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PERSPECTIVE

ADVANCES IN GLYCEROL-FREE AND LOW-GLYCEROL CRYOPRESERVATION OF RED BLOOD CELLS

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Abstract

This review recounts the advances in cryopreservation technologies of red blood cells (RBC). The limitations of traditional glycerol-based methods and the latest developments in cryopreservation with no or less glycerol are elaborated. Although the use of glycerol at high-concentrations effectively prevents ice crystal formation, the associated osmotic stress damages RBC. Moreover, the cumbersome and time-consuming deglycerolization process not only increases operational costs, but also poses the risks of contamination and additonal cell damage, thereby limiting the application of frozen blood for emergency situations. To overcome these challenges, novel strategies have focused on rapid freezing with no or less glycerol, new permeable cryoprotectants and the synergistic use of non-permeable macromolecular/saccharide protectants (such as trehalose and hydroxyethyl starch). These approaches aim to achieve more efficient and safer preservation through multi-faceted protective mechanisms via the reduction of ice crystal damage, osmotic stress and oxidative damage. New strategies demonstrate significant advantages in streamlining processes (eliminating or simplifying deglycerolization steps), improving cell recovery and quality (low hemolysis) and enhancing in vivo efficacy. However, challenges such as long-term storage stability, scale-up production costs, standardization and regulatory approval remain critical issues to be addressed before clinical translation can be realized. Future research needs to focus on optimizing cryoprotectant formulations, elucidating molecular mechanisms, establishing a standardized quality control system and clinical validation, thereby revolutionizing blood inventory management.

Keywords: cryopreservation; cryoprotectants; glycerol-free CPAs; low-glycerol CPAs; red blood cells.

INTRODUCTION

In the early stages of transfusion therapy, the short shelf life of fresh blood necessitated that donors and recipients lie side-by-side for transfusion. Subsequently, technical advancements allowed red blood cells (RBCs) to be stored under refrigerated conditions at 1°C to 6°C for up to 6 weeks. While such advancements allowed for improved blood availability, the limited storage duration underscores the ongoing importance of enhancing preservation techniques for RBCs in transfusion medicine and clinical research (1). Cryopreservation of RBCs thus emerges as a crucial method that extends shelf life, alleviates seasonal blood shortages, meets demands for rare blood types, and provides sufficient blood reserves for special transfusion scenarios and emergencies such as wars and natural disasters (2, 3, 4).

At present, cryopreservation of RBCs involves high costs and requires specially trained personnel for clinical application. To reduce freezing damage, appropriate cryoprotective agents (CPAs) must be added (5). Despite its promise for RBC storage, cryopreservation can lead to significant RBC loss (6) due to various biological damage mechanisms. RBCs undergo physical changes during freezing and thawing due to ice formation and solute concentration in both intracellular and extracellular environments. Osmotic damage arises from the osmotic stress upon the CPA addition and removal and solute concentration during freezing, resulting in dehydration and cell lysis. Mechanical damage results from cell membrane rupture, and can be lethal to cells due to ice crystal penetration, particularly upon thawing when large ice crystals form by recrystallization. These challenges highlight an urgent need to develop more efficient, biocompatible CPAs and feasible cryopreservation protocols for RBCs (7).

Currently glycerol is the only clinically approved CPA for RBC cryopreservation and is widely used (8); by promoting vitrification, it prevents ice crystal formation (3). Glycerol pretreatment also facilitates the preservation of RBCs via freeze-drying. Specifically, Zhou et al. reported that glycerol pretreatment enhances the recovery rate of freeze-dried RBCs and maintains the higher activities of key antioxidant enzymes as compared to untreated groups (9). The sustained high enzyme activity after extended storage further demonstrates the protective effect of this method (9). However, glycerol has drawbacks such as high toxicity, difficulty in removal, and the presence of harmful residues (10), which limit its application in clinical practice and other fields. Glycerol can also have side effects such as RBC deformation and hemolysis; therefore, it must be removed through multiple gradient washing steps before transfusion. Nevertheless, the gradient washing still leads to approximately 15% RBC hemolysis and about 1% residual intracellular glycerol (3). Other studies have indicated adverse morphological changes in RBCs after glycerol cryopreservation (3, 11), including the detrimentally-reduced RBC deformability and impaired function. Consequently, the search for novel CPAs with minimal side effects and easy removability has become a research hotspot.

