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When I need you most: frozen red blood cells for transfusion

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1. An alternative storage strategy

Storage of red blood cells (RBCs) at extremely low temperatures (at −65 to −196 °C) is an alternative to liquid hypothermic storage (at 4-6 °C). It offers prolonged cellular longevity through halting of biological activities [1]. Freezing, however, is associated with irreversible damage to living cells [2,3]. Depending on the cooling rate, ice crystals may be formed extracellular (slow rate) or intracellular (high rate). Moreover, passage of cells along the intermediate temperature zone (approx. between -10 to -60 °C) damages the membrane permeability [4]. Consequently, cryoprotective additives are pivotal, with nontoxic glycerol being the most widely used for RBC cryopreservation. [5]. It enters cells via facilitated transport and limits ice crystal formation, solute effects and dehydration [6].Its concentration varies according to the freezing protocol: at slow cooling rates a high glycerol concentration is used to counteract osmotic imbalance, while at high cooling rates glycerol is needed at lower concentration [7]. Two methods are developed accordingly for clinical use: the high glycerol method (HGM) and the low glycerol method (LGM). In the first one (used in North America), RBCs are frozen slowly (1-3 °C/min), stored at temperatures between -60 and −80 °C in 40-50% w/v glycerol and thus, thawing is long [1]. The LGM protocol (used in Europe) is based on the rapid freeze (>100 °C/min) of RBCs and their cryopreservation at temperatures below −140 °C after addition of 15-20% w/v glycerol [8]. Regardless the method of choice, cryopreservation includes three obligatory steps: a) Glycerolization and cooling, b) Storage and, c) Thawing and deglycerolization needed to prevent intravascular osmotic hemolysis of glycerol-laden RBC on contact with plasma [9] and consequently, free Hb toxicity [10].

Time plays a central role in determining the quality of cryostored RBCs, but unlike hypothermic storage, the right processing time and other parameters of sample preparation account more for it compared to the storage duration *per se* [11,12]. Rapid thawing is necessary to prevent ice crystal growth (“recrystallization”) upon warming [13], and RBCs must be deglycerolized shortly after that because glycerol interferes with the metabolism of liquid-stored RBCs. This is not as easy as it seems due to high intracellular concentration of glycerol and its slow rate of osmosis relatively to water. The osmotically stressful process of glycerol removal can be as damaging to RBCs [14] as the cooling/thawing steps.

Finally, however, the quality of frozen RBCs is in compliance with international transfusion regulations and guidelines [7]. RBCs are currently frozen within 7 days after donation, stored up to 10 years (or longer [11]) and are available for transfusion within 1, 7 or 14-21 days (depending on freezing method, glycerol concentration and additive solution) after thawing and washing [15]. Washing was initially characterized by long processing time that hampered the actual use of RBCs in emergency setting. Moreover, it posed significant risks for bacterial contamination that substantially shorted the postthaw storage period. The introduction of fully closed automated cell processors made the glycerolization/deglycerolization steps shorter and safer and rendered

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frozen RBCs more utilizable for clinical practice [16,17]. To date, however, cryopreserved RBCs are infrequently implemented in transfusion medicine, being mostly used in special transfusion circumstances [18]. What's keeping us from moving on to this promising technology? Are there any good reasons to take this step?

2. To freeze or not to freeze

The main advantage of frozen RBCs is their impressively extended storage life. Since blood donation is voluntary, an "eternal" stock of RBC units could be especially useful in periods of supply-and-demand imbalances [19]. In emergency situations the need for blood is immediate. Unfortunately, even with the latest technology, it takes about an hour to prepare a frozen unit for transfusion [20]. In addition, the shelf-life of thawed RBCs is shorter compared to that of refrigerated RBCs, while the need for specialized instruments and processing raises substantially the cost compared to the liquid preservation [21].

Despite those practical drawbacks, cryopreservation is useful in maintaining an inventory of phenotypically rare RBCs [22]. Blood centers around the world and international rare donor networks collect and cryopreserve RBCs of uncommon blood types [23]. Storage of autologous blood for imminent transfusion is also benefitted by cryopreservation. Despite the associated risks [24], autologous transfusion may be a choice for patients susceptible to the negative effects of allogenic transfusion, including patients with alloantibodies, surgical tumors and kidney transplants [21]. Frozen autologous RBCs seem to be superior to the refrigerated RBCs, in regard to net gain in Hb post transfusion [25].

