demonstrate that the quenching of Hb autofluorescence takes place at Hb concentrations > 10 g/L. The observed quenching of fluorescence in concentrated solutions of Hb is a specific example of inner-filter effect that takes place in concentrated fluorophore solutions. Non-linear relationship between fluorescence and Hb concentration at concentrations > 330 g/L can be explained by numerous difficulties when working with extremely viscous Hb solutions: pipetting errors, rapid drying of concentrated Hb samples that are in contact with air, and non-linearity of fluorescence detection by the instrument at such high concentrations. Further evidence for Hb self-quenching in this study is higher absolute value of fluorescence for lysed RBCs than intact RBCs (Figure 3-3). Although in both cases the same amount of Hb is added to the same volume of a solution, when Hb is entrapped inside intact RBCs, its concentration is much higher (normal range is 310-370 g/L for adult blood and 300-360 g/L for cord blood) [42] than in Hb solution prepared with lysed RBCs. Inner filter effect may also explain the non-linearity of fluorescence increase as a function of RBC concentration in Figure 3-9. Initially, the intensity of fluorescence increases with increasing concentration of fluorophore (RBCs), however, after reaching a certain critical fluorophore concentration, fluorescence slowly levels out.

The Hb self-quenching phenomenon observed in the present study can explain the direct relationship between RBC autofluorescence and RBC volume.

When RBC swells in hypotonic solutions, intracellular Hb concentration decreases, which results in increase in fluorescence. Similarly, when RBC shrinks in hypertonic solutions, intracellular Hb concentration increases, which causes self-quenching and, hence, decrease in fluorescence. Hirsch et al. showed that there is no dependence of intrinsic Hb fluorescence upon Hb concentration at concentrations higher than ~0.16 mM Hb [32]. However, they only tested Hb concentrations up to 1.4 mM Hb, which is equivalent to approximately 0.01 g/L (considering that molar mass of Hb tetramer is 64.458 g/mol [43]). In this study, concentration quenching of Hb fluorescence was observed at much higher concentrations (>10 g/L). Moreover, the differences in results could be explained by the fact that, unlike in this study, Hirsch et al. (1980) used front face fluorescence optics [32]. Front-face fluorometry is designed for samples with high viscosity, and emission intensity of the fluorophore is almost independent of the total absorbance of the sample [44].

Differences in fluorescence kinetics of cord and adult RBC

My results indicated several differences between the osmotic responses of adult and cord RBCs. Compared with adult RBCs, cord RBCs reached an equilibrium volume in anisotinic NaCl solutions more slowly. This is consistent with data published by Agre et al. (1994), who showed that cord RBCs are approximately 1.3-1.4 times less permeable to water compared with adult RBCs [9]. I also

observed that the fluorescence intensity measured with cord RBCs is higher than that of adult RBCs. In this regard, Hirsch et al. (1980) reported that the intensity of emitted fluorescence from HbF is 1.5-2 times larger compared with HbA, perhaps due to an additional tryptophan residue present in the HbF structure [32].

This method is easy to perform, does not require any treatment of cells with fluorescent dyes, and overcomes the limitations of traditional methods. Of particular importance is the ability of this method to measure osmotic permeability of RBCs from umbilical cord blood to water and cryoprotectants, data critical for designing an optimized cryopreservation protocol for cord RBCs [4-7, 45]. Such a protocol would facilitate long-term storage of cord RBCs without a decrease in quality, and would potentially result in a superior blood product for intrauterine and neonatal transfusions. The method will also be used to expand present understanding of changes in RBC permeability during hypothermic storage. Moreover, the method has numerous clinical applications. Erythrocyte permeability is altered in many hereditary hematological disorders, such as sickle cell anemia [46], hereditary spherocytosis, stomatocytosis, and xerocytosis [47], as well as infectious diseases like malaria [48]. Improved methods to measure RBC permeability may result in faster diagnosis and better understanding of these disorders.

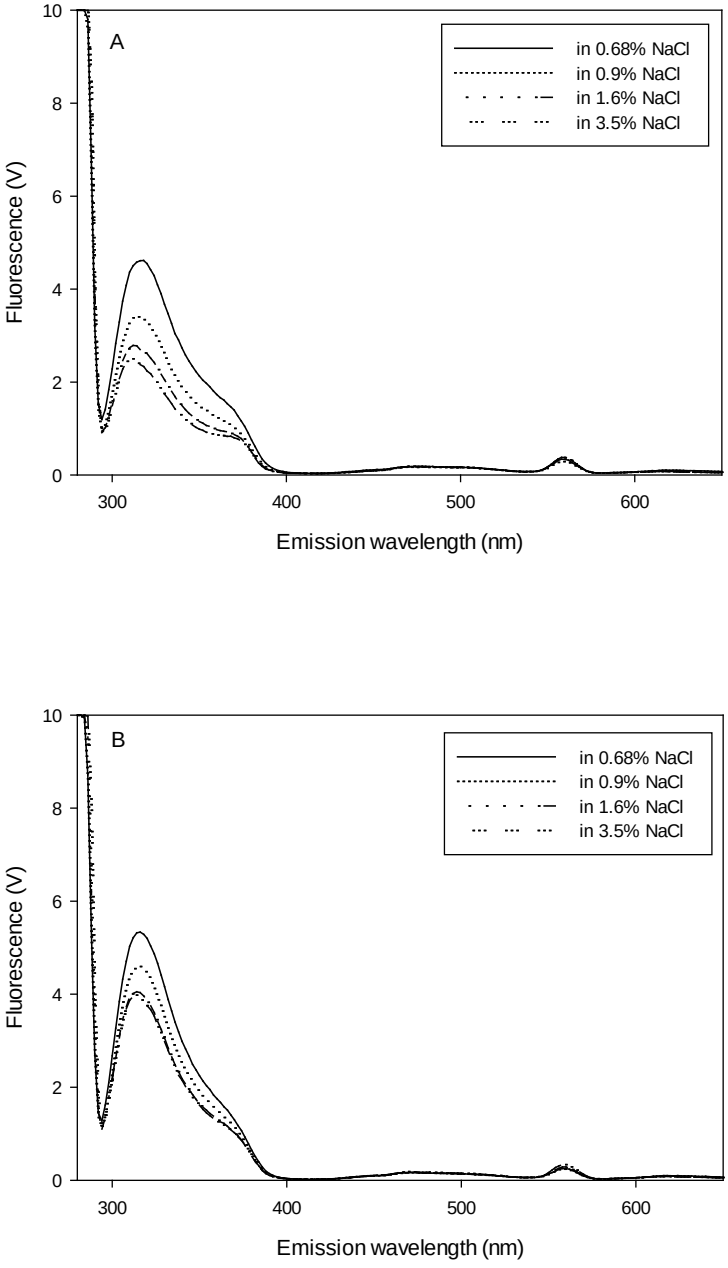

Figure 3-1. Emission spectra for RBC autofluorescence. Emission spectra of adult (A) and cord RBC (B) autofluorescence were determined on stopped-flow analyzer. RBCs were suspended in NaCl solution of the following concentrations: 0.68% (w/v), 0.9% (w/v), 1.6% (w/v), and 3.5% (w/v). Fluorescence was excited at 280 nm, and the range of emission wavelengths (280 to 650 nm) was scanned with 2 nm step. Figures show one representative sample for adult and cord RBCs.

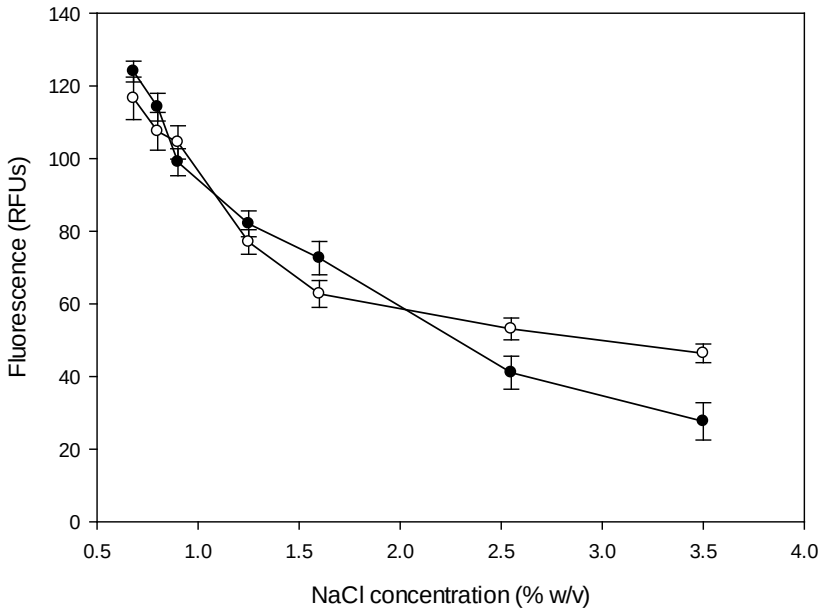

Figure 3-2. Equilibrium autofluorescence of adult (●) and cord (○) RBCs in NaCl solutions of different tonicity. RBCs were suspended in NaCl solutions of the following concentrations: 0.68% (w/v), 0.8% (w/v), 0.9% (w/v), 1.25% (w/v), 1.6% (w/v), 2.55% (w/v), and 3.5% (w/v). Samples were equilibrated at room temperature for approximately 5 minutes. On spectrofluorometer, fluorescence was excited at 280 nm, and emission was measured at 340 nm with 325 nm filter. Autofluorescence of NaCl buffers was also measured and subtracted from RBC autofluorescence as a background. Data is mean ± SEM of three samples, each measured in triplicate. RFUs – Relative Fluorescence Units.

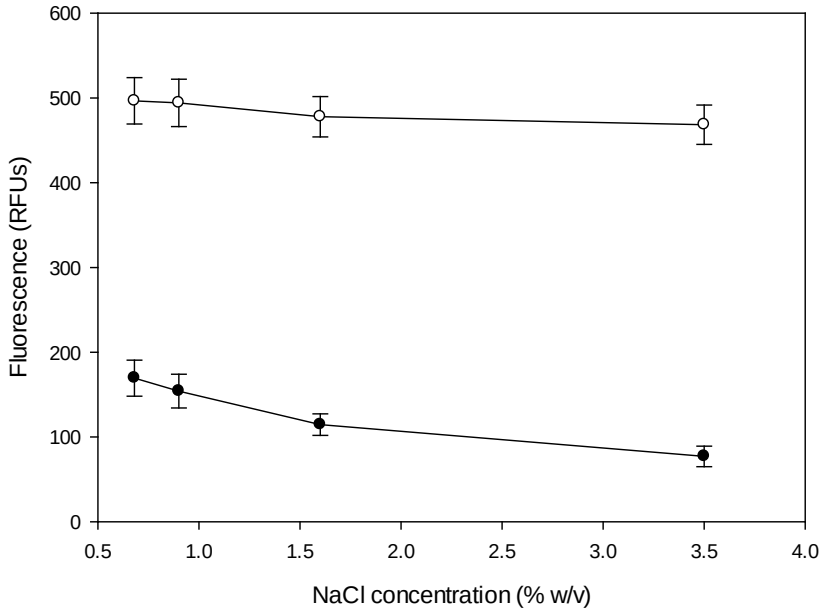

Figure 3-3. Equilibrium autofluorescence of adult RBCs (●) and adult hemoglobin (○) in NaCl solutions of different tonicity. See Materials and Methods section for preparation of adult hemoglobin (lysed RBCs). Autofluorescence was measured in the same fashion as for intact RBCs. Data is mean ± SEM. N=3, each sample measured in triplicate.

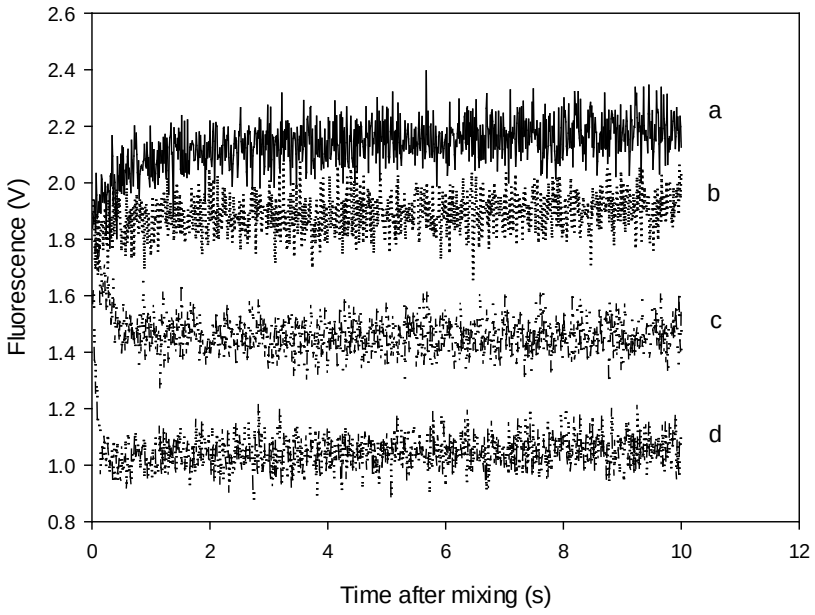

Figure 3-4. Kinetics of autofluorescence of adult RBCs upon exposure to NaCl solutions of the following concentrations: a - 0.68% (w/v), b - 0.9% (w/v), c - 1.6% (w/v), and d - 3.5% (w/v). To achieve these extracellular NaCl concentrations, RBCs suspended in 0.9% (w/v) NaCl were rapidly mixed 1:1 with 0.46% (w/v), 0.9% (w/v), 2.3% (w/v), and 6.1% (w/v) NaCl, respectively. In the stopped-flow system, fluorescence was excited at 280 nm, and emission was acquired at 314 nm. Autofluorescence kinetics of NaCl buffers was also measured and subtracted from RBC autofluorescence as a background. Figure shows one representative adult RBC sample. Data points are means of tree replicates.

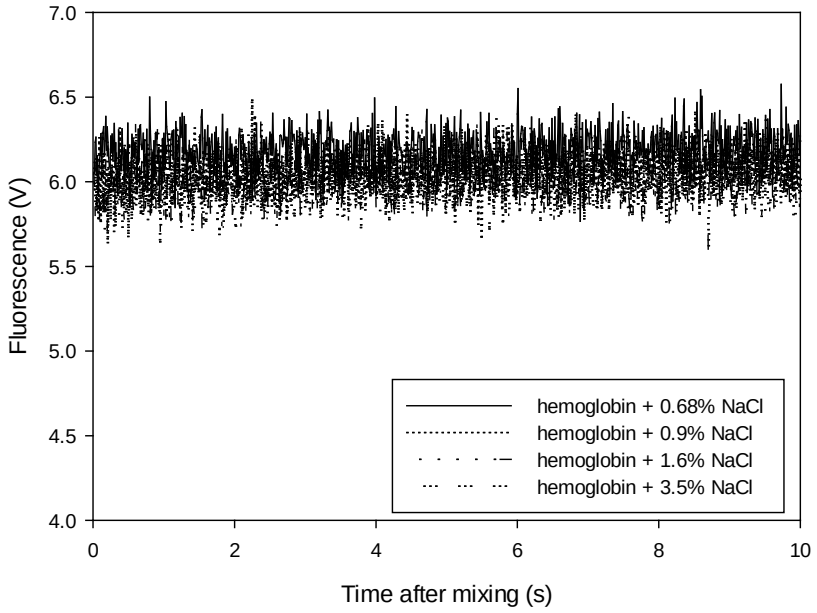

Figure 3-5. Kinetics of autofluorescence of adult hemoglobin upon exposure to NaCl solutions of the following concentrations: 0.68% (w/v), 0.9% (w/v), 1.6% (w/v), and 3.5% (w/v). See Materials and Methods section for preparation of adult hemoglobin (lysed RBCs). Autofluorescence was measured in the same fashion as for intact RBCs. Figure shows one representative adult hemoglobin sample. Data points are means of tree replicates.

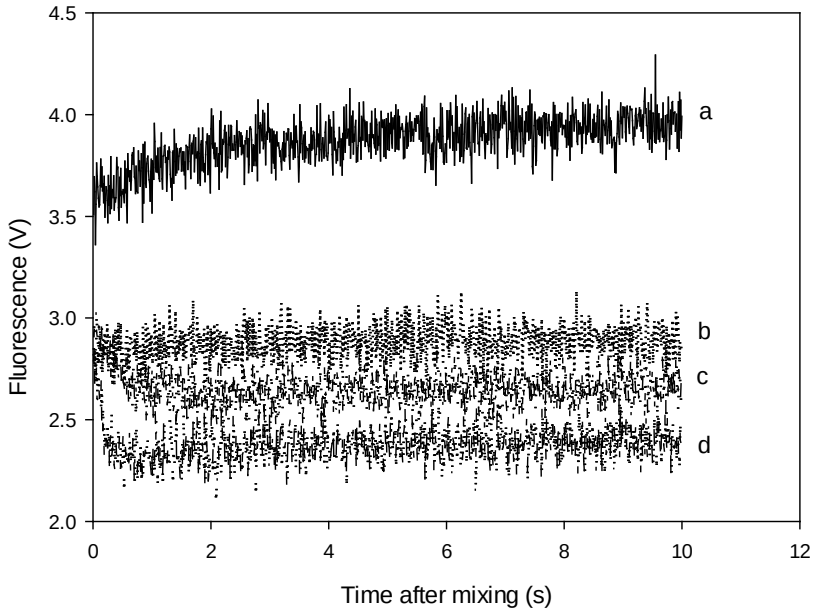

Figure 3-6. Kinetics of autofluorescence of cord RBCs upon exposure to solutions of different tonicity. Kinetics of cord RBC autofluorescence was recorded upon exposure to NaCl solutions of the following concentrations: a - 0.68% (w/v), b - 0.9% (w/v), c - 1.6% (w/v), and d - 3.5% (w/v). To achieve these extracellular NaCl concentrations, RBCs suspended in 0.9% (w/v) NaCl were rapidly mixed 1:1 with 0.46% (w/v), 0.9% (w/v), 2.3% (w/v), and 6.1% (w/v) NaCl, respectively. In the stopped-flow system, fluorescence was excited at 280 nm, and emission was acquired at 315 nm. Autofluorescence kinetics of NaCl buffers was also measured and subtracted from RBC autofluorescence as a background. Figure shows one representative adult RBC sample. Data points are means of three replicates.

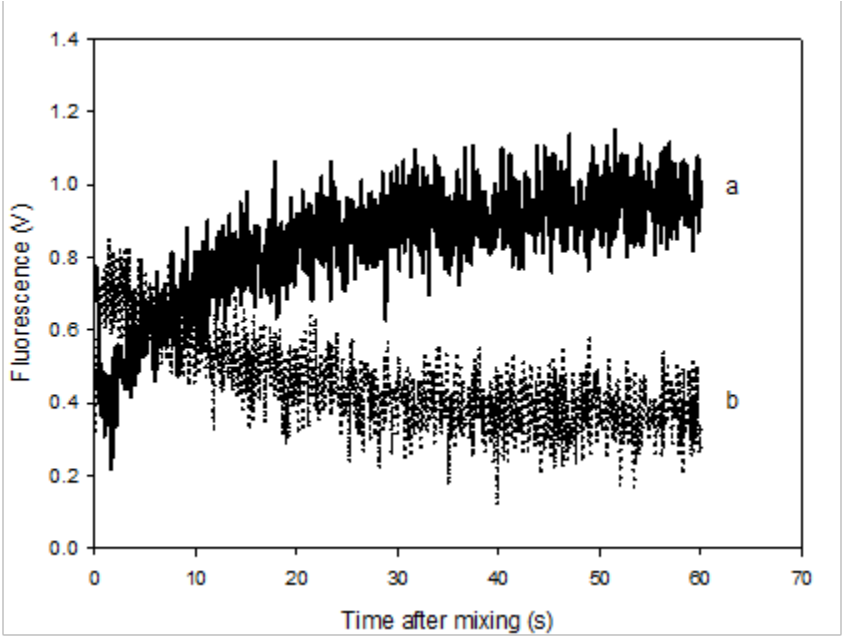

Figure 3-7. Rapid kinetic measurements of adult RBC autofluorescence upon addition and removal of glycerol. (a) – RBCs were exposed to 1.75% (w/v) glycerol. This was achieved by rapid 1:1 mixing of RBCs suspended in 0.9% (w/v) NaCl with 3.5% (w/v) glycerol. (b) – RBCs loaded with 3.5% (w/v) glycerol were rapidly mixed 1:1 with 0.9% (w/v) NaCl. In the stopped-flow system, fluorescence was excited at 280 nm, and emission was acquired at 314 nm. See Materials and Methods section for more details on how the curves were generated. Each curve demonstrates one representative adult RBC sample.

