Rheologic changes of hypothermic preserved red blood cells
Henkelman, Sandra

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Chapter 5

Utilization of cryopreserved red blood cells in transfusion medicine

Sandra Henkelman
Gerhard Rakhorst
Reindert Graaff
Johan W.M. Lagerberg

Submitted
5.1. Introduction

The ability to store RBCs outside the body has been regarded as a life-saving practice for many years. More recently, the usage of refrigerated stored RBCs in transfusion medicine has been under extensive evaluation. During refrigerated storage RBCs progressively deteriorates and infusion of prolonged stored RBCs has been linked to adverse clinical outcome in terms of postoperative infections, length of hospital stay and mortality. Although the majority of these studies are prone to selection bias due to a retrospective study design, concerns regarding the infusion of aged RBCs still remains and a restrictive transfusion strategy is currently being favored. The latter concerns have revived the interest in cryopreservation. Storage of RBCs at ultra-low temperatures halts the cellular metabolism and subsequently prevents the progressive cellular deterioration that has been linked to adverse clinical outcome.

Initially, cryopreservation appeared a promising approach for maintaining RBCs viable for prolonged periods of time. Yet, the clinical applicability of cryopreserved RBCs (commonly known as frozen RBCs) was hampered by the expensive, time-consuming and less efficient nature of this preservation method. The subsequent unfamiliarity with regard to the quality of cryopreserved RBCs has also limited clinical usage. However, ongoing scientific and technological advancement has made cryopreserved RBCs more utilizable for clinical practice. This could be advantageous especially in situations where refrigerated stored RBCs are less desired. In this review the utilization of cryopreserved RBCs in modern transfusion practice will be discussed.

5.2. Cryopreservation methods

Storage of RBCs at ultra-low temperatures ceases the biological activity of RBCs which enables them to be preserved for prolonged periods of time. In order to minimize freezing damage, cryoprotective additives are pivotal. In general, either high concentrations of cryoprotective additives or rapid freezing rates are necessary to prevent cell damage. Along the years different non-permeating and permeating additives for the cryopreservation of RBCs have been investigated. Non-permeating additives such as hydroxyethyl starch and polyvinylpyrrolidone, as well as a variety of glycols and sugars appeared promising because it was proposed that removal from thawed RBCs prior to transfusion was not
required.\textsuperscript{15-20} To date, the lack of data that support safe usage of non-permeating additives have prevented these cryoprotectants from being licensed.\textsuperscript{11,12} Conversely, the permeating additive glycerol is well known for its ability to protect RBCs at ultra-low temperatures. The concentration of glycerol that is necessary to protect the RBCs is dependent on the cooling rate and the storage temperature.\textsuperscript{14} At slow cooling rates, ice formation will occur extra-cellular. As ice forms, the solute content of the unfrozen fraction becomes more concentrated. The resulting osmotic imbalance causes fluid to move out of the RBC and intracellular dehydration occurs. However, at rapid cooling rates the RBC cytoplasm becomes super-cooled and intracellular ice formation occurs, which subsequently can lead to mechanical damage (Figure 5.1). Glycerol protects the RBCs by slowing the rate and extent of ice formation while minimizing cellular dehydration and solute effects during freezing.\textsuperscript{11}

\textbf{Figure 5.1.} RBC changes in response to cooling rate. The snowflakes indicate the presence of ice crystals in the extra- and/or intracellular environment. Printed with permission of Scott et al.\textsuperscript{11}
To date, there are two freezing methods approved for the storage of RBCs. On the one hand, RBCs can be frozen rapidly (i.e. > 100°C/ min) by the low-glycerol method (LGM). With this method, RBCs are frozen with a final concentration of approximately 20% glycerol (wt/vol) at temperatures below -150°C. On the other hand, RBCs can be frozen slowly (i.e. ~1-3°C/ min) by the high-glycerol method (HGM). With this method, RBCs are frozen with a final concentration of approximately 40% (wt/vol) glycerol at temperatures between -65°C and -80°C. Overall, RBC preservation can be extended to at least ten years if the correct storage temperature is guaranteed.

