Chapter 1

Introduction and aim of the thesis
1.1 Introduction

Blood transfusion experiments were practiced as early in the 17th century. However, it was the discovery of the ABO blood groups in the early 1900 that boosted transfusion medicine. Blood transfusion practice has considerably improved since then and nowadays refrigerated stored red blood cells (RBCs) are routinely infused to compensate for excessive blood loss or to correct for abnormal hemoglobin content. Yet, during refrigerated storage the RBCs undergo structural and biochemical alterations, collectively referred to as the storage lesion, which could compromise their function after infusion. Particularly, alterations in the RBC flow properties (i.e. rheologic properties) may impede the RBCs to properly function in the microcirculation. This chapter describes, from a rheologic perspective, the RBC characteristics and the cellular changes that are observed during refrigerated blood bank storage. In addition, cryopreservation of RBCs will be discussed as an alternative approach to counteract storage induced lesions and thus to extend the shelf life of RBCs.

Characteristics of RBCs

RBCs are the most abundant cells in the blood. Adult humans have approximately 20-30 trillion circulating RBCs and on an average 2.6 million human RBCs are produced every second by the red bone marrow and released into the blood via marrow sinusoids. RBCs have unique structures and properties which enable them to distribute oxygen and collect carbon dioxide in the body. Mature RBCs are shaped as biconcave discs (Figure 1.1) that lack nuclei, mitochondria and other cell organelles. Yet, the RBCs are rich in hemoglobin (Hb), an iron-containing protein that is able to reversible bind oxygen and carbon dioxide.

Figure 1.1. Human red blood cells under physiological conditions.
In the RBC, energy is provided via the anaerobic glycolysis pathway. In this pathway glucose is broken down in pyruvate with among others adenine-triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) as intermediate metabolites. Subsequently, pyruvate is converted to lactate and transported out of the cell. ATP as an energy source is essential for the overall functioning of the RBC. Loss of ATP has been associated with shape changes, enhanced cation permeability, rigid cell membranes, altered surface expression of phospholipids, microvesiculation and decreased RBC viability. Furthermore, ATP released by the RBC is a potent vasodilatation signaling molecule that adapts the blood flow according to local needs. Recently, the ATP content was also correlated with the oxygenation capacity of the RBC. In the RBC binding of 2,3-DPG to the Hb induces a conformation state which will release oxygen from the Hb. Due to the high binding affinity of 2,3-DPG for deoxygenated Hb, oxygen is released in regions with low oxygen tension. Loss of 2,3-DPG will increase the oxygen affinity to the Hb which may hamper the oxygen delivery to the tissues.

The RBC membrane is composed of a lipid bi-layer and an underlying viscoelastic cytoskeleton. This bi-layer consists of a layer of proteins which, due to the sialic acid groups, bears a net negative charge. The viscoelastic cytoskeleton enables the RBC to adapt their shape to maintain the flow behavior in the microvessels.

RBCs in the microcirculation

The microcirculation consists of a network of arterioles, capillaries and venules with a diameter of less than 100 µm. In the capillaries exchange of oxygen and carbon dioxide between circulating RBCs and the surrounding tissues takes place. Most microcirculatory vessels are embedded within an organ. Adequate flow in the microcirculation is thus a requisite for normal organ perfusion and function. The RBC rheologic properties (i.e. the ability of RBC to aggregate, deform and adhere to endothelial cells) are important determinants of the flow behavior of blood and subsequently the oxygenation of the microvascular environment. The ability of RBCs to form linear aggregates (Rouleaux: Figure 1.2) or more complex three dimensional aggregates in regions with low shear rate is a phenomenon that has been studied for decades. RBC aggregation is depending on opposing shear forces. On the one hand RBC aggregation is counteracted by the repulsive force between negatively charged
cells as well as the force generated by the blood flow. On the other hand, RBC aggregation
is promoted by the presence of plasma proteins, most importantly fibrinogen, or by high
molecular weight polymers. 18,19 At present there are two theories proposed to explain the
RBC aggregation mechanism: a bridging and a depletion theory. 20 In the bridging theory, it
is hypothesized that macromolecules adsorbed onto the RBC surface form bridges between
adjacent RBCs, whereas in the depletion theory RBC aggregation is induced due to an
osmotic gradient difference between the polymer concentration near the RBC surface
versus the suspending medium. Although RBC aggregation markedly enhances the blood
viscosity at low shear rate, 21 the physiological role of this process is still elusive. Under
normal physiological conditions, RBC aggregates are easily dispersed by the rise in blood
flow rate. However, in certain pathologic states such as cardiovascular disorders, sepsis,
pre-eclampsia, diabetes mellitus, chronic renal failure, rheumatoid arthritis, inflammation,
hypertension, thalassemia and sickle cell disease, 18,22-26 stronger and or larger aggregates
are formed which are more resistant to dispersion by the blood flow. Essentially, enhanced
RBC aggregation has the potential to impair the blood flow in the microcirculation and
contribute to the occlusion of micro-vessels. 26,27