LIMITATIONS OF GLYCEROL AS A RBC CRYOPROTECTANT

Glycerol damage to red blood cells

Glycerol, as a permeable cryoprotectant, increases the total solute concentration within red blood cells, thereby reducing ice crystal formation during cryopreservation. Glycerol can penetrate the red blood cell membrane primarily via aquaporins (12, 13). When glycerol enters cells, it protects red blood cells from freezing damage; but it may also trigger and alter protein interactions in cytoplasm and cytoskeleton. As a polar molecule, glycerol at high concentrations changes the ionic strength and dielectric constant of aqueous solutions (14, 15). Additionally, the osmotic effects of glycerol within red blood cells may lead to membrane or cytoskeletal damage.

Lin et al. demonstrated significant RBC morphological changes upon glycerol loading, a notably the increase in mean corpuscular volume (MCV) and red cell distribution width-standard deviation (RDW-SD). Even after repeated washing procedures, MCV and RDW-SD values remained high as compared to the fresh blood, with RDW-SD demonstrating the largest increase (11.2%) (3). The elevated parameters suggests that glycerol causes irreversible alterations or its residue remain within red blood cells, which raises concerns for blood transfusion therapy. The effect of glycerol as a cryoprotectant on irreversible damages to RBC proteins and cellular structures has to be re-visited. Furthermore, high concentrations of glycerol are potentially fatal (10).

Deglycerolization of cryopreserved RBCs

Prior to deglycerolization, the process of freezing and storage of cryopreserved RBCs do not significantly affect metabolic activity, as evidenced by no significant changes in pH or lactate concentration (3). However, subsequent to the deglycerolization washes, the intracellular pH decreases. The decrease is likely initiated by changes in cell membrane due to the repeated deglycerolization washing processes. Membrane alterations, in turn, lead to a dysfunction of cation pumps (16), thereby altering intracellular homeostasis (3) and ultimately contributing to the observed drop in intracellular pH.

GLYCEROL-FREE AND LOW-GLYCEROL CRYOPRESERVATION

Rapid freezing protocol with less glycerol

To circumvent the operational complexity and potential toxicity issues associated with high glycerol concentrations, recent attention was increasingly paid to new methods using low-glycerol concentration or even glycerol-free

approaches for RBC preservation. To this end, studies have introduced numerous novel non-permeating protectants that act synergistically with glycerol, significantly reducing the required glycerol concentration while improving the efficiency and safety.

Liu et al (5) used medium-molecular-weight sodium hyaluronate (MSH) for RBC preservation. MSH acted as an effective cryoprotectant and increased the post-thaw RBC recovery rate from 33.1 ± 5.8% (without MSH) to 63.2 ± 3.5% (with MSH alone). A significant synergistic protective effect was observed when MSH was combined with low concentration glycerol (5% v/v), achieving the 92.3 ± 6.0%, cell recovery and normal cell property and function. Similarly Zhao et al. (17) reported that the combination of TAPS (tris (hydroxymethyl) aminomethane-3-propanesulfonic acid) with 5% (v/v) glycerol achieved an RBC recovery of 88.4 ± 0.6%, comparable to the recovery of 89.2 ± 0.2% for the traditional 20% glycerol method. The findings suggest a synergistic mechanism exists between novel cryoprotectants and permeating CPAs like glycerol.

Novel, highly efficient protectants that may potentially replace glycerol have been actively pursued. Liu et al (18) first reported the potential of N-(Tris (hydroxymethyl) methyl) glycine (Tricine) in sheep red blood cell cryopreservation, which dramatically increased the recovery of red blood cells from 19.5 ± 1.8% to 81.2 ± 8.5%. Tricine not only possesses excellent osmoregulatory capacity to alleviate cell dehydration, but also inhibits ice recrystallizatio to reduce mechanical damage and scavenges reactive oxygen species (ROS) to mitigate oxidative stress. Similarly, Sui et al (19) demonstrated that betaine can serve as a core protective component for blood cryopreservation. By combining betaine with othere membrane stabilizers (such as sucrose and glycine) and employing a rapid freezing protocol, a recovery rate of red blood cells exceeding 91% was achieved. Another notable advantage of this protocol is that, because betaine has favorable biocompatibility and osmotic properties, the protectants can be efficiently removed post-thaw using a simple one-step washing method, simplifying the operational procedure and helping to ensure transfusion safety.