RBC units are preferentially thawed in a shaking water bath of about 36 to 42°C. The general consensus is that thawing should be done rapidly in order to prevent ice crystal growth (so called re-crystallization) upon warming. Once thawed, a deglycerolization washing procedure is performed to reduce the glycerol content in the RBC prior to infusion. This is necessary, since incomplete deglycerolized RBCs will swell and lyse upon infusion, resulting in hemolytic transfusion reactions and renal failure.

The deglycerolization washing process causes to some extent osmotic stress to the RBC which results in cellular losses. Yet, the deglycerolization washing process is also advantageous in that it 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. Hence, in the absence of leukofiltration, the deglycerolization process reduces the leukocyte count to a mean of 1 x 10^7 leukocytes per RBC storage unit. In addition, buffycoat depletion before cryopreservation further reduces the leukocytes count to a mean of 1-3 x 10^6 leukocytes per unit, which in some countries may even eliminate the need to leukofiltrate RBC units. It became also apparent that after post-thaw washing the immunogenicity of the residual leukocytes was reduced. So although frozen storage results in a RBC unit with a lower yield, the remaining RBCs contain less detrimental substances as compared to refrigerated stored RBCs. Altogether, transfusions of cryopreserved RBCs are associated with less febrile transfusion reactions, human leukocyte antigen alloimmunization as well as occurrences of TRALI and SIRS.

If the glycerolization and deglycerolization of RBCs is performed in open systems, the post-thaw storage time is limited to 24-hours due to the potential risk of bacterial contamination. Yet, with the implementation of the ACP-215 device (Haemonetics, Braintree, MA), glycerol could be added and removed via an automated closed system, which minimized the risk of bacterial contamination. As a result, the post-thaw storage
time of RBCs could be extended to 7 days when stored in SAGM solution and to 14 days when stored in AS-3.\textsuperscript{31,37,38}

Cryopreserved RBCs are less efficient due to the cellular losses that occur during the processing procedure. This cell loss was more pronounced in the HGM cryopreserved group (\textsim 10-20\%) since these RBCs required more extensive washing. However, despite the higher yield of RBCs with the LGM method, it was recognized that HGM cryopreserved RBCs could tolerate wide fluctuations in temperature during freezing and were more stable during post-thaw storage.\textsuperscript{13,39} In addition, HGM cryopreserved RBCs did not require liquid nitrogen which eased storage and transportation conditions. Consequently, the HGM is currently the most applicable RBC freezing method in Europe and the United States.

### 5.3. Quality of cryopreserved RBCs

Cryopreservation prolongs the longevity of RBCs. However, once thawed the shelf life of RBCs is limited. Cryopreserved RBCs have to meet certain guidelines (Table 5.1)\textsuperscript{21,22}. Yet, these guidelines do not specifically reflect the ability of the RBCs to function after infusion. Cryopreservation subjects RBC to a range of chemical, thermal and mechanical forces, which might affect their oxygen delivering capacity after infusion.

The quality of HGM cryopreserved RBCs is primarily dependent on the pre-freeze and post-thaw storage time, as well as on the anticoagulant and additive solution used.\textsuperscript{40} The duration of frozen storage per se minimally attributes to cellular damage.\textsuperscript{41,42} In order to limit storage induced lesions, refrigerated stored RBCs need to be frozen as soon as possible. According to the AABB, RBCs collected in CPDA-1 need to be frozen within 6 days, whereas in Europe the RBCs are preferably frozen within 7 days after collection.\textsuperscript{21,22} It is possible to freeze prolonged or outdated refrigerated stored RBCs, provided that the RBCs have been rejuvenated prior to freezing in order to restore the metabolic status of the cell (i.e. ATP, 2,3-DPG and Hb p50 levels).\textsuperscript{43-46} After deglycerolization even the outdated rejuvenated RBCs showed acceptable quality up to seven days of post-thaw storage in AS-3.\textsuperscript{36}

