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Review

Innovations in red blood cell preservation

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ABSTRACT

The global infrastructure supporting nearly 100 million transfusions annually relies on the ability to store red cell concentrates (RCCs) for up to 42 days at hypothermic temperatures or indefinitely at low sub-zero temperatures. While these methods are generally effective, there is both an opportunity and, in specific settings, a need to refine storage techniques that have remained largely unchanged since the 1980s. Recent research has identified ways to address limitations that were not fully understood when these methods were first implemented in blood banks, with much of it focusing on modifying conventional storage strategies, while some studies explore alternative approaches. In this review, we explore the current state of RBC preservation and the future prospects for advancing both short- and long-term storage strategies.

1. Introduction

Over the past 40 years, the field blood banking has remained largely unchanged, with its most significant advancements occurring in the first half of the 20th century. Karl Landsteiner's discovery of the ABO blood group system in 1901 transformed transfusion from an experimental therapy into an established treatment, igniting the initial efforts to develop short-term blood banking strategies [1]. By anticoagulating plasma with citrate, it became possible to separate donors from recipients spatially, while the addition of glucose supported metabolism and enabled temporal separation [2–5]. Using citrate-dextrose solutions, RBCs could remain viable for transfusion for up to 26 days of storage at hypothermic temperatures (1–6 °C), leading to the establishment of the first blood bank in France towards the tail-end of World War I [4,5]. The long-term preservation of RBCs became feasible several decades later following the serendipitous discovery in 1949 that glycerol could mitigate freezing injury [6–8]. The first-ever transfusion of frozen blood took place in 1951, and by the time the U.S. entered the Vietnam War, the methods for processing and transfusing cryopreserved blood were sufficiently robust to allow for widespread use [7,9].

In the ensuing two decades, these early versions of short- and long-term preservation strategies were iteratively refined, leading to the widespread implementation of the hypothermic storage and cryopreservation methods still in use today [10–12]. While later changes to blood product manufacturing practices have led to notable improvements in the quality and efficacy of stored red cell concentrates (RCCs), the core storage methods remain unchanged [13].

Despite minimal practical advancements in the RBC preservation methods employed by blood banks, the past two decades have seen prolific efforts to improve on the existing approaches. Generally, this research has focused on three interrelated avenues: (1) characterizing potential limitations to the current strategies, (2) identifying ways to improve them, and (3) developing alternative strategies. Here, we aim to discuss each as it relates to both the short- and long-term preservation methods of RBCs.

2. Short-term storage of RBCS

The extrusion of mitochondria, development of robust antioxidant systems, and sole reliance on anaerobic glycolysis, permit mammalian RBCs to maintain hemoglobin in a reduced state, even in the presence of high oxygen concentrations (~16 mM) [14]. These adaptations not only contribute to their unique physiology but also confer a certain resilience against the stressors of hypothermic storage—a capability most non-mammalian, organelle-containing RBCs, and nucleated cells lack [15–17]. Human RBCs are thus one of the few widely used therapeutic cell types for which cryopreservation is not a necessity but rather an option.

Additive solutions have been designed to counteract the biochemical changes caused by hypothermia, including disruptions in energy and redox metabolism and cation homeostasis [18]. Refrigerated storage durations range from 21 to 49 days, depending on the solution and

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regional regulations (see Ref. [19] for a detailed review and Table 1 for an overview of major RBC storage solutions). Beyond the allotted storage period, the accumulated hypothermic storage injuries (referred to as the "storage lesion" in transfusion literature) are considered potentially detrimental to patients (a summary of critical RBC parameters changing throughout storage can be found in Fig. 1) [2].

Growing evidence suggests that injury occurring within the permitted storage period is not entirely without risk, with some epidemiological studies reporting adverse clinical outcomes associated with RBCs stored within these durations (see Refs. [23–26] for in vivo animal studies, Refs. [27–31] for meta-analyses, Refs. [32–35] for retrospective studies, and Refs. [36, 37] for commentaries on this topic). That said, the significance of the storage lesion remains a subject of debate for two reasons: (1) few studies have examined transfusions at the extreme ends of the storage period, and (2) any outcome associated with storage lesion would largely depend on the patient's pathology and the volume of blood transfused [36,37]. Although no notable changes to transfusion practices have thus been implemented based on the so-called "age of blood" effect, several initiatives have emerged to mitigate the storage lesion. These have primarily focused on: (a) developing new storage solutions to counteract detrimental biochemical changes, (b) promoting redox homeostasis through additive supplementation or modulation of storage conditions, and (c) lowering the storage temperature.

2.1. *Minimizing pH-dependent metabolic Sequalae using novel storage solutions*

ATP and 2,3-DPG are the major metabolic correlates of post-transfusion recovery (PTR) and oxygen delivery capacity, respectively [38,39]. After 42 days of storage (the permitted storage duration in a majority of North American and European jurisdictions), ATP levels lie at ~50 % of the initial values, while 2,3-DPG levels are almost entirely depleted [2]. Although both metabolites are rapidly replenished following transfusion, with a ~ 50 % return to baseline within 8 h, acute impairments in PTR or oxygen delivery can be particularly detrimental to critically ill patients requiring immediate oxygen offloading [40–42]. In such cases, pre-transfusion rejuvenation is both impractical and unable to reverse the 'irreversible' storage injuries which begin to manifest after day-21 [43]. Applying simple biochemical principles to better maintain energy homeostasis during storage thus presents a more effective alternative.

A reduction in pH negatively impacts glycolysis, compromising energy homeostasis and leading to decreased production of ATP and 2,3-DPG. This effect is due to the pH-dependent regulation of glycolysis through the allosteric control of phosphofructokinase (PFK), which converts fructose-6-phosphate to fructose 1,6-bisphosphate [44]. A drop of 0.1 in pH causes a 10 % reduction in PFK activity, thereby reducing glycolytic flux [44]. When red blood cells (RBCs) are first placed in storage, their starting pH is around 7.0, down from the physiological pH of 7.35 due to the addition of acidic citrate phosphate dextrose (CPD) and the acidity of conventional storage solutions [19]. As pH drops below 7.0, hemoglobin becomes increasingly ineffective as a buffer [45]. This, combined with the buildup of lactic acid throughout storage, causes pH levels to fall to around 6.5, approaching the point where glycolytic flux nearly ceases at the end of the storage period [19]. Intuitively, raising the starting intracellular pH can promote glycolysis while improving tolerance to lactic acid buildup, with hemoglobin more effectively buffering the resulting acidity.