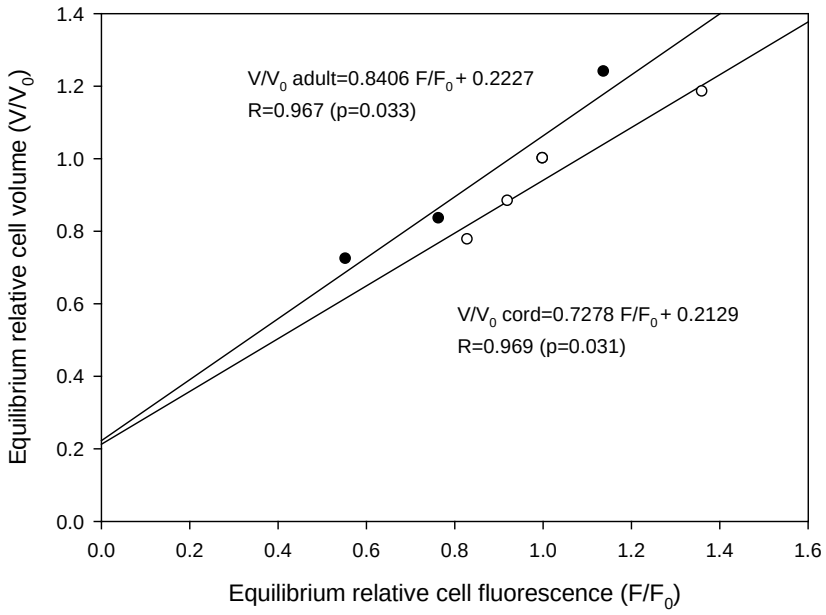

Figure 3-8. Correlation between RBC volume and RBC autofluorescence. Equilibrium RBC volumes in NaCl solutions of various tonicities were measured on Coulter Counter (see Materials and Methods section for details on the procedure). Equilibrium RBC autofluorescence in NaCl solutions of various tonicities was obtained using stopped-flow system. See Materials and Methods section for details on calculating relative volume and relative autofluorescence. Regression line was fitted to adult (●) and cord (○) RBC data. The strength of the relationship between relative RBC volume and relative RBC autofluorescence was assessed through Pearson's correlation analysis in SPSS Statistics Software. N=2 (one cord and one adult RBC sample). R – Pearson's correlation coefficient.

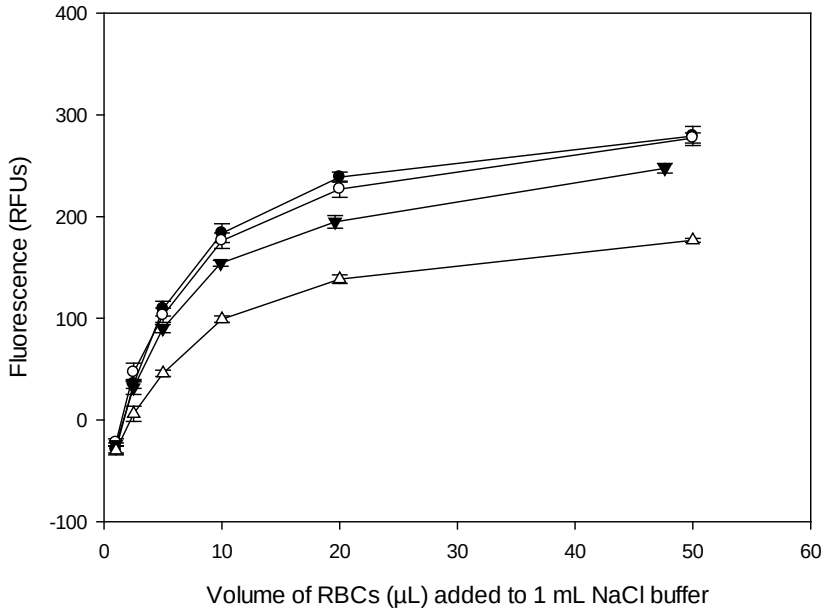

Figure 3-9. Equilibrium autofluorescence of adult RBCs as a function of RBC concentration. Various volumes of adult RBCs (55-65% hematocrit) were suspended in ● - 0.68% (w/v) NaCl, ○ - 0.9% (w/v) NaCl, ▼- 1.6% (w/v) NaCl, and Δ - 3.5% (w/v) NaCl . Samples were equilibrated at room temperature for approximately 5 minutes. On spectrofluorometer, fluorescence was excited at 280 nm, and emission was measured at 340 nm with 325 nm filter. Autofluorescence of NaCl buffers was also measured and subtracted from RBC autofluorescence as a background. Data points are means of three replicates. RFUs – Relative Fluorescence Units.

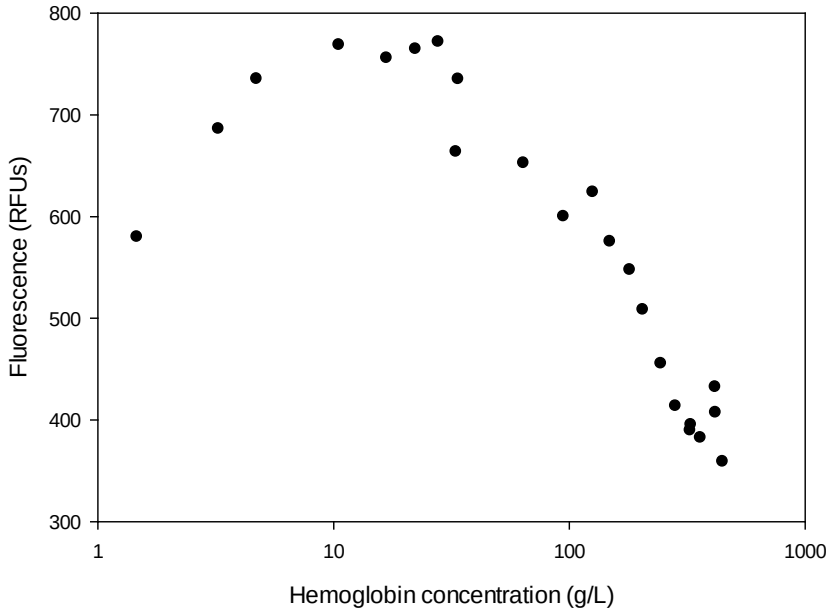

Figure 3-10. Autofluorescence of hemoglobin solution as a function of hemoglobin concentration. Various concentrations of adult hemoglobin were prepared as described in Materials and Methods section. Hemoglobin concentration in each dilution was measured by Drabkin's method. Fluorescence intensities of hemoglobin dilutions were then measured on spectrofluorometer (λex 280 nm, λem 340 nm, cut off 325 nm).

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Chapter 4

Osmotic parameters of red blood cells from umbilical cord blood

4.1 Introduction

The practice of transfusions of RBCs derived from umbilical cord blood to treat neonatal anemia has recently gained a lot of interest [1-10]. Fetal and neonatal anemias are among the most serious complications of pregnancy and postnatal development. The most commonly used treatments for anemia in fetuses and newborns are transfusions of red blood cells (RBCs), either intrauterine [11] or intravenous [12-15], to help replace the lost RBCs of the fetus or neonate. RBCs used in intrauterine and neonatal (intravenous) transfusions are derived from adult donors [11-16]. Adult RBCs are different from those present in the blood of a fetus or neonate [17-22]. The practice of administering adult RBC transfusions to premature infants has been associated with a number of complications, such as retrolental fibroplasia [23-25] and bronchopulmonary dysplasia [26-28], usually caused by the delivery of unnecessarily high amounts of oxygen to tissues.

Neonatal RBCs obtained from umbilical cord blood (cord RBCs) may offer a superior alternative for intrauterine and neonatal transfusions [29, 30]. Cord RBCs are usually discarded during isolation of stem cells from cord blood [31-33]. Due to the high concentration of fetal hemoglobin (HbF), which is practically absent in adult RBCs, cord RBCs have a potential to deliver more

physiological amounts of oxygen to fetal and neonatal tissues upon transfusion [34]. A number of studies have demonstrated that transfusions of autologous cord RBCs are both safe and effective in the treatment of anemic neonates [1-7].

Unlike RBCs from adult blood, cord RBCs deteriorate quickly during traditional storage at 1-6°C [3, 35] and would benefit from low temperature preservation. Despite several reports describing the effect of cryopreservation on cord RBCs [36, 37], an effective cryopreservation method for cord RBCs still needs to be developed.

Cryopreservation – preservation of cells and tissue at subzero temperatures below which biochemical reactions do not occur – is an effective means to preserve the quality of biological material for clinical transplantation and research purposes [38]. To achieve successful cryopreservation of cells, efforts typically target avoidance of the two primary causes of damage, termed freezing injury. If cells are cooled slowly, the formation of ice crystals in the extracellular space increases the concentration of extracellular solutes which causes the osmotic efflux of water from the cell, concentrating intracellular solutes. This is a potential source of cell damage due to solute toxicity. If cells are cooled rapidly, there is not enough time for water to leave the cell, which leads to the supercooling of the cell cytoplasm and the formation of ice crystals inside the cell [39]. To prevent damage to cells during exposure to such low temperatures, cryoprotectants are used. Glycerol is a common cryoprotectant used in cryopreservation of red blood cells [40].

To design effective cryopreservation procedures for cord RBCs, it is critical to know the osmotic parameters of these cells [41-44]. A cell undergoes a number of osmotic changes during cryopreservation. Addition and removal of cryoprotectant, as well as freezing and thawing, cause changes in solute concentration inside and outside the cell and induce flux of water and permeating solutes across the cell membrane. Such changes in intra- and extracellular osmolality result in corresponding changes in cell volume. A number of osmotic parameters define the movement of water and solutes across the cell membrane. Hydraulic conductivity (L_p) and osmotic permeability to solutes (P_s) describe the rate at which these substances cross the cell membrane and are typically determined by measuring the rate of cell volume changes when placed in an anisotonic environment. The osmotically inactive fraction (*b*) is the fraction of the cell volume that does not participate in the osmotic response of the cell. Arrhenius activation energy (E_a) describes the temperature dependence of the membrane permeability to water and solutes [45]. These osmotic parameters are used in mathematical modelling to predict optimized cryopreservation protocol for the cells of interest [46-48].

It has been reported, that, compared to adult RBCs, cord RBCs are less permeable to water [49], have higher activation energy for osmotic water permeability [49], and are less permeable to some solutes, in particular common cryoprotectant glycerol [50]. Reports on cord RBC osmotic parameters are very limited at present. Moreover, being measured with different experimental techniques and under different experimental conditions, the absolute values of

cord RBC osmotic parameters differ significantly between reports and, therefore, can not be used for mathematical modelling.

The objective of this study is to measure and compare the osmotic parameters of adult and cord RBCs, such as osmotically inactive fraction, permeability to water and glycerol, and Arrhenius activation energies for these processes.

4.2 Materials and Methods

Experimental samples

I used two sources of RBCs for this study: RBCs from peripheral blood of adult donors (adult RBCs), and RBCs from umbilical cord blood (cord RBCs). The Canadian Blood Services Network Center for Applied Development in Vancouver provided adult RBCs for my experiments. RBCs were leukocyte-reduced, stored in CPD (citrate-phosphate-dextrose) anticoagulant and SAGM (saline-adenine-glucose-mannitol) preservative at 1-6°C, and were used in experiments within 15 days of collection. The hematocrit of adult RBCs was standardized to 60 ± 2%, if necessary, by the removal of supernatant or addition of saline. The Alberta Cord Blood Bank supplied cord RBCs, a waste product after stem cell isolation from umbilical cord blood. Cord blood collected from a placenta was stored at room temperature for up to 38 hours prior to stem cell isolation, which was previously

shown not to result in a decrease in RBC quality (Chapter 2) [51]. The leftover cord RBC product was washed with saline 3 times by centrifugation at 2200 g, 4°C for 5 minutes to remove any residual pentastarch used in the stem cell isolation from cord blood [52]. The hematocrit of cord RBCs was then adjusted to 60 ± 2% by the addition of saline to the RBC pellet. Cord RBCs were stored in our laboratory at 1-6°C and used in experiments within 24 hours of isolation from cord blood. Ethics approval for the study was obtained from the University of Alberta Health Research Ethics Board (Biomedical Panel) and Canadian Blood Services Research Ethics Board.

Experimental solutions

Sodium chloride (NaCl) solutions were prepared by diluting 12% (w/v) NaCl stock solution (Baxter, Deerfield, IL, USA) with distilled water to yield final concentrations (% (w/v)) of 0.68, 0.9, 1.6, and 3.5. Phosphate-buffered saline (PBS) solutions were prepared by diluting 10x PBS solution (Calbiochem, Gibbstown, New Jersey) with distilled water to 0.5x, 1x, 3x, 5x, and 7x PBS. 5% (w/v) glycerol in 1x PBS solution was prepared by diluting 50 g glycerol (99.5+%, Sigma Aldrich, Inc., St. Louis, MO, USA) and 100 mL 10x PBS with distilled water to 1 L.

Osmolality of experimental solutions was measured with a freezing-point depression osmometer Osmette (Precision Systems Inc., Natick, Massachusetts).

Prior to each experimental run, the osmometer was calibrated with 290 mmol/kg Opti-Mole standard (Wescor, Inc., Logan, Utah) and 1500 mOsm/kg standard (Precision Systems Inc., Natick, Massachusetts).

Hemolysis of RBCs in experimental solutions

RBC hemolysis (membrane damage) was measured in experimental PBS and glycerol solutions. 50 μL RBCs were pipetted into 1 mL experimental solution and were allowed to equilibrate at room temperature for approximately 5 minutes. RBC hemolysis was determined by spectrophotometric measurement of total and supernatant cyanmethemoglobin according to Drabkin's method [53, 54]. Controls for total hemoglobin were prepared from Stanbio Tri-Level Hemoglobin controls (Stanbio Laboratory, Boerne, TX, USA). The hematocrit of the RBC sample was measured, using a microhematocrit centrifuge (Hettich, Tuttlingen, Germany), as the ratio of the volume occupied by packed RBCs to the volume of a whole RBC sample [55].

Measurement of RBC volume kinetics on stopped-flow

Changes in RBC volume upon exposure to different osmolalities were determined by monitoring changes in intrinsic hemoglobin fluorescence intensity according to

the method described in the Chapter 3. In a SX20 stopped-flow reaction analyser (Applied Photophysics, Ltd., Leatherhead, UK), RBC suspension was rapidly mixed with equal volume of anisotonic experimental solution to induce osmotically-driven changes in RBC volume. RBC fluorescence intensity, which is directly related to RBC volume, was recorded simultaneously as a function of time after mixing.

The RBC suspension for stopped-flow experiments was prepared by adding 20 μL RBCs to 1 mL of 1x PBS (final osmolality of 287 mOsm/kg). To determine hydraulic conductivity, RBCs were exposed to 0.75x, 2x, 3x, and 4x PBS solution (final osmolalities of 214, 562, 832, and 1112 mOsm/kg, respectively). To achieve these extracellular PBS concentrations, RBC suspension (in 1x PBS) was rapidly mixed in a 1:1 ratio with 0.5x, 3x, 5x, and 7x PBS, respectively. 1000 data points were collected during the 10 s period immediately following mixing. As a control, RBCs exposed to 1x PBS (osmotic equilibrium conditions) were assessed.

To determine glycerol permeability, RBCs were exposed to 2.5% (w/v) glycerol (final osmolality of 578 mOsm/kg) by rapid 1:1 mixing with 5% (w/v) glycerol in 1x PBS solution. 2000 data points were collected during 120 s period immediately following mixing to allow for the complete equilibration of glycerol on both sides of RBC membrane. As a control, RBCs were mixed 1:1 with 1x PBS (osmotic equilibrium conditions) and fluorescence measured as a function of time.

Background fluorescence of buffer solutions (without RBCs) was also measured. All samples were measured in triplicate. Prior to each experimental run, the stopped-flow circuit was flushed with the solution of the same osmolality as the one to be tested. This was done to prevent cross-contamination between experimental solutions and maintain the accurate osmolality during each experimental run.

SX20 instrument settings. The sample cell was illuminated at 280 nm, and emission was measured at 314 nm (adult RBCs) or 315 nm (cord RBCs). See Chapter 3 for the information of how these optimized emission wavelengths for adult and cord RBCs were determined. Excitation and emission slit widths were set to 3 mm (equivalent to a wavelength bandwidth of 13.95 nm). Total stopped-flow drive volume was set to approximately 120 μL. The 20 μL optical cell had a 10 mm pathlength and 1 ms dead time (during which mixing occured). Fluorescence was expressed in Volts (V). Data were acquired using ProData SX software (Applied Photophysics, Ltd., Leatherhead, UK).

To determine Arrhenius activation energy, measurements of the kinetics of osmotically-induced changes in RBC volume were performed at 3 different temperatures (mean ± SEM): 3.8 ± 0.01°C (3.5-4.0°C range), 19.4 ± 0.06°C (18.6-21.0°C range), and 35.2 ± 0.02°C (34.8-35.6°C range) for adult RBCs; 3.7 ± 0.01°C (3.5-3.9°C range), 20.4 ± 0.03°C (19.7-21.2°C range), and 35.3 ± 0.01°C (35.1-35.7°C range) for cord RBCs. Temperature of the stopped-flow system was controlled through an attached water-filled circulator (CH/P temperature control

system, Forma Scientific, Marietta, Ohio). Syringes containing RBC samples and experimental solutions were also equilibrated in a water bath, set to experimental temperature, for a minimum of five minutes before each run.

Conversion from fluorescence to volume

1. *Normalization of fluorescence kinetics curves (background subtraction)*

   For analysis of hydraulic conductivity, fluorescence of PBS buffer was averaged over 10 seconds. This value was then subtracted from each data point on the curve representing fluorescence of RBCs mixed with this buffer.

   For analysis of glycerol permeability, fluorescence of 2.5% (w/v) glycerol and 1x PBS buffers was averaged over 10 seconds. These values were then subtracted from fluorescence of RBCs mixed with 2.5% (w/v) glycerol and 1x PBS (osmotic equilibrium conditions, control), respectively.

2. *Conversion from fluorescence to volume*

   A linear regression was used to convert relative fluorescence ($\frac{F}{F_0}$) to relative volume ($\frac{V}{V_0}$).

$$\frac{V}{V_0} = m\frac{F}{F_0} + c, \qquad \text{(Eq. 4-1)}$$

where V is the equilibrium RBC volume at an experimental osmolality, V_0 is the isotonic RBC volume, F is the equilibrium RBC fluorescence at an experimental osmolality, and F_0 is the isotonic RBC fluorescence, m is the slope, and c is the y intercept.

Equilibrium RBC volumes in anisotonic NaCl solutions were measured on Coulter Electronic Particle Counter (ZB1, Coulter Electronics, Inc., Hialeah, FL, USA) equipped with a pulse-height analyzer (The Great Canadian Computer Company, Spruce Grove, AB, Canada) [56]. Isotonic volume of RBCs was measured on Coulter A^{c}T Series Analyzer (Beckman Coulter, Inc., Brea, California). Equilibrium RBC autofluorescence in NaCl solutions of various tonicities was obtained using stopped-flow system. Data for adult and cord RBCs were fit to equation 4-1 to derive values for m and c.

For adult RBCs, m=0.8406 and c=0.2227. For cord RBCs, m=0.7278 and c=0.2129 (see Chapter 3, Figure 4-8).

For analysis of hydraulic conductivity, each data point on the normalized curve representing fluorescence of RBCs mixed with PBS was converted to RBC volume using equation 4-1.

Analysis of glycerol permeability was performed in three steps. First, each data point on the normalized curves representing fluorescence of RBCs mixed

with 2.5% (w/v) glycerol and 1x PBS was converted to RBC volume using equation 4-1 (Figure 4-4 A). Second, each data point on the osmotic equilibrium curve (RBCs + 1x PBS) was subtracted from each corresponding data point on the curves representing fluorescence of RBCs mixed with 2.5% (w/v) glycerol (RBCs + 2.5% glycerol) (Figure 4-4 B). Third, isotonic RBC volume was added to each data point on the curve generated in step 2 (Figure 4-4 C).