In recent years more knowledge about the quality of HGM cryopreserved RBCs has become available. During frozen storage, the ATP and 2,3-DPG content is preserved. Yet, the length of pre-freezing storage time at 4°C was an important predictor of the ATP and
2,3-DPG content after deglycerolization.\textsuperscript{36,42} The RBC ATP content is important for the overall functioning of the cell. Loss of ATP has been associated with rigid cell membranes, echinocyte shape change, enhanced cation permeability, exposure of phosphatidylserine on the RBC surface, microvesiculation, loss of vasodilatation control and decreased RBC viability.\textsuperscript{47-55} After deglycerolization the RBCs have a high ATP content when stored in AS-3 or SAGM additive solution. Nevertheless, during post-thaw storage the ATP content gradually declines. This decline was more prominent in AS-3 due to the diminished glycolytic activity induced by the lower pH of this storage solution.\textsuperscript{38}

In the RBC binding of 2,3-DPG to the Hb induces a conformation state which will release oxygen from the Hb. In regions with low oxygen tension, oxygen is released from the Hb due to the high binding affinity of 2,3-DPG for deoxygenated Hb. Loss of 2,3-DPG will increase the affinity of oxygen to the Hb which may hamper the oxygen delivery to the tissues. Due to the low pH of the storage media a considerable loss of 2,3-DPG content was observed already after one week of refrigerated RBC storage.\textsuperscript{2} By limiting this pre-freezing storage time, higher 2,3-DPG values could be obtained post-thaw.\textsuperscript{56} Nevertheless, transfusing RBCs with low 2,3-DPG content appeared not to be detrimental to tissue oxygenation.\textsuperscript{57-59} Presumably, because the RBC 2,3-DPG content can be replenished hours following infusion,\textsuperscript{60-62} or because in hypoxic regions oxygen can still be released from the RBCs despite the low 2,3-DPG content.

The ability of RBCs to adhere to the vascular endothelium is an important determinant of the flow behavior of blood and subsequently the oxygenation of the micro-vascular environment.\textsuperscript{63,64} Under physiological conditions, the adherence of RBCs to the vascular endothelium is negligible. Yet, structural changes in the RBC membrane may promote adherence to ECs and impair the microcirculatory blood flow. PS expression on the RBC surface mediates adherence of RBCs to ECs and might trigger RBC clearance from the circulation.\textsuperscript{65-67} In contrast, surface expression of CD-47, a marker of self, prevents RBCs from being engulfed by phagocytes.\textsuperscript{68} In general PS exposure and loss of CD-47 expression on the RBC surface as well as membrane microvesiculation are all apoptotic signals and therefore determining factors for the lifespan of the RBCs.\textsuperscript{59,70} HGM cryopreserved RBCs that were post-thaw stored in SAGM additive solution showed no significant changes in PS exposure, CD-47 expression and membrane microvesiculation when the pre-freezing storage time was limited to three days. However, surface expression of PS and microvesiculation was observed when longer pre-freezing storage times were used.\textsuperscript{56}
Previously, it has been shown that the freeze-thaw process makes the RBC membrane permeable to cations, which result in RBC swelling and subsequently hemolysis.\textsuperscript{38,71,72} However, post-thaw storage of RBCs in AS-3 media, which contains the impermeable solute citrate, prevented cell swelling and limited the level of hemolysis during post-thaw storage.\textsuperscript{38} Altogether, it can be concluded that good post-thaw quality of HGM cryopreserved RBCs can be obtained when the appropriate storage conditions pre-freeze, frozen and post-thaw were used.