Although elevating the starting intracellular pH can help mitigate the loss of ATP and 2,3-DPG by promoting glycolysis, this strategy requires careful balancing of bisphosphoglycerate mutase (BPGM) activity. At a pH above 7.2–7.3, BPGM acts as a mutase, producing 2,3-DPG from 1,3-BPG at the expense of 1 ATP molecule [46,47]. Below this pH range, BPGM becomes a phosphatase, consuming 2,3-DPG and converting it into 3-phosphoglycerate (3-PG), a glycolytic intermediate [46,47]. As pH declines, the phosphatase activity of BPGM increases, accelerating 2,3-DPG breakdown [48]. This results in 2,3-DPG loss of approximately 10 % from baseline levels within the first week and 90–100 % by six weeks [49]. While this phosphatase activity depletes 2,3-DPG, it supports ATP production by halting the ATP-consuming synthesis of 2,3-DPG and converting 2,3-DPG into 3-PG, promoting glycolytic flux.

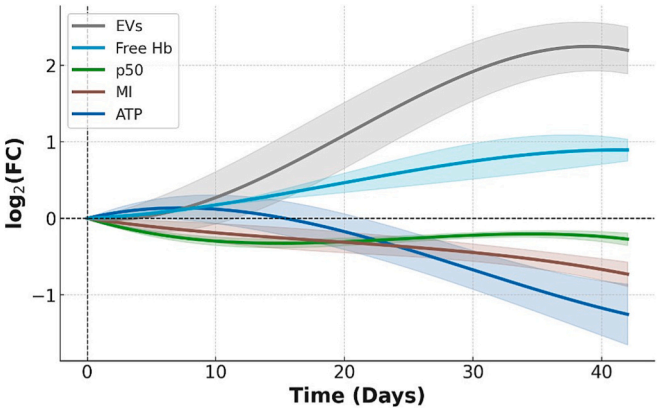

Fig. 1. Summary of changes in key RBC quality parameters over 42 days of hypothermic storage. Data from a Canadian Blood Services (CBS) quality monitoring program were used to calculate the $\log_2$ fold change ($\log_2$(FC)) in the indicated parameters, relative to baseline values measured at 5 days post-collection ($n = 20$). Shaded regions indicate the 95 % confidence intervals. Parameters include: (1) the number of extracellular vesicles (EVs) per μL, (2) free hemoglobin concentration in the supernatant (g/L), (3) oxygen-carrying capacity as indicated by the p50 value (the pO_2 in mmHg at which hemoglobin is 50 % saturated with oxygen), (4) morphology index (MI), and (5) ATP levels (μmol/g Hb). Methods for parameter measurements are detailed in Refs. (20, 21, 22).

Table 1
Composition of major licenced and experimental hypothermic RBC storage solutions.[1]

		CPDA-1	SAGM	MAP	AS-1	AS-3	AS-5	AS-7	*E*-sol5	PAGGSM	PAG3M
pH Buffers	NaCl	–	150	85	154	70	150	–	–	72	–
	$NaHCO_3$	–	–	–	–	–	–	26	–	–	–
	Na_2HPO_4	–	–	–	–	–	–	12	20	16	8
	NaH_2PO_4	16	–	6.03	–	–	–	–	–	8	8
Osmoregulator	Mannitol	–	30	80	41	–	45.5	55	41	55	–
Iron / Calcium Chelator	Citric acid	16	–	0.95	–	2	–	–	–	–	–
	Sodium Citrate	90	–	–	–	23	–	–	25	–	–
	Sodium Gluconate	–	–	–	–	–	–	–	–	–	40
Metabolic substrates	Glucose	160	45	40	111	55	45	80	111	47	47
	Adenine	2	1.25	1.5	2	2	2.2	2	2	1.4	1.4
	Guanosine	–	–	–	–	–	–	–	–	1.4	1.4
pH		5.6	5.7	5.7	5.5	5.8	5.5	8.5	8.4	6	8.7
Osmolarity (mOsm/kg)		365	376	324	462	295	310	237	301	287	278

[1] Concentrations are all in mM.

Consequently, stored RBCs initially experience an increase in ATP levels due to 2,3-DPG breakdown, provided the pH remains sufficiently high to sustain glycolysis [19]. A decline in 2,3-DPG is preferable, as ATP is essential for maintaining ion gradients, redox homeostasis, and membrane phospholipid asymmetry, thereby countering the irreversible changes in RBC rheology that are strongly linked to reduced post-transfusion recovery (PTR) [50,51]. An upper intracellular pH limit of 7.2 is therefore optimal.

In alignment with these principles, novel storage solutions have been developed to raise the initial pH, reduce or eliminate chloride to enhance intracellular pH via the chloride shift, and increase phosphate concentrations to provide additional buffering capacity [52,53]. These include additive solution-7 (AS-7, also known as SOLX® or EAS-81), erythrosol-5 (*E*-SOL5), and phosphate-adenine-glucose-guanosine-saline-mannitol (PAG3M), the compositions of which are detailed in Table 1 [54]

Each of these novel solutions demonstrates significant improvements in RBC storage quality compared to conventional solutions (see Ref. [55] for a characterization of the metabolomic profiles of RBCs stored in these solutions). ATP levels exhibit a similar trend to those observed in conventional solutions, beginning with a production phase, transitioning to a brief plateau, and concluding with a consumption phase [55]. However, in these novel solutions, the production phase is prolonged due to heightened glycolytic activity [55]. Retention of 2,3-DPG differs among the solutions: AS-7 and *E*-SOL5 maintain higher levels for up to 35 days before converging with levels seen in conventional solutions [55]. In contrast, PAG3M exhibits a unique increase in 2,3-DPG levels that parallels ATP production, with levels remaining relatively unchanged over six weeks of storage [55]. While the mechanism underlying this metabolic peculiarity remains unclear, it is hypothesized that the guanosine-dependent rise in ribose-5-phosphate via the purine salvage pathway stimulates late-stage glycolysis downstream of the Luebering-Rapoport shunt, thereby sustaining 2,3-DPG production [55].

Despite the clear benefits of these solutions, none are currently in widespread use in blood banks. AS-7 is the only formulation that has received FDA approval, while the others remain experimental and are not yet commercially available [56]. The reluctance to implement these solutions stems from the absence of conclusive evidence linking storage lesions to adverse clinical outcomes, as well as practical challenges associated with glucose caramelization during heat sterilization at pH levels above 6. However, as RBC transfusion practices continue to evolve (discussed in Section 4), the adoption of these additive solutions warrants renewed consideration, particularly given that the sterilization challenge can be addressed through proposed two-pack systems, in which glucose and alkaline salts are stored separately [57].