Red blood cell cryopreservation has moved beyond the high concentration glycerol only method towards the protocols combining low-concentration glycerol with novel molecules (e.g., MSH, TAPS, Tricine, betaine). The core concept is functional complementarity among protectants that encompass osmoregulation, inhibition of ice formation, antioxidation and membrane stabilization. By the combination of protective functions across multiple agents, the need for high concentrations of any single protectant is diminished, thereby mitigating individual toxicities while enhancing overall protection. The approach reduces the toxicity and procedural complexity while matching or surpassing the cryopreservation efficacy of traditional methods, representing an important trend towards safer and more convenient red blood cell cryopreservation technology.

Small-molecule permeable cryoprotectants

The development of novel small-molecule protectants for non-vitrification cryopreservation and the exploration of synergistic multiple protection strategies have become an important new research direction. Hu et al. used fulvic acid (FA) for RBC cryopreservation (20). FA improve cell cryopreservation by inhibiting ice crystal growth during freezing, reducing recrystallization during thawing. FA's ice-control capability stems from its strong binding with water molecules according to the study on water migration characteristics. Furthermore, FA is taken up by RBCs and is primarily localized in cell membrane, suggesting a potential membrane stabilization mechanism (21). When used alone, FA tripled the recovery of sheep RBCs as compared to the hydroxyethyl starch.

Among glycine derivatives, dimethylglycine (DMG) has demonstrated significant efficacy as a cryoprotectant. Hu et al reported that DMG increased the post-thaw RBC recovery from 11.6 ± 1.4% to 72.2 ± 1.2% (19). DMG reduced mechanical damage by inhibiting ice crystal formation and recrystallization uypon warming. Additionally, it scavenges ROS and maintains endogenous antioxidant enzyme activities to mitigate oxidative damage during storage.

Zhao et al (17) studied the feasibility and mechanism of TAPS in RBC cryopreservation. The addition of TAPS resulted in a post-thaw RBC recovery of 79.1 ± 0.7% with cell integrity of exceeding 97%. Mechanistically TAPS operates through two primary pathways: first, binding water molecules to inhibit ice recrystallization, and second, scavenging ROS to provide antioxidant protection. TAPS can be effectively removed by direct washing, and key physiological parameters of the washed RBCs —

hemolysis, membrane ATPase activity, and hemoglobin content — showed no significant differences as compared with fresh RBCs.

Yang et al (21) compared three permeable proline, glycine and taurine. These protectants vary in preventing osmotic damage, and exhibit different capacities to inhibit ice formation, thereby mitigating intra- and extracellular ice damage. Proline consistently performed the best, both in the osmoregulatory capability by preventing osmotic damage and in the inhibition of ice formation. This superior performance was observed in cryopreserving sheep RBCs and four other cell types (21).

Therefore, novel small molecule protectants (such as FA, DMG, TAPS, and proline) have shown their potential in RBC cryopreservation. They act by multiple mechanisms: inhibiting ice-induced damage, alleviating oxidative stress, and enhancing membrane stability.

Non-permeating macromolecules/saccharides

Non-permeating macromolecules and saccharides have some advantages. They can significantly reduce the reliance on permeating cryoprotectants, thereby advancing the development of glycerol-free cryopreservation techniques. Trehalose is a non-toxic and highly effective compatible protective agent. It provides significant protection to cells under stressful conditions such as freezing, desiccation, heat shock, oxidation, and high osmotic pressure (22). Particularly, trehalose has shown great promise in RBC cryopreservation. Dou et al reported that the L-proline/trehalose combination method is a promising glycerol-free cryopreservation method for RBCs, a potential alternative to the current time- and labor-intensive method (7). However, trehalose does not readily penetrate the RBC membrane (23). To overcome this permeation barrier and enable intracellular delivery, recent work has focused on developing novel deliverry methods. Stefanic et al pioneered the use of colloidal hydroxyapatite nanoparticles stabilized with AEP/HMP to facilitate trehalose entry into RBCs. This strategy increased the cryopreservation survival of RBCs to 91% (2). Nanoparticles involves the temporary alteration of the lipid bilayer structure, thereby promoting transmembrane transport of trehalose (2). Qin et al (23) developed a core-shell structured alginate hydrogel microfibre encapsulation technique. The method utilized trehalose as the sole cryoprotectant, achieving a high recovery of 95% and overcoming the limitation requiring trehalose to be present on both sides of cell membrane simultaneously and improved freezing efficiency by enhancing heat transfer.