### Table 5.1. Requirements of cryopreserved RBCs

<table>
<thead>
<tr>
<th>Variable</th>
<th>European Guidelines *</th>
<th>AABB guidelines **</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolyse</td>
<td>&lt; 0.8 %</td>
<td>&lt; 1.0 %</td>
</tr>
<tr>
<td>Volume</td>
<td>&gt; 185 ml</td>
<td>-</td>
</tr>
<tr>
<td>Hb content</td>
<td>&gt; 36 g/unit</td>
<td>-</td>
</tr>
<tr>
<td>HCT</td>
<td>0.65-0.75 %</td>
<td>-</td>
</tr>
<tr>
<td>Post-thaw recovery</td>
<td>-</td>
<td>≥ 80 %</td>
</tr>
<tr>
<td>24-hour posttransfusion survival</td>
<td>-</td>
<td>≥ 75 %</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>&lt; 0.1 x10^9 cells/unit</td>
<td>-</td>
</tr>
<tr>
<td>Osmolarity</td>
<td>&lt; 340 mOsm/l</td>
<td>-</td>
</tr>
</tbody>
</table>

* European guidelines\textsuperscript{22}

** AABB guidelines\textsuperscript{21}

### 5.4. Utilization of cryopreserved RBCs

Currently, cryopreserved RBCs are primarily used for controlling an inventory in situations where the RBC availability is limited or unpredictable. Yet, cryopreserved RBCs are useful in a variety of clinical settings. The utilization of cryopreserved RBCs in transfusion medicine will be described in more detail below.
Storage of rare RBCs

Cryopreservation is currently used for preserving RBCs with rare blood phenotypes. In general, a blood group is regarded as rare if the RBC phenotype has a frequency of approximately 1 in 100-1000 or less in the general population. Refrigerated stored RBCs need to be as soon as possible, ideally within 6 to 7 days. However, cryopreservation of RBCs even beyond the regulated expiration date is still possible for exceedingly rare RBC phenotypes.

Cryopreservation of rare RBCs is advantageous to patients for whom compatible blood is not instantly available. Over the years, numerous countries in Europe, America and Asia have set up frozen rare RBC banks. In Europe, most notably are the blood banks in Amsterdam, Birmingham and Paris, which house a large collection of cryopreserved rare RBC units. Most cryopreserved rare RBC units are for national use. This is because the RBC unit usually needs to be thawed in the donor blood center, imposing a time limit in which the RBCs must be transfused. Nevertheless, when no compatible blood can be found via the national blood banks, it is general practice to appeal to countries abroad. Although usage of cryopreserved rare RBCs is extremely costly and the international shipment is usually cumbersome, it has been lifesaving for a variety of patients.

Military blood bank

In military combat massive blood loss is a major cause of death. Having RBCs available in the military theater at all times is therefore of vital importance. Previously, an inventory of RBCs was difficult to maintain in combat areas due to the unpredictable demand and the limited shelf life of refrigerated stored RBCs. Yet, cryopreserved RBCs are a valuable blood resource due to the prolonged storage time. Cryopreserved RBCs have been used in the military theater since the Vietnam war. Although back then the processing of cryopreserved RBCs was still in its early stages, it was already concluded that usage of cryopreserved RBCs in combat casualty care was technically feasible and clinically acceptable. Ongoing scientific and technological advancement have made cryopreserved RBCs become a more utilized blood product in modern military operations. Especially the Dutch army has been using cryopreserved RBCs in theater operations with great success. This was also demonstrated at the conflicts in Afghanistan, where already 1360 cryopreserved RBC units have been transfused by the Dutch military blood
Usage of frozen RBCs