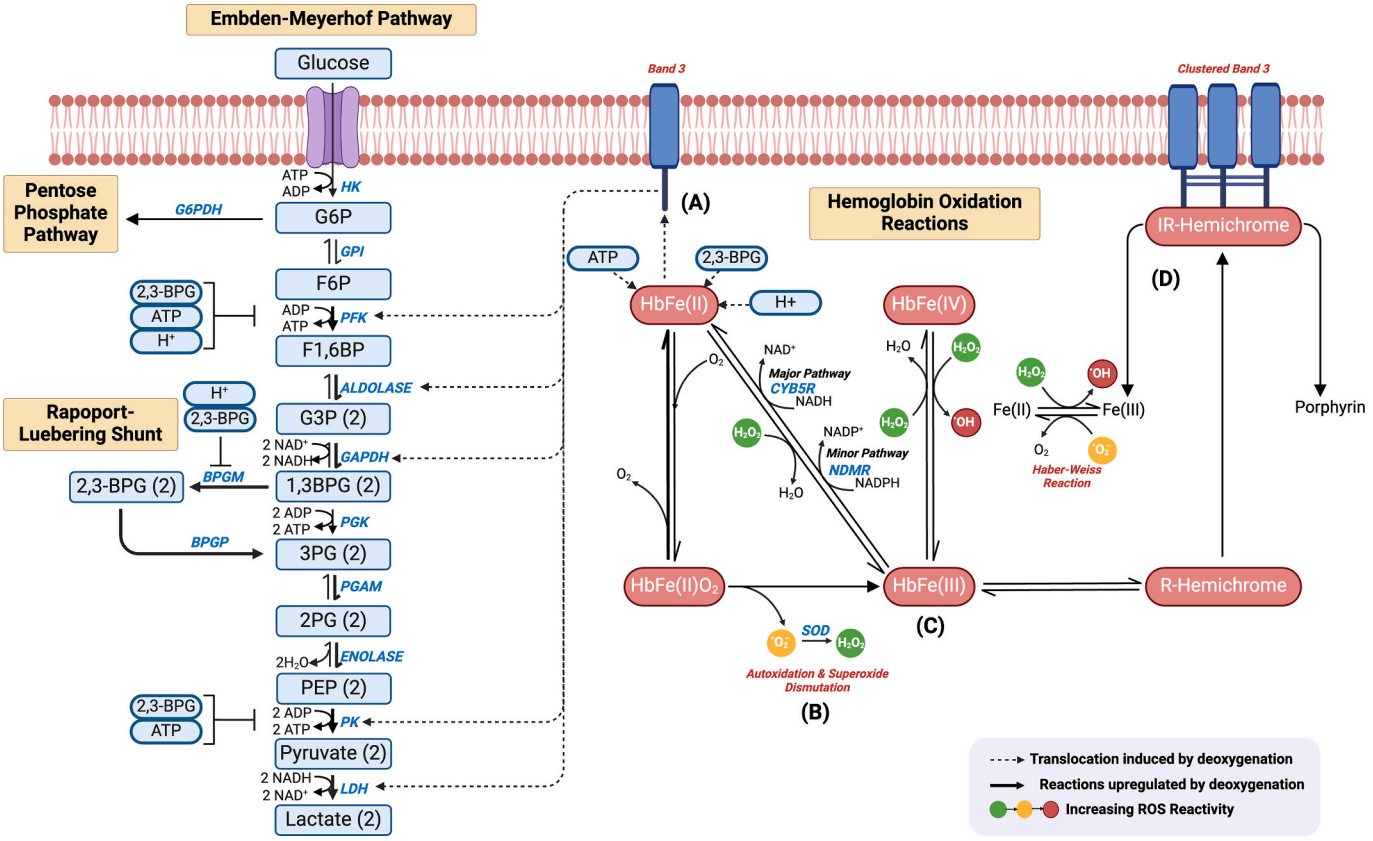

Fig. 2. Interplay between hemoglobin (Hb) deoxygenation, metabolism, and Hb oxidation reactions. (A) In the absence of oxygen, ferrous Hb increases flux through the Embden-Meyerhof pathway by 45 % through: (1) competitively binding to the cytoplasmic domain of band 3 to displace PFK, aldolase, GAPDH, PK, and LDH; and (2) binding to H+, 2,3-BPG, and ATP, reducing the glycolytic inhibition caused by these heterotropic Hb effectors. All bolded arrows indicate reactions upregulated by deoxygenation. (B) The autoxidation of ferrous oxygenated Hb is the primary source of superoxide anions and H_2O_2, as superoxide is quickly converted to H_2O_2 by superoxide dismutase (SOD). (c) Ferric Hb (metHb) can be reduced back to ferrous Hb through NADH-dependent cytochrome *b*5 reductase (CYB5R) or NADPH-dependent metHb reductase (NMDR). The reverse reaction can be fueled by H_2O_2, but in the absence of oxygen, this reaction is not preferred due to the lack of Hb autoxidation to generate significant H_2O_2. If not reduced, metHb may react with H_2O_2 to form highly reactive ferryl Hb or degrade into reversible (R-) hemichromes due to ferric iron destabilization. (C) Irreversible (IR-) hemichromes form when structural changes block reversion to ferric Hb, causing them to bind to the cytoplasmic domain of band 3. This promotes oxidation, band 3 cluster formation, and the binding of autologous IgG antibodies, leading to clearance. Breakdown of the heme moiety in hemichromes results in release of the porphyrin ring and ferric iron which can be reduced into ferrous iron with superoxide. Ferrous iron and H_2O_2 function as a Fenton reagent to fuel production of highly reactive hydroxyl radicals through the Haber-Weiss reaction.

2.2. Simultaneous control of hemoglobin redox reactions and energy homeostasis through anaerobic storage

Oxidative stress is a major contributor to the decline in RBC function during storage, primarily because non-enzymatic oxidant-generating reactions are not as temperature-sensitive as enzyme-dependent antioxidant pathways [58]. Since there is a close link between redox balance and energy homeostasis as detailed in Fig. 2, many of the novel alkaline, chloride-free solutions help reduce oxidative stress [55]. However, addressing energy homeostasis alone is insufficient to ameliorate oxidative injuries over the six-week storage period. Therefore, alternative strategies have focused on targeting the root cause—molecular oxygen—through taking advantage of RBCs' unique reliance on anaerobic metabolism. By bubbling sterile nitrogen, helium, or argon gas through the unit for 5–6 cycles, oxygen levels can be reduced to as low as 2–3 % over six weeks of storage when the units are kept in an oxygen-depleted chamber with palladium catalysts [59].

