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Original Article

Establishment and application of a novel method for thawing frozen red blood cells

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ABSTRACT

This study aimed to develop a novel method for thawing frozen red blood cell (FRBC) samples for transfusion compatibility testing. Fifteen samples were selected from voluntary blood donors at the Henan Red Cross Blood Center. After being washed with saline, samples were stored in a −80 ℃ freezing environment for 15 months using a FRBC preservation solution. The dialysis method was used to optimize the deglycerolization process. Following thawing, RBCs were stored at 2–8 ℃ for 75 days, during which cell morphology, antigenicity, antigen intensity, and hemolysis indicators were evaluated. The optimized deglycerolization method achieved a cell recovery rate over 75%. Thawed RBCs maintained intact morphology and stable blood group antigen expression during 75-day storage at 2–8 ℃ with no significant reduction in antigenicity or antigen intensity. The results suggest that a new method for thawing FRBCs be successfully established. The thawed RBCs exhibit stable quality and are suitable for clinical compatibility testing, such as ABO/RhD blood typing, Rh typing, and cross-matching.

Keywords: frozen red blood cell, glycerol, blood type, blood transfusion compatibility testing

INTRODUCTION

In recent years, the escalating clinical demand for blood products, coupled with a growing scarcity of blood resources, has presented severe challenges to clinical transfusion practices. This critical situation stems primarily from a reduction in the donor population and significant regional and seasonal imbalances in supply and demand[1]. Consequently, some regions face the situation of limiting blood collections during peak donation periods due to concerns about the short shelf life of conventional red blood cells (RBCs) and potential wastage, while experiencing severe shortages during off-peak seasons. This directly threatens the lives of transfusion-dependent patients. To address this structural dilemma, an increasing number of blood centers in China have established frozen RBC (FRBC) reserve banks for routine use and rare blood type libraries (such as Rh-negative and para-Bombay types). Additionally, standards such as the "Blood Storage Requirements" and the "Expert Consensus on

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Quality Evaluation Indicators for Frozen Red Blood Cells" have been progressively introduced to promote the clinical application of FRBCs.

FRBCs, also known as frozen-thawed-deglycerolized RBCs (FTDRBCs), are specialized RBC preparations cryopreserved at ultra-low temperatures using glycerol as a cryoprotectant. These cells are thawed and subsequently washed to remove glycerol based on clinical needs. However, current regulations in China, as outlined in the "Blood Storage Requirements," stipulate a strict 24-hour post-thaw storage limit for deglycerolized RBCs[2]. Furthermore, the "Clinical Blood Transfusion Technical Management Guidelines" require that pre-transfusion compatibility testing must include ABO blood group and RhD antigen typing of donor blood, followed by cross-matching before transfusion. If no suitable recipient is identified for a thawed FRBC unit within 24 hours, the thawed cells must be discarded. The short post-thaw viability period contributes to blood resource wastage, along with associated human and material costs. Furthermore, the deglycerolization process is highly complex, and both freeze-thaw injury and mechanical damage during cryopreservation and thawing can compromise RBC surface antigen integrity. To address these challenges, the study introduced a novel method for thawing FRBCs that simplifies the thawing and deglycerolization procedures, reduces processing time, and enables previously cryopreserved donor samples to be thawed and effectively stored in RBC preservation solution for up to 75 days. When compatibility testing between the thawed donor samples and the recipient's samples is successful, the donor's FRBCs can be thawed and deglycerolized. Following successful quality control, these units can be transfused within the 24-hour timeframe stipulated by the "Blood Storage Requirements".