Figure 1.2. RBC Rouleaux formation in the microcirculation.
The ability of RBCs to deform due to applied shear stress enables these cells to adapt their size to squeeze through narrow capillaries. It also allows RBCs to reduce the blood viscosity at high shear rate, so that blood remains fluid even at a high hematocrit. The RBC deformability is depending on the geometry of the cell, the viscosity of the cytoplasm as well as on the viscoelastic properties of the cytoskeleton. Alterations in these properties can make the RBC more rigid and impair or block the blood flow in the microcirculation. Ultimately, rigid RBCs will be sequestered by the spleen and destroyed. The RBC deformability is thus essential for adequate tissue perfusion and cell survival.

Reduced RBC deformability has been observed in sepsis, diabetes mellitus, malaria, hypertension, thalassemia and sickle cell disease.

The adherence of RBCs to the vascular endothelium is, under physiological conditions, negligible. However, structural changes in the RBC membrane may promote adherence to endothelial cells (ECs) and impair the microcirculatory blood flow. Adherence of RBCs to ECs is mediated by expression of phosphatidylserine (PS) on the RBC surface. PS expression also triggers recognition by macrophages which clears the RBCs from the circulation. Enhanced RBC-EC interaction has been observed in malaria, diabetes mellitus, thalassemia, sickle cell disease.

In summary, changes in the rheologic properties of RBCs may form a circulatory risk by hindering adequate tissue perfusion and contributing to ischemia or even infarction. It is therefore not surprisingly that flow disturbances in the microcirculation are closely associated with morbidity and mortality. These findings highlight the importance of RBC rheologic properties as functionality parameters in transfusion medicine.

Usage of the Laser-assisted Optical Rotational Cell Analyzer

The laser-assisted optical rotational red cell analyzer (LORCA; R&R Mechatronics, Zwaag The Netherlands) is a useful device to determine the aggregation and deformation behavior of RBCs ex vivo. In general, a laser beam is directed through the RBC solution and RBC aggregation is determined by means of backscattered light. Initially, the RBC suspension is sheared to disperse pre-existing RBC aggregates. After several seconds the shear is halted and the light reflection from the suspension is recorded over a certain timeframe and subsequently analyzed by a computer program. As RBCs start to form side to side aggregates (Rouleaux) the intensity of the backscattered light decreases.
exponentially. The aggregation behavior of RBCs is described by the aggregation index (AI), which depends both on the kinetics and extent of aggregation. The RBC deformability can be determined at various fluid shear stresses by means of laser diffraction analysis. In general, a laser beam is directed through a sheared diluted RBC solution and the diffraction pattern produced by the deformed cells is analyzed by a computer program. Under the influence of shear stress, RBCs will gradually deform from a biconcave to ellipsoid shape. Based upon the geometry of the diffraction pattern the elongation index (EI) is calculated, in which an increased EI at any given shear stress indicates greater cell deformation.

**RBC Senescence**

Human RBCs have an in vivo life span of approximately 120 days after which they are selectively removed from the circulation by macrophages. This mechanism is essential since it prevents Hb release from aged RBCs directly in the circulation. Aged RBCs are characterized by cell shrinkage, microvesiculation and PS exposure at the cell surface. Aged RBCs also have a decreased ability to deform and an enhanced tendency to aggregate. RBC injury due to energy depletion, osmotic shock or oxidative stress reduces the life span of the RBC. Refrigerated storage also induces cellular changes in the RBC that diminish the life span and possibly the functionality of RBCs.

**1.2. Refrigerated storage of RBCs**

Worldwide RBCs are routinely infused in order to compensate for excessive blood loss as well as to improve the oxygen carrying capacity. Annually, developed countries transfuse approximately one RBC storage unit for every 20 inhabitants. Yet, recently RBC transfusions have been under intensive evaluation. This is mainly because infusion of prolonged refrigerated stored RBCs have been linked to adverse outcome in terms of postoperative infections, length of hospital stay, multiple organ dysfunction syndrome (MODS), transfusion related acute lung injury (TRALI), cancer progression and even mortality.
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Requirements of refrigerated stored RBCs

To date RBCs are routinely stored at 2-6°C for a maximum of 5 to 6 weeks, depending on the preservation solution used. Notably, in the Netherlands RBCs are refrigerated and stored in saline-adenine-glucose-mannitol (SAGM) solution for a maximum of 5 weeks. International guidelines require that the hemolysis in a refrigerated RBC storage unit must remain below allowable levels (i.e., 0.8% in Europe and 1% in The United States) and that at least 75% of the infused RBCs must still circulate 24 hours after infusion.\(^{57,58}\) Interestingly, these guidelines do not specifically reflect the RBC ability to function after infusion.