One of the major sources of oxidative injury during hypothermic storage results from hemoglobin-dependent autoxidation reactions, many of which are detailed in Fig. 2 [60]. Molecular oxygen can transfer an electron to ferrous (Fe^{2+}) heme iron, generating a superoxide anion and converting oxyhemoglobin (oxyHb) into methemoglobin (metHb), with the heme iron left in a ferric state (Fe^{3+}) [61]. Due to the minimal activity of cytochrome-b5-reductase, the enzyme responsible for reducing metHb at low temperatures, metHb eventually denatures into irreversible hemichromes and, ultimately, releases free iron [61]. Breakdown of the heme moiety in hemichromes results in release of the porphyrin ring and ferric iron which can be reduced into ferrous iron with superoxide [61]. Ferrous iron and H_2O_2 function as a Fenton reagent to fuel production of highly reactive hydroxyl radicals through the Haber-Weiss reaction [62,63].

In addition to minimizing hemoglobin autoxidation reactions, anaerobic storage improves energy homeostasis in stored RBCs through capitalizing on the relationship between hemoglobin oxygenation and metabolism (this relationship is shown in Fig. 2) [59]. In its T-state (i.e., deoxygenated form, HbT), hemoglobin competitively binds to the N-terminus of the cytoplasmic domain of Band 3 (CDB3), displacing key glycolytic enzymes such as PFK, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aldolase, and lactate dehydrogenase (LDH) [64,65]. The resulting disinhibition of these enzymes leads to approximately a 45 % increase in glycolytic flux and a 66 % decrease in flux through the pentose phosphate pathway (PPP) compared to oxygenated RBCs [66,67]. This metabolic shift is further supported by HbT's high affinity for 2,3-DPG, ATP, and H+ ions, which reduces the negative feedback typically caused by heightened glycolytic activity (note: the binding of HbT to H+ ions facilitates intracellular alkalinization) [68]. Moreover, heightened glycolytic activity helps maintain a high ATP:AMP ratio, which suppresses AMP deaminase activity in the purine salvage pathway and limits the production of hypoxanthine [69]. Because hypoxanthine can contribute to hydrogen peroxide production in circulation, elevated levels of hypoxanthine in blood products are linked to reduced post-transfusion recovery (PTR), underscoring the importance of minimizing its accumulation [70,71]. Consistent with these in vitro observations, in vivo models have demonstrated faster oxygen off-loading and improved post-transfusion survival when RBCs are stored under anaerobic conditions [72–75].

These positive findings have spurred the first multi-center, randomized, controlled cross-over study to test conventional vs. anaerobically stored RBCs in patients with transfusion-dependent hematological malignancies [76]. Initial transfusions began in late 2024.

2.3. Reducing storage temperatures to alleviate hypothermic injury

Over the past 100 years, myriad changes to storage solutions, storage containers, and RCC manufacturing methods have taken place; however, storage temperature has remained a constant aspect of RBC storage practices [13]. Within the allowable storage temperature range of 1–6 °C (note: this applies to the US, other jurisdictions use a range of 2–8 °C), recent studies using precision-controlled freezers have shown that storing RBCs at the lower end of this range offers metabolic benefits [77]. Thus, some have proposed that the lower temperature threshold may be unnecessary, provided nucleation (i.e., ice formation) is prevented [78]. Unfrozen storage within the −10–0 °C range could offer an effective strategy to attenuate metabolic sequelae in stored RBCs and potentially extend allowable storage durations.

Rather than relying on colligative agents to suppress the freezing point, the few studies exploring the storage of RBCs at high sub-zero temperatures have instead focused on maintaining the cells in a metastable, supercooled liquid state below the freezing point while taking measures to prevent nucleation (i.e., ice formation) [78–80]. Since the air-liquid interface is the most likely site for nucleation, strategies such as de-gassing the storage container or applying an immiscible hydrocarbon and/or alcohol mixture can be employed to reduce the probability of nucleation. The latter "surface sealing" approach alters the structure of water molecules at the interface, restricting their ability to form a stable crystal lattice, and thereby more effectively preventing nucleation [78]. As the probability of nucleation increases with the degree of supercooling, (i.e., the difference between the freezing point and storage temperature), this would be the preferable method if a storage temperature towards the lower end of the −10–0 °C range is chosen.

Efforts to supercool RBCs have demonstrated that while lowering the temperature can reduce metabolic injury, its impact on storage quality is not uniformly positive; thus, adjustments to the storage solutions are required to make this storage modality viable [78–80]. For instance, Isiksacan et al. (2024) show that while RBCs stored at −5 °C show slower depletion of glycolytic intermediates (including ATP and 2,3-DPG) due to the expected decline in metabolic rates, they also exhibit higher levels of hemolysis over a 6–10-week period compared to samples stored at hypothermic temperatures [80]. However, beyond the 10-week mark, hemolysis levels in RBCs stored at conventional hypothermic temperatures begin to surpass those stored at −5 °C, presumably due to ATP depletion in the latter [80].

A potential mechanism underlying the "acute" increase in hemolysis may involve oxidative injury. The same study suggests that although ROS-generating reactions are slowed at supercooled temperatures, the antioxidant capacity of RBCs declines and lipid peroxidation increases [80]. In keeping with the principle that reduced temperatures suppress ROS-generating reactions more than ROS-scavenging ones, antioxidants that offer only marginal protection in refrigerated RBCs become significantly more effective in supercooled RBCs [80]. However, addressing oxidative injury alone is insufficient to fully mitigate the acute damage and achieve storage quality equivalent to or better than that of refrigerated RBCs within the first 10 weeks [80]. This underscores the need for further research to identify and address additional mechanisms of injury in supercooled RBCs.

3. Long-term storage of RBCS

The resilience of RBCs to hypothermic storage stressors also extends to cryopreservation. Lacking intracellular organelles and capable of tolerating osmotic fluctuations up to twice their isotonic volume, RBCs exhibit greater resistance than nucleated cells to the two primary mechanisms of freezing injury outlined in Fig. 3A: *slow cooling* injury (caused by excess solute concentration in the extracellular unfrozen fraction) and *rapid cooling* injury (resulting from intracellular ice formation) [81,82] (see Ref. (83) for a detailed review of these mechanisms). However, the low membrane packing and order parameters that grant RBC membranes their high flexibility also make them particularly susceptible to dimethyl sulfoxide (DMSO)—the most commonly used cryoprotective agent (CPA)—due to its potent pore-forming properties [84,85]. Consequently, RBCs are the only clinical cell type