Another issue is related to the storage lesion and its potential clinical consequences [26]: do we "freeze" the storage lesion along with the RBCs? It seems that the answer is yes, since the storage lesion itself appears to be limited to the short pre-freezing period [27]. Thus, classic biochemical defects and cellular aggregability are not as evident in the cryopreserved RBCs compared to the refrigerated RBCs, while cellular deformability is equal between the two strategies [12,28]. Moreover, cryopreservation does not induce the appearance of stress or removal markers (e.g. caspase activation [28], CD47 antigen expression [29]), neither promotes microvesiculation [30].

Despite that, cryopreservation itself affects the ion homeostasis and integrity of RBCs [28]. The deglycerolization/washing steps leave cells with decreased intracellular pH [31] and increased susceptibility to osmotic hemolysis [12]. It seems that the initial pause of the storage lesion is followed by an acceleration of it post thawing, which limits the shelf-life of thawed RBCs. Cryoinjuries or subhemolytic damages of deglycerolized RBCs could weaken their capability of enduring storage stress [32]. In spite of that, the increased extracellular Hb observed immediately post thawing [31] is removed by the subsequent washing steps, along with other soluble biological response modifiers (like cytokines and potassium [7]) linked to side effects of transfusion [26].

Finally, there is laboratory and clinical evidence that transfusion of cryopreserved RBCs is both safe and effective in several recipient settings, including trauma patients [33] and acute sickle cell disease [34]. In particular, tissue oxygenation was found superior in both obese [35] and stable trauma [36] patients receiving cryopreserved RBCs compared to those receiving refrigerated RBCs. Frozen RBCs further present lower levels of inflammatory markers [19]. The rapid extracellular hemolysis observed following transfusion of autologous cryopreserved RBCs is not accompanied by increased production of proinflammatory cytokines [37].

3. New entries in the cryofield

The main challenges in cryopreservation include, but are not limited to a) the endurance of RBCs while cooling/thawing (intermediate temperature zone effect) [4], b) crystallization during cooling and recrystallization when thawing [13], and c) significant hypo-osmotic hemolysis during deglycerolization [31]. Several novel cryo-protocols and cryoprotectants have been tested for the optimization of "frozen blood techniques" the last 5-10 years. By using interrupted graded freezing, Poisson et al. [38] demonstrated that post-thaw RBC integrity of glycerol-preserved RBCs was progressively decreased between -25 and −30 °C while addition of non-permeating small molecule ice recrystalization inhibitors, like aryl-glycosides, synergistically improved RBC integrity up to 55%.

Besides glycerol, several non-permeating (polyvinylpyrrolidone, glycols, sugars) or permeating additives have been proposed to replace or accompany glycerol's role in RBC integrity. In line with the current trend of using natural products for "medical" purposes, replacement of glycerol with zwitterionic betaine, a natural plant cryoprotectant, resulted in about 80% of post-thaw RBC integrity rate [39]. A combination of other natural cryoprotectants, such as the permeating L-proline and the non-permeating trehalose, limited the degree of post thaw hemolysis by decreasing the freezing points and inhibiting ice crystal formation [40]. Trehalose is an "old player" in this "cryo-game", but still relevant as its synergistic effect with hydroxyethyl starch is comparable to that of glycerol [41]. At the same time, attempts of increasing trehalose uptake in RBCs via liposomes [41] or apatite nanoparticles [42] seem promising in terms of RBC recovery. In addition, when using dextran as the extracellular protectant, pre-freeze incubation of human RBCs with various sugars (e.g. glucose) resulted not only in differential modulation of the postthaw recovery, but also in equal post-transfusion recovery in a mouse model [43]. Finally, synthetic mimics of anti-freeze proteins (including the FDA approved -for dietary use- polyvinyl alcohol) are being investigated [44] but none of them has been licensed for clinical use.