Subtraction of equilibrium curve in step 2 was done because even under osmotic equilibrium conditions (no anticipated change in RBC volume and, hence, fluorescence) RBC fluorescence was increasing over time in a stopped-flow optical cell (Figure 4-4 A). As a result, over 120 seconds acquisition time fluorescence of RBCs in 1x PBS and 2.5% glycerol kept increasing, never reaching plateau. This can potentially be caused by the gradual settling of RBCs in an optical cell. As a result, increase in RBC concentration in the bottom part of RBC suspension can lead to an increase in fluorescence intensity (see Figure 3-9 for demonstration of this phenomenon). To adjust for this gradual increase in fluorescence intensity and to be able to see RBC fluorescence reach plateau once RBCs are in osmotic equilibrium with glycerol, the above-mentioned adjustment was performed.

Addition of isotonic cell volume in step 3 was done because subtraction of equilibrium curve from kinetics curve often brings the resulting curve to zero or negative value. Adding isotonic cell volume preserves the right shape of the kinetics curve, while bringing it back to the right RBC volume range.

Determination of osmotic parameters

Osmotically inactive fraction

Osmotically inactive fraction of adult RBCs was taken from the literature [57] and was equal to 0.51 ± 0.02. Osmotically inactive fraction of cord RBCs was calculated using Boyle-van't-Hoff equation:

$$\frac{V}{V_0} = (1 - b)\frac{\pi_0}{\pi} + b\,, \qquad \text{(Eq. 4-2)}$$

where V is the equilibrium cell volume at an experimental osmolality (π), V_0 is the isotonic cell volume at an isotonic osmolality (π_0), and b is the osmotically inactive fraction of the cell volume. To determine osmotically inactive fraction of cord RBCs, equilibrium volumes of cord RBCs were measured at different osmolalities. All measurements were done at room temperature. Boyle-van't-Hoff plot was then created, which is the equilibrium relative RBC volume ($\frac{V}{V_0}$) as a function of inverse relative osmolality ($\frac{\pi_0}{\pi}$). Osmotically inactive fraction of cord RBC volume was determined from y-intercept of the linear regression line [57].

Equilibrium volumes of cord RBCs in NaCl solutions were determined on a Coulter Electronic Particle Counter. RBCs were diluted in 10 mL of 0.68%, 0.9%, 1.6%, and 3.5% (w/v) NaCl to approximately 20000 cells/mL and were

allowed to equilibrate at room temperature for at least 5 min. Current pulses, proportional to cell volumes, were measured as RBCs were passing through a 50 μm aperture. Measurements were made in triplicate in each experimental solution. At each NaCl concentration, 5 μm latex beads (Beckman Coulter, Inc., Fullerton, CA, USA) with known bead volume were used for calibration. Thereafter, calculated calibration factor was used to convert instrument output data to RBC volumes [45].

Hydraulic conductivity and permeability to glycerol

The following equation by Jacobs and Stewart describes the rate of cell volume change when the cell is exposed to anisotonic conditions and was used to calculate hydraulic conductivity [43, 45]:

$$\frac{dV}{dt} = L_p ART\left(\left(C_s^i - C_s^e\right) + \left(C_i^i - C_i^e\right)\right), \quad \text{(Eq. 4-3)}$$

where V is the cell volume (μm³), t is the time (min), L_p is the hydraulic conductivity (μm/min/atm), A is the cell surface area, either constant at a specified value, or area of a sphere with volume of the cell (μm²), R is the gas constant (L-atm/mole/K), T is the absolute temperature (K), C_s^i, C_s^e are the intracellular, extracellular concentration of permeant solute (Moles), C_i^i, C_i^e are the intracellular, extracellular concentration of impermeant solute (Moles).

Glycerol movement across cell membrane as a function of time was calculated using the following equation [43, 45]:

$$\frac{dS}{dt} = P_s A\left(C_s^e - C_s^i\right), \qquad \text{(Eq. 4-4)}$$

where S is the Moles of glycerol, t is the time (min), P_s is the glycerol permeability (μm/min), A is the cell surface area, either constant at a specified value, or area of a sphere with volume of the cell (μm²), C_s^i, C_s^e are the intracellular, extracellular concentration of glycerol (Moles).

Experimental data on the kinetics of RBC volume in PBS and glycerol solutions was fit to equations (4-3) and (4-4) using the least squared method in Excel Solver. The following assumptions were made: solutions are dilute, the density of water is 1 kg/m^3, cell surface area is calculated from the volume of the cell, and the reflection coefficient is zero (meaning there is no interaction between water and glycerol transport).

Arrhenius activation energies

Arrhenius activation energy (E_a) describes the temperature dependence of hydraulic conductivity and solute permeability. E_a was determined from the slope

of the plot of the natural logarithm of L_p or P_s as a function of the inverse temperature ($\frac{1}{T}$) [43, 45]:

$$L_p or\ P_s = k * exp\left(\frac{-E_a}{RT}\right), \quad \text{(Eq. 4-5)}$$

where L_p is the hydraulic conductivity (μm/min/atm), P_s is the solute permeability (μm/min), k is the fitting constant, R is the gas constant (l-atm/mole/K), E_a is the activation energy for L_p or P_s (kcal/mol), and T is the absolute temperature (K).

Comparison between L_p (μm/min/atm) and P_f (cm/s)

The majority of published papers report values for RBC osmotic water permeability as P_f (cm/s). P_f can be determined from the following equation:

$$\frac{dV/V_0(t)}{dt} = P_f \times SAV \times MVW \times \left\{\left[C_{in(t=0)}/\frac{V}{V_0}(t)\right] - C_{out}\right\}, \quad \text{(Eq. 4-6)}$$

where $V/V_0(t)$ is relative volume of the vesicle as a function of time, P_f is osmotic water permeability in cm/s, SAV is the vesicle surface area to volume ratio,

MVW is the molar volume of water (18 cm^3/mol), $C_{in(t=0)}$ is the initial intravesicular osmolality, and C_{out} are the extravesicular osmolality [58].

L_p (μm/min/atm) can also be converted to P_f (cm/s) using the equation below [59]:

$$P_f = L_p \times (273.15 + °C) \times 7.5926 \times 10^{-6} \quad \text{(Eq. 4-7)}$$

To evaluate the impact of the method of analysis (L_p vs P_f), I analyzed a small subset of my RBC volume kinetics data using both equation (4-3) and (4-6). L_p and P_f were determined for one adult RBC sample (ran in triplicate) at three experimental temperatures (4.0, 19.0, and 35.2°C). To determine P_f, experimental data on the kinetics of RBC volume in PBS solutions was fit to equation (4-6) using the least squared method in Excel Solver. L_p was calculated using equation (4-3) and converted to P_f values using equation (4-7).

Statistical analysis

Differences between experimental groups were analyzed with Mann-Whitney non-parametric test (hemolysis data), Student's T-test and one-way analysis of variance (ANOVA) (other data). Significance level was set to 0.05. The strength

of the relationship between RBC hemolysis and extracellular osmolality, hydraulic conductivity and experimental temperature, hydraulic conductivity and extracellular osmolality, and glycerol permeability and experimental temperature were investigated using correlation analysis (Pearson's correlation coefficients were calculated). Statistical analysis was performed using IBM SPSS Statistics software (version 19.0, SPSS, Inc., IBM Corporation, Armonk, New York).

Contributions of others

Personnel of Alberta Cord Blood Bank provided cord RBC samples for this study. Dr. Andy Holt (Department of Pharmacology, University of Alberta) granted me the access to the stopped-flow system for the duration of this study. Dr. Locksley McGann was involved in data analysis and calculations of hydraulic conductivity, glycerol permeability, and Arrhenius activation energy. Dr. Tom Churchill provided help with statistical analysis of the data.

4.3 Results

Integrity of red cells in experimental solutions

Upon equilibration in 0.75 – 4x PBS solutions, the range for hemolysis was 0.53-0.96% for adult RBCs and 0.83-1.5% for cord RBCs. Hemolysis significantly

increased with the osmolality of the solution for cord RBCs (r=0.580, p=0.023), however not for adult RBCs (r=0.326, p=0.255). No difference was found between the level of hemolysis of cord and adult RBCs in PBS solutions. In 2.5% (w/v) glycerol solution, adult RBCs showed lower hemolysis compared to cord RBCs (0.44 ± 0.02% vs 0.93 ± 0.17%, p=0.05) (Table 4-1).

Osmotic parameters of adult and cord RBCs

Osmotically inactive fraction of cord RBCs

The average isotonic cell volume was 93.8 ± 1.1 fL (mean ± SEM) for adult RBCs and 115.1 ± 1.5 fL (mean ± SEM) for cord RBCs as determined using Coulter A^cT Series Analyzer. Using measured equilibrium volumes of cord RBCs at different osmolalities, Boyle van't Hoff plot was created (Figure 4-1). Osmotically inactive fraction of cord RBCs determined from this plot was equal to 0.56 ± 0.04. The slope of the linear regression line was equal to 0.51 ± 0.05.

Hydraulic conductivity

Figure 4-2 shows a representative plot for the adult RBC volume kinetics upon exposure to 2x PBS at 3.8ºC. As can be seen from the plot, RBC volume reaches osmotic equilibrium within approximately 0.01 minute (0.6 second). Table 4-2 shows values for hydraulic conductivity of adult and cord RBCs in 562 mOsm/kg

(2x PBS) at three different experimental temperatures. Hydraulic conductivity increased with temperature in both adult RBC ($r=0.586$, $p<0.001$) and cord RBCs ($r=0.725$, $p<0.001$). Statistical analysis showed that hydraulic conductivity of cord and adult RBCs was the same at 3.8°C ($p=0.83$) and 19.9°C ($p=0.075$). Cord RBCs were more permeable to water at 35.3°C ($p<0.001$).

Table 4-3 shows values of hydraulic conductivity of adult and cord RBCs over the range of experimental osmolality (214-1112 mOsm/kg) measured at room temperature. Hydraulic conductivity of cord RBCs was significantly lower than that of adult RBCs in 214 mOsm/kg (0.75x PBS, $p<0.001$) and 1112 mOsm/kg (4x PBS, $p=0.009$). Hydraulic conductivities were equal in 562 mOsm/kg (2x PBS, $p=0.075$) and 832 mOsm/kg (3x PBS, $p=0.401$). Hydraulic conductivity significantly increased with extracellular osmolality in adult ($r=0.381$, $p<0.001$) and cord ($r=0.402$, $p<0.001$) RBCs. Increase in hydraulic conductivity was observed in 214-832 mOsm/kg (0.75x – 3x PBS) for adult RBCs and in 214-562 mOsm/kg (0.75x – 2x PBS) for cord RBCs. In 1112 mOsm/kg (4x PBS), hydraulic conductivity slightly dropped for adult ($p=0.004$) and cord RBCs ($p<0.001$).

Comparison between P_f values a) calculated using Zeidel's et al. method [58] and b) calculated by converting L_p values to cm/s is shown in Table 4-4. Converting L_p from μm/min/atm to cm/s always resulted in P_f value that was 50% larger than the one calculated using Zeidel's approach. This difference in methods

is important to know for future comparison between my data and the published literature.

Arrhenius activation energy for hydraulic conductivity was 4.8 kcal/mol for adult RBCs and 5.4 kcal/mol for cord RBCs (Figure 4-3). No statistically significant difference was found between activation energies for adult and cord RBCs ($p=0.275$).

Permeability to glycerol

Figure 4-4 C shows a representative plot for the adult RBC volume kinetics upon exposure to 2.5% (w/v) glycerol in 1x PBS solution at 20ºC. The first part of the curve represents RBC shrinking (V/V_0 decreases with time), as water leaves the cell. The second part of the curve represents RBC swelling (V/V_0 increases with time), as glycerol enters the cell, followed by plateau, as glycerol reaches equilibrium on both sides of RBC membrane. As can be seen from the plot, water efflux from RBC is complete within approximately 0.01 minute (0.6 second), and glycerol flux into RBC is complete within approximately 0.6 minutes (36 seconds).

Table 4-5 shows values of glycerol permeability ($P_{glycerol}$) for adult and cord RBCs at three experimental temperatures. Permeability to glycerol increased with temperature for both adult ($r=0.837$, $p<0.001$) and cord RBCs ($r=801$, $p<0.001$). $P_{glycerol}$ of cord RBCs was higher than that of adult RBCs at 3.8°C

($p<0.001$). Permeabilities of adult and cord RBCs to glycerol were equal at 20.2°C ($p=0.079$) and 35.3°C ($p=0.435$).

Arrhenius activation energies for glycerol permeability were 11.5 kcal/mol and 9.7 kcal/mol for adult and cord RBCs, respectively (Figure 4-5). Activation energy for cord RBCs was significantly lower than activation energy for adult RBCs ($p=0.0003$).

4.4 Discussion

In this study I determined osmotic permeabilities of cord and adult RBCs to water and the common cryoprotectant glycerol, as well as the Arrhenius activation energy for these processes. For this, I used a newly developed method that records fast kinetics of osmotically-induced RBC volume changes via intrinsic RBC hemoglobin fluorescence.

Integrity of red cells in experimental solutions

A RBC hemolysis assay demonstrated that ~99% RBCs were intact in experimental PBS and glycerol solutions and were thus able to respond to changes in extracellular osmotic gradient by changing cell volume. In 2.5% (w/v) glycerol

solution, hemolysis of cord RBCs was slightly increased compared with adult RBCs. Since cord RBCs are known to be more mechanically fragile than adult RBCs [60], increased hemolysis could have been the result of damage during processing, that involved triple centrifugation. It can also be due to the fact that cord RBCs are less stable during hypothermic storage (1-6°C) compared with adult RBCs, which results in higher levels of hemolysis [3, 35], especially in the absence of a hypotonic preservation solution.

Osmotically inactive fraction

The average isotonic cell volumes obtained for adult and cord RBCs in my study were in a good agreement with values reported in the literature [61]. I confirmed the well-established fact that volume of cord RBCs is larger than of adult RBCs. I also determined the osmotically inactive fraction for cord RBCs (b=0.56 ± 0.04), which, to my knowledge, has not been previously reported. Cord RBCs were found to have larger osmotically inactive fraction compared to that reported for adult RBCs (b=0.41-0.51) [57, 62, 63].

Temperature- and osmolality- dependence of osmotic parameters

The permeabilities of adult and cord RBCs to water and glycerol were strongly dependent on temperature. Particularly, hydraulic conductivity and glycerol permeability significantly increased with temperature in both adult and cord RBCs (Tables 2 and 5). Increase of water [45, 64, 65] and cryoprotectant permeability [43, 64] with temperature has also been previously reported for a variety of cell types and serves as the basis for the determination of Arrhenius activation energy. My results also suggested osmolality-dependence of hydraulic conductivity.

Literature data describing osmolality-dependence of hydraulic conductivity is controversial. Ross-Rodriguez showed that hydraulic conductivity of TF-1 cells did not change significantly with extracellular osmolality [45]. Rich at al. reported that hydraulic conductivity of human and dog erythrocytes decreased with increase in osmolality of the medium. Particularly, hydraulic conductivity of human erythrocytes decreased from 1.87 cm^3/dyne sec in 199 mOsm solution to 0.76 cm^3/dyne sec in 516 mOsm solution [66]. Liu et al. demonstrated the opposite trend in the relationship between hydraulic conductivity and extracellular osmolality in canine RBCs. Their data suggested that hydraulic conductivity increased when osmolality increased (r=0.35). Increase in hydraulic conductivity of dog RBCs with increase in osmolality was observed in 220-360 mOsm solutions. However, in 445 mOsm solution, a slight drop in hydraulic conductivity was observed [64]. My data is consistent with

observations of Liu et al. I showed that hydraulic conductivity of RBCs increased with increase in osmolality ($r=0.381$, $p<0.001$ for adult RBCs and $r=0.402$, $p<0.001$ for cord RBCs) over the range of experimental osmolalities. However, in PBS solution of very high osmolality (1112 mOsm/kg or 4x PBS), hydraulic conductivity decreased compared to values in lower PBS concentrations. The threshold of osmolality where hydraulic conductivity starts decreasing differs between these two studies. This may be attributed to the difference in the source of RBCs (dog vs human).

There are two possible reasons for such decrease in RBC L_p at very high osmolalities. Firstly, Farrant and Woolgar showed that at high extracellular concentrations of NaCl (1200 mmol/kg sodium) RBCs start leaking potassium and taking up sodium ions [67]. If this is the case, efflux of water from RBC upon exposure to 1112 mOsm/kg PBS solution will be counter-balanced by an influx of water into RBCs in the direction of osmotic pressure gradient, caused by increasing concentration of intracellular sodium ions. As a result, cell shrinking will be slower, and this will be reflected by lower value for hydraulic conductivity. Secondly, 4x PBS has been reported to be a lower threshold for RBC osmotic tolerance [68]. In solution of such high osmolality, osmotically-induced shrinking of RBCs can be limited (cell approaches its osmotically inactive volume) which, again, will be reflected by lower value for hydraulic conductivity.

Hydraulic conductivity and its activation energy

Differences in hydraulic conductivity between adult and cord RBCs varied depending on the experimental conditions (temperature and extracellular osmolality). Cord RBCs showed lower L_p than adult RBCs when subjected to extreme swelling or shrinking (in 214 and 1112 mOsm/kg solutions, Table 4-3). At the same time, when subjected to more normal range of cell volume changes (in 562 and 832 mOsm/kg solutions, Tables 2 and 3), L_p of cord RBCs was equal or higher than of adult RBCs (depending on the temperature).

Literature shows that, overall, hydraulic conductivity of fetal RBCs is lower than that of adult RBCs. Using stopped-flow by light scattering technique, Agre et al. measured hydraulic conductivity of adult and fetal RBCs (from neonates 28-40 weeks gestational age) upon doubling isotonic osmolality at 37°C. They reported that fetal RBCs were 1.4 times less permeable to water compared to adult RBCs. Reduced osmotic water permeability of fetal RBCs was correlated with lower expression of aquaporin CHIP protein – the major water transporter – in their membranes [49]. Sjolin determined hydraulic conductivities of adult and cord RBCs by measuring light scattering of cells suspended in prehemolytic and hemolytic NaCl concentrations. He reported that RBCs from cord blood were approximately 1.8 (hemolytic NaCl concentrations) and 2.5 (prehemolytic NaCl concentrations) less permeable to water compared to adult RBCs [69].

Possible link between deformability and hydraulic conductivity

In this study, water permeabilities were studied over a wider range of experimental conditions (temperature, extracellular osmolality) compared to the two studies mentioned above. Therefore, I was able to provide additional information and more in-depth understanding of the differences in this parameter between cord and adult RBCs. The fact that cord RBC L_p was lower than adult under conditions causing extreme cell volume exertions can be explained not only by the lower expression of aquaporins in cord RBC membrane [49], but also by the differences in erythrocyte deformability between cord and adult blood. It is possible that extreme swelling or shrinking introduced too much stress on cord RBC membrane and, being less deformable, cord RBCs could not reply to osmotic pressure gradient by the same extent of cell volume changes as adult RBCs could.

Studies show that unfractionated cord RBCs have the same [70, 71] or higher (if measured within one hour of cord blood collection) deformability [72] compared to adult RBCs. I did not measure deformability of cord and adult RBCs for this particular study, however previous data acquired in our laboratory suggests that cord RBCs used in this study had lower deformability compared to adult RBCs. In particular, the average deformability for cord RBCs was 0.446 ± 0.030 (mean ± SD) (Chapter 2) [51] and the average deformability for adult RBCs was 0.599 ± 0.034 (mean ± SD) [73]. There are several other reasons for lower deformability of cord RBCs used in this study. Cord RBC population is very heterogeneous compared to adult. When cord RBCs are separated by density, a

fraction of extremely dense erythrocytes (oldest, bottom) exhibit lower deformability than adult erythrocytes of the densest fraction, and least dense (youngest, top) cord erythrocytes are more deformable than least dense adult erythrocytes [70, 71]. When cord blood is processed for stem cell isolation, the middle (buffy coat) fraction containing leukocytes is removed for storage and with it a certain volume of RBCs from the top fraction (most deformable) is also removed [52]. Therefore, the leftover RBC product that I worked with potentially had a higher proportion of very dense, and therefore, less deformable RBCs compared to normal cord RBC population. Also, cord RBCs are known to assume echinocytic shape within hours after cord blood collection [72] and echinocytes have been shown to be less deformable than normal spheroid cells [74]. Since in this study RBCs were stored for up to 62 hours prior to being used in experiments, such extended storage time could have caused echinocytosis and, therefore, adversely affect rheological properties (deformability) of cord red cells.

Other factors that could have affected permeability of cord RBCs and cause discrepancies between my results and those reported in the literature are conditions of cord blood storage and processing, such as time, temperature, centrifugation speed, addition of pentastarch. Gestational age of neonate (pre-term, full term) was not controlled in this study and could have also affected the properties of red cell membrane.