In addition, various cryoprotective materials, encompassing natural polysaccharides, synthetic polymers, and liposome composites, have been explored. Hu et al (20) pioneered the use of inulin, a natural polysaccharide, demonstrating an increase in RBC recovery from 11.8 ± 1.4% to 81.9 ± 0.4%. Inulin was found to promote vitrification due to its high viscosity and high glass transition temperature, while also providing membrane stabilization and inhibiting ice recrystallization. The molecular modeling analysis indicated that inulin's protective efficacy surpassed glycerol and hydroxyethyl starch (HES). Building upon the concept of polymeric cryoprotectants, Deller et al (24) developed a cryopreservation strategy based on HES and the ice recrystallization inhibitor poly(vinyl alcohol) (PVA). The method was effective at -20°C without glycerol or dimethyl sulfoxide (DMSO). The addition of 0.1 to 0.5% PVA increased cell recovery up to 75% under rapid freezing conditions in liquid nitrogen. Stoll et al. (25) investigated the synergistic effects of various liposomes combined with trehalose and HES. RBCs treated with DOPC liposomes, trehalose, and HES in combination achieved an immediate post-thaw recovery as high as 97.8%, with 81.8% viability maintained after 96 hours of post-thaw incubation. This indicated significant synergistic protection during the freezing and thawing processes.

Non-permeating macromolecules and saccharide facilitate RBC cryopreservation through various innovative approaches such as nanoparticle-based delivery, microfiber-based encapsulation, and liposome-based strategies. These approaches not only improve cell recovery, but also overcome some challenges associated with traditional methods.

COMPARATIVE ASSESSMENT

RBC cryopreservation technology has consistently revolved around four core elements: preservation efficacy, operational convenience, clinical safety and cost-effectiveness (26). While the high glycerol method (HGM) is technologically mature and regulatory-approved, it has notable limitations. Specifically, issues such as the toxicity of high glycerol concentration, the cumbersome deglycerolization

process, and patient side effects compromise clinical safety and operational convenience.

Based on existing data, we conducted a systematic comparison of five representative RBC cryopreservation methods The analysis spanned multiple dimensions, including cryoprotectant composition, freezing/thawing protocols, in vitro quality indicators, relevant functional attributes, suitability for various storage conditions, and potential for clinical translation (see Table 1). The study aims to provide guidance for the future development of this field. Primary differences among various methods lie in CPA composition and how CPAs are used, which correlated to the protective mechanisms and the downstream process steps.

Synergistic cryoprotection

The traditional HGM employs glycerol at a high concentration (40%). Protection is achieved primarily through glycerol permeation across cell membrane, thereby depressing the intracellular freezing point and inhibiting the formation of large ice crystals. However, the very high concentrations of glycerol required alters intracellular ionic strength and protein interactions, thereby potentially damaging cell structure and function. Consequently, glycerol's high osmolarity necessitates complex, time-consuming, multi-step washing to remove glycerol before transfusion. Recent advancements in cryopreservation have explored diverse strategies to mitigate cryo-damage and reduce glycerol dependence.