bank without any shortages or transfusion reactions reported. Hence, the Dutch military
blood bank only uses type O leukodepleted RBCs in the theater in order to improve the
effectiveness and decrease the chance of clerical errors. In this regard, leukodepleted type O
whole blood is processed into RBCs and frozen within 24 hours after donation. Additionally, the short pre-storage time reduces storage induced lesions to a minimum.
Cryopreserved RBC units are transported to combat areas on dry ice. These RBCs have
been frozen in polyvinyl chloride plastic (PVC) bags inside polyester plastic bags which
were placed in rigid cardboard boxes. In the past, PVC bags that were stored at -80°C and
subjected to transportation had a breakage incidence of approximately 6.7%. Due to the
use of an additional vacuum sealed over-wrap bag, the breakage incidence of cryopreserved
RBC units subjected to transportation is currently negligible. Subsequently, all in theater
storage, thawing and washing procedures are performed in a temperature controlled blood
bank container, designed by the Dutch army. Thawed and washed RBCs are ultimately
preserved in AS-3 and can be refrigerated stored at 2-6 °C for a maximum of 14 days.
Usage of cryopreserved RBCs in combat casualty care offers a better inventory control.
This is because cryopreserved RBC units can be prepared either on demand or in advance,
thereby providing a continuous RBC supply even when standard refrigerated stored RBCs
cannot be replenished on time. Furthermore, cryopreserved RBCs may be advantageous to
prolonged refrigerated stored RBCs. In combat hospitals, it is not uncommon to receive
refrigerated stored RBC units with a mean storage age of 27 days on arrival. Compared
to prolonged refrigerated stored RBCs, cryopreserved RBCs have less detrimental
substances that can cause transfusion reactions. Nevertheless, randomized-controlled trials
comparing transfusion of cryopreserved RBCs with refrigerated stored RBCs have still to
be performed. Cryopreserved RBCs are undeniable more costly. Hence, a unit of
cryopreserved RBCs cost approximately twice the amount of a refrigerated stored RBC
unit. In addition, thawing and washing of cryopreserved RBC units requires skilled
personnel and takes up about 70-120 minutes. Yet despite these disadvantages,
stockpiling a frozen RBC inventory proved to be an efficient and safe blood resource in
combat casualty care.
Blood shortages

Blood shortages due to natural or civil disasters as well as due to seasonal shortages can pose a major health challenge. However, in emergency blood management planning cryopreserved RBCs are rarely implemented. This is primarily due to the longer processing time of cryopreserved RBCs, making it difficult to prepare large quantities of RBC units within a given time frame. Cryopreserved RBCs are also less implemented during times of blood shortages, due to the improved emergency procedures of blood centers with regard to refrigerated stored RBCs.91-94

A main strategy of the emergency procedures is to mobilize stocks of refrigerated stored RBC through coordination with nearby blood centers. Hence, blood centers will usually have a 2-3 day supply of refrigerated stored RBC units on hand. This strategy could compensate for RBC shortages as long as the local stocks of the nearby blood centers are repleted appropriately. After a disaster the influx of blood donations is often increased because of the altruistic response of the public. Although this influx of blood donations could be used to replenish local stocks, this is true for only part of the donated blood. Notably, part of the donated blood is non-transfusible due to the higher reactive screening tests and sometimes inadequately processing procedures.95-99 In this regard, blood centers that do send refrigerated stored RBC units could face the risk of becoming under-supplied themselves.

Recently, it was demonstrated that managing a frozen RBC reserve could be useful in emergencies scenarios.100 In this regard, cryopreserved RBCs would serve as a bridge-over supply during short term RBC deficits until support by the blood centers could be re-established. Despite the complexity and costs of implementing and maintaining a frozen RBC reserve, it was concluded that the benefits of self-sufficiency outweighs the disadvantages.

Autologous transfusion

Cryopreserved RBCs have occasionally been used for preoperative autologous storage.101 In general, preoperative autologous RBC transfusion offers advantage above allogeneic RBC transfusions in that it prevents immunosuppression and infectious disease transmission, while it reduces postoperative infections and subsequently length of hospital stay.102,103 During the last couple of years preoperative autologous RBCs usage has been
questioned and its demand has declined. This is predominantly due to the improved safety of allogeneic RBCs as well as due to the organizational and logistic hurdles, the higher disposal rate and the more costly nature associated with autologous RBCs transfusions.\textsuperscript{103-107} Additionally, the beneficial effect of preoperative autologous RBC donation has been compromised by the short time period between the last donation and the planned surgical procedure. As a result, patients often develop anemia before the surgery and are more likely to receive transfusion.\textsuperscript{105,106,108}

In order to avoid anemia, RBCs need to be harvested months in advance of the expected use so that the Hb level of the patient can be restored. Yet, prolongation of the time period between the last donation and the surgical procedure is hampered by the short storage time of refrigerated stored RBCs. In contrast, cryopreservation enables storage of RBCs for years, which allow RBCs to be donated far in advance of the surgical procedure\textsuperscript{109} without affecting its quality. Usage of cryopreserved RBCs has an additional advantage in that less detrimental substances, that have accumulate during storage and which may affect transfusion outcome, are transfused.