Comparison between hydraulic conductivity (L_p) and osmotic water permeability (P_f)

The majority of published papers report values of adult RBC osmotic water permeability as P_f in cm/s. Table 4-6 summarizes the literature data on adult and cord RBC permeability. In order to compare my data to published data, I converted L_p values from μm/min/atm to cm/s using equation (4-7). In this study, P_f of adult RBCs was 0.017 ± 0.001, 0.040 ± 0.003, and 0.056 ± 0.003 cm/s at 4.0, 19.0, and 35.2°C, respectively; P_f of cord RBCs was 0.023 ± 0.003, 0.052 ± 0.006, and 0.078 ± 0.004 cm/s at 4.0, 19.0, and 35.2°C, respectively. As can be seen, my values for osmotic water permeability are about 1.5 times higher than the majority of published reports. I have previously determined that conversion from μm/min/atm to cm/s results in 50% increase in P_f value (Table 4-4). Therefore, accounting for the impact of this conversion, it is reasonable to conclude that my values for hydraulic conductivity permeability of RBCs are in good agreement with published data.

Arrhenius activation energy for hydraulic conductivity was found to be statistically the same for cord and adult RBCs (5.4 kcal/mol for cord and 4.8 kcal/mol for adult, $p>0.05$). Agre et al. measured activation energy for osmotic water transport for adult and cord RBC ghosts at 8-39°C using stopped-flow by light scattering technique. They found that activation energy for cord RBCs was 1.3 times higher than for adult RBCs ($p<0.01$) [49]. My values for activation energies for cord and adult RBCs are also in agreement with those reported in the

literature. In particular, reported activation energies for osmotic water permeability were 3.6 ± 0.4 kcal/mol [58], 3.3 ± 0.4 kcal/mol [75], and 4.6 ± 0.8 kcal/mol [49] for adult RBCs, and 6.0 ± 0.5 kcal/mol for cord RBCs [49]. Low activation energies such as these (around 5 kcal/mol on average) are correlated with presence of aquaporins in the cell membrane [76, 77].

Glycerol permeability and its activation energy

There were also differences in glycerol permeability between adult and cord RBCs. Particularly, cord RBCs were more permeable to glycerol at 4°C. At other investigated temperatures (20°C and 35°C), glycerol permeabilities of cord and adult RBCs were equal (Table 4-5). Published studies on permeability of cord RBCs to glycerol are limited. Moore reported that the average 50% hemolysis time in 0.3 M glycerol is approximately twice longer for fetal RBCs than for adult RBCs. He concluded that adult RBCs are twice more permeable to glycerol compared to fetal RBCs, the difference presumably caused by the higher percentage of lecithin in adult erythrocyte membrane [50]. In this study Moore used a glycerol lysis test to estimate cell permeability to glycerol. In this test, erythrocytes are exposed to hypotonic salt solution (about 0.3% NaCl) containing 0.3 M glycerol. Glycerol prevents rapid flux of water inside the cell in the direction of osmotic gradient, and, as both glycerol and water slowly enter the cell, hemolysis is monitored as a function of time. This method has been

originally developed as the test for osmotic fragility, specifically used as a screening test for hereditary spherocytosis [78] – a condition in which increased osmotic fragility of erythrocytes is caused by mutations in cytoskeletal proteins. Bautista et al. used the same test to show that it takes fetal RBCs longer to lyse in 0.3 M glycerol than adult RBCs, and interpreted this as a measure of decreased osmotic fragility of fetal erythrocytes [79]. In the glycerol lysis test, it would make sense that the time to hemolysis is determined by membrane permeability not only to glycerol, but also to water (since water enters the cell from extracellular hypotonic solution), which would be a confounding factor. If permeability of cell membrane to water in hypotonic solution is lower for cord than for adult RBCs (and that is what my data shows, Table 4-3), it will take longer for water to enter cord RBC and cause lysis. This would result in seemingly decreased permeability of the cell to glycerol.

Previously it was thought that glycerol, as well as other small molecules, predominantly cross cell membrane by diffusion through lipid bilayer [80]. However, this understanding has recently changed. It was found that glycerol transport across human erythrocyte membrane occurs through specialized channels called aquaglyceroporins [81]. Therefore, the differences in glycerol permeability between cord and adult RBCs need to be interpreted in the light of these new recent findings. Although expression of aquaglyceroporins has not yet been evaluated in fetal RBC, based on my data it is reasonable to assume that differences in glycerol permeability would imply differences in expression and/or function of these channels between fetal and adult erythrocytes.

Several studies reported values for $P_{glycerol}$ of adult RBCs. Naccache and Sha'afi measured glycerol permeability coefficient of adult RBCs exposed to 0.3M glycerol in hypotonic saline solution at 19-24°C using a combination of hemolysis and stopped-flow technique. They reported $P_{glycerol}$ as $0.58 \pm 0.04 \times 10^{-5}$ cm/s, which is equal to 3.48 μm/min [82]. Mazur and Miller estimated glycerol permeability coefficient for adult RBCs using 1) time to 50% hemolysis in hypotonic saline solutions containing 1 and 2 M glycerol, and 2) time it took RBCs in isotonic saline solution containing 1 or 2 M glycerol to undergo osmotic shock upon tenfold dilution with isotonic saline buffer. They reported glycerol permeability coefficients as 2.5×10^{-4} cm/min at 20°C and 0.9×10^{-4} cm/min at 0°C, which is equal to 2.5 μm/min and 0.9 μm/min, respectively [83]. My values for glycerol permeability of adult RBCs are higher than those presented in two reports mentioned above (8.49 ± 0.87μm/min at 20°C). This can be caused by significant differences in the methods used to estimate $P_{glycerol}$. In particular, Naccache's and Mazur's methods were based on measuring time to RBC lysis in hypotonic solutions in the presence of various amounts of glycerol. The limitations of this methodological approach have been discussed above.

Arrhenius activation energy for glycerol permeability was found to be lower for cord than for adult RBCs (9.7 kcal/mol for cord vs 11.5 kcal/mol for adult, $p<0.05$). Activation energy of 11.5 kcal/mol is slightly higher than 7.2 kcal/mol reported by Mazur and Miller as an activation energy for glycerol permeability for adult RBCs [83]. Since activation energy describes the temperature dependence of glycerol permeability [45], cells with higher value of

activation energy (adult RBCs) will experience faster decline in glycerol permeability as the temperature drops during cooling and faster increase in glycerol permeability as the temperature rises during thawing.

This study provides values for osmotic parameters of cord and adult RBCs, such as osmotically inactive fraction, water and glycerol permeability, and activation energies for these processes. Osmotic parameters were determined over a broad range of experimental temperatures and osmolalities, supplementing data from other studies in which osmotic parameters were determined under very specific and limited experimental conditions. Moreover, this data adds to a very limited published data on cord red cell osmotic permeability to water and glycerol. I conclude that cord RBCs have larger osmotically inactive fraction compared to adult RBCs. Hydraulic conductivity and osmotic permeability to glycerol of cord RBCs differ from those of adult RBCs depending on experimental conditions, such as temperature and osmolality. In future, these osmotic parameters can be used in mathematical modelling to predict optimized cryopreservation protocol for cord RBCs.

Table 4-1. Hemolysis (%) of adult and cord RBCs in experimental solutions.

Source of RBCs	Experimental solution					
	0.75x PBS (~214 mOsm/kg)	**1x PBS (~287 mOsm/kg)**	**2x PBS (~562 mOsm/kg)**	**3x PBS (~832 mOsm/kg)**	**4x PBS (~1112 mOsm/kg)**	**2.5% (w/v) glycerol (~578 mOsm/kg)**
Adult	0.96±0.15	0.53±0.18	0.81±0.04	0.93±0.05	0.96±0.06	0.44±0.02
Cord	1.1±0.17	0.83±0.12	1.1±0.14	1.4±0.28	1.5±0.30	0.93±0.17*

Values are means ± SEM (n=3). *p=0.05 vs adult

Table 4-2. Hydraulic conductivity (L_p) of adult and cord RBCs at various temperatures

Source of RBCs	**Experimental temperature (**°C)		
	3.8 ± 0.01	**19.8 ± 0.06**	**35.3 ± 0.01**
Adult	8.19 ± 0.38	18.11 ± 1.51	23.80 ± 1.48
Cord	10.96 ± 1.48	23.16 ± 2.51	33.13 ± 1.53*

L_p values were obtained upon exposure of RBCs to 2x PBS (562 mOsm/kg) solution at 3 different experimental temperatures. *$p<0.05$ vs adult. Values are means ± SEM (n=8-15 for adult RBCs, n=7 for cord RBCs).

Table 4-3. Hydraulic conductivity (L_p) of adult and cord RBCs in various osmolalities

Source of RBCs	Experimental osmolality (mOsm/kg)			
	214	**562**	**832**	**1112**
Adult	9.51 ± 0.35	18.11 ± 1.51	23.76 ± 0.70	16.94 ± 0.20
Cord	6.66 ± 0.22*	23.16 ± 2.51	24.50 ± 0.54	16.18 ± 0.18*

L_p values were obtained at room temperature upon exposure of RBCs to four different concentrations of PBS solution. *$p<0.05$ vs adult. Values are means ± SEM (n=8-15 for adult RBCs, n=7 for cord RBCs).

Table 4-4. Comparison between calculated and converted values for osmotic water permeability (P_f).

P_f value	**Experimental temperature (**°C)		
	4.0	**19.0**	**35.2**
Calculated according to Zeidel et al.	0.012 ± 0.001	0.018 ± 0.002	0.027 ± 0.005
Converted from L_p	0.018 ± 0.001	0.027 ± 0.004	0.040 ± 0.008

"Calculated" P_f values were determined using equation (4-6) from Zeidel et al [58]. "Converted" P_f values were determined by converting corresponding L_p values into cm/s using equation (4-7). Data is presented for one adult RBC sample, ran in triplicate. Values are mean ± SEM.

Table 4-5. Permeability of adult and cord RBCs to glycerol ($P_{glycerol}$) at various temperatures

Source of RBCs	**Experimental temperature (**°C)		
	3.8 ± 0.01	**19.9 ± 0.06**	**35.3 ± 0.01**
Adult	1.24 ± 0.19	8.49 ± 0.87	19.96 ± 1.60
Cord	2.30 ± 0.31*	10.46 ± 0.63	21.98 ± 0.03

$P_{glycerol}$ values were obtained upon exposure of RBCs to 2.5% (w/v) glycerol in 1x PBS solution at 3 different experimental temperatures. *$p<0.05$ vs adult. Values are means ± SEM (n=8 for adult RBCs, n=7 for cord RBCs).

Table 4-6. Literature values for hydraulic conductivity and its activation energy for adult and cord RBCs.

Source	Method	Value
Osmotic water permeability values for adult RBCs		
Agre, P. et al., J. Clin. Invest., 1994 [49]	Stopped-flow by light scattering	0.037 ± 0.007 (P_f ± SD) cm/s at 37°C
Zeidel, M.L. et al., Biochemistry 1992 [58]	Stopped-flow fluorimetry with carboxyfluorescein diacetate	0.040 ± 0.009 (P_f ± SEM) cm/s
Mlekoday, H.J. et al. J. Gen. Physiol. 1983 [84]	Stopped-flow by light scattering	0.2 cm/s
Sidel, V.W and Solomon, A.K. J. Gen. Physiol. 1957 [85]	Stopped-flow by light scattering	0.23 ± 0.03 cm^4/osm., sec
Liu, L. et al. J. Comp. Physiol. B 2011 [86]	Stopped-flow by light scattering	0.029 ± 0.004 (P_f ± SEM) cm/s
Sjölin, Acta paediatrica Supplement, 1954 [69]	Light scattering: *Hemolytic NaCl concentrations* *Prehemolytic NaCl concentrations*	 0.0084 cm/s; 0.022 cm/s 0.016 cm/s
Osmotic water permeability values for fetal RBCs		
Agre, P. et al., J. Clin. Invest., 1994 [49]	Stopped-flow fluorimetry by light scattering	17-28 wk: 0.029 ± 0.009 (P_f ± SD) cm/s at 37°C 28 wk-term: 0.026 ± 0.006 (P_f ± SD) cm/s at 37°C
Sjölin, Acta paediatrica Supplement, 1954 [69]	Light scattering: *Hemolytic NaCl concentrations* *Prehemolytic NaCl concentrations*	 0.0047 cm/s; 0.012 cm/s 0.0066 cm/s
Activation energy values for osmotic water permeability for adult RBCs		
Agre, P. et al., J. Clin. Invest., 1994 [49]	Stopped-flow by light scattering	4.6 ± 0.8 kcal/mol at 8-39°C
Zeidel, M.L. et al., Biochemistry 1992 [58]	Stopped-flow fluorimetry with carboxyfluorescein diacetate	3.6 ± 0.4 kcal/mol
Vieira, F.L. et al. J. Gen. Physiol. 1970 [75]	Rapid reaction continuous flow system analysis for tritiated water (THO) radioactivity	3.3 ± 0.4 kcal/mol at 7-37°C
Activation energy values for osmotic water permeability for fetal RBCs		
Agre, P. et al., J. Clin. Invest., 1994 [49]	Stopped-flow by light scattering	17-28 wk: 6.7 ± 0.4 kcal/mol at 8-39°C 28 wk-term: 6.0 ± 0.5 kcal/mol at 8-39°C

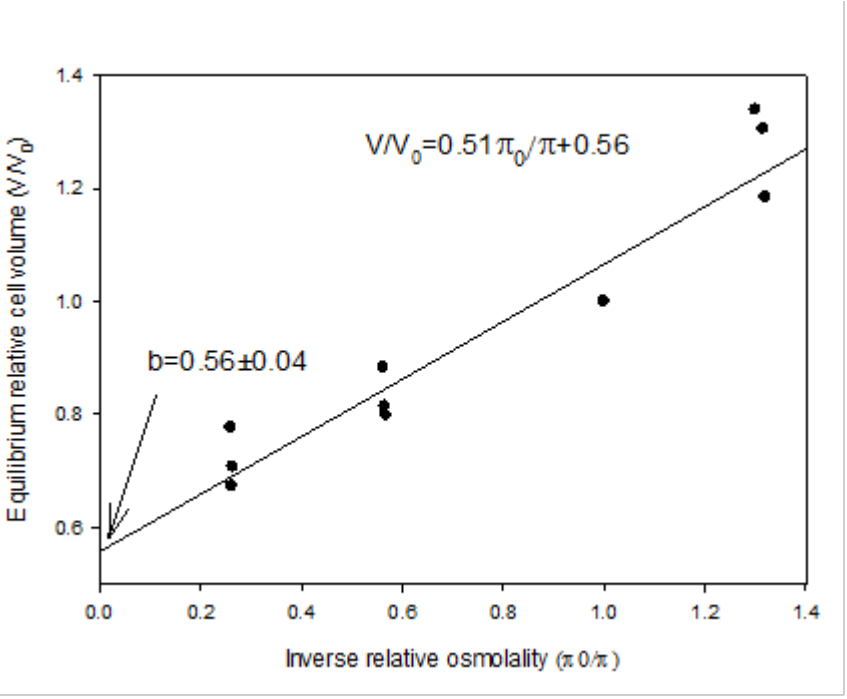


Figure 4-1. Boyle-van't-Hoff plot for cord RBCs. Data points are equilibrium relative cell volumes in various sodium chloride concentrations (0.68, 0.9, 1.6, and 3.5% (w/v)). Osmotically inactive fraction (b) was determined form y-intercept of the linear regression fit to the data. N=3 cord RBC samples.

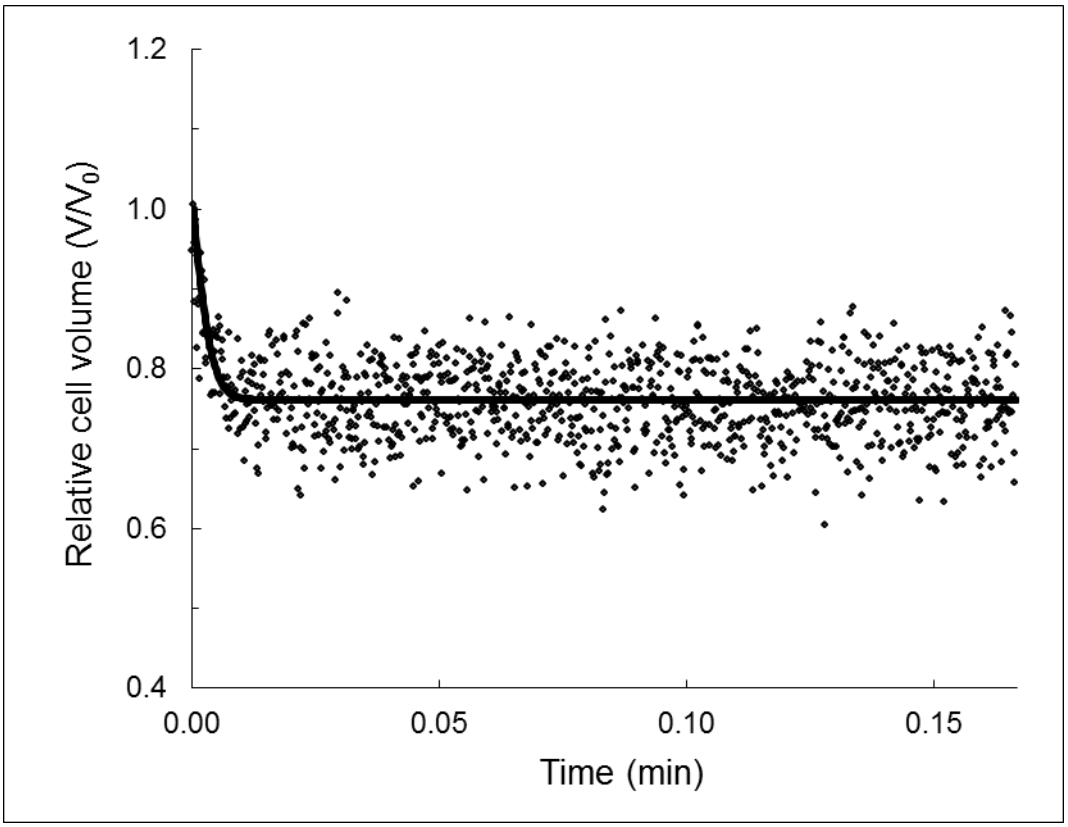


Figure 4-2. Representative plot of the kinetics of adult RBC volume upon exposure to 2x PBS at 4ºC. Rhombuses are the relative cell volumes and solid line is the fitted curve to data.