Wagner et al (27) developed the low glycerol biochemical stabilization method (LC-V) utilizing 5% glycerol combined with high-viscosity agents such as HES and dextran, and biochemical stabilizers like nicotinamide. This approach creates a high-viscosity environment to mitigate ice damage while simultaneously employing membrane/metabolic stabilization to reduce cryo-damage through a multi-mechanism synergistic actions. Similarly focusing on low glycerol level. Wang et al (28) used a gelatin-alginate microencapsulation protocol, comprising 5% glycerol and 500mM trehalose within gelatin-alginate microcapsules. The method leverages the "water replacement" hypothesis for membrane stabilization and provides physical protection against ice-induced mechanical damage via microencapsulation, with the controlled microenvironment established by microcapsules simplifying post-thaw removal of cryoprotectants. Beyond the low-glycerol approaches, several glycerol-free protocols have also emerged. Gao et al (26) introduced an ice-affinity glycopeptide and trehalose method, employing an ice-affinity glycopeptide and trehalose. The glycopeptide anchors to cell membrane, actively inhibiting ice growth, while trehalose concentrates around the cell, forming a vitrified protective layer; these two mechanisms synergistically prevent cryo-damage. Another glycerol-free option is the trehalose + HES method, which uses 290 mM trehalose and 24.5% HES as its basis components. Optionally, liposomes can be added for direct membrane stabilization, and when utilized as a triple combination (trehalose, HES, and liposomes), this protocol demonstrates high-efficiency preservation and significantly reduces hemolysis (25). Sui et al (19) developed a betaine-based glycerol-free method, centered on 15% betaine as the primary osmoregulant, further supplemented with various membrane stabilizers. This method offers excellent biocompatibility and significantly simplifies the post-thaw process, requiring only a single-step dilution for cryoprotectant removal, a streamlined approach particularly advantageous for resource-limited settings. Current strategies for RBC preservation reflect a trend toward low- or no-glycerol strategies leveraging multiple mechanisms.

Simplification of freeze/thaw protocols

The tates of cooling and warming directly influence the formation and growth of ice crystals, thereby impacting the success of cryopreservation. These rates affect nucleation, ice growth, and recrystallization. Nucleation is the initial step during freezing, where molecules arrange to form a crystalline structure, generating crystal nuclei. Once nuclei form, additional water molecules accrete on their surfaces, leading to the growth of larger ice crystals. Recrystallization typically occurs during thawing, where larger ice crystals grow at the expense of smaller ones (10).

Table 1. Different red blood cell cryopreservation protocols.

Parameter	Feature/ method (Reference)					
	Traditional high glycerol (HGM) (30)	Gel-alginate microencapsulation (28)	Trehalose + HES ± liposomes (25)	Ice-binding glycopeptide + trehalose (26)	LC-V low glycerol (27)	Betaine + membrane stabilizers (glycerol-free) (19)
Protectant composition	Glycerol 40%	5% Glycerol + 0.5 M trehalose + microcapsules	290 mM trehalose + 24.5% HES; liposomes (0.5-7 mM)	Glycopeptide (1.0-4.0 mg/mL) + 0.36 M trehalose	5% glycerol + 2% Me_2SO + 5% HES + 5% dextran (+ biochemical stabilizers)	Betaine + stabilizers (e.g., trehalose, sucrose)
Glycerol level	High	Low (5%)	Glycerol-free	Glycerol-free	Low (5%)	Glycerol-free
Freezing protocol	Slow (1°C/min)	Direct LN_2	Direct LN_2	Direct LN_2	-80°C (non-programmed)	Direct LN_2
Thawing protocol	37°C water bath	37°C water bath	37°C water bath	37°C water bath	40°C water bath (fast)	37°C water bath (fast)
Washing required	Yes	Yes	No	Yes	Yes	No
Recovery rate	80% (Initial)	80%	97.8%	74%	Comparable to 40% gycerol	90%
ATP Level	Degrades over storage	Similar to fresh cells	Minor acceptable change	-	-	-
Morphology/ stability	Good morphology & osmotic fragility	Improved deformability with DOPC	Mostly biconcave, reversible changes	Better osmotic fragility than 40% glycerol	Good morphology integrity	-
In vivo survival	75%	90% in mice (24 h)	In vitro only	In vitro only	In vitro only	In vitro only
Storage duration	Long-term (LN_2)	Long-term (LN_2)	Short-term post-thaw (e.g., 96 h)	≥6 months (LN_2)	≥8 weeks (-80°C)	Long-term (LN_2)
Cost/ complexity	High (equipment/time)	Potentially lower cost	Low/moderate (no wash needed)	Uses synthetic peptide	Aims for simplicity	Simple, no special equipment
Regulatory status	Approved (FDA, EU)	Not clinically approved	Experimental	Research phase	Experimental	Research phase

HGM employs slow freezing (1°C/min), facilitating adequate cell dehydration during cooling to avoid lethal intracellular ice formation, albeit at the risk of increased solution-effects injury. The low glycerol biochemical stabilization method adopts a non-programmed cooling rate of 1-5 °C/min to -80°C, positioning it as an intermediate approach between slow and ultra-rapid freezing. In contrast, protocols by (19, 26, 28) and the trehalose/HES method (25) utilize ultra-rapid freezing (direct immersion in liquid nitrogen), aiming to bypass the critical temperature zone for ice formation, achieving vitrification or generating extremely fine ice crystals to minimize cryodamage. Consequently, ultra-rapid freezing, as employed in these methods, reduces the dependence on CPAs permeability, thereby forming a fundamental basis for glycerol-free and low-glycerol strategies.