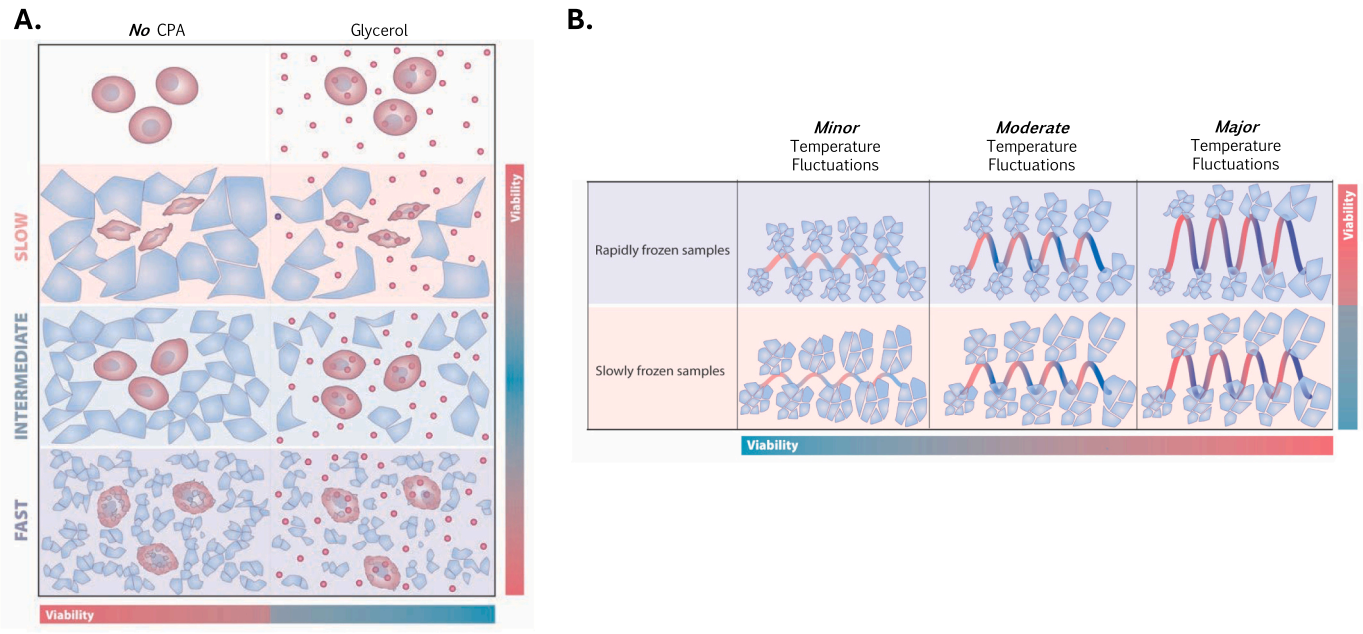

Fig. 3. The role of freezing parameters, cryoprotective agents (CPAs), and storage conditions on frozen cell recovery. (A) In conventional cryopreservation protocols, extracellular ice forms before intracellular ice due to the absence of nucleating (ice-forming) agents in the cell cytoplasm. As extracellular ice grows, solutes are excluded, increasing the concentration of solutes in the remaining unfrozen extracellular fraction. This creates a chemical potential imbalance between the inside and outside of the cell, which drives equilibration either through osmotic dehydration or a cytoplasmic phase transition. Freezing too fast prevents osmotic dehydration, leading to damaging intracellular ice formation. Conversely, freezing too slowly results in excessive osmotic dehydration, which is too is harmful. Optimal cell recovery requires a balanced cooling rate that minimizes these risks. CPAs like glycerol improve recovery by lowering the amount of ice at any given temperature, reducing osmotic dehydration, and decreasing the chance of intracellular ice formation. (B) The stability of frozen cells are largely determined by the initial cooling rate and the extent of temperature fluctuations during storage. Higher temperatures provide more thermal energy, increasing the risk of ice recrystallization during routine handling. Rapid cooling produces small, thermodynamically unstable ice crystals, which are more prone to recrystallization during storage or temperature fluctuations.

cryopreserved using glycerol instead of DMSO.

Developed in the 1960s and 1970s, the so-called high and low glycerol methods—used predominantly in North America and the EU, respectively—remain the only RBC cryopreservation strategies implemented in blood banks today (Table 2) [86–91] Each has distinct limitations. As its name suggests, the high glycerol method (HGM) requires a higher glycerol concentration (~40 % wt/v), making glycerolization and deglycerolization more time-consuming and complex. In contrast, the lower glycerol concentrations in the low glycerol method (LGM; 15–20 % wt/v) simplifies these steps but requires a rapid cooling rate (60 °C/min vs. 1 °C/min for HGM) to prevent slow cooling injury in RBCs. This rapid cooling rate increases susceptibility to ice recrystallization (i.e., a mechanism whereby large ice crystals grow at the expense of smaller, more thermodynamically unstable ice crystals), requiring use of lower storage temperatures (LGM: -196 °C; HGM: −80 °C) that complicate transport and storage logistics [90–93]. Despite their respective advantages and drawbacks, both methods are more costly and logistically demanding than hypothermic storage, restricting RBC cryopreservation primarily to military settings, rare blood programs, and preoperative autologous transfusions [94].

Table 2
Overview of the high glycerol method (HGM) and low glycerol method (LGM) for RBC cryopreservation.

	HGM (40 % *w/v*)	LGM (15–20 % w/v)
Cooling Rate	1C/min	~60C/min
Cooling Method	Mechanical freezer	Liquid nitrogen
Storage Temperature	−80C to −65C	−120C to −196C
Transportation Method	Dry Ice	Nitrogen Vapour
Deglycerolization Time	~60 min	~30 min
Impact of Temperature Fluctuations	Low (minimal recrystallization)	High (extensive recrystallization)

The longstanding challenges associated with RBC cryopreservation have driven numerous efforts to improve current practices. Key strategies include: (1) developing more efficient methods of glycerol removal, (2) exploring alternative CPAs to reduce required glycerol concentrations, and (3) investigating experimental long-term preservation methods that eliminate glycerol entirely. When discussing alternative CPAs, it is important to acknowledge several studies presenting CPAs capable of freezing RBCs without glycerol, which we have chosen not to discuss due to limited insight into the mechanisms of cryoprotection and the freezing conditions being non-replicable on full-sized units. Nonetheless, we recognize the potential of these compounds and provide an overview of these studies in Table S1.

3.1. *Improving the present standards of RBC cryopreservation*

3.1.1. *Increasing the efficiency of Deglycerolization*

Implementation of the semi-automated ACP-215 cell processor (FDA-approved in 2003) represents a significant advancement in deglycerolization strategies compared to earlier methods [95]. Deglycerolization with the now-retired COBE-2991 cell processor required manual intervention at two steps of the dilution process, a task that is fully automated by the ACP-215 [96]. Additionally, the COBE-2991 operated as an 'open system', requiring units to be disposed within 24 h of deglycerolization due to the risk of bacterial contamination [96]. The ACP-215's closed system design eliminates this concern, allowing thawed units to be stored for up to 21 days (a limit established to eliminate accumulation of excess hypothermic injury) [20,97–99]. Despite these advancements, significant opportunities remain to reduce processing time, lower costs, and improve equipment portability.