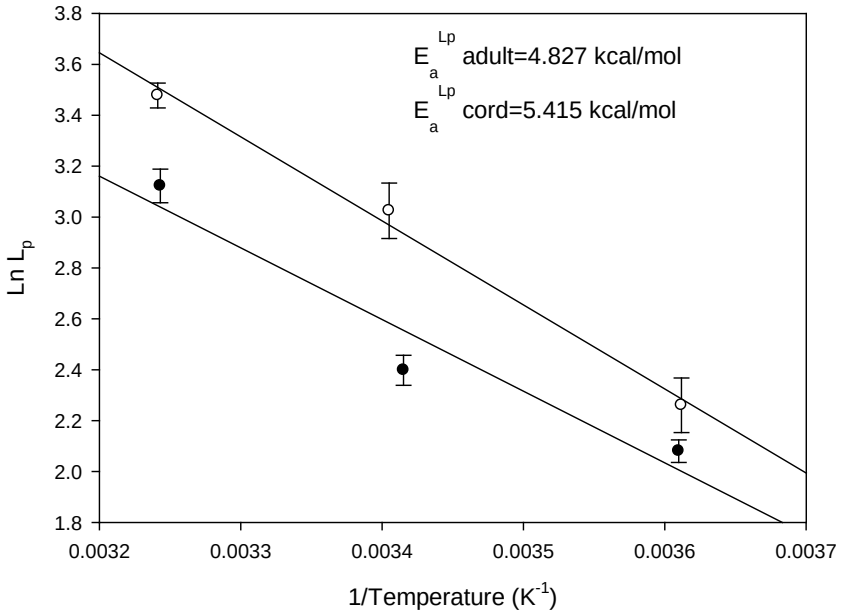
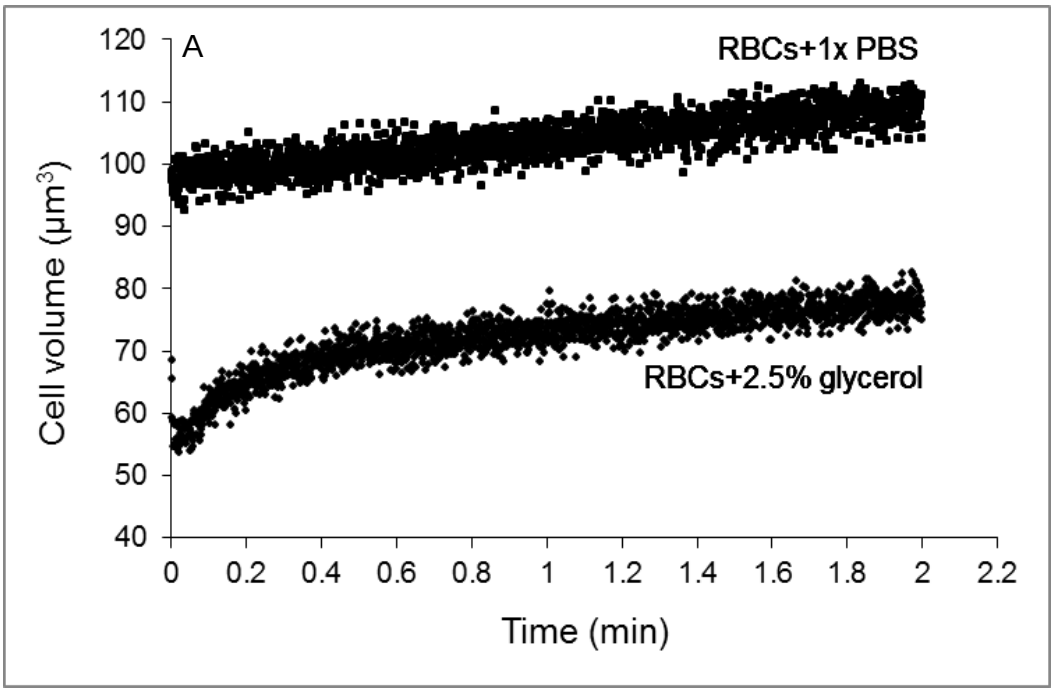
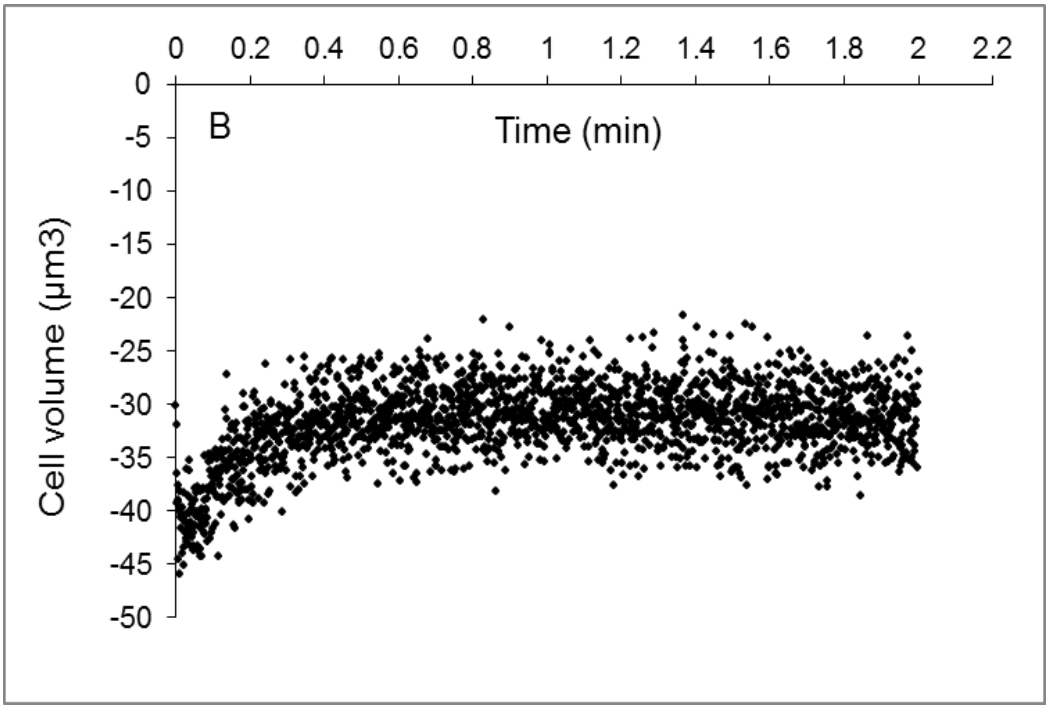


Figure 4-3. Arrhenius plot of the natural logarithm for hydraulic conductivity (L_p, μm/min/atm) of adult (●) and cord (○) RBCs as a function of inverse absolute temperature (K^{-1}). Activation energies (Ea) were calculated from the slope of the linear regression fits to the data. Values are means ± SEM (n=8 for adult RBCs, n=7 for cord RBCs).


A
RBCs+1x PBS
RBCs+2.5% glycerol
Cell volume (µm3)
Time (min)
120
110
100
90
80
70
60
50
40
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2


B
Time (min)
Cell volume (µm3)
0
-5
-10
-15
-20
-25
-30
-35
-40
-45
-50
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2

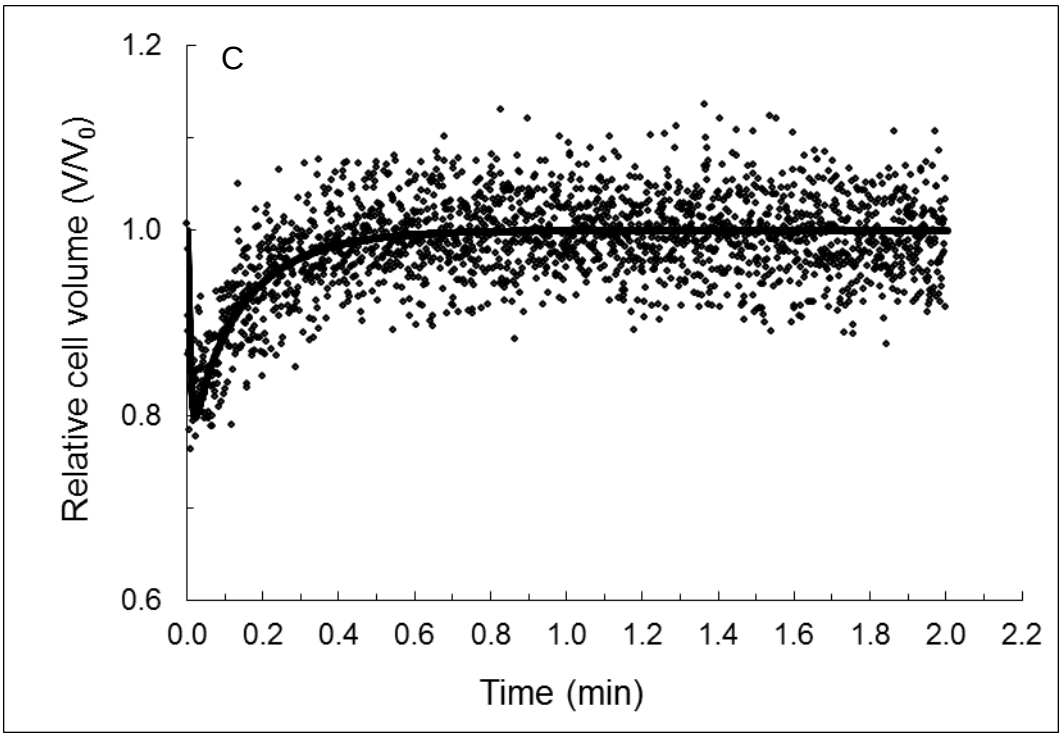


Figure 4-4. Representative plot of the kinetics of adult RBC volume upon exposure to 2.5% (w/v) glycerol at 20°C. **A**. Curves representing kinetics of RBC volume after mixing with 1x PBS (RBCs + 1x PBS) and 2.5% (w/v) glycerol (RBCs + 2.5% glycerol). **B**. Curve generated after RBCs + 1x PBS curve was subtracted from RBCs + 2.5% glycerol curve. **C**. Curve generated after isotonic RBC volume was added to each data point on the curve generated in Figure B. Rhombuses are the relative cell volumes and solid line is the fitted curve to data.

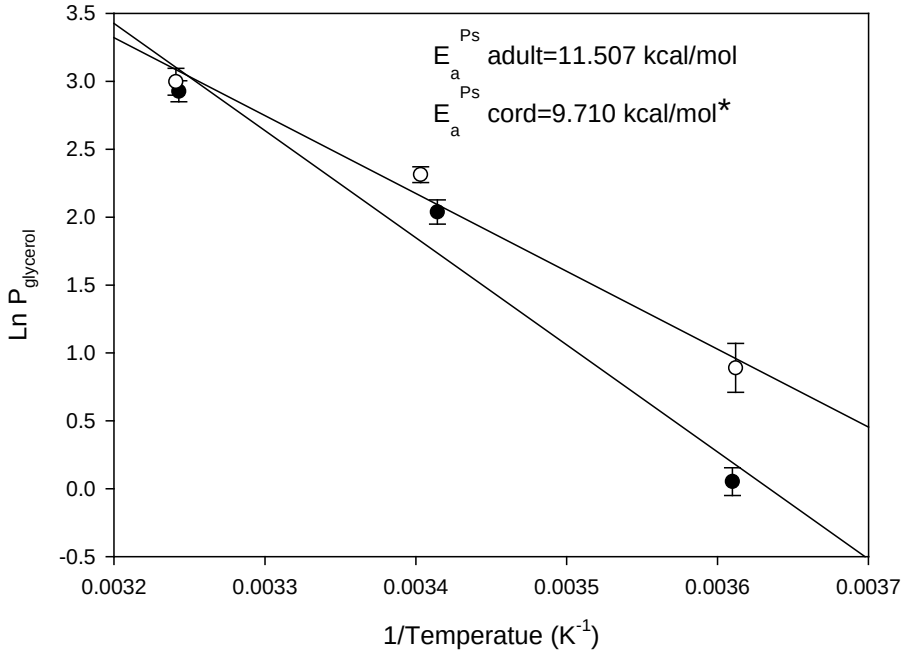


Figure 4-5. Arrhenius plot of the natural logarithm for glycerol permeability ($P_{glycerol}$, μm/min) of adult (●) and cord (○) RBCs as a function of inverse absolute temperature (K^{-1}). Activation energies (Ea) were calculated from the slope of the linear regression fits to the data. Values are means ± SEM (n=8 for adult RBCs, n=7 for cord RBCs). * $p<0.05$ vs adult.

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Chapter 5

Osmotic tolerance limits of cord and adult red blood cells.

5.1 Introduction

During cryopreservation, cells are subjected to many osmotic stresses. Osmotic pressure gradients are formed during addition and dilution of cryoprotectant (CPA), as well as during freezing and thawing. When a permeable CPA is added to the cell, the cell first dehydrates as water leaves the cell in the direction of the osmotic pressure gradient, and then returns back to its isotonic volume as CPA gradually enters the cell, followed by water. To remove permeable CPA, cells are suspended in the media that does not contain this CPA. Upon such dilution, the cell first swells, as water enters the cell in the direction of osmotic pressure gradient, and then returns to it former volume as CPA leaves the cell, followed by water. The same osmotic stresses affect the cell during freezing and thawing. Formation of ice outside the cell causes concentration of extracellular solutes and cell dehydration. Upon thawing, free water enters the cell from the outside to dilute concentrated intracellular solutes, and cell expands in volume.

Cell damage can take place during all these steps. Swelling of the cell beyond its critical volume in hypotonic solutions leads to cell lysis. Excessive shrinking of the cell in hypertonic solutions also results in cell damage. There have been a number of theories explaining the cause of cell injury in hypertonic

solutions. 1) Meryman proposed the “minimum cell volume hypothesis”, according to which there is a minimum cell volume, shrinking beyond which causes rupture of the cell membrane [1]. As concentration of extracellular solutes increases, cell volume decreases. Once the certain minimum cell volume is reached, failure of the cell to shrink beyond this minimum volume causes inward osmotic gradient across the cell membrane, which is balanced by the mechanical resistance of the cell. Once this osmotic gradient reaches critical level, stress imposed on the cell membrane causes its rupture. 2) Cell damage in hypertonic solutions was also explained by the loading of normally impermeant solutes inside the cells at very high extracellular osmolalities. Lovelock was the first to observe that cells, exposed to high concentrations of sodium chloride (NaCl) undergo lysis upon return to isotonic conditions [2]. The mechanism of such hypertonic damage has later been explained by Farrant and Woolgar, who showed that at high extracellular concentrations of NaCl (1200 mmol/kg sodium) red blood cells (RBCs) start leaking potassium and taking up sodium ions [3]. Such accumulation of intracellular sodium can lead to hemolysis upon resuspension in hypotonic or even isotonic solutions [4]. 3) The hypothesis of “expansion induced cell lysis” was proposed by Steponkus for plant cells [5]. According to Steponkus, cell dehydration causes endocytotic vesiculation of plasma membrane. Due to such irreversible loss of cell membrane material, cell surface area is decreased and cell cannot expand to its original isotonic volume upon its return to isotonic environment, which results in cell lysis. 4) Finally, Muldrew and McGann originally proposed the “osmotic rupture hypothesis” to explain the mechanism of

intracellular ice formation. According to this theory, cell membrane is ruptured by the water flux that results from an increasing osmotic pressure gradient across the cell membrane during cell dehydration.

Gilmore et al. defined the osmotic tolerance limits of the cell as “the extent of volume excursions a cell can withstand before the irreversible loss of function occurs” [6]. When cryopreservation procedures are developed, efforts mostly focus on avoiding damage due to exposure to low temperatures. Less attention is given to avoiding excessive cell volume excursions during loading and unloading of CPAs. Knowledge of osmotic tolerance limits of the cell is crucial for the development of effective CPA addition/removal procedures, since special care needs to be taken not to exceed critical maximum and minimum volumes of the cell [4].

In Chapter 4 I determined osmotic parameters of cord RBC membrane (osmotically inactive fraction, hydraulic conductivity, osmotic permeability to glycerol, and corresponding activation energies for these processes). The information on the minimum and maximum tolerable volumes of cord RBCs is still missing and is required for future design of an effective cryopreservation protocol. Therefore, the objective of this study was to determine osmotic tolerance limits of cord and adult RBCs.

5.2 Materials and Methods

Overall study design

I tested the osmotic tolerance of cord RBCs by exposing them to saline solutions of different molality. There were three treatment groups of RBCs: 1) fresh, 2) in the presence of 40% glycerol, non-frozen, and 3) in the presence of 40% glycerol, frozen-thawed. For fresh RBCs, osmotic tolerance was tested 1) upon addition of anisotonic saline that resulted in shrinking or swelling of RBCs 2) upon return to isotonic conditions. For RBCs in the presence of 40% glycerol, I only induced RBC swelling by addition of lower glycerol concentrations and osmotic tolerance was tested upon return to isotonic conditions. Percent hemolysis was determined using Harboe method as a marker of RBC damage. Information on osmotic tolerance limits of adult RBCs was collected by Ratih Lucianti in Dr. Adam Higgins' laboratory, Oregon State University, OR, USA. Only data on adult RBCs cryopreserved in the presence of 40% glycerol is available up to date. The schematic experimental design is shown on Figure 5-1.

Experimental samples

Cord RBCs were obtained from the Alberta Cord Blood Bank, as a waste product, after stem cell isolation from umbilical cord blood. Cord blood collected from a

placenta was stored at room temperature for up to 43 hours prior to stem cell isolation, which was previously shown not to cause a decrease in RBC quality (Chapter 2) [7]. Leftover cord RBC product was washed with saline once by centrifugation at 2200 g, 4°C for 5 minutes. The hematocrit of cord RBCs was then adjusted to 60±2% by the addition of saline to the RBC pellet. Cord RBCs were stored in our laboratory at 1-6°C and used in experiments within 24 hours of isolation from cord blood. Ethics approval for the study was obtained from the University of Alberta Health Research Ethics Board (Biomedical Panel) and Canadian Blood Services Research Ethics Board.

Experimental solutions

For osmotic tolerance study of fresh RBCs.

Phosphate-buffered saline (PBS) solutions of the following molalities were prepared in-house: 34 mmol/kg NaCl + 12.5 mmol/kg sodium phosphate dibasic (Na_2HPO_4), 51 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, 67 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, 131 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, 171 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, 777 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, 1584 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, and 2391 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4. Also, 11.5 mmol/kg glucose + 12.5 mmol/kg Na_2HPO_4 solution was prepared in-house. As an example, 34 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 was prepared by adding together 100 mL distilled water,

0.2 g NaCl (Sigma Aldrich, Inc., St. Louis, MO, USA), and 0.177 g Na_2HPO_4 (Sigma Aldrich, Inc., St. Louis, MO, USA). For other solutions, only the amount of NaCl was adjusted and the amount of Na_2HPO_4 was kept the same. pH of all solutions was adjusted to 7 with 1 N hydrochloric acid (HCl), 6 N HCl, or 6 N sodium hydroxide (NaOH).

For osmotic tolerance study of RBCs in the presence of 40% glycerol.

Phosphate-buffered glycerol-saline solutions of the following molalities were prepared: 1672 mmol/kg glycerol + 41 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, 2291 mmol/kg glycerol + 63 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, 2899 mmol/kg glycerol + 86 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, 6500 mmol/kg glycerol + 206 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, and 7384 mmol/kg glycerol + 222 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4. As an example, 1672 mmol/kg glycerol + 41 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 solution was prepared by adding together 100 mL distilled water, 15.4 g glycerol (99.5+%, Sigma Aldrich, Inc., St. Louis, MO, USA), 0.24 g NaCl, 0.177 g Na_2HPO_4. For other solutions, only the amount of glycerol and NaCl was adjusted and the amount of Na_2HPO_4 was kept the same. pH of all solutions was adjusted to 7 with 1 N HCl, 6 N HCl, or 6 N NaOH.

RBC glycerolization, freezing and thawing procedures

Glycerolization

Cord RBCs were glycerolized by the addition of 6.5 mL Glycerolyte 57 solution (Baxter Corporation, Missisauga, Ontario) to 4.075-4.225 g packed RBCs to achieve final glycerol molality of approximately 6500 mmol/kg H_2O and NaCl molality of approximately 206 mmol/kg.

Prior to glycerolization, cord RBCs were washed once with isotonic buffered saline at 2200 g, 4°C, for 5 minutes. Most of the supernatant was removed to obtain RBC pellet with approximately 90% hematocrit. The weight of RBC pellet was measured and adjusted to 4.075-4.225 g. This was done to make sure RBC mass is in the appropriate range for the protocol and the desired final molalities of glycerol and non-permeating solute are maintained. Glycerolyte 57 (at room temperature) was added to RBC pellet in two steps. In the first step, 1.5 mL of Glycerolyte 57 was added over three minutes, while tube with RBCs was constantly gently agitated by hand to ensure proper equilibration of glycerol. After that RBCs were left to equilibrate at room temperature for five minutes. In the second step, 5 mL of Glycerolyte 57 was added to RBCs over three minutes with constant gentle agitation. After the second addition of glycerol, cord RBCs were either directly used for osmotic tolerance experiments, or cryopreserved according to the following procedure.

Cryopreservation

Glycerolized RBCs were transferred into cryovials (Thermo Fisher Scientific, Roskilde, Denmark) and frozen by placing them in a -80°C freezer to achieve a freezing rate of approximately 1°C per minute. Cord RBCs were left in a frozen state for at least 48 hours before being used in osmotic tolerance experiment. Cryovilas with RBCs were thawed by placing them into the 37°C water bath (VWR, manufactured by Sheldon Manufacturing, Inc., Cornelius, OR, USA) for one minute. Thawed RBCs were analyzed immediately.

Testing osmotic tolerance limits of RBCs

Osmotic tolerance limits of fresh RBCs

0.1 mL of cord RBC suspension, with hematocrit of about 60%, was placed into each of eight 50 mL plastic conical tubes. While tubes were being shaken in a Gyrotory Water Bath Shaker (New Brunswick Scientific, Edison, NJ, USA) at room temperature, 1 mL of anisotonic saline solution of various osmolalities was added to the 0.1 ml RBCs. This was done to achieve various final concentrations of non-permeating solute in RBC suspension and, hence, to induce a range of RBC volume changes. V_w/V_{w0} is the relative volume of the cell water upon exposure to anisotonic solution, where V_w is the volume of water in the cell upon exposure to anisotonic solution, and V_{w0} is the initial volume of water in the cell. $V_w/V_{w0} = 1$ means cell water volume remains unchanged (isotonic conditions),

$V_w/V_{w0} > 1$ means there is an influx of water into the cell that causes cell swelling, and $V_w/V_{w0} < 1$ means there is an efflux of water from the cell that causes cell shrinking. The expected volume change was calculated in Dr. Higgins' laboratory using a MATLAB model that is based on the two-parameter model for membrane transport [8]. Two coupled differential equations (describing the flux of water and CPA into the cell) were programmed into MATLAB. Using information on adult RBC membrane permeability to water, isotonic molality, final molality of the environment cells were exposed to, and exposure time, the model was used to calculate the flux of water into the cell as a function of time and, therefore, V_w. To get a value for the osmotic volume change of the cell, V_w was divided by V_{w0}.