Most protocols employ rapid thawing in a 37-40°C water bath to minimize recrystallization during warming, when small ice crystals can regroup and grow, potentially rupturing cells. This rapid process ensures the cryopreserved samples traverse the hazardous temperature range quickly and uniformly and is a key step toward high recovery.

Post-thaw RBC quality attributes in vitro

Post-thaw recovery and post-wash hemolysis are considered the most direct indicators for evaluating the success of a cryopreservation protocol (3). HGM typically demonstrates a post-thaw recovery of greater than 80% and a post-wash hemolysis of less than 1%. However, the washing process lasts 60 minutes or more and can lead to approximately 15% cell loss.

The LC-V low glycerol biochemical stabilization protocol (27) reported a post-thaw hemolysis of 11.9 ± 4.5%. This value exhibited no statistical difference from the value reported for the 40% glycerol method, suggesting a similar level of cryoprotection performance under the conditions studied. The microencapsulation protocol (28) reported a recovery between 50% and 80% with low hemolysis. However, the interpretation of the later study requires caution, as the reported recovery might refer to microcapsule integrity rather than direct erythrocyte recovery, or it could be influenced by specific counting or detection methodologies. Despite the low recovery, the protocol noted normal erythrocyte morphology and osmotic fragility, indicating preserved cell quality within the surviving fraction. The betaine-based glycerol-free protocol achieved a post-thaw, pre-wash recovery exceeding 90%. Recovery remained above 90% after a simplified wash, as well as low hemolysis. This method demonstrates the capability of glycerol-free formulations to provide high recovery. Furthermore, the "one-step dilution removal" strategy inherent to this protocol mitigates cell loss typically associated with traditional washing procedures. The trehalose/HES/liposomes protocol (25) reported a post-thaw recovery of 97.8%. For the DOPC combination, this recovery was maintained at 81.8% after 96 hours, with hemolysis reported as less than 3%. These results suggest a notable protective effect from the combination of trehalose, HES, and liposomes.

ATP, the primary energy currency of RBCs (29), is directly linked to cell survival and function. Studies on novel low-glycerol or glycerol-free (low/no-glycerol) cryopreservation protocols have investigated their impact on metabolic activity. For instance, the gelatin-alginate protocol developed by Wang et al. (28) maintained ATP levels not significantly different from fresh cells right after cryopreservation. In contrast, ATP levels in RBCs preserved by the conventional HGM are known to gradually degrade during storage. Similarly, the trehalose/HES protocol (25) demonstrated good metabolic activity, suggesting that trehalose may contribute to energy support during preservation.

Beyond metabolic status, membrane integrity is a critical indicator of cell quality. The gelatin-alginate protocol (28) also showed well-preserved expression of surface markers CD47 and CD235a, indicating the maintenance of membrane integrity. Furthermore, maintaining physiological morphology is essential for RBC function. Protocols developed by Wagner et al (27) and Sui et al (19) both successfully preserved the biconcave discocyte morphology of RBCs after cryopreservation.The mechanical properties of RBCs, such as osmotic fragility and deformability, are also crucial for their in vivo function. For cells preserved using Wagner et al.'s protocol (27), osmotic fragility was better than that of the HGM. The gelatin-alginate protocol (28) further demonstrated osmotic fragility identical to fresh cells. Moreover, the trehalose/HES protocol (25) showed enhanced deformability.

Collectively, the studies indicate that several novel low/no-glycerol cryopreservation protocols are effective in maintaining normal cell

morphology, membrane stability, and mechanical function, with some methods demonstrating comparable or even superior outcomes compared to the traditional high-glycerol method.