Promising strategies to improve deglycerolization efficiency involve the use of hollow fiber dialysis filters or microfluidic devices, where the cell-containing stream flows adjacent to a wash stream, with the two

separated by a semipermeable membrane [100–103]. A key advantage of these approaches is the ability to model the transport of water, glycerol, sodium chloride, and associated cell volume changes using known parameters such as solution viscosity, solute flux across plasma and semipermeable membranes, and the dimensions of the two streams [100,104]. This modeling enables precise optimization of variables like cell concentrations, diluent volume, flow rates, and temperatures, to maximize glycerol removal efficiency while maintaining cells within their osmotic tolerance limits. Thanks to a higher surface area-to-volume ratio, shorter diffusion distances, and precise flow control to maintain laminar conditions, microfluidics can achieve much more efficient deglycerolization, with some studies showing complete glycerol removal within 3 min (compared to 30 mins – 60 mins with advanced hollow fiber dialysis filters) [101,104].

Despite these advantages, scaling microfluidic platforms to process 200–300 mL of frozen RBCs remains a significant challenge. To address this, three-stream designs, where a cell-laden stream is flanked by wash streams, have shown to increase CPA removal rates and throughput [105]. Although efforts to further scale up microfluidic systems to process frozen RBCs are limited, microfluidic networks with hundreds of parallel channels are now being used for high-throughput drug screening, and their application to RBC cryopreservation could pave way for the ambitious goal of real-time deglycerolization during transfusion [106].

3.1.2. Methods to reduce glycerol concentrations

3.1.2.1. Protecting against ancillary sources of cryoinjury. The so-called "two-factor model" of freezing injury provides a foundational framework for designing cryoprotective agent (CPA) formulations to attenuate osmotic stress and intracellular ice formation, the primary causes of cell death during freezing [83]. However, as secondary sources of cryoinjury, such as ice recrystallization, do not directly fall within the bounds of this model, they were historically overlooked. That said, conventional CPAs do indirectly attenuate recrystallization through increasing solution viscosity [107–109]. Consequently, any effort to reduce CPA concentrations will increase susceptibility to recrystallization, even when the primary mechanisms of cryoinjury outlined in the two-factor model are adequately controlled [110]. This, in part, explains why temperature fluctuations are more detrimental to RBCs frozen using the LGM than those frozen using the HGM [111] (note: Fig. 3B demonstrates the relationship between freezing rate and injury in response to temperature fluctuations). To perturb recrystallization that could become more damaging with reduced glycerol concentrations or simply minimize the impact of deleterious temperature fluctuations during routine storage and handling, emerging ice recrystallization inhibitors (IRIs) that actively prevent the transfer of water molecules between ice crystals offer a promising solution [112–117].

Several classes of easily synthesizable compounds exist with ice recrystallization inhibition properties that recapitulate that of antifreeze proteins found in nature, among which polyvinyl alcohol (PVA) and select synthetic amphipathic, highly hydrated carbohydrates are the most potent candidates applied to RBCs [112–114]. Deller et al. (2015) demonstrated that low concentrations of PVA (0.5 mg/mL) were able to improve RBC recovery when the cells were thawed slowly, a condition that exacerbates injury resulting from recrystallization [114]. Capicciotti et al. (2015) similarly indicate that the benefits of an amphipathic carbohydrate, in this case in response to an intermittent warming event to exacerbate recrystallization [112]. More importantly, their study showed that while RBC survival was comparable across conditions in the absence of warming, subjecting cells to intermittent warming reduced survival in the 15 % glycerol condition, whereas no decline was observed in RBCs frozen with either 40 % glycerol or 15 % glycerol supplemented with an IRI. These findings highlight the increased susceptibility to recrystallization when glycerol concentrations are reduced, while underscoring the protective role of IRIs in mitigating this effect.

3.1.2.2. 3.1.2.1 Deep eutectic solvents. Eutectic solvents are mixtures of hydrogen bond donors and acceptors with a freezing point lower than the combined freezing points of their individual components, a phenomenon frequently observed in cold-tolerant and anhydrobiotic organisms [118,119]. While suppression of the freezing point is not the sole mechanism by which CPAs mitigate cryoinjury, it is critical towards successful cryopreservation [120]. In fact, eutectic mixtures of compounds that individually offer minimal cryoprotection, like glucose, urea, and proline, have demonstrated comparable outcomes to conventional cryopreservation methods [121]. Glycerol is a common component of naturally occurring eutectic mixtures, where it is often combined with polyhydric alcohols, polyols, sugars, and amino acids—many of which are conventionally or have been experimentally employed in cryopreservation [122–125].

In the context of reducing glycerol concentrations for RBC cryopreservation, it is important to identify not only the optimal constituents but also their precise ratios to achieve a 'deep' eutectic mixture—defined as the specific ratio at which the mixture reaches its minimum melting point [126]. Promising constituents for a glycerol-containing eutectic mixture include disaccharides such as sucrose and trehalose, as well as amino acids like proline and isoleucine [122,127]. Certain combinations of these constituents have demonstrated promising post-thaw RBC recovery in scenarios where glycerol alone proves inadequate (i.e., glycerol concentrations of 5–7.5 %) [128,129]. However, consideration for the molar ratios which satisfy the definition of a deep eutectic mixture has not yet been explored for RBCs.

3.2. Experimental alternatives to conventional cryopreservation

3.2.1. Improving desiccation tolerance through promoting intracellular delivery of cell-impermeable sugars

Freeze-drying (lyophilization) has the potential to revolutionize red blood cell (RBC) storage and transport by enabling long-term preservation at room temperature, alleviating logistical challenges, particularly in military and remote settings [130]. However, replicating the natural desiccation tolerance seen in anhydrobiotic organisms remains a significant challenge, with sperm cells being the only mammalian cells that can be reproducibly lyophilized with success to date [131]. Attempts to reduce RBC water content by over 90 %— sufficient to halt metabolism at room temperature—achieve recovery rates of 70–85 %, adequate for reagent RBCs used in hemagglutination testing but insufficient for transfusion applications. [132–135].