Addition of anisotonic saline solution (containing non-permeating solutes NaCl and Na_2HPO_4) was done according to the following treatment groups:

a. 0 mmol/kg saline solution (distilled water) → final concentration of ~10.5 mmol/kg NaCl, $V_w/V_{w0}=14$
b. 34 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 → final concentration of ~41 mmol/kg NaCl + ~12.5 mmol/kg Na_2HPO_4, $V_w/V_{w0} = 2.5$
c. 51 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 → final concentration of ~56 mmol/kg NaCl + ~12.5 mmol/kg Na_2HPO_4, $V_w/V_{w0} = 2.0$
d. 67 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 → final concentration of ~71 mmol/kg NaCl + ~12.5 mmol/kg Na_2HPO_4, $V_w/V_{w0} = 1.7$

e. 131 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 (isotonic buffered saline) → final concentration of 131 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, $V_w/V_{w0} = 1.0$

f. 777 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 → final concentration of ~731 mmol/kg NaCl + ~12.5 mmol/kg Na_2HPO_4, $V_w/V_{w0} = 0.2$

g. 1584 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 → final concentration of ~1481 mmol/kg NaCl + ~12.5 mmol/kg Na_2HPO_4, $V_w/V_{w0} = 0.1$

h. 2391 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 → final concentration of ~2231 mmol/kg NaCl + ~12.5 mmol/kg Na_2HPO_4, $V_w/V_{w0} = 0.067$

After addition of anisotonic saline, samples were left to equilibrate at room temperature for five minutes, while being agitated on a shaker plate. After that, 0.1 mL of RBC suspension was removed from each tube for hemolysis testing. The remaining samples were brought back to isotonic conditions by the addition of appropriate volume of hypotonic buffered solution (11.5 mmol/kg glucose + 12.5 mmol/kg Na_2HPO_4), hypertonic buffered solution (171 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4), or isotonic buffered saline (131 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4). Following that, the volume of all samples was brought to 20 mL by the addition of appropriate volume of isotonic buffered saline. RBC samples were tested for hemolysis.

Osmotic tolerance limits of RBCs in the presence of 40% glycerol

0.1 mL of glycerolized cord RBC suspension, whether fresh or previously frozen (to imitate RBCs frozen and thawed using standard high glycerol – slow cooling method), with hematocrit of about 35%, was placed into each of five 50 mL plastic conical tubes. While tubes were being shaken in a Gyrotory Water Bath Shaker at room temperature, 1 mL of anisotonic glycerol-saline solution of various molalities was added to the 0.1 ml RBCs. $(V_w+V_{glyc})/V_{w0}$ is the relative volume of water+glycerol in the cell upon exposure to anisotonic solution, where V_w+V_{glyc} is the volume of water and glycerol in the cell upon exposure to anisotonic solution, and V_{w0} is the initial volume of water in the cell. $(V_w+V_{glyc})/V_{w0} = 1$ means cell water and glycerol volume remain unchanged (isotonic conditions), $(V_w+V_{glyc})/V_{w0} > 1$ means there is an influx of water or glycerol into the cell that causes cell swelling. The expected volume change of the cell was calculated using the same approach as for fresh RBCs (see previous section). The known permeabilities of adult RBC membrane to water and glycerol were used to calculate the flux of both water and glycerol into the cell as a function of time and, therefore, V_w and $V_{glyc.}$ To get a value for the osmotic volume change of the cell, V_w+V_{glyc} was divided by V_{w0}.

In this experiment, I could induce only swelling of RBCs, since shrinking would require extremely high glycerol concentrations. Addition of anisotonic glycerol-saline solutions (containing permeating solute glycerol and non-permeating solutes NaCl and Na_2HPO_4) was done according to the following treatment groups:

a. 0 mmol/kg solution (distilled water) → final concentration of 373 mmol/kg glycerol + 12.5 mmol/kg NaCl, $(V_w+V_{glyc})/V_{w0}$=13
b. 1672 mmol/kg glycerol + 41 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 → final concentration of 1988 mmol/kg glycerol + 50 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, $(V_w+V_{glyc})/V_{w0}$ = 2.5
c. 2291 mmol/kg glycerol + 63 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 → final concentration of 2575 mmol/kg glycerol + 70 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, $(V_w+V_{glyc})/V_{w0}$ = 2.0
d. 2899 mmol/kg glycerol + 86 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 → final concentration of 3141 mmol/kg glycerol + 90 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, $(V_w+V_{glyc})/V_{w0}$ = 1.7
e. 6500 mmol/kg glycerol + 206 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4 → 6500 mmol/kg glycerol + 206 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4, $(V_w+V_{glyc})/V_{w0}$ = 1.0

After addition of anisotonic glycerol-saline solutions, samples were left to equilibrate at room temperature for five minutes, while being agitated on a shaker plate. Samples were brought back to isotonic conditions by the addition of appropriate volume of hypertonic solution (7384 mmol/kg glycerol + 222 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4) or isotonic solution (original solution composition of 6500 mmol/kg glycerol + 206 mmol/kg NaCl + 12.5 mmol/kg Na_2HPO_4). After that, the volume of all samples was brought to 20 mL by the addition of appropriate volume of isotonic glycerol-saline solution and concentration of supernatant hemoglobin in RBC samples was tested.

Testing percent hemolysis using Harboe method

Supernatant hemoglobin concentration in RBC samples was measured by the method of Harboe [9, 10]. The method is based on the direct measurement of oxyhemoglobin absorbance at 415 nm. The interfering absorbances are also measured at 380 and 450 nm and later subtracted from oxyhemoglobin absorbance.

RBC samples were centrifuged at 2200 g, 4°C for 10 minutes, supernatant was collected and diluted 1:10 in distilled water. Absorbance of thus diluted samples was measured on spectrophotometer SPECTRA max PLUS 384 microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) at 415, 380, and 450 nm and hemoglobin concentration in supernatant was calculated using SoftMax Pro software (Molecular Devices Corporation, Sunnyvale, CA, USA) according to the equation below:

$$Hemoglobin\ \left(\frac{g}{L}\right) = (167.2 \times A_{415} - 83.6 \times A_{380} - 83.6 \times A_{450}) \times \frac{1}{100} \times F, \qquad \text{(Eq. 5-1)}$$

where A_{415} - absorbance of the solution at 415 nm,

A_{380} - absorbance of the solution at 380 nm,

A_{450} - absorbance of the solution at 450 nm, and

F - dilution factor. For 1:10 dilution of supernatant in water, dilution factor equals 10. Dilution factor has to be adjusted accordingly if higher or lower supernatant dilution is prepared.

Standards for supernatant hemoglobin concentration were prepared from Stanbio Tri-Level Hemoglobin controls (Stanbio Laboratory, Boerne, TX, USA). Control I was prepared by diluting 4 μL Stanbio Control Level I in 2000 μL distilled water (dilution factor 501). Control II was prepared by diluting 20 μL Stanbio Control Level II in 2000 μL distilled water (dilution factor 101). Control III was prepared by diluting 50 μL Stanbio Control Level III in 2000 μL distilled water (dilution factor 41).

Percent hemolysis was determined from calibration curve. The calibration curve was created for each RBC sample by measuring supernatant hemoglobin concentration of this sample at 0, 50, and 100% hemolysis. For 0% hemolysis, I measured absorbance of distilled water. 100% hemolysis sample was prepared in by adding an appropriate volume of distilled water to RBCs, maintaining the same dilution as in experimental treatment group for which 100% hemolysis control is being created. After water was added, diluted sample was transferred into cryovial and subjected to three freeze-thaw cycles by immersing it into liquid nitrogen and subsequent thawing at 37°C until no ice crystals were visible. After that RBCs were considered to be completely lysed. 50% hemolysis sample was prepared by mixing equal volumes of distilled water and 100% hemolysis sample. Absorbance of three hemolysis standards was measured and calibration curve was created to represent hemoglobin concentration (g/L) as a function of hemolysis (%).

Statistical analysis

Comparison between adult and cord RBCs, as well as between different treatment groups, was performed by Mann-Whitney non-parametric test in SPSS software (version 12.0, SPSS Inc., Chicago, IL, Texas). Significance level was set to 0.05.

Contributions of others

Personnel of Alberta Cord Blood Bank provided cord RBC samples for this study. Ratih Lucianti and Dr. Adam Higgins (Oregon State University, OR, USA) designed the experiments and provided data on osmotic tolerance limits of adult RBCs.

5.3 Results

Osmotic tolerance limits of fresh RBCs

I chose 10% hemolysis as an upper threshold for the osmotic tolerance, based on the recent study by Liu et al. [11]. Figure 5-2 shows osmotic tolerance limits for fresh cord RBCs. Upon exposure to 10.5-731 mmol/kg Nacl (V_w/V_{w0} = 14-0.2), cord RBCs showed similar hemolysis in anisotonic solution and upon return to

isotonic solution. However, at extracellular NaCl molalities of 730 mmol/kg and higher ($V_w/V_{w0} \leq 0.2$), hemolysis of cord RBCs increased significantly ($p=0.001$) upon return to isotonic conditions. When fresh cord RBCs remained in anisotonic solution, upper and lower osmotic tolerance limits were 1.7 and 0.1 times isotonic cell water volume (extracellular NaCl molalities 71 and 1481 mmol/kg, respectively). When, after exposure to anisotonic solution, cord RBCs were transferred back to isotonic conditions, lower osmotic tolerance limit was 0.2 times isotonic cell water volume (extracellular NaCl molality 731 mmol/kg) (Figure 5-2).

Osmotic tolerance limits of RBCs in the presence of 40% glycerol

Osmotic tolerance of glycerolized frozen-thawed cord RBCs was lower then glycerolized non-frozen cord RBCs (Figure 5-3). After cord RBCs were loaded with 40% (w/v) glycerol, their upper limit of osmotic tolerance was 1.7 times their original water volume (extracellular molality 3141 mmol/kg glycerol and 90 mmol/kg NaCl). After glycerolized cord RBCs were subjected to freezing, hemolysis in isotonic solution was similar to glycerolized non-frozen RBCs ($p=0.690$). However, these cells were not able to withstand swelling to 1.7 times their original water volume without significant lysis ($p=0.016$ vs non-frozen RBCs) (Figure 5-3). It was not possible to obtain the data on lower osmotic

tolerance limit for glycerolized cord RBCs, because, to induce shrinkage, glycerol concentrations much higher than 40% (w/v) would be required.

Comparison of osmotic tolerance limit between cord and adult RBCs

After exposure to 40% (w/v) glycerol without freezing, cord RBCs looked more resistant to osmotic lysis than adult RBCs when swollen to 2.5 times their original water volume (Figure 5-4). Data for adult RBCs was taken from Meryman and Douglas [12]. Since values for adult RBC hemolysis are approximated from the figure, it was not possible to perform statistical analysis on this data subset.

Figure 5-5 shows the comparison of osmotic tolerance of glycerolized frozen-thawed cord RBCs (my data) and adult RBCs (data obtained from Ratih Lucianti and Dr. Adam Higgins, Oregon State University, OR, USA). After exposure to 40% (w/v) glycerol with subsequent freezing and thawing, cord RBCs appeared more resistant to swelling than adult RBCs. This trend was more apparent in the lower range of molalities ($(V_w+V_{glyc})/V_{w0} > 2$, cells swell to twice their original water volume). Statistically significant difference between cord and adult RBC hemolysis was only observed when cells were swollen to 2.5 times their original water volume ($p=0.036$). At smaller degrees of swelling ($(V_w+V_{glyc})/V_{w0} < 2$), cord RBCs showed higher hemolysis than adult RBCs (although not statistically significant).

5.4 Discussion

In this Chapter I report the osmotic tolerance limits for cord and adult RBCs, fresh and in the presence of 40% glycerol (to simulate RBCs that are subjected to standard high glycerol – slow cooling cryopreservation procedure). My major findings were that 1) upper and lower osmotic tolerance limits of cord RBCs are $1.7V_0$ and $0.2V_0$, respectively (V_0 is isotonic volume of water in the cell), and 2) cord RBCs are generally more osmotically tolerant than adult RBCs. The majority of published studies on this topic report hypotonic NaCl concentration required to produce 50% hemolysis in RBC population as a measure of osmotic fragility. This study reports values for RBC hemolysis over the broad range of NaCl concentrations, investigating RBC resistance to shrinking as well as swelling.

Osmotic tolerance of cord RBCs

My osmotic tolerance data shows that fresh cord RBCs can swell up to 1.7 times their original water volume and shrink up to one-fifth of their original water volume without significant hemolysis. In other words, for cord RBCs to remain intact, molality of extracellular NaCl should not exceed the range between 70 and 730 mmol/kg (Figure 5-2). Cord RBCs that have been cryopreserved in the presence of 40% (w/v) glycerol can withstand even lesser degree of swelling (Figure 5-3). Relative to cryopreservation, this means that cord RBCs are more

likely to be damaged during post-thaw removal of glycerol by means of salt solutions, than by an abrupt addition of glycerol prior to freezing. Therefore, caution has to be exercised not to exceed the critical maximum volume of these cells during cryopreservation processing [11].

My data on osmotic tolerance of cord RBCs is in general agreement with the published literature. Godal et al. in 1980 reported that fresh adult RBCs undergo 50% hemolysis in ~0.45% NaCl, which is equal to 75 mmol/kg [13]. The same is true for cord RBCs in my experiment, as can be seen from Figure 5-2 (hemolysis at $V_w/V_{w0}=2$, which occurs at 56 mmol/kg NaCl, is approximately 50%). Bautista et al., however, reported that 50% hemolysis in full-term neonatal and adult RBCs occurs at lower concentration of NaCl (~0.37%) [14]. Another article by Linderkamp et al. states that swelling index (maximum volume that RBC can reach without lysis divided by isotonic RBC volume) is 1.66 ± 0.03 (SD) for cord RBCs and 1.64 ± 0.05 (SD) for adult RBCs [15]. This also agrees with my finding that cord RBCs can increase their water volume up to 70% compared to their original water volume (Figure 5-2).

Shrunken RBCs hemolyze upon return to isotonic conditions

The difference in hemolysis of RBCs in hypertonic solution and after return to isotonic environment confirms observation of Lovelock. He found that damage in RBCs subjected to excessive shrinking in extremely hypertonic solutions is not

immediately obvious when cells remain in hypertonic solution, but shows itself when cells are returned to isotonic solution. Lovelock suggested that RBC becomes permeable to sodium ions in solutions with high ionic strength, and cell hemolysis in this case results from the dilution stress caused by an excessive intracellular osmotic pressure [2].

Cord RBCs show different resistance to swelling than adult RBCs

My data seem to suggest, that cord RBCs show slightly higher osmotic tolerance compared to adult RBCs when swollen to more than twice their original water volume; however, at smaller degrees of swelling, cord RBCs may be less osmotically resistant (Figures 5-5 and 5-6). However, several factors prevent me from drawing this conclusion with certainty. In Figure 5, hemolysis of cord RBCs in 40% (w/v) glycerol exposed to hypotonic solutions was compared to adult RBC data obtained by Meryman and Douglas [12]. Meryman and Douglas used 5 M glycerol. In my experiment, I used 40% (w/v) glycerol, which is equal to aproximately 4.3 M. Although these values are quite similar, these two sets of data can be compared only with a limited accuracy. In Figure 5-5, hemolysis of cord RBCs frozen and thawed in 40% (w/v) is compared to adult RBCs that underwent the same treatment (data by Lucianti). Both experiments were done following the same procedure and the same relative cell water volumes were achieved upon addition of glycerol-NaCl solutions. It is worth to mention,

however, that adult RBCs contained ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, instead of citrate-phosphate dextrose (CPD) used for cord RBCs in my experiment. Godal et al. reported that osmotic fragility of RBCs is greater when EDTA is used as an anticoagulant compared to heparin [13]. Therefore, increased hemolysis of adult RBCs in hypotonic glycerol-NaCl solutions can partly be caused by EDTA.

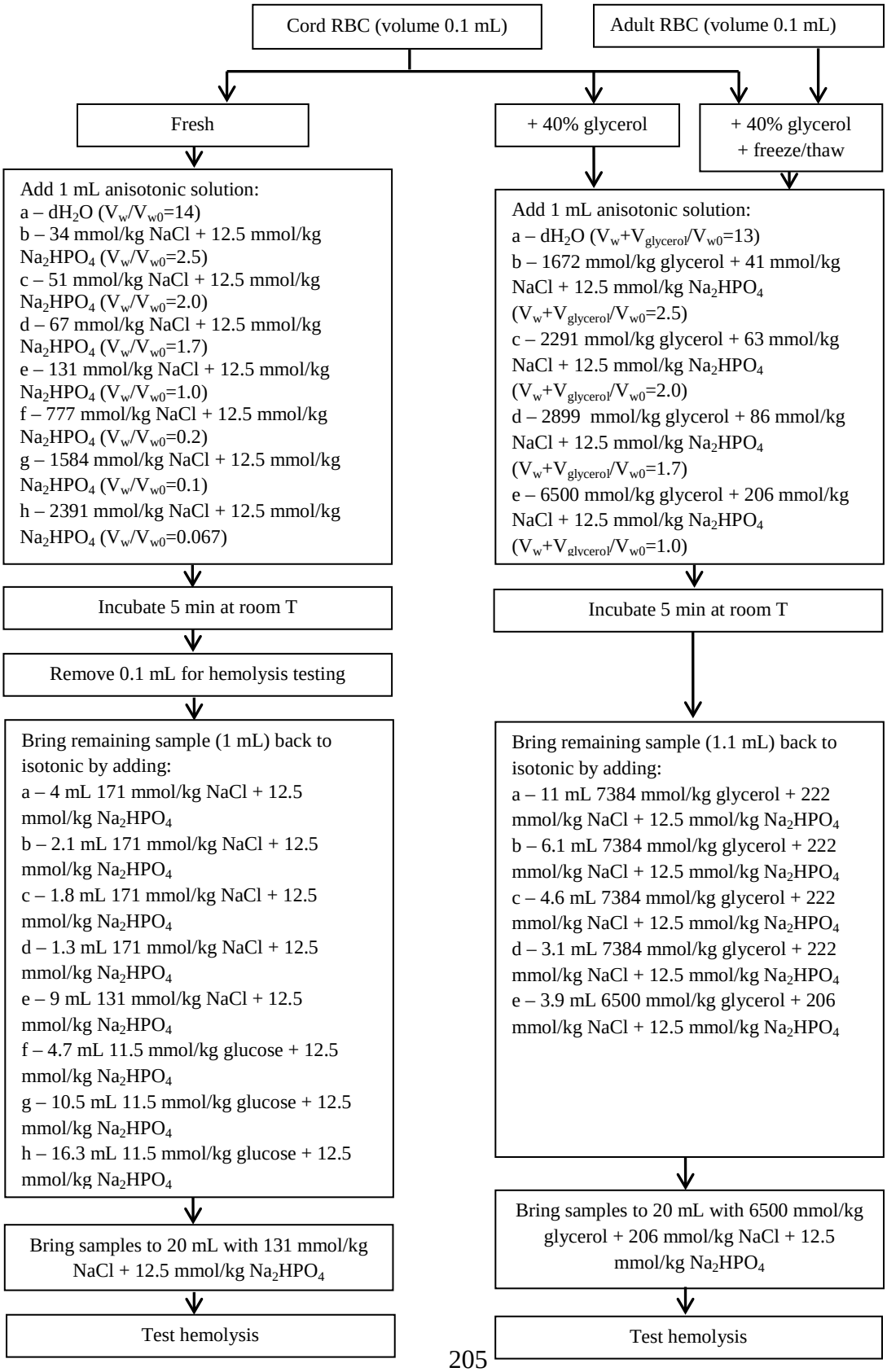
Lucianti's (Figure 5-5) and Meryman and Douglas's data (Figure 5-4) on osmotic tolerance of adult RBC in the presence of 40% (w/v) glycerol are consistent with one another.