Economic, procedural and regulatory aspects

The disadvantage of the HGM lies in its high cost and cumbersome procedures (requiring specialized equipment, consumables, and lengthy washing steps). Four novel protocols discussed above appear to target this weakness. Among these, the betaine with membrane stabilizers protocol (no washing required) and the rehalose/HES protocol (no washing; uses common reagents) may show greater potential for simplifying the process and reducing costs. The remaining two protocols still require washing steps; however, because of their lower glycerol concentration, their washing process may be faster and simpler than with HGM.

Despite these advantages, a significant challenge remains for all novel protocols that are currently in the experimental research stage. Only the HGM is approved by regulatory authorities in the US and EU for clinical use. Advancing these protocols requires the completion of large-scale safety/efficacy validation, and the establishment of stringent quality control standards for future clinical translation.

Based on the analysis for the five aforementioned new methods, two trends in RBC cryopreservation technology are evident: the reduction and eventual replacement of glycerol, as well as the diversification and synergism of protective mechanisms. The progression from high-glycerol (40%) to low-glycerol (5%) and finally to glycerol-free formulations aims to eliminate glycerol-related toxicity and simplify the complex washing process. Concurrently, the protective strategy has evolved from relying solely on glycerol to explore multiple mechanisms. These include osmotic protection via agents like low-concentration glycerol or betaine; ice crystal inhibition via HES or polymers; membrane stabilization via trehalose, sucrose, or liposomes; biochemical regulation via stabilizers (e.g., antioxidants or chelators); and physical protection via microencapsulation (e.g., by encapsulating cells in protective matrices). This synergistic approach can achieve improved preservation while reducing or eliminating glycerol.

CHALLENGES AND OUTLOOK

Compared to the traditional high-glycerol protocol, new approaches offer potential advantages, including simplifying the deglycerolization process, enhancing post-thaw cell quality, and meeting the demands of specific clinical applications. Despite these potential advantages, the translation from laboratory research into routine clinical application faces several challenges. These include a need for more robust evidence, such as sufficient long-term storage data and expanded in vivo studies. Furthermore, translational hurdles persist, including unverified feasibility and cost-effectiveness for large-scale production, and rigorous regulatory approval requirements. To address these challenges, future research needs to develop novel cryoprotectants, optimize composite formulations, identify advanced carriers and smart materials, and standardize protocols with personalized strategy.

A challenge limiting the widespread clinical adoption of novel cryopreservation agents and techniques is their higher cost. Glycerol is inexpensive and stable. It has well-established supply chain and has been widely used for clinical blood banking (30). In contrast, novel protective agents — such as sugars (e.g., trehalose), polymers (e.g., sodium hyaluronate, biomimetic polymers), other small molecules (e.g., dimethylglycine), and ice recrystallization inhibitors — has higher costs (5, 7, 20, 31). Materials like biomimetic block copolymer worms and nanoparticle–antifreeze protein complexes remain confined to laboratory-scale production (30, 31 32, 33), with complex synthesis processes posing major challenges for industrial-scale manufacturing and quality control (7 5). Therefore, achieving cost control while maintaining high protective efficacy is crucial for future large-scale application.

The long-term stability of glycerol-free and low-glycerol systems, particularly for extended durations, remains a critical aspect that has not been fully validated. The traditional 40% glycerol system has been demonstrated to be stable for over ten years at −80 °C. In contrast, data for most glycerol-free and low-glycerol systems currently only cover periods ranging from several months to two years. Despite advances where sodium hyaluronate improves intra- and extracellular osmotic balance via a water reservoir effect, and encapsulation methods using hydrogel or gelatin-alginate microcapsules significantly enhance

RBC) recovery even at low glycerol concentrations, it remains difficult to fully avoid issues such as the degradation of membrane skeletal proteins, disruptions in energy metabolism, and damage from recrystallization during prolonged freezing (25, 32 28, 34). Particularly in large-volume blood bag storage, heterogeneous ice crystal distribution and localized stress damage become more pronounced. Therefore, the evidence base for the long-term stability of these advanced systems remains insufficient.