Another field of research focuses on slight modification of the standard procedure in favor of a) RBC stability mainly during deglycerolization, b) method simplification and c) special needs. For instance, omission of prefreeze removal of excess glycerol [45] or spliting of blood units into pediatric units and consequent adjustment to a higher hematocrit [46] resulted in increased stability and low hemolysis and potassium, respectively. Furthermore, since deglycerolization leads to RBC loss, optimization of the existing protocols through reducing the washing duration by applying a mathematical formula [47], changing NaCl concentration for postwashing [48], and introducing a new dilution-filtration system [49] may represent promising alternatives. When small volumes of blood need to be frozen (mainly for future serological tests), droplet freezing, a method for RBC preservation in small, individual droplets is common. This technique minimally affects RBC antigenicity [50], while the recovery of polyvinylpyrrolidone supplemented RBCs is acceptable [51]. Last, but not least, freeze drying (lyophilization) of RBCs has been suggested as an alternative way to overtake problems like the complicated transportation of frozen samples. Such a task is far from simple because it presupposes novel lyophilization techniques and combination of CPAs [52]. However, it would allow the storage of low weight samples at room temperature.

4. What next

The uncontested usefulness of cryopreservation in special transfusion circumstances and the increasing needs for blood transfusion globally have reawakened the interest of the scientific community in frozen blood. However, we know but the basics about its biochemistry, efficacy and effects. RBC assessment following cryopreservation has to be expanded from classic (though necessary) *in vitro* analyses (hemolysis etc.) [11,12,31] and recovery [53] to equally important *ex vivo* and *in vivo* biological activities and physiological functions (e.g. vasoreactivity, flow dynamics, metabolism). Those aspects have been recently examined in a new cryopreservation protocol optimized to serve RBC research purposes [8]. The contemporary era of transfusion medicine has been marked by the introduction of new concepts (e.g. updated quality criteria, donor variation effects) and new analytical tools,

including the so-called “omics” technologies, which have offered better understanding of the RBC storage lesion and its clinical relevance. In-depth analysis of the biochemical and metabolic properties of frozen RBC may reveal currently unknown strengths/ weaknesses and possibilities for improvement. A metabolomics analysis recently revealed that rejuvenation is very effective for old stored refrigerated RBCs following cryopreservation, due to an impressive metabolic reprogramming [54], suggesting the possibility of extending storage for precious RBC units at outdate.

Current evidence suggested the presence of RBC sub-populations (e.g., oldest cells [31]) less resilient to cryopreservation-related injuries. The high variability in the glycerol permeability of the RBC membrane, and thus in the hemolysis after deglycerolization, between donors and within cells from the same donor [55], is further indicative of samples with low responsiveness to cryopreservation. In similarity to the currently known donor effects on the refrigerated storage lesion [56], small-scale studies at first, and then large donor-recipient cohorts studies are needed to understand the impact of donor factors on the efficacy and safety of frozen RBCs. Clinical trials and head-to-head comparisons between refrigerated and cryopreserved RBCs [33,35,36] are further needed to support the suitability of the latter for transfusion in the surgical, pediatric, and massively/repeatedly transfused patients. Deep understanding of those effects will be necessary in developing optimized cryopreservation protocols and standardized processing systems that may allow expansion of frozen RBC repository to common clinical settings and personalization of blood donation.

Such contemporary research, however, would not be enough to define the utilization of frozen RBCs in the future. Refrigerated storage is nowadays better understood and continuously improved. Information systems and lists of available donors, for example, are increasingly used globally to manage the phenotypically rare units within the inventory of liquid RBCs. Moreover, while frozen RBCs stored for long can be used to balance inventory in times of crisis, severe safety issues (absence of up-to-date testing) would prohibit their use in a potential emerging pathogen crisis. A series of similar considerations and logistics could restrain companies from investing in frozen RBC technologies, even though such a prospect would mark a substantial shift in transfusion practices. No doubt, a blood banking system supported by both refrigerated and frozen RBCs would be unique by its capacity to offer the highest-quality components to patients, free of stochastic variations in donor availability and transfusion needs. By today’s standards, however, the popularity of frozen RBCs in the future is not an easy guess for neither transfusion clinicians nor researchers.

Conflicts of interest: none

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