Quality of refrigerated stored RBCs

Although storage at 4°C slows down the biochemical processes in the RBC, cellular metabolism is not completely suppressed at these temperatures. During refrigerated storage a variety of changes have been observed that could compromise the RBCs to function after infusion. These changes include decreased concentrations of 2,3-DPG, ATP and membrane sialic acid content, translocation of PS to the cell surface, oxidative injury to membrane lipids and proteins, shape change to spheroechinocytes, membrane blebbing and accumulation of potassium, free HB, cytokines, bioactive lipids and (pro-coagulant) microvesicles in the RBC storage unit.\(^{59-63}\)

The RBCs rheologic properties also become impaired during refrigerated storage. RBCs demonstrate an increased tendency to aggregate, diminished deformability and augmented adhesiveness to ECs starting already from the second week of storage.\(^{64-68}\) These alterations could hamper the RBCs to properly function in the microcirculation. Especially, since the majority of the RBC units that are infused have a lifespan that exceeds 2 weeks of storage.\(^{69-71}\) Yet, studies which propose a detrimental association with the infusion of long-term refrigerated stored RBCs are often biased and not adequately corrected for known confounding factors.\(^{72-76}\) These factors include among others the number of RBC units transfused, mixture of RBCs of different durations, severity of illness and the utilization of leukoreduction.
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Leukofiltration of RBCs

In most developed countries, leukoreduction of RBC units prior to storage is a mandatory practice. Initially leukoreduction was implemented to prevent HLA alloimmunization, however by reducing the leukocyte count from a mean of $10^9$ to $10^6$ cells per RBC unit, less detrimental substances such as cytokines, proteolytic enzymes and oxygen free radicals accumulate in the RBC storage unit.\textsuperscript{77,80} Notably, leukoreduction reduces the risk of HLA alloimmunization, febrile non-hemolytic transfusion reactions, immunomodulation and transmission of infectious agents.\textsuperscript{81-85} It has been reported that leukoreduction prior to RBC storage diminish the extent of storage induced RBC lesions.\textsuperscript{86,87} Hence, a lower degree of hemolysis, microvesiculation, potassium leakage and osmotic fragility was observed during refrigerated RBC storage as compared to their non-leukoreduced counterparts.\textsuperscript{80,88,89} In addition, leukoreduced refrigerated stored RBCs showed negligible PS exposure and adhesiveness to ECs with storage time.\textsuperscript{89-91} Interestingly, it was also observed that oxygen free radicals and enzymes derived from activated leukocytes can compromise the aggregation and deformation behavior of RBCs.\textsuperscript{86,92,93} Despite these findings, the aggregability and deformability (i.e. rheologic features) of leukoreduced refrigerated stored RBCs are yet to be determined.

In general, leukoreduction has been widely regarded as a beneficial practice to transfusion medicine.\textsuperscript{81,94,95} However, whether leukoreduced refrigerated stored RBCs are functional in the microcirculation or might be deleterious to transfusion outcome is still unclear.\textsuperscript{15,96-99} As a result concerns regarding the infusion of prolonged stored RBCs are ongoing and a more restrictive transfusion policy is currently being favored.\textsuperscript{100,101} Alternatively, storage of RBC at ultra-low subzero temperatures halts the biochemical processes in the cell and could therefore represent a promising approach to circumvent storage induced lesions and subsequently to extend the shelf life of preserved RBCs.

1.3. Cryopreservation of RBCs

Since the discovery in the 1950s that glycerol protects RBCs from freezing injury, cryopreserved RBCs have been under intensive investigation.\textsuperscript{48} Currently cryopreserved RBCs (commonly known as frozen RBCs) are primarily used for controlling an inventory in situations where the RBC availability is limited or unpredictable. Such as is the case for
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storage of RBC with rare blood groups or for usage in military settings. Occasionally cryopreservation is used to preserve autologous RBCs.

Freezing methods
To date, there are two freezing methods approved for the preservation of RBCs. On the one hand, RBCs can be frozen rapidly using a low-glycerol method (LGM) with a final concentration of approximately 20% glycerol (wt/vol) at temperatures below -140°C. On the other hand, RBCs can be frozen slowly using a high-glycerol method (HGM), allowing storage of RBC units with a final concentration of approximately 40% (wt/vol) glycerol at temperatures between -65°C and -80°C. Cryopreserved RBCs can be preserved up to at least ten years if the correct storage temperature is guaranteed. The HGM cryopreserved RBCs tolerate wide fluctuations in temperature during freezing and are more stable during post-thaw storage. In addition, HGM cryopreserved RBCs did not require liquid nitrogen which eased storage and transportation conditions. Therefore, the HGM is currently the most applicable RBC freezing method in Europe and the United States.