The primary challenge in lyophilization is preserving molecular organization as water is removed. In membranes, lipid head groups rearrange to maximize contact with water, potentially forming non-lamellar structures incompatible with the bilayer's semipermeable properties [136]. For proteins, dehydration destabilizes their secondary, tertiary, and quaternary structures [137]. Destabilization of hemoglobin's tetrameric structure plays a significant role in the overall injury RBCs experience during desiccation (accounts for ~13 % of the total bound water in RBCs) [138,139]. Autoxidation rates resulting from this destabilization peak when the water content reaches ~16 % of its original value, then decline as oxygen solubility decreases due to rising salt concentrations in the remaining water [140].

To stabilize proteins and lipids during desiccation, additives are required that mimic the molecular properties of water while restricting molecular motion in the desiccated state to prevent degradation [141]. Trehalose, a disaccharide and kosmotrope, excels at this. It possesses a high glass transition temperature (i.e., the point at which a liquid becomes an amorphous solid without undergoing a true liquid-to-solid phase change) and displaces water molecules from biomolecular hydration shells by forming preferential interactions with polar functional

groups [142–145].

While the prevalence of trehalose in anhydrobiotic organisms across all domains of life is a testament to its lyoprotective properties, its use in lyophilization of biological material was historically challenged by its membrane impermeability [146,147]. Classical membrane permeabilization techniques, including sonoporation, electroporation, and thermal/osmotic shock, can achieve sufficient trehalose loading in under an hour [148,149]. However, these methods often result in significant hemolysis (>5 %) due to permanent membrane or cytoskeletal damage and uncontrolled solute flux [150,151]. The need to balance the damage caused by loading methods with the amount of trehalose delivered within acceptable timeframes has undoubtedly contributed to historically poor RBC recovery following lyophilization [152].

To minimize membrane injury during loading, researchers have developed amphipathic polymers that promote intracellular trehalose delivery with minimal membrane stress [153–157]. Although many such polymers are less efficient than classical approaches—requiring 7–24 h for sufficient loading—Chen et al. report a notable exception, achieving trehalose loading within just 15 min using a pseudopeptidic polymer containing long alkyl side chains that mimic membrane-anchoring fusogenic proteins [153]. Additionally, membrane-permeable trehalose-containing nanocapsules and membrane-permeable trehalose derivatives have been developed, though these also require several hours to reach equilibrium concentrations [158–161]. Notably, many of these strategies have yet to be applied in RBC lyophilization, presenting promising opportunities for future research (see Ref. (147) for a review of novel trehalose loading strategies).

3.2.2. *Low-temperature Vitrificaiton as an alternative to cryopreservation*

Like lyophilization, low-temperature vitrification preserves biological material by achieving an amorphous liquid state [162]. However, rather than removing water, vitrification relies on rapid cooling to reach the glass transition temperature without undergoing a phase change, allowing for low-temperature preservation without ice formation [162]. Once vitrified, the sample must be rapidly warmed to prevent devitrification (i.e., ice formation within the vitrified system) when temperatures rise above the glass transition point [163]. Achieving the necessary cooling and warming rates to prevent nucleation in an isotonic salt solution is challenging; therefore, CPAs are required to elevate the glass transition temperature, facilitating vitrification at more practical rates [164–166].

Vitrification becomes increasingly challenging with larger systems due to limitations heat transfer and the higher probability of stochastic events like ice nucleation [162,167]. These challenges have hindered its application to entire units of RBCs or bulk compositions of therapeutic cell types. To address this challenge, an effective vitrification method for large-volume cell suspensions involves dispensing small, cell-laden droplets at a high flow rate using a syringe pump [168–170]. These droplets are either introduced directly into liquid nitrogen or onto a pre-cooled plate with high thermal conductivity on the liquid nitrogen surface, ensuring rapid cooling and vitrification [168–170]. This technique, successfully applied to cells such as hepatocytes and oocytes, allows for adequate recovery while minimizing the need for high CPA concentrations, as much faster cooling rates can be achieved in droplets [168,170]. Although similar to historical droplet preservation methods proposed for RBC storage in immunohematology testing, those approaches involved freezing rather than vitrification, as the resulting high levels of hemolysis (20–40 %) were deemed acceptable as long as antigen reactivity was maintained [171–174].

Achieving vitrification in highly concentrated cell suspensions, such as red cell concentrates (RCC), is inherently more challenging than dilute suspensions because cell membranes can serve as sites for heterogeneous nucleation [175,176]. This necessitates careful optimization of droplet size and CPA composition to achieve vitrification while preventing devitrification. Additionally, striking this balance is crucial for identifying the most practical approach, as larger droplet sizes increase vitrification throughput but require higher CPA concentrations, which in turn reduce throughput during pre- and post-storage processing. To our knowledge, no studies have systematically explored this trade-off, and research on droplet vitrification of RBCs remains limited [177,178]. One notable study is that of El Assal et al. (2014), where a CPA cocktail consisting of 9 % (*v*/v) ectoine, 25 μg/mL trehalose, and 1 % (v/v) PEG, along with droplet sizes in the nanoliter range (~0.14 nL), achieved post-thaw hemolysis of <1.5 % [178]. While these findings are promising, the use of nanoliter-sized droplets presents significant feasibility challenges for clinical-scale cryopreservation, limiting the practicality of this approach for large-volume applications.

4. The role of storage in evolving paradigms of RBC transfusions

Two major advancements are shaping the future of RBC transfusion practices: the use of alternative plasticizers in storage containers and the adoption of more comprehensive donor-recipient matching strategies. Several of the novel storage methods discussed may impact both areas.

4.1. *Transitioning away from DEHP*

Since 1955, polyvinyl chloride (PVC) blood bags have been plasticized with di(2-ethylhexyl) phthalate (DEHP) to address PVC's inherent brittleness, as the material, despite being durable, inert, and inexpensive, requires plasticizers for flexibility [179]. As DEHP is not covalently bound to the PVC matrix, it leaches into the stored blood during hypothermic storage, with levels over six weeks of storage increasing ~12-fold [180]. Ample studies have shown that the incorporation of DEHP into the RBC membrane and cytoplasm improves retention of rheological properties throughout storage [181–183]. AuBuchon et al. (1988) in fact reported a significant 24 % increase in post-transfusion recovery when DEHP was present in 35-day old RBCs [184]. However, in response to mounting evidence of DEHP's endocrine-disrupting and ecotoxicological properties, the European Union has enacted legislation to phase out all DEHP-containing products by May 2025 [185–187]. While this decision will primarily impact European blood banks, global non-DEHP movements are expected to make DEHP prohibitively scarce and costly worldwide [188].

To mitigate potential compromises in RBC storage quality during the transition away from DEHP, two main strategies can be considered: shortening allowable storage durations or implementing alternative measures to ensure non-DEHP storage remains comparable. Shortening shelf-life is likely impractical; a recent survey of 16 blood banks across North America, Europe, and Asia found that only 3 respondents would accept a shelf-life of less than 28 days, where differences between DEHP and non-DEHP storage would be less apparent [188].