Literature data comparing osmotic tolerance of neonatal and adult RBCs is conflicting. Lindercamp et al. showed that the swelling indexes (maximum volume the cell can achieve without lysis) of neonatal and adult RBCs are the same [15]. Bautista et al. reported osmotic tolerance of RBCs decreased with age, so that RBCs of pre-term neonates are the most osmotically resistant, followed by full-term neonates, and then adults [14]. In particular, they showed that RBCs of full-term neonates and adults have similar NaCl concentration that causes 50% hemolysis. However, RBCs from full-term neonates have higher difference in NaCl concentration that causes 80% and 20% hemolysis (another marker of osmotic resistance), and longer lysis time based on glycerol lysis test.

In conclusion, I determined the limits of osmotic tolerance of cord RBCs: fresh, in the presence of 40% glycerol, and cryopreserved with 40% glycerol according to the standard high glycerol – slow cooling procedure. Cord RBCs were found to be generally more tolerant to osmotic swelling than adult RBCs,

however the difference in tolerance between these two types of cells depends on the extent of swelling. This information will be applied in future for the development and optimization of cryopreservation method for cord RBCs, with particular focus on the procedure for addition and removal of CPA.

Figure 5-1. Experimental design for osmotic tolerance experiments



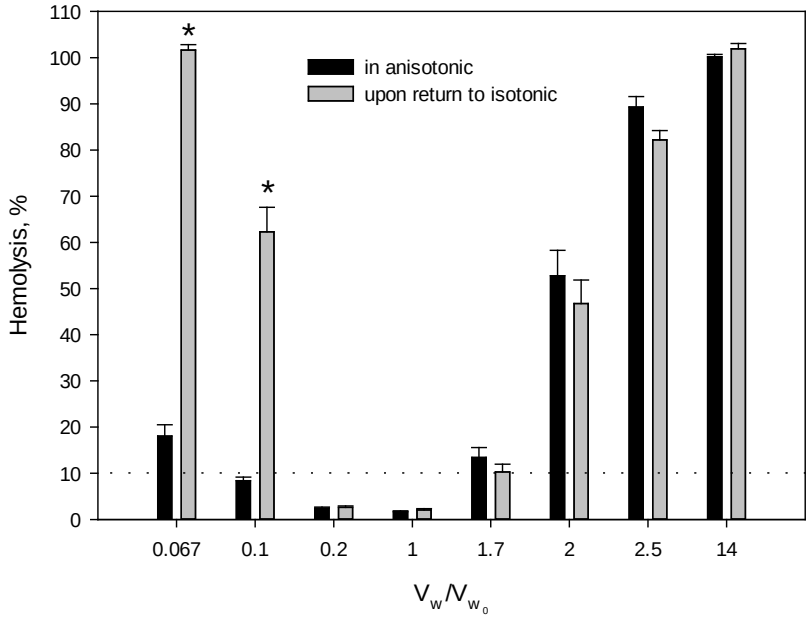


Figure 5-2. Hemolysis of fresh cord RBCs upon exposure to various molalities of non-permeating solute (NaCl). Hemolysis was measured in anisotonic solution and upon return to isotonic environment. V_w/V_{w0} is the relative water volume of RBCs upon exposure to anisotonic solution. Mean ± SEM, n=7. * $p<0.05$ vs hemolysis in anisotonic (Mann-Whitney test).

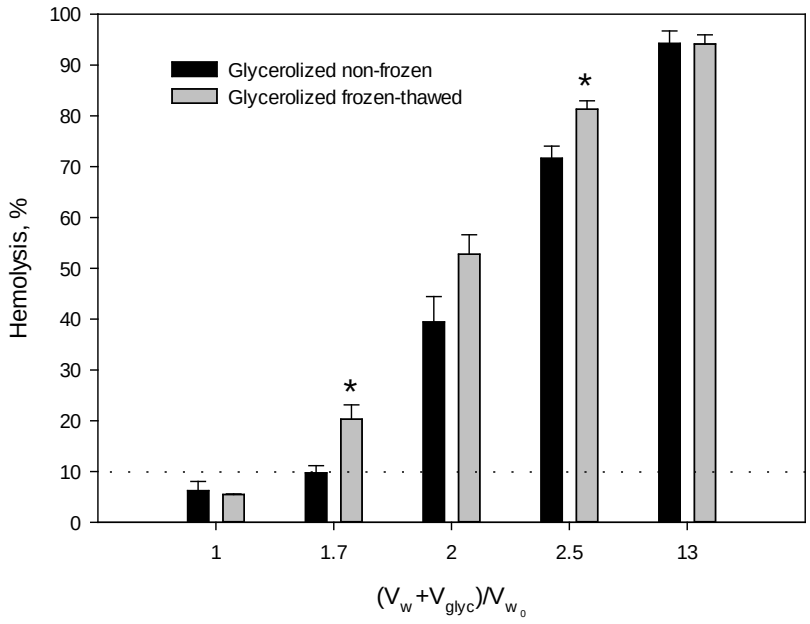


Figure 5-3. Hemolysis of cord RBCs in the presence of 40% (w/v) glycerol upon exposure to various molalities of permeating and non-permeating solute (glycerol-saline). After addition of 40% (w/v) glycerol, RBCs were either tested immediately (non-frozen), or cooled at 1ºC/minute to -80ºC, left for at least 48 hours, and subsequently thawed at 37ºC (frozen-thawed). V_w+V_{glyc}/V_{w0} is the relative water+glycerol volume of RBCs upon exposure to anisotonic solution. Mean ± SEM, n=5. * $p<0.05$ vs non-frozen (Mann-Whitney test).

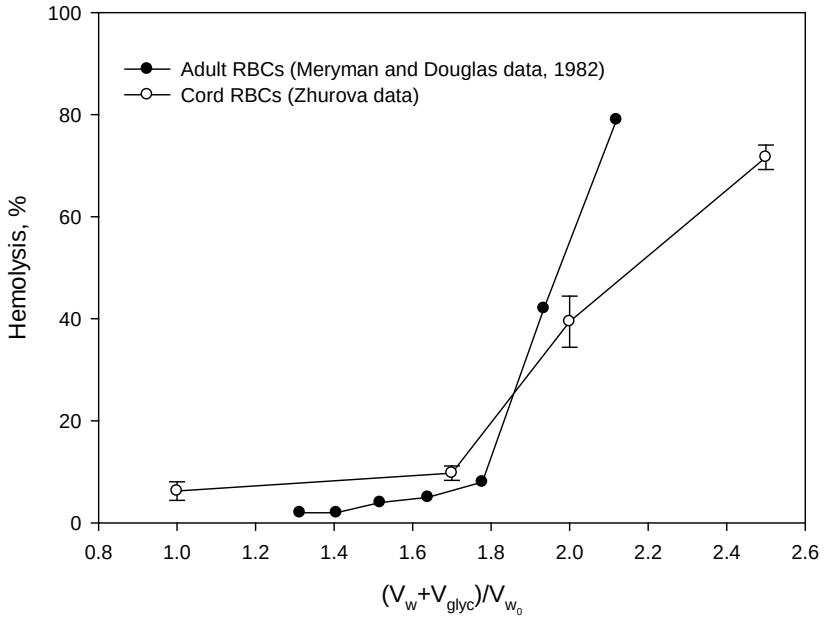


Figure 5-4. Hemolysis of fresh cord and adult RBCs in the presence of glycerol upon exposure to solutions of various molalities. V_w+V_{glyc}/V_{w0} is the relative water+glycerol volume of RBCs upon exposure to anisotonic solution. Cord RBCs were loaded with 40% (w/v) glycerol (equivalent to ~4.3 M) and different relative cell volumes were achieved by exposure to various concentration of permeating and non-permeating solute (glycerol-saline). Data for adult RBCs was taken from Meryman and Douglas [12]. Adult RBCs were loaded with 5 M glycerol and different relative cell volumes were achieved by exposure to various concentrations of non-permeating solute (NaCl). Values for adult RBC hemolysis are approximate. Mean ± SEM, n=5 cord RBC samples, number of adult RBC samples is unknown.

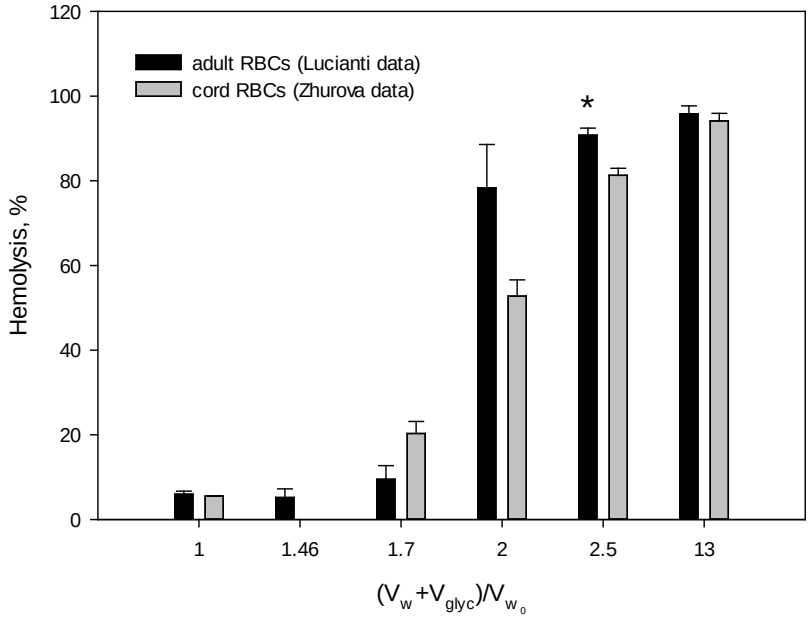


Figure 5-5. Hemolysis of cord and adult RBCs frozen in the presence of 40% (w/v) glycerol upon exposure to various molalities of permeating and non-permeating solute (glycerol-saline). After addition of 40% (w/v) glycerol, both types of RBCs were cooled at 1ºC/minute to -80ºC, left for at least 48 hours, and subsequently thawed at 37ºC. V_w+V_{glyc}/V_{w0} is the relative water+glycerol volume of RBCs upon exposure to anisotonic solution. Data for adult RBCs was obtained from Ratih Lucianti and Dr. Adam Higgins, Oregon State University, OR, USA. Mean ± SEM, n=5 cord RBC samples and 3 adult RBC samples. * $p<0.05$ vs cord (Mann-Whitney test).

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Chapter 6

Cryopreservation of cord and adult red blood cells using high glycerol – slow cooling method

6.1 Introduction

Despite several reports describing the effect of cryopreservation on cord red blood cells (RBCs) [1, 2], an effective cryopreservation method for cord RBCs still needs to be developed. Cryopreservation may prevent the deterioration of cord RBCs seen during liquid storage and maintain a high quality of cord RBC unit for use in transfusion. Also, for the small volume top up transfusions in preterm and neonatal patients [3], it would be possible to cryopreserve cord RBCs from a single donor in small aliquots and, by thawing one at a time, perform multiple transfusions from a single source. This would limit the exposure of the neonate to multiple donors and the concomitant adverse effects.

In Chapter 4 of this thesis, I have demonstrated that cord RBCs are different from adult RBCs in regards to a number of osmotic parameters (such as permeability to water and glycerol, as well as activation energies for these processes). In Chapter 5 I showed that osmotic tolerance of cord RBCs also differ from that of adult RBCs. Since these are major determinants of RBC response to cryopreservation [4-7], I hypothesize that the conventional cryopreservation method for adult RBCs (high glycerol – slow cooling) will result in poorer

recovery of cord RBCs. My objective is to compare the recovery of cord and adult RBCs cryopreserved using the standard high glycerol – slow cooling method.

6.2 Materials and Methods

Overall study design

The cryogenic experimental procedure was designed to parallel the method used to freeze adult RBCs for transfusion, but was performed on a smaller scale. Adult and cord RBC samples were cryopreserved in parallel in accordance with the standard high glycerol – slow cooling method [8]. After glycerolization, samples were either left unfrozen in glycerol to evaluate the hemolytic effect of glycerol addition and glycerol toxicity, or cryopreserved and left at -80ºC for at least 48 hours. Freeze-thaw-wash recovery and percent hemolysis were assessed immediately after deglycerolization. Since RBC membrane damage develops with time, percent hemolysis was also assessed after storage for 24 hours at 1-6ºC.

Preparation of cord and adult RBC samples

Cord RBCs were obtained from Alberta Cord Blood Bank as a waste product after stem cell isolation from cord blood. Only fresh cord RBCs, isolated from cord

blood within 24 hours post cord blood collection, were used for experiment. To remove any residual pentastarch used during cord blood processing [9], cord RBC concentrate was centrifuged at 2200 g, 4°C for 5 minutes and supernatant was discarded. Equal volume of 0.9% sodium chloride (NaCl) solution was then added to cord RBC pellet. Suspension was centrifuged again, supernatant discarded, and RBC pellet re-suspended in 0.9% NaCl to approximately 60% hematocrit. Hematocrit is the fraction of the solution occupied by RBCs [10].

Whole peripheral blood of volunteer donors was freshly collected into EDTA anticoagulant. To isolate adult RBCs, whole blood was centrifuged at 2200 g, 4°C for 5 minutes, supernatant and buffy coat layer (containing leukocytes and platelets) were discarded. For the first wash, equal volume of 0.9% NaCl was added to RBC pellet, tube was centrifuged, and supernatant was discarded. The second wash was performed following the same procedure. After the second wash was complete, RBC pellet was re-suspended in 0.9% NaCl to approximately 60% hematocrit.

Glycerolization and freezing of RBCs

2.5 mL of RBCs were transferred to 50 mL plastic conical tube. Glycerolyte 57 solution (Baxter Corporation, Missisauga, Ontario) and RBC tube were warmed in 37°C water bath for 10 minutes. Glycerolyte 57 solution (total of 4 mL) was added to RBCs to final glycerol concentration of 35% (w/v) in two steps. First, 1

mL of Glycerolyte 57 was added to RBCs drop wise during 60 seconds and left to incubate at room temperature for 10 minutes. After that, the remaining 3 mL of Glycerolyte 57 were added to RBCs at once and left to incubate at room temperature for 10 minutes. Samples were either left unfrozen in glycerol ('glycerolized control'), or cooled at 1ºC/min to -80ºC by placing tubes with glycerolized RBCs into -80°C freezer. Cryopreserved samples were left frozen at -80ºC for at least 48 hours.

Thawing and deglycerolization of RBCs

Tube containing cryopreserved RBCs was thawed in a 37ºC water bath until the last bit of ice melted. Cryopreserved RBCs and 'glycerolized controls' were deglycerolized by a series of washes with decreasing concentrations of NaCl solutions, to facilitate glycerol efflux from RBCs. For the first wash, 1.5 mL of 12% NaCl were added to glycerolized RBCs drop wise during 60 seconds and left to equilibrate at room temperature for 3 minutes. After that, 10 mL of 1.6% NaCl were added to RBCs at once, tube was centrifuged at 3000 g, 4°C for 8 minutes, and supernatant was carefully aspirated not to disturb RBC pellet and discarded. For the second wash, 10 mL of 1.6% NaCl were added to RBCs at once, tube was centrifuged at 3000 g, 4°C for 6 minutes, and supernatant was discarded. For the final wash, 10 mL of 0.9% NaCl with 0.2% dextrose were added to RBCs at once, tube was centrifuged at 3000 g, 4°C for 7 minutes, and supernatant was discarded.

After the final wash was complete, RBC pellet was mixed with residual supernatant, which would normally result in approximately 60% hematocrit, or resuspended in 0.9% NaCl, if further dilution was necessary.

Freeze-thaw-wash recovery

50 mL plastic conical tube was weighed before and after 2.5 mL of RBCs were transferred into it, and the *pre-frozen net weight of RBCs* was calculated by subtracting the weight of empty tube from the weight of tube with RBCs. After deglycerolization was complete, tube with RBCs was weighed again, and the *post-deglycerolized net weight of RBCs* was calculated by subtracting the weight of empty tube from the weight of tube with deglycerolized RBCs. Hematocrits (Hct) of untreated RBC sample (*final pre-deglycerolized Hct*) and of deglycerolized RBC sample (*final post-deglycerolized Hct*) were measured using the microhematocrit centrifuge (Hettich, Tuttlingen, Germany) as the ratio of the volume occupied by packed RBCs to the volume of a whole RBC sample [10]. RBC recovery was calculated as the percentage of RBCs left in the sample after deglycerolization according to the following formula [11]:

$$Recovery\ (\%) = \frac{[Post-Deglycerolized\ Net\ Weight\ (g)] \times \left[Final\ Post-Deglycerolized\ Hct\ \left(\frac{L}{L}\right)\right]}{[Pre-Deglycerolized\ Net\ Weight\ (g)] \times \left[Final\ Pre-Deglycerolized\ Hct\ \left(\frac{L}{L}\right)\right]} \times 100$$

(Eq. 6-1)

Percent hemolysis

RBC hemolysis was determined by spectrophotometric measurement of total and supernatant cyanmethemoglobin according to Drabkin's method [12]. Controls for total hemoglobin were prepared from Stanbio Tri-Level Hemoglobin controls (Stanbio Laboratory, Boerne, TX, USA). Hematocrit of RBC sample was measured using the microhematocrit centrifuge (Hettich, Tuttlingen, Germany) as the ratio of the volume occupied by packed RBCs to the volume of a whole RBC sample [10].

Statistical analysis

Comparison between adult and cord RBCs, as well as between different treatment groups, was performed by Mann-Whitney non-parametric test in SPSS software (version 12.0, SPSS Inc., Chicago, IL, Texas). Significance level was set to 0.05.

Contributions of others

Personnel of Alberta Cord Blood Bank provided cord RBC samples for this study.

6.3 Results

After cryopreservation, freeze-thaw-wash recoveries of cord RBCs and adult RBCs were both within the clinically acceptable range of ≥ 80%. The recovery of cord RBCs was lower than that of adult RBCs after the glycerolization step and after the complete cryopreservation process ($p=0.016$ and 0.032, respectively). It was interesting to see that the recovery of RBCs after the glycerolization step alone (without freezing and thawing) was the same as recovery after complete cryopreservation process ($p=0.290$ for cord RBCs and 0.064 for adult RBCs) (Figure 6-1).

There was no difference in hemolysis of cord RBCs and adult RBCs in untreated samples, after glycerolization step, after the complete cryopreservation process, and 24 hours after both treatments. However, for both cord and adult RBCs hemolysis increased significantly 24 hours after deglycerolization and cryopreservation (Figure 6-2).

6.4 Discussion

In this Chapter, I compared post-thaw recovery and hemolysis of cord and adult RBCs cryopreserved according to the standard high glycerol – slow cooling method. After cryopreservation using standard high-glycerol slow-cooling method, the recovery of cord RBCs was slightly, although significantly, lower compared to adult RBCs (Figure 6-1). This proves my original hypothesis that the conventional cryopreservation procedure for adult RBCs will result in poorer recovery of cord RBCs.

The post-thaw recovery (Figure 6-1) and hemolysis (Figure 6-2) of cord and adult RBCs were both within the clinically acceptable limits (≥ 80% recovery [13] and < 0.8% hemolysis [14]). It is important to keep in mind, that cryopreservation and processing of RBC samples were performed manually in an open system (tubes), unlike clinical cryopreservation and processing of RBC samples that are performed automatically in a closed system (blood bags). Manual processing in an open system inevitably causes higher RBC loss. It is reasonable to expect even higher post-thaw recovery if RBCs are cryopreserved and processed in a closed system. Low post-thaw hemolysis of RBCs is due to the fact, that the majority of free hemoglobin is removed with supernatant during deglycerolization stage.

Addition and removal of glycerol appeared to be the primary cause of RBC damage, since red cell recoveries after addition and removal of glycerol

were the same as recoveries after complete cryopreservation procedure (Figure 6-1).

Hemolysis of deglycerolized RBCs significantly increased after 24 hours of storage in saline (Figure 6-2). This signifies, that RBC membrane was compromised to a certain extent during addition and removal of glycerol, as well and freeze-thaw treatment, and damage developed over time. This observation is in agreement with standard guidelines for using cryopreserved RBCs for transfusion, which state that deglycerolized RBCs must be stored for only 24 hours at 1-6°C prior to being transfused [13]. My value for hemolysis of adult RBCs 24 hours post-thaw was higher (2.13 ± 0.68%, mean ± SD) than the one obtained for adult RBC units cryopreserved using Canadian Blood Service's current procedure and processed on the COBE blood cell processor (0.57 ± 0.15%, mean ± SD) [15]. Such difference can be explained by the small volume of RBC samples in my experiment, and different nature of samples (open tubes vs blood bags).