MATERIALS AND METHODS

Sample collection and selection

From the perspective of clinical application and RBC quality control, a cohort of 15 samples from the Henan Red Cross Blood Center was selected for this study. In addition to the common A_1, B, O, and A_1B blood types, the selection specifically focused on six ABO subtypes: one A_2, one Bel, one AB_3, one Bw, and two B(A) phenotypes, as well as five RhD variants: one heterozygote for DEL1227A (Asian-type DEL) and weak D15, one RHD-CE(3-6)-D, two weak D15, and one RHD-CE(2-9)-D. All samples were obtained from healthy adult volunteers recruited through the Henan Red Cross Blood Center, and the study was approved by the Ethics Committee of the Henan Red Cross Blood Center (Approval No. 2023008). All 15 samples were accurately typed using serological methods and genotyping. A 10 mL sample of fresh peripheral blood was drawn from each volunteer and stored in sterile heparin sodium anticoagulant tubes. The samples were transported to the laboratory under cold chain conditions (2–8 ℃) within 48 hours.

Reagents and consumables

The following reagents and consumables were utilized in this study: sterile plastic sampling tubes and MD77-14000D dialysis tubing (Beckman Biotech, Indianapolis, IN, USA); sodium chloride (AR grade, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China); 0.9% sodium chloride solution (Jimin Health Management Co., Ltd., Tai Zhou, Zhejiang, China); Anti-A (mouse IgM, clone: BIRMA-A), Anti-B (mouse IgM, clone: LB-2), Anti-AB (mouse IgM, clone: ES-15/ES-4), Anti-D (human IgM, clone: RUM-1), Anti-E (human IgM, clones: MS80/MS258), Anti-C (human IgM, clone: MS24), Anti-e (human IgM, clones: MS62/MS69), Anti-c (human IgM, clone: MS33), and Anti-IgG+C_3d (rabbit polyclonal, BRIC-8) from Millipore (Burlington, MA, USA); Anti-H (plant lectin), Anti-A_1 (plant lectin), RBC preservation solution, FRBC preservation solution (containing 57% glycerol), ABO blood group reverse typing cards, and anti-human globulin (anti-IgG+C_3d) detection cards from Jiangsu ZoJiWat Bio-Pharmaceutical Co., Ltd. (Jiangyin, Jiangsu, China); Rh phenotype cards from Jiangsu LIBO Medicine Biotechnology Co., Ltd. (Jiangyin, Jiangsu, China); and a plasma-free hemoglobin detection kit (colorimetric method) from Beijing Ruierda Biotechnology Co., Ltd. (Beijing, China).

Equipment and instruments

The following equipment and instruments were utilized in this study: an ME303E/02 electronic balance (accuracy: 0.001 g; Mettler Toledo, Columbus, OH, USA); 2420 and KA-2200 serological centrifuges (Kubota Corporation, Tokyo, Japan); an LB-3000 centrifuge (Wuxi Yuanbo Biotechnology Co., Ltd., Jiangyin, Jiangsu, China); a T6 new century UV-visible spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China); a CX21FS1C biological microscope (Olympus Corporation, Tokyo, Japan); an HH-420 multi-purpose constant-temperature water bath (Changzhou Yidu Instrument Co., Ltd., Changzhou, Jiangsu, China); and a CX-C constant-temperature oscillating

incubator (Junchi Technology Co., Ltd., Shenzhen, Guangdong, China).

RBC pre-treatment

Upon receipt, fresh peripheral blood samples were centrifuged at 1000 g for 5 minutes. Following centrifugation, all samples were visually inspected. The RBCs exhibited bright red without discoloration or clotting. The plasma was clear and free of hemolysis. The plasma and buffy coat layers were then separated, labeled, and stored at –20 ℃ or lower.

RBC cryopreservation

The remaining RBCs were washed twice with 0.9% sodium chloride solution to remove residual plasma components and fragmented RBCs, followed by a single wash with RBC preservation solution to provide metabolic substrates for long-term cryopreservation. The supernatant was then discarded after centrifugation. A sterile test tube was weighed using an electronic balance (recorded as mass 1). Then, 2 mL of FRBC preservation solution was added to a sterile plastic collection tube. After securing the cap, the tube was reweighed (mass 2). Subsequently, 2 mL of RBCs were added to the same tube in a slow-to-fast manner within 20 seconds (approximately 120 drops/min). The tube was capped, gently inverted several times for mixing, and appropriately labeled before being weighed again (mass 3). Following weighing, the samples were equilibrated at room temperature for 10 minutes, transferred to a 4 ℃ refrigerator for 1 hour, and then placed at –20 ℃ overnight. Finally, they were transferred to a –80 ℃ ultra-low temperature freezer for long-term storage. The FRBCs were stored at –80 ℃ for 15 months.