Challenges related to process complexity and clinical operability are prominent in blood cryopreservation. The deglycerolization process in traditional glycerol-based systems is cumbersome and time-consuming, often leading to significant RBCs loss. While glycerol-free and low-glycerol methods aim to reduce the washing burden by minimizing required washing steps, they frequently involve additional processing, such as hydrogel microfiber encapsulation or nanocarrier introduction. These supplementary procedures increase the operational complexity for routine blood bank workflows and demand specialized automated equipment (23, 31, 33 25, 33, 35). Furthermore, even slight variations in factors like freeze–thaw rates, CPA concentration, and osmotic pressure control can significantly impact preservation outcomes (34), resulting in considerable interlaboratory variability, poor reproducibility, and lack of standardization.

With regard to safety and regulation, novel materials, such as nanoparticle-antifreeze protein complexes and synthetic polymers, exhibit ideal protective properties. However, their safety profile remains insufficiently elucidated. Specifically, their immunogenicity, toxicological risks, and in vivo metabolic pathways require further clarification (17, 31, 34 18, 33, 36). In clinical transfusion settings, any adverse reactions triggered by residual amounts of these novel materials, acting as protectants or carrier materials, may pose significant patient safety risks. Current international guidelines for transfusion and blood management are primarily established around the traditional high-glycerol cryopreservation system. Consequently, unified standards for safety validation of emerging protocols are lacking, which not only increases the difficulty of clinical translation but also hampers comparability between studies.

Individual variation in red blood cell characteristics, arising from diverse donor populations or specific pathological states, further complicates the optimization of preservation protocols. Red blood cells from these differing sources exhibit significant differences in sensitivity to freeze-thaw damage. For instance, red blood cells from donors with sickle cell trait are more susceptible to morphological and functional freeze-thaw damage; even after modifications to hypotonic washout procedures, their post-thaw quality often remains suboptimal (33 35). Given these differences, developing personalized preservation protocols tailored to specific populations and disease states warrants further investigation.

Future development pathways are becoming clearer. Firstly, the development of novel, low-cost cryoprotectants is crucial. Metabolic engineering or synthetic biology methods for the large-scale production of naturally low-toxicity molecules can significantly reduce costs (6 7). Secondly, the introduction of composite (or combinatorial) cryoprotectant formulations can reduce reliance on single, high-cost components. Existing research indicates that combining low concentrations of glycerol with trehalose or sodium hyaluronate can maintain high cell viability while reducing the total amount of cryoprotectants required, thereby demonstrating clinical feasibility.

Further research in the multi-component synergistic protection strategies is of great importance. A single cryoprotectant is often insufficient to address the multiple challenges arising during cryopreservation, including osmotic stress, ice crystal damage, and oxidative stress. Combinations such as liposome–trehalose–hydroxyethyl starch (23 25) or polyampholyte–trehalose–DMSO systems (35 37) have shown promising results. However, a better understanding of the intricate mechanisms governing their efficacy and interactions is still lacking. Therefore, future studies need to integrate omics technologies and molecular simulation methods to elucidate the interaction mechanisms of different protectants in membrane stabilization, osmoregulation, and ice inhibition, thereby guiding the optimization of composite systems.

Moreover, functional and safety validation of new cryopreservation systems urgently needs strengthening. Evaluation metrics should extend beyond hemolysis rate and morphology to encompass oxygen-carrying capacity, ATP levels, membrane antigen stability, and immunologic safety. Animal models and

preclinical studies must comprehensively assess the long-term in vivo survival of thawed RBCs and potential adverse reactions to ensure the ultimate safety and efficacy for clinical use.

Furthermore, process optimization and standardization aligned with clinical needs are indispensable. Glycerol-free and low-glycerol preservation systems must be deeply integrated with existing automated freeze-thaw platforms to establish integrated workflows enabling full automation from protectant addition to freezing, thawing, and residual cryoprotectant removal (28 30). This should be accompanied by promoting the establishment of international quality control standards and technical guidelines to support multi-center and multi-scenario clinical applications.

CONCLUSION

Glycerol-free and low-glycerol RBC cryopreservation technology is a approach to overcome the limitations inherent in the traditional high-glycerol concentration methods, including the complex and time-consuming deglycerolization process prior to transfusion. The advanced approach demonstrates significant potential in streamlining processes, improving safety and improving transfusion efficiency, but significant challenges remains to be addressed such as high costs, unknown long-term stability, as well as unproved safety and efficacy for regulatory approval.

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