Potential limitation of this study is the fact that adult peripheral blood was collected in EDTA anticoagulant, whereas umbilical cord blood was collected in CPD anticoagulant. Godal et al. reported that osmotic fragility of RBCs is greater when EDTA is used as an anticoagulant compared to heparin [16]. In future, anticoagulant in adult and cord blood units needs to be standardized (for example, CPD) in order to compare recoveries of adult and cord RBCs with better certainty.

Conceptual model of cord RBC damage during cryopreservation

Information on the differences in osmotic parameters and cryopreservation recovery between adult and cord RBCs can be synthesized into a model of cord RBC damage during cryopreservation. This model attempts to answer the question why traditional high glycerol – slow cooling cryopreservation method results in lower recovery of cord RBCs compared to adult RBCs. For convenience, cryopreservation process is divided into four steps – addition of glycerol, freezing, thawing, and deglycerolization – and possible causes of cord red cell damage during each of these steps are discussed.

1. *Addition of glycerol*

Glycerol (~57% solution) was added to RBCs at 37°C dropwise, with constant agitation, in two steps. Final concentration of glycerol inside and outside the cells was ~40%. Since permeability to glycerol ($P_{glycerol}$) of cord RBCs is equal to adult RBCs at 37°C, the rate of glycerol entry into the cell and final concentration of glycerol inside RBCs should be equal for cord and adult RBCs. Upon addition of cryoprotectant, cell first shrinks, and later, as glycerol gradually permeates the cell, returns to its original volume. It is possible that cord RBCs are less osmotically tolerant to shrinking compared to adult RBCs, however at this point I do not have experimental data to verify this assumption (osmotic tolerances of cord vs adult RBCs were only

compared during cell swelling). Another possible cause of cell damage during this step is glycerol toxicity. If glycerol at high concentrations is more toxic for cord RBCs than for adult RBCs, this explains lower post-thaw recovery of the former. Glycerol toxicity for cord and adult RBCs must be investigated in future in order to test this hypothesis.

2. Freezing

After RBCs had been equilibrated with ~40% glycerol, they were cooled at approximately 1°C/min to final storage temperature of -80°C. Cooling at 1°C/min is considered a slow cooling. During slow cooling, ice crystals form outside the cell and cause efflux of water from the cell in the direction of osmotic gradient. As the result, cell can be damaged by dehydration and increased concentration of intracellular solutes. Although intracellular cryoprotectant glycerol significantly reduces the magnitude of such damage, it can still occur to a certain extent. Prior to freezing, concentration of non-permeating solutes inside and outside RBC is ~450 mOsm/kg. However, when ice forms outside the cell, osmolality of non-permeating solutes increases significantly. I have previously shown that at salt concentration from 300 to ~1000 mOsm/kg, hydraulic conductivity (L_p) of cord and adult RBCs is equal; at salt concentrations > 1000 mOsm/kg, L_p of cord RBCs is less than of adult RBCs. This means that, depending on what extracellular salt concentration is reached upon ice formation outside the cell, water will leave cord RBCs at the

same or slower rate compared to adult RBCs. Activation energy for hydraulic conductivity is also similar for cord and adult RBCs, which means that both cell types will experience similar rate of decrease of hydraulic conductivity as the temperature drops. If that is the case, cord RBCs have the same or lower likelihood of damage by increased concentration of extracellular solutes. Therefore, it is not likely that freezing step on its own causes more damage to cord RBCs than to adult RBCs.

3. Thawing

After RBCs had been stored at -80°C for a certain amount of time, they were rapidly thawed by immersion into 37°C water bath until the last bit of ice melted. During thawing process, as extracellular ice melts, water enters the dehydrated cell trying to dilute intracellular salts, concentrated during freezing step. I have determined that cord RBCs are more permeable to water than adult RBCs at 37°C. Even though the influx of water into cord RBCs upon thawing will probably be more rapid than into adult RBCs, final RBC volume upon rehydration will be determined by the concentration of intracellular solutes and not by the velocity of water entry into the cell. Therefore, it is unlikely that excessive damage to cord RBCs is caused during this step.

4. Deglycerolization

Upon thawing, glycerol was removed from RBCs by a series of washes with decreasing concentrations of sodium chloride (NaCl) solutions, to facilitate glycerol efflux from RBCs. NaCl solutions were added to RBCs at room temperature, and RBCs were subsequently centrifuges at 4°C. I have previously determined, that $P_{glycerol}$ of cord RBCs is equal (at room temperature) or higher (at 4°C) compared to adult RBCs. This means that the same degree of deglycerolization would be achieved for cord and adult RBCs, since glycerol would leave cord RBCs at the same or even faster rate. The damage during this deglycerolization step, however, can be caused the difference in osmotic tolerance between cord and adult RBCs. When NaCl solution is added to red cell containing glycerol, cells first swells as a result of water moving into the cell trying to dilute intracellular glycerol, and then, as glycerol leaves the cell, cell gradually returns to its initial volume. My previous data suggests that, although, after glycerolization with subsequent freezing and thawing, cord RBCs were more resistant to swelling than adult RBCs, this increased resistance was mostly observed when cells were forced to swell to twice or more their initial water volume. At smaller degrees of swelling, cord RBCs showed higher hemolysis than adult RBCs (although not statistically significant). Therefore, swelling of RBCs upon dilution with NaCl solutions can potentially cause more damage to cord RBCs and result in larger cell loss.

In conclusion, these results demonstrated that high glycerol – slow cooling cryopreservation method in its original form results in lower recovery of cord RBCs. Optimization of this cryopreservation method for cord RBCs is required in future. Addition and removal of glycerol is the primary factor of RBC injury during cryopreservation and, therefore, particular attention must be given to optimization of this process to improve cord RBC yield. Proposed model attempts to link cord RBC osmotic parameters to their lower recovery after cryopreservation. Future work needs to be undertaken in order to establish direct causation between individual osmotic parameters and cryopreservation outcome for cord RBCs.

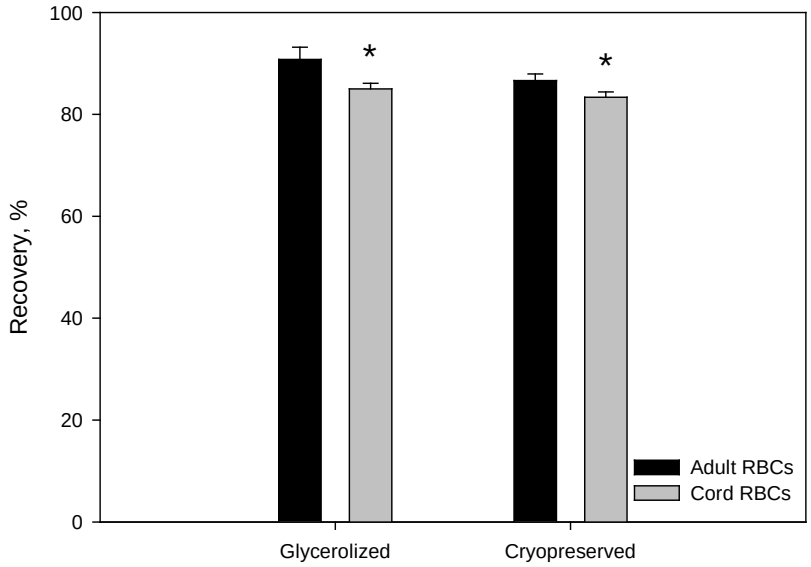

Figure 6-1. The recovery of cord RBCs and adult RBCs after glycerolization and cryopreservation using standard high glycerol – slow cooling method. Mean ± SEM, n=8 adult RBC samples and 10 cord RBC samples. * $p<0.05$ vs adult (Mann-Whitney test).

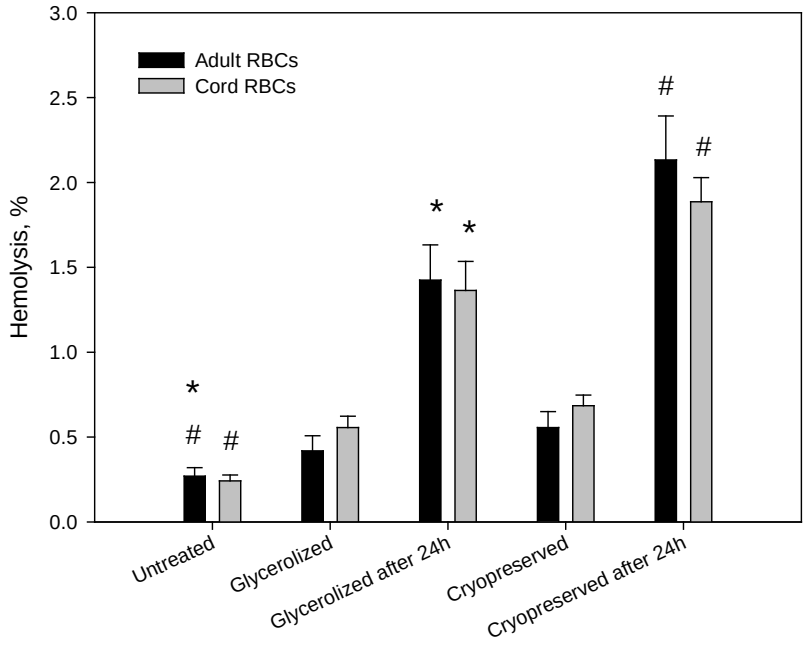

Figure 6-2. The hemolysis of cord RBCs and adult RBCs after glycerolization and cryopreservation using standard high glycerol – slow cooling method. Mean ± SEM, n=8 adult RBC samples and 10 cord RBC samples. * $p<0.05$ vs glycerolized, # $p<0.05$ vs cryopreserved (Mann-Whitney test).

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Chapter 7

General discussion and conclusions

7.1 Summary of results

The work presented in this thesis was conducted to understand the physiology of RBCs derived from umbilical cord blood (cord RBCs) from a cryobiological perspective with an attempt to relate this knowledge to the development of the successful cryopreservation technique for these cells. Investigations were conducted on several levels, starting from assessing the initial quality of cord RBCs after their isolation from umbilical cord blood and ending by the determination of cryobiological parameters of cord RBC membrane.

In Chapter 2, I assessed the quality of cord RBCs upon their isolation from cord blood. Cord blood collected upon delivery can be stored at room temperature for up to several days, with little known about how these storage conditions affect the quality of RBCs, which are currently treated as waste. Therefore, it was important to study the effect of cord blood storage conditions on cord RBCs to ensure that only a high quality cord RBC product was used in subsequent cryopreservation studies. Assays included traditional assessment of integrity and function of RBCs, as well as novel predictors of *in vitro* quality. I found that there was no significant decrease in quality measures of cord RBCs during the first 65 hours of whole cord blood storage at room temperature. However, the ratio of

cord blood volume to anticoagulant volume in cord blood collection bag appeared to be important and too much anticoagulant adversely affected quality measures of cord RBCs. Therefore, cord blood:anticoagulant ratio needs to be optimized in future in order to ensure that a good quality cord RBCs are preserved.

In Chapter 3, I developed a novel method to measure rapid osmotically-induced changes in RBC volume. Many traditional methods that measure kinetics of cell volume are not suitable for fast-responding RBCs, and several methods that are currently used for RBCs (stopped-flow fluorimetry and stopped-flow by light scattering) have limitations. My new method is fast and easy to perform, does not require addition of fluorescent dyes to RBCs, and is based on my finding that there is a direct relationship between RBC volume and intrinsic fluorescence of intracellular hemoglobin. This method involves rapid mixing of RBCs with anisotonic solution in stopped-flow system and simultaneous measurement of intrinsic hemoglobin fluorescence as a function of time after mixing. RBC shrinking causes decrease in hemoglobin fluorescence and RBC swelling – increase in hemoglobin fluorescence. As a result, changes in RBC volume upon exposure to anisotonic solutions can be accurately measured via changes in intrinsic hemoglobin fluorescence. This phenomenon occurs due to the self-quenching of RBC hemoglobin at high intracellular concentrations, which is a specific example of inner-filter effect that takes place in concentrated fluorophore solutions. The rate of cell volume change in anisotonic solutions of permeating and non-permeating solutes is key to determining permeability of cell membrane to these solutes. The ultimate goal of this method's development was to use it to

determine cryobiological parameters of cord RBCs (permeability to water and solutes, as well as activation energies for these processes). The method also has numerous clinical applications. It may result in faster diagnosis and better understanding of hematological diseases, in which erythrocyte permeability is altered, such as sickle cell anemia, hereditary spherocytosis, stomatocytosis, xerocytosis, and malaria.

In Chapter 4, I measured the osmotic parameters of cord and adult RBCs, such as osmotically inactive fraction of cell volume, hydraulic conductivity and osmotic permeability to common cryoprotectant glycerol, and Arrhenius activation energies for water and glycerol permeability. Osmotic parameters of adult RBCs are well characterized in the literature, however, published data on osmotic parameters of cord RBCs is limited and, being obtained by different experimental methods, controversial. These parameters are critical for the development of a cryopreservation technique for cord RBCs since they determine cell behavior during cryopreservation. To measure permeabilities of RBC membrane, I applied the new method developed in Chapter 3. I was able to determine values for cord RBC osmotically inactive fraction, cord and adult RBC hydraulic conductivity and osmotic permeability to glycerol with corresponding activation energies, as well as characterize the dependence of permeability parameters on temperature and extracellular solute concentration. I found that cord RBCs have a larger osmotically inactive fraction compared to that reported for adult RBCs. Contrary to the common belief that cord RBCs are always less permeable to water and glycerol than adult RBCs, I found that permeability of

cord RBCs differs from that of adult RBCs depending on the experimental conditions. In particular, cord RBC permeability to water can be lower, equal, or higher than in adult RBCs depending on temperature and extracellular osmolality. Likewise, permeability of cord RBCs to glycerol can be higher or equal to adult RBCs at different temperatures. Cord and adult RBCs had similar value of activation energy for hydraulic conductivity, however cord RBCs had lower activation energy for glycerol permeability. In future, this information on osmotic parameters can be used in mathematical modelling to predict cryopreservation parameters (such as cooling and warming rates, concentration of cryoprotectant, etc.) for cord RBCs.

In Chapter 5, I continued the investigation of osmotic parameters and determined osmotic tolerance limits of cord RBCs. Knowledge of osmotic tolerance limits of the cell is important when adding and removing cryoprotectant, taking care not to exceed critical volume of the cell and cause cell damage. Osmotic tolerance was tested for non-frozen and frozen-thawed RBCs, in the absence and in the presence of glycerol. Cord RBCs appeared to be more osmotically resistant compared to adult RBCs. I found that for fresh cord RBCs upper and lower osmotic tolerance limits are $1.7V_0$ and $0.2V_0$, respectively (V_0 is isotonic water volume of the cell). Cord RBCs frozen with glycerol could not withstand swelling to 1.7 times their original water volume without significant lysis. Data on osmotic tolerance limits of cord RBCs, combined with my previous knowledge of cord RBCs osmotic parameters, provides key information for the

future development and optimization of the cryopreservation procedure for cord RBCs, in particular, steps involving the addition and removal of cryoprotectant.

In Chapter 6, I examined the feasibility of using the standard high glycerol – slow cooling method to cryopreserve cord RBCs. Since I demonstrated that there are differences in cord and adult RBC osmotic parameters – key determinants of cell response to cryopreservation, it was reasonable to assume that these cells will show different recovery when subjected to the standard adult RBC cryopreservation procedure. Indeed, after high glycerol – slow cooling cryopreservation procedure in its original form, cord RBCs showed lower recovery compared to adult RBCs. The primary damaging factor appeared to be not the freezing process itself, but the addition and removal of glycerol. I proposed a model that describes how differences in osmotic parameters can be linked to differences in cryopreservation outcome, and why standard high glycerol – slow cooling cryopreservation method results in poorer recovery of cord RBCs. Information on osmotic tolerances, critical volume, and glycerol permeabilities of cord RBCs obtained in this thesis is invaluable and will be used in future to optimize the process of addition and removal of glycerol, as well as other parts of a cryopreservation protocol, and improve the post-thaw recovery of cord RBCs.

My original hypothesis states that "Since the osmotic properties of RBCs, such as membrane permeability to water and cryoprotectant and osmotic tolerance limits, determine cell recovery after cryopreservation, differences in osmotic properties of cord and adult RBCs will result in poorer recovery of cord RBCs

after the conventional cryopreservation procedure for adult RBCs". All information obtained from this study gave me enough evidence to conclude that there is a **correlation** between differences in cord and adult RBC osmotic parameters and differences in cord and adult RBC recovery after cryopreservation. However, more work is required in order to establish direct **causation** between individual osmotic parameters and post-cryopreservation recovery of cord RBCs.

7.2. Significance of this work to cryobiology and transfusion medicine

This work has made a number of important contributions to the fields of cryobiology and transfusion medicine.

1. *New knowledge of the quality of red cells in umbilical cord blood*

   I characterized the quality of red blood cells present in umbilical cord blood and evaluated its dependence on conditions of cord blood collection, processing and storage. Cord RBCs have been previously treated as waste and sub-product of stem cell isolation, with nothing known about their quality and the conditions affecting the pre-isolation storage.

2. *Improved procedure for cord blood collection*

I demonstrated the importance of cord blood:anticoagulant volume ratio in cord blood collection bag for the quality of red cells. Currently, all cord blood collection bags have a fixed volume of anticoagulant and cord blood:anticoagulant ratio varies significantly from collection to collection, depending on the amount of cord blood collected from a placenta. Based on these findings, I made a recommendation for further investigation and adjustment of blood:anticoagulant ratio in future.

3. *New method to monitor osmotic volume changes in fast responding cells*

I have developed a new method to monitor fast kinetics of osmotic volume changes of RBCs, which is based on volume-dependent changes in intensity of intracellular hemoglobin fluorescence. There are a number of methods available to monitor the rate of osmotically-induced volume changes in living cells. Only few of those methods are suitable for fast responding RBCs, but have a number of limitations. This method does not require any specific RBC preparation, such as addition of fluorescent dyes, and, therefore, is fast and easy to perform. My new method has numerous applications both within and outside the field of cryobiology. It provides a new and effective tool for measurement of osmotic permeability of red cell to water and solutes with the purpose of incorporating this knowledge into design of cryopreservation techniques for human and other mammalian erythrocytes. Future clinical applications of this method include faster

diagnosis and better understanding of many hematological diseases, in which erythrocyte permeability is altered.

4. *New data on osmotic parameters of cord RBCs*

In this thesis, osmotic parameters of cord RBCs, such as osmotically inactive fraction, hydraulic conductivity, osmotic permeability to glycerol, corresponding activation energies for hydraulic conductivity and glycerol permeability, and osmotic tolerance limits have been reported. Literature contains only very limited data on some of the osmotic parameters of cord RBCs and, considering the importance of these parameters for the development of cryopreservation method for cord RBCs, these new acquired knowledge is of great value. Secondly, by comparing osmotic parameters of cord and adult RBCs, I also challenged common beliefs that cord RBCs are always less permeable to water and glycerol. I showed that the difference between cord and adult RBC permeabilities varies depending on temperature and extracellular osmolality. Finally, by demonstrating that hydraulic conductivity increases with extracellular osmolality, I provided more evidence to potentially resolve the current controversy in the literature around the osmolality-dependence of hydraulic conductivity.

5. *First attempt to cryopreserve cord RBCs using routine technique for adult RBCs*

In this thesis I demonstrated the first attempt to cryopreserve cord RBCs by using routine method for adult RBC cryopreservation and evaluated the effect of this

method on cord RBC post-thaw recovery. Since cord RBCs showed lower recovery than adult RBCs, I synthesized all previously obtained information on osmotic differences between cord and adult RBCs and proposed a model for the potential causes of damage to cord RBCs during this cryopreservation procedure.

In conclusion, this thesis provides all pieces of a puzzle required for the development of effective storage method for cord RBCs, starting from their initial quality upon isolation from umbilical cord blood and ending with cryobiological parameters of cord RBC membrane. Due to numerous potential therapeutic benefits of cord RBC transfusions to fetuses and neonates and the absence of effective storage technique for these cells, this work is of great value. Future studies need to be undertaken to create an effective pre-freeze storage and cryopreservation procedures for cord RBCs. All information on cord RBC osmotic parameters and cryopreservation outcome reported herein needs to be combined in future with the goal to create and optimize a cryopreservation technique that would preserve good quality and result in high post-thaw recovery of these unique cells. If a successful storage technique for cord RBCs is developed, RBCs from umbilical cord blood can be turned from a waste product into a superior blood product for intrauterine and neonatal transfusions. This will potentially overcome numerous side effects of adult RBC transfusions to fetuses and neonates and significantly improve prenatal and postnatal medical care.