Thawing of RBCs

The sterile sampling tube was retrieved from a –80 ℃ freezer and completely thawed in a 37 ℃ water bath. Glycerol was then removed *via* dialysis using MD77-14000D dry dialysis tubing. This tubing, made of regenerated cellulose, features a semipermeable membrane with selective permeability that allows glycerol to freely diffuse through the micropores into the external dialysate while retaining the cells within the bag (glycerol's molecular weight ≈ 92 Da, significantly lower than the tubing's nominal molecular weight cutoff of 14 000 Da). The MD77-14000D dry dialysis tubing was cut into 15 segments, each approximately 10 cm in length. These segments were boiled in distilled water for 10 minutes and subsequently rinsed with distilled water to remove any manufacturing preservatives, such as glycerol and sulfides. One end of each dialysis bag was securely sealed with a dialysis clip, followed by transfer of the thawed cell-glycerol mixture into the bags. The opposite end was also sealed, ensuring that at least 50% of the bag's volume remained unoccupied. The sealed dialysis bags were then fully submerged in separate containers filled with 1 L of 2.5% sodium chloride solution. These containers were placed on a shaker and agitated gently at 20 rpm for 1 hour. Following dialysis, the bags were individually removed, and surface liquid was dried. The dialysis clips were then detached, and the cells were transferred into the original sterile sampling tube using a pipette. A 1.6% sodium chloride solution was added slowly at a 1 : 1 volume ratio and mixed thoroughly. The mixture was centrifuged at 1000 g for 1 minute, and the supernatant was discarded. The cells were subsequently washed 2–3 times with 0.9% sodium chloride solution until the supernatant became clear. After a final wash with RBC preservation solution and supernatant removal, the tube was capped and weighed (mass 4). Subsequently, 3% and 1% RBC suspensions were prepared using RBC preservation solution. The cell recovery rate was calculated as follows: Recovery rate (%) = [(mass 4 – mass 1) / (mass 3 – mass 2)] × 100%. The preservation efficacy of the aforementioned thawed suspensions was evaluated after storage at 2–8 ℃ for 75 days.

Cell morphology assessment

A drop of 1% RBC suspension was placed near one end of a microscope slide. Using a spreader slide held at a 30°–45° angle, the suspension was smoothly and uniformly drawn across the length of the slide to form a thin, even blood film. Cell morphology was subsequently observed under a microscope.

Direct antiglobulin test[3]

The direct antiglobulin test was performed using both tube and column agglutination methods with 3% and 1% RBC suspensions, respectively. For the tube method, 50 μL of anti-IgG+C_3d reagent was added to a test tube, followed by 50 μL of RBC suspension. The mixture was gently agitated and centrifuged at 1000 g for 15 seconds. The results were interpreted after centrifugation. For the column agglutination method, 50 μL of RBC suspension was added to the appropriate well of an antiglobulin (anti-IgG+C_3d) detection card. The card was then centrifuged in an LB-3000 centrifuge at 900 rpm for 2 minutes, followed by 1500 rpm for 3 minutes. Results were interpreted following centrifugation.

RBC antigen detection

RBC surface antigens were serologically tested using both tube and column agglutination methods, employing 3% and 1% RBC suspensions, respectively. For the tube method, 50 μL of anti-A, anti-A_1, anti-B, anti-AB, anti-H, anti-D, anti-E, and anti-C antibody reagents were separately added to individual test tubes, followed by 50 μL of RBC suspension. The contents were mixed thoroughly and then centrifuged at 1000 g for 15 seconds. Results were interpreted after centrifugation. For the column agglutination method, 50 μL of RBC suspension was added to the gel columns of an ABO reverse typing reagent card. Subsequently, 25 μL of anti-A, anti-A_1, anti-B, anti-AB, anti-H, anti-D, anti-E, and anti-C antibody reagents were individually added. The card was then centrifuged in an LB-3000 centrifuge at 900 rpm for 2 minutes, followed by 1500 rpm for 3 minutes. Results were interpreted following centrifugation.

Determination of RBC antigen intensity

RBC antigen strength was assessed for selected samples of A_1, B, O, and A_2 types. Both tube and column agglutination methods were employed using 3% and 1% RBC suspensions, respectively. Serial twofold dilutions were prepared in 0.9% sodium chloride solution using 150 μL of anti-A (titer 256–512), 75 μL of anti-B (titer 256–512), and 75 μL of anti-H (titer 16–32). RBC samples of A_1 and A_2 subtypes were reacted with anti-A, those of the B subtype with anti-B, and those of the O subtype with anti-H. For the tube method, 50 μL of the diluted antibody reagent was added to a test tube, followed by 50 μL of RBC suspension. The contents were mixed and then centrifuged at 1000 g for 15 seconds. Results were interpreted after centrifugation. For the column agglutination method, 50 μL of RBC suspension was added to the gel columns of an ABO reverse typing reagent card, followed by the addition of 25 μL of the diluted antibody reagent. The card was centrifuged using an LB-3000 centrifuge at 900 rpm for 2 minutes, then 1500 rpm for 3 minutes, after which the results were interpreted.

Hemolysis detection[4]

The hemolysis rate was determined using a plasma-free hemoglobin detection kit (colorimetric method), following the manufacturer's instructions.

RESULTS

Cell recovery rate

The post-thaw cell recovery rate was calculated according to the methodology described in Section 2.5. All 15 samples demonstrated a recovery rate of ≥ 75%. Further details are presented in ***Table 1***.

Table 1 **Cell recovery rate results**

Sample type	Mass 1 (g)	Mass 2 (g)	Mass 3 (g)	Mass 4 (g)	Recovery rate (%)
A_1	5.5	7.5	9.4	7.0	79.7
B	5.6	7.6	9.6	7.3	85.0
O	5.2	7.4	9.5	7.0	85.7
A_1B	5.5	7.7	10	7.3	78.3
A_2	5.4	7.7	10	7.3	82.6
Bel	5.4	7.6	10	7.4	83.3
AB_3	5.5	7.7	10	7.3	78.3
Bw	5.4	7.7	10.1	7.4	83.3
B(A)-1	5.2	7.4	9.6	7.0	81.8
B(A)-2	5.3	7.6	9.8	7.1	81.8
Heterozygote for DEL1227A (Asian-type DEL)	5.4	7.6	10	7.4	83.3
RHD-CE(3-6)-D	5.5	7.6	9.8	7.4	86.4
Weak D15-1	5.7	8.0	10.4	7.7	83.3
Weak D15-2	5.4	7.6	9.7	7.1	81.0
RHD-CE(2-9)-D	5.4	7.8	9.7	7.0	84.2

Cell morphology assessment

Upon thawing, the cellular membranes of all 15 samples appeared smooth and intact with characteristic biconcave disc shape, showing no signs of deformation, spiculation, or other abnormalities. This well-preserved cellular morphology, free from fragmentation or distortion, was maintained after 75 days of storage. Representative cell morphology is illustrated in ***Fig. 1***.

Direct antiglobulin test

Following thawing and after 75 days of storage, all

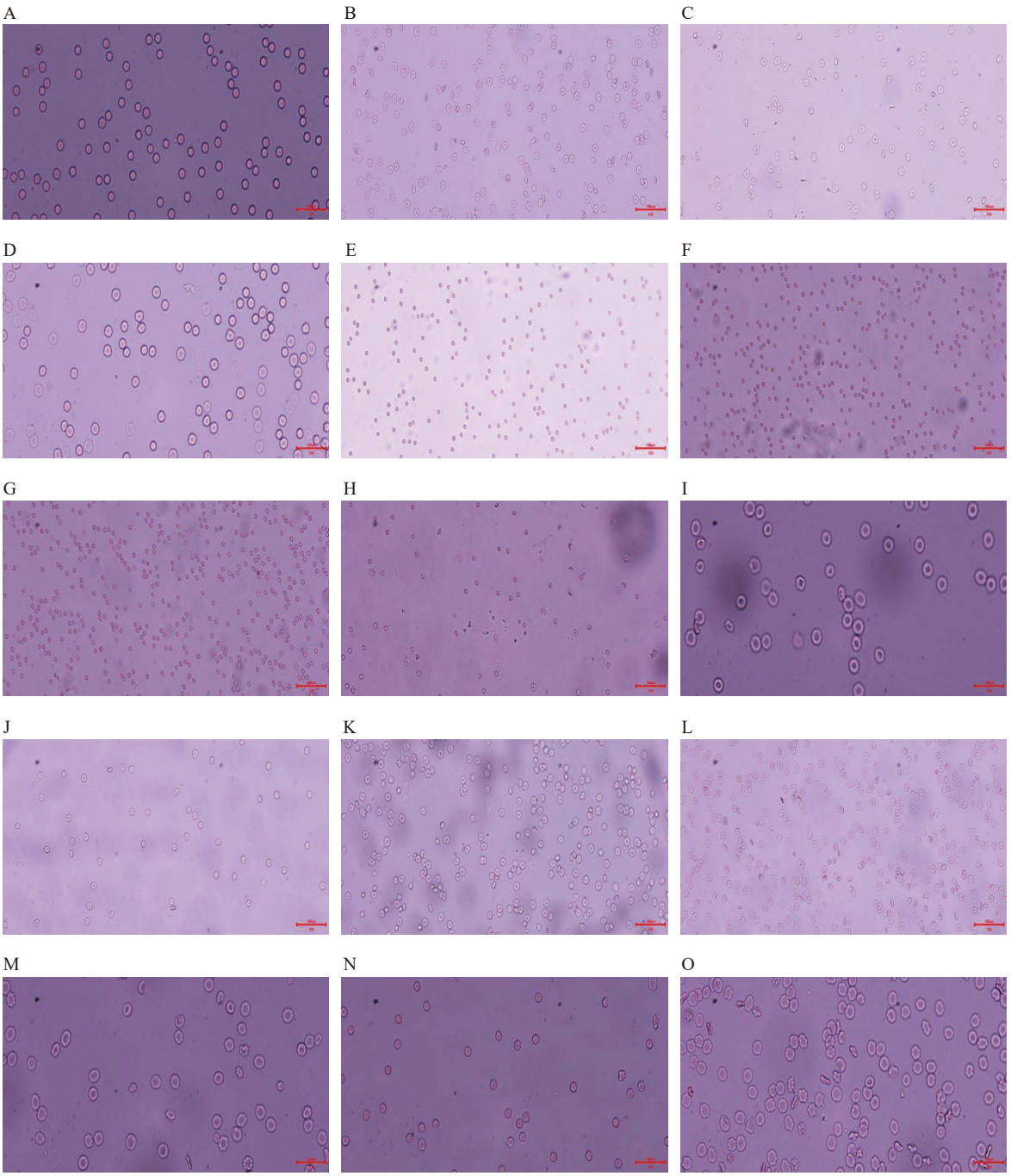

***Fig. 1* Microscopic images of red blood cells (RBCs) from 15 samples after storage at 2–8 °C for 75 days.** A: The cell morphology of A_1 type RBC samples; B: The cell morphology of B type RBC samples; C: The cell morphology of O type RBC samples; D: The cell morphology of A_1B type RBC samples; E: The cell morphology of A_2 type RBC samples; F: The cell morphology of Bel type RBC samples; G: The cell morphology of AB_3 type RBC samples; H: The cell morphology of Bw type RBC samples; I: The cell morphology of B(A)-1 type RBC samples; J: The cell morphology of B(A)-2 type RBC samples; K: The cell morphology of RBC samples with heterozygous DEL1227A (Asian-type DEL) genotype; L: The cell morphology of heterozygous RHD-CE (3-6)-D type RBC samples; M: The cell morphology of the weak D15-1 type RBC samples; N: The cell morphology of the weak D15-2 type RBC samples; O: The cell morphology of RHD-CE(2-9)-D type RBC samples.

15 samples yielded negative results in the direct antiglobulin test, as assessed by both tube and column agglutination methods. No aggregation or auto-agglutination of cells was observed.

RBC antigenicity testing

This study included four routine ABO blood samples, six ABO subgroup samples, and five RhD variant samples. Prior to cryopreservation, their RBC

surface antigens were serologically characterized following the guidelines outlined in "Identification of Discrepant ABO Forward and Reverse Typing" and "Weak D Testing Methods" from *Red Blood Cell Serological Techniques*. After 15 months of storage at –80 ℃, the samples were thawed and their RBC surface antigens were evaluated serologically. Subsequently, these thawed cells were stored at 2–8 ℃ for an additional 75 days and tested again using identical serological methods. All three sets of detection results showed complete concordance in positivity/negativity outcomes with no significant variations in agglutination strength. These findings align with the specifications for "Serological Reactions of A and B Subtypes" and "D Testing" specified in the 20th edition of the *AABB Technical Manual* (***Table 2***)[5–6].

Table 2 **Antigenicity tests of RBCs before freezing, after thawing, and after storage at 2–8 °C for 75 days**

A_1	Anti-A			Anti-A_1			Anti-B			Anti-AB			Anti-H			Anti-D			Anti-E			Anti-C			Anti-c			Anti-e		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
	4+	4+	4+	4+	4+	4+	0	0	0	4+	4+	3+	0	0	0	4+	4+	4+	3+	3+	3+	3+	3+	3+	3+	2+	2+	3+	3+	3+
B	0	0	0	0	0	0	4+	4+	4+	4+	4+	3+	0	0	0	4+	4+	4+	0	0	0	3+	3+	2+	0	0	0	4+	3+	3+
O	0	0	0	0	0	0	0	0	0	0	0	0	4+	4+	4+	4+	4+	4+	2+	2+	2+	3+	3+	3+	3+	3+	3+	3+	3+	3+
A_1B	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	0	0	0	4+	4+	4+	0	0	0	3+	3+	3+	0	0	0	3+	3+	3+
A_2	4+	4+	4+	0	0	0	0	0	0	4+	4+	3+	4+	3+	3+	4+	4+	4+	3+	3+	3+	3+	3+	3+	0	0	0	3+	3+	3+
Bel	0	0	0	0	0	0	0	0	0	0	0	0	4+	4+	4+	4+	4+	4+	0	0	0	3+	3+	3+	0	0	0	3+	3+	3+
AB_3	4+	4+	4+	4+	4+	4+	1+	1+	1+	4+	4+	4+	3+	3+	3+	4+	3+	3+	3+	2+	2+	3+	3+	3+	3+	2+	2+	3+	3+	3+
Bw	0	0	0	0	0	0	w+	w+	w+	0	0	0	4+	3+	3+	4+	4+	4+	0	0	0	3+	3+	3+	0	0	0	3+	2+	2+
B(A)-1	1+	1+	1+	0	0	0	4+	4+	4+	4+	4+	3+	3+	3+	3+	4+	4+	4+	0	0	0	3+	3+	3+	2+	2+	2+	3+	3+	3+
B(A)-2	1+	1+	1+	0	0	0	4+	4+	4+	4+	4+	4+	3+	3+	3+	4+	4+	4+	3+	3+	3+	3+	3+	3+	3+	3+	3+	0	0	0
Heterozygote for DEL1227A (Asian-type DEL)	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0	0	0	3+	3+	3+	4+	4+	4+	4+	3+	3+	3+	3+	3+
RHD-CE(3-6)-D	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0	0	0	0	0	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Weak D15-1	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0	0	0	3+	3+	3+	4+	4+	3+	4+	3+	3+	3+	3+	3+
Weak D15-2	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0	0	0	4+	4+	0	0	0	0	4+	4+	4+	4+	4+	4+
RHD-CE(2-9)-D	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0	0	0	0	0	0	4+	4+	4+	4+	3+	3+	4+	4+	4+

"0" indicates a negative result with no agglutination; "1+" to "4+" represent varying strengths of positive agglutination reactions. "I" represents the RBC antigen test results before sample freezing; "II" represents the RBC antigen test results after the sample was taken out and thawed after being frozen for 15 months; "III" represents the RBC antigen test results after being stored at 2–8 ℃ for 75 days. RBCs: red blood cells.

Determination of RBC antigen intensity

According to the criteria in RBC antigen intensity (titer) of the *Guidelines for the Registration Review of Human Red Blood Cell Reverse Grouping Reagents*, anti-A (titer: 1 : 256 – 1 : 512) must agglutinate A_1 and A_2 type RBCs with a strength ≥ 3+ at a maximum dilution ≥ 1 : 8, and ≥ 1+ at maximum dilutions ≥ 1 : 64 and 1 : 32, respectively; anti-B (titer: 1 : 256 – 1 : 512) must agglutinate B type RBCs with a strength ≥ 3+ at a maximum dilution ≥ 1 : 8, and ≥ 1+ at a maximum dilution ≥ 1 : 32; anti-H (titer: 1 : 16 – 1 : 32) must agglutinate O type RBCs with a strength ≥ 3+ at a maximum dilution ≥ 1 : 1, and ≥ 1+ at a maximum dilution ≥ 1 : 2. Based on these requirements, A_1, A_2, B, and O type samples were selected from 15 samples for antigen strength determination. The results, presented in ***Table 3***, demonstrated no significant difference in antigen intensity among the three testing timepoints: before freezing, after thawing, and following 75 days of post-thaw storage at 2–8 ℃. All samples consistently met the specified antigen strength requirements, indicating that antigens on RBCs remained stably expressed without rapid deterioration after 15 months of cryopreservation,

Table 3 **RBC antigen intensity test results**

	Agglutination strength ≥ 3+ at a maximum dilution			Agglutination strength ≥ 1+ at a maximum dilution		
	Pre-freezing	Post-thaw	75 days	Pre-freezing	Post-thaw	75 days
A_1	1 : 64	1 : 64	1 : 32	1 : 512	1 : 512	1 : 512
A_2	1 : 32	1 : 32	1 : 32	1 : 256	1 : 256	1 : 256
B	1 : 32	1 : 32	1 : 32	1 : 512	1 : 256	1 : 256
O	1 : 2	1 : 2	1 : 2	1 : 16	1 : 8	1 : 8

RBC: red blood cell

thawing *via* the present protocol, and subsequent storage at 2–8 ℃ for 75 days.

Hemolysis indicators

Following thawing and subsequent storage at 2–8 ℃ for 75 days, the cells maintained a bright red color without visible aggregation or darkening indicative of hemolysis. The supernatant displayed varying shades of yellow-brown, and a faint red interface was observed between the cells and preservation solution. Hemoglobin concentrations ranged from 0.19 to 0.26 g/L (***Table 4***).

Table 4 **Plasma-free hemoglobin test results after 75 days of storage at 2–8 °C**

Hemoglobin concentration (g/L)														
A_1	B	O	A_1B	A_2	Bel	AB_3	Bw	B(A)-1	B(A)-2	Asian-type DEL	RHD-CE(3-6)-D	Weak D15-1	Weak D15-2	RHD-CE(2-9)-D
0.23	0.21	0.19	0.24	0.25	0.21	0.26	0.22	0.25	0.24	0.26	0.21	0.24	0.23	0.22

DISCUSSION

Research on blood cryopreservation originated in the 1940s. In 1949, Polge C *et al*. first discovered that glycerol could effectively protect RBCs from freezing damage[7]. Subsequently, in September 1987, the U.S. Food and Drug Administration (FDA) approved the cryopreservation of glycerized RBCs at –80 ℃ for up to 10 years. Further validation was provided in 2000, when Valeri CR *et al*. confirmed that deeply frozen RBCs remained transfusion-suitable after 37 years of storage[8–9]. Currently, blood banks in China employ FRBC preservation techniques for rare blood types such as Rh-negative and para-Bombay phenotypes. However, given persistent blood resource shortages, this technology is increasingly being applied to the preservation of conventional blood types. The establishment of a standardized FRBC bank represents a strategic measure to mitigate seasonal and regional blood shortages, optimize blood resource allocation, and ultimately enhance the efficient utilization of blood resources and emergency response capabilities.

While the broad application of FRBCs faces several technical challenges, such as the complexity of the deglycerolization process, high preparation costs, and cryopreservation-induced cellular damage, the most critical issue is the conflict between their short post-thaw storage period and the demands of emergency blood supply. Current Chinese regulations, as stipulated in the "Blood Storage Requirements", limit the post-thaw storage of deglycerolized RBCs to 24 hours. These cells must be transfused within this timeframe and maintained strictly at 2–6 ℃ throughout transportation. These requirements severely restrict the clinical application of FRBCs in China, leading to limited transport timeliness and a narrow window for their use. Moreover, a proportion of transfusion recipients, particularly those undergoing long-term transfusions for conditions such as sickle cell anemia or thalassemia, develop unexpected RBC alloantibodies. Alloimmunization rates in these patients range from 14% to 50%. In such cases, thawed and deglycerolized FRBCs may be unusable due to incompatible cross-matching. This, combined with the extremely short transfusion window, results in the discard of RBCs and significant wastage of human resources, materials, and precious blood products.

To address these challenges, the present study introduced a novel approach integrating materials science with biology. This method utilized a semipermeable dialysis membrane, using a 2.5% sodium chloride solution as the dialysate. An osmotic pressure gradient across the membrane allows glycerol and other solutes to gradually diffuse out of the membrane while retaining the cells within the dialysis bag. This technique offers notable advantages in simplicity and operational convenience. The process primarily requires only a centrifuge for cell washing, eliminating dependence on large and expensive instruments and enhancing cost-effectiveness. Compared to conventional gradient washing methods for glycerol removal, this process is significantly streamlined and more practical. It significantly reduces the thawing time, mitigating osmotic shock-induced damage and hemolysis, as well as the potential harm to antigens caused by excessive mechanical washing. Following thawing using this method, cells resuspended in preservation solution maintained viability for up to 75 days, with no significant changes in morphology, antigenicity, or antigen strength compared to pre-freezing levels. The novel thawing method for FRBCs presented in this study effectively preserves RBC surface antigens. It ensures stable expression even for antigens with low surface abundance and reduces the mechanical damage to cell surface antigens commonly associated with conventional thawing techniques.

The primary value of this thawing method lies in its efficiency as a laboratory procedure for processing FRBCs. Donor RBC samples can be tested and then cryopreserved alongside reserved blood units. During

periods of blood supply shortage, these frozen samples can be thawed, resuspended in preservation solution, and used for transfusion compatibility testing. Once a suitable recipient is identified, the corresponding RBCs can then be thawed, thereby avoiding the wastage that typically occurs with conventionally thawed RBCs, which must be used within 24 hours but are often discarded due to cross-matching incompatibility. Furthermore, this method provides a technical foundation for supporting research on RBC surface antigens, developing standardized reference materials for panel cells, establishing rare blood type reagent RBC banks, and conducting quality control assessment of forward and reverse grouping reagents for various blood types.

Author contributions

Conceptualization, Zebin Hu and Shoufang Qu; Methodology, Zebin Hu; Software, Yi Wu; Validation, Zebin Hu and Chen Cao; Formal Analysis, Yi Wu; Investigation, Chen Cao; Resources, Zebin Hu; Data Curation, Chen Cao; Writing – Original Draft Preparation, Zebin Hu and Chen Cao; Writing – Review & Editing, Buqiang Wang and Shoufang Qu; Visualization, Chen Cao; Supervision, Buqiang Wang and Shoufang Qu; Project Administration, Zebin