Although the transmission of infectious diseases is currently very low and the benefit of preoperative autologous RBC transfusions is questioned, new pathogens keep emerging\textsuperscript{110-113} and with the implementation of cryopreservation, preoperative autologous RBC transfusions could become more attractive again.

**Usage when refrigerated stored RBCs are less desired**

Cryopreserved RBCs could be advantageous in situations where refrigerated stored RBCs are less desired. This could be the case for patients who receive transfusions frequently, such as patients with thalassemia, sickle-cell disease or with certain types of glucose-6-phosphate dehydrogenase deficiency. This is because frequent transfusions with refrigerated stored RBCs are a risk of human leukocyte antigen alloimmunization. Hence, the development of antibodies against infused RBC surface antigens is of considerable medical importance because it will result in hemolytic transfusion reactions.\textsuperscript{114,115} In contrast, usage of cryopreserved RBCs reduces the risk of alloimmunization due to a reduction in the number of leukocytes by the deglycerolization washing procedure.\textsuperscript{12,13}

Cryopreserved RBCs may also be helpful as part of the routine RBC inventory because of its advantages over prolonged refrigerated stored RBCs. As mentioned earlier, transfusion
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of prolonged refrigerated stored RBCs is associated with occurrences of TRALI and SIRS.\textsuperscript{12,35} Although the etiology of TRALI and SIRS remains incompletely understood, it is recognized that substances that accumulate in the supernatant of refrigerated stored RBCs are involved in the pathogenesis of these syndromes.\textsuperscript{116-120} Ultimately, these syndromes will lead to an increased hospitalization and subsequently higher burden on the healthcare costs. Cryopreserved RBCs on the other hand have less than 5\% cytokines and biologically active substances in the supernatant, which make them the ideal blood product to prevent TRALI and SIRS.\textsuperscript{35,121}

In line with this, cryopreserved RBCs are also useful for patients with immunoglobulin A (IgA) deficiency. These patients usually have undetectable IgA and high titer of class specific anti-IgA. Transfusion of only a small amount of blood can cause severe anaphylactic reactions due to the presence of IgA in plasma.\textsuperscript{40} Since cryopreserved RBCs are extensively washed, these blood products are especially recommended for patients with IgA deficiency.\textsuperscript{13,40,122}

5.5. Conclusion

In transfusion medicine the balance between the RBC availability and demand is variable. However due to the perishable nature of refrigerated stored RBCs, hospitals often maintain only a minimal RBC reserve to maximize the efficiency while minimizing the cost of wastage. This policy also means that hospitals will be more vulnerable to RBC shortages due to fluctuations in the RBC availability and or demand. Cryopreserved RBCs may be helpful as part of the routine refrigerated stored RBC inventory. Hence, having cryopreserved RBCs available in civilian blood banks and or hospitals could result in better blood management and patient care need. Today, cryopreserved RBCs are still infrequently implemented in transfusion medicine. This is mainly because of the expensive nature of this preservation method. Although, the higher costs of cryopreserved RBCs are of major concern, the cost difference with regard to refrigerated stored RBCs is often overrated. This is because the costs of treating and managing adverse events are not taken into account, indicating that the total cost of a refrigerated stored RBC unit would be substantial higher than currently is represented.\textsuperscript{123} Cryopreserved RBCs are also less efficient and more time-consuming, however ongoing scientific and technological advancement has made cryopreserved RBCs more utilizable for
clinical practice. Notably, more knowledge about the quality of cryopreserved RBCs could further expand its use in clinical practice.

In the foreseeable future the overall use of cryopreserved RBCs could expand as a result of a change in RBC supply and demand, due to a shift of increasingly older patient population.\textsuperscript{124,125} For now, we showed that cryopreservation of RBCs is already useful in a variety of clinical settings. Especially, since cryopreserved RBCs are available, save, in compliance with European and US guidelines and can be used effectively.

References


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