The search for alternative non-phthalate plasticizers has identified bis(2-ethylhexyl) terephthalate (DEHT; a structural isomer of DEHP) and 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) as promising candidates [189,190]. Studies suggest that PVC-DEHT and PVC-DINCH bags containing AS-1, AS-3, PAGGSM, and SAGM solutions provide storage outcomes comparable to those achieved with DEHP-containing bags [190–192]. While storage quality, particularly with SAGM, appears marginally inferior based on hemolysis and hematological parameters, these differences are unlikely to be clinically significant [188,190–192]. Certainly, the transition away from DEHP presents an opportunity to leverage next-generation storage solutions to prevent any exacerbation of storage-related injuries with alternative non-phthalate plasticizers. However, to our knowledge, no such efforts have been made.

4.2. *Towards more personalized practices in RBC transfusions*

In patients requiring regular transfusions, the use of extended phenotype matching to mitigate the risk of alloimmunization offers

unique insight into how more comprehensive donor-recipient matching strategies could lead to population-specific blood shortages. Sickle cell disease (SCD)—the most common indication for regular transfusions—is particularly prevalent among individuals of African descent, with 8 % carrying the sickle gene and 1 in 625 having SCD [193]. Significant disparities in the prevalence of specific Rh, Duffy, Kell, and Kidd blood groups exist between individuals of African descent and other ethnic backgrounds, particularly Caucasians, mean that SCD patients often require blood from donors of the same ethnic background [194]. This can be a challenge in countries where individuals of African descent are a minority. In the UK, approximately 15,000 individuals have SCD, with an estimated 80 % being of African descent, while only 2 % of the donor population – ~19,000 individuals – are of African descent. [195]. Although blood group variability between SCD patients and the donor population are certainly not going to be a perfect match, this example underscores how shortages can arise if a minority population requires blood from a minority population.

Similar in principle is the growing drive to match donor RBC metabolic biomarkers and donor-specific single nucleotide polymorphisms (SNPs) associated with post-transfusion recovery (PTR) to the recipient's pathology or demographic profile [196–198]. This notion is supported by the intersection of findings from recent large-scale studies showing that donor characteristics—such as sex, age, ethnicity, lifestyle, and iatrogenic intake—affect RBC parameters relevant to PTR [33,199–202]. Implementing comprehensive donor-recipient matching aligned with these findings would require multifaceted changes to the transfusion infrastructure.

New storage paradigms discussed in this article would aid in supporting this theoretical transfusion network. For example, since the metabolic phenotype of RBCs varies between donors and influences the progression of the hypothermic storage lesion, minimizing storage-induced stress could attenuate donor-dependent differences and potentially expand the pool of units suitable for specific recipients [202]. Seasonal surpluses and shortages of certain blood products, particularly high-demand blood types, could become more common than they are now; thus, implementing 'intermediate-term' storage strategies, such as supercooling, could help mitigate these fluctuations [203,204]. Additionally, lowering costs and improving the feasibility of long-term blood preservation would facilitate stockpiling reserves for anticipated crises and reduce waste of unused units when supply significantly exceeds demand [205,206].

5. Conclusion and future considerations

Although clinical practice in RBC preservation has seen limited change in the past four decades, technological advancements have allowed the research landscape continues to rapidly evolve. As multi-omics techniques become more robust and accessible, the characterization of cellular injury in response to storage-induced stressors continues to gain depth and precision, allowing for more comprehensive, 'systems biology' perspectives [207]. A key benefit in this regard will be the eventual development of systematic -omics frameworks that can broadly and quantitatively characterize specific functional domains relevant to post-transfusion recovery, akin to the 'redoxomics' and 'immunomics' frameworks developed for nucleated cells [208,209]. Alongside these advances, more direct methods of assessing RBC function are emerging, enabled primarily by lab-on-a-chip devices that offer real-time, physiologically relevant interpretations of post-transfusion recovery (PTR) [210]. Together, these innovations will not only improve preservation standards but also deepen our understanding of donor-to-donor heterogeneity in blood products; an increasingly critical consideration, given evidence that factors like age, sex, ancestry, and environmental exposure influence metabolic and biophysical properties. By integrating these scientific insights into clinical practice, we open the door to a more robust transfusion infrastructure and, potentially, improved transfusion outcomes.

Practice points

- Short-term preservation of RCCs for up to six weeks at hypothermic temperatures causes quality deterioration, which evidence suggests could negatively impact transfusion outcomes in critically ill recipients. Reducing the storage duration to prevent this decline would either strain the blood supply or increase waste, depending on the local supply-demand balance. Therefore, optimizing storage solution composition to minimize RBC quality deterioration within the six-week period is a more favorable approach, and several such solutions have now been developed.
- Half of unused RCCs are discarded due to lack of usage before the six-week expiry, leading to financial losses estimated at $4.4 million to $113.9 million, based on discard rates ranging from 0.27 % to 6.7 % across the United States (at an average cost of $250 per unit). Strategies to extend storage durations without incurring significant additional costs or processing challenges could substantially reduce these losses. One promising approach under development is storage in an unfrozen state at high sub-zero temperatures.
- The high cost and inefficiency of current clinical RCC cryopreservation methods restrict their use to rare blood type collection programs and military settings. Expanding long-term RCC storage beyond these contexts could be achieved by reducing glycerol concentrations (alleviating processing challenges) or lyophilization (alleviating freezer capacity limitations in blood banks). Such advancements would facilitate the development of national blood stockpiles, which are particularly crucial for disaster preparedness in underserved regions where the balance between supply and demand is precarious.

Research agenda

- Advancing Hypothermic Storage: Translate innovations in hypothermic red cell concentrate (RCC) storage, such as alkaline, chloride-free solutions and hypoxic storage conditions, into routine blood banking practices to enhance storage quality and extend shelf life.
- Developing 'Intermediate' Storage Paradigms: Develop alternative storage strategies that provide semi-long-term preservation, bridging the gap between conventional hypothermic storage and cryopreservation. These paradigms should not introduce significant processing complexity and help addressing blood supply challenges in resource-limited or emergency scenarios.
- Optimizing Long-Term Preservation: Reduce the cost and improve the efficiency of long-term RCC preservation by lowering the concentrations of glycerol required for cryopreservation. Additionally, advance the development of cost-effective alternatives, such as lyophilization, to make long-term storage more accessible and sustainable.

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Declaration of competing interest

The authors have no conflicts of interest to disclose.

Appendix A. Supplementary data

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