Deglycerolization of thawed RBCs
Usage of glycerol as a cryoprotectant requires an intensive deglycerolization washing procedure post-thaw in order to reduce the glycerol concentration within the cell. This is necessary, since incomplete deglycerolized RBCs will cause hemolytic transfusion reactions and renal failure after infusion. Washing also considerably reduces the amount of detrimental substances such as bioactive lipids, microparticles, cytokines, potassium and free Hb as well as leukocytes from the RBC unit. As a result, transfusion of cryopreserved RBCs are associated with less febrile transfusion reactions, alloimmunization as well as occurrences of TRALI and systemic inflammatory response syndrome (SIRS).

Requirements of cryopreserved RBCs
Preservation of RBCs at ultra-low subzero temperatures ceases the biological activity of the cells which enables them to be preserved for years. However, once thawed the shelf life of RBCs is limited. Deglycerolized RBCs are primarily stored in SAGM preservation
solution for up to 48 hours or in AS-3 preservation solution for up to 14 days. Cryopreserved RBCs need to be deglycerolized to reduce the residual glycerol content to below 1%. Furthermore, international guidelines require that the hemolysis in the RBC units must remain below allowable levels (i.e. 0.8% in Europe and 1% in The United States) and that the RBC post-thaw recovery after deglycerolization (i.e. freeze-thaw-wash recovery) must exceed 80%. Also, at least 75% of cryopreserved RBCs must still circulate 24-hours after infusion. However, these guidelines also do not reflect the ability of RBCs to function after infusion. Hence, frozen storage subjects RBC to a range of chemical, thermal and mechanical forces, which might affect their oxygen delivering capacity after infusion.

**Quality of thawed RBCs**

The quality of HGM cryopreserved RBCs is dependent on the pre-freeze and post-thaw storage time, the anticoagulant and additive solution used as well as on the biological variation among RBC units. The cooling process per se only minimally induces cellular damage when high glycerol concentrations were used. In general, RBCs intended for frozen storage are refrigerated stored prior to processing. The 2,3-DPG content of refrigerated stored RBCs rapidly declines due to the low pH of the storage solution. By limiting this pre-freezing storage time, higher 2,3-DPG values could be obtained post-thaw. Yet, the 2,3 DPG content continue to diminish during postthaw storage. Cryopreserved RBCs maintain high ATP content following post-thaw storage in SAGM and AS-3. However, during postthaw storage also the ATP content gradually declines. This decline is more prominent in AS-3 due to the lower pH of this storage solution. When the pre-freezing storage time was limited to three days no significant changes in PS exposure, CD-47 expression and membrane microvesiculation was observed with HGM cryopreserved RBCs that were post-thaw stored in SAGM solution. In the past the rheologic properties of cryopreserved RBCs have only briefly been addressed. It was demonstrated that the aggregability and deformability of LGM cryopreserved RBCs were diminished. Yet, the rheologic properties of HGM cryopreserved RBCs are still to be determined.
Implementation of new freezing technology

For routine clinical usage, cryopreserved RBCs must be safe, effective and reasonably priced. Cryopreserved RBCs are more expensive, inefficient and time consuming than refrigerated stored RBCs.48 However, ongoing technological advances could potentially expand the utilization of cryopreserved RBCs in transfusion medicine. A promising approach is the usage of a liquid Bio-freezer (Supachill, Lubbock, USA) with a high heat absorption capacity. This Bio-freezer consists of a reservoir of fluid in which samples are immersed. Consequently, the heat is rapidly dissipated from the sample and less freezing damage is observed. This Bio-freezer has been successfully used to freeze bovine sperm cells with a high post-thaw quality.122,123 The rate of heat release during freezing is also an important contributor to the post-thaw quality of RBCs.124 Traditional freezing of RBCs at –25°C in a mechanical freezer, normally requires high glycerol solutions to minimize ice formation.125 However, usage of high concentrations of cryoprotectants also exert detrimental effects on the cell.126 The rapid heat exchange of the liquid Bio-freezer, might allow RBCs to be frozen with lower glycerol concentrations at higher temperatures. The latter could reduce the osmotic stress and subsequently the freeze-thaw-wash related cell loss. This characteristic could make the Bio-freezer particular interesting for cryopreservation of RBCs.

1.4. Aim of this thesis

The aim of this thesis is to gain a better understanding of the RBC quality from a rheologic perspective in transfusion medicine. Alternatively, the utilization of cryopreservation for long-term storage of RBCs will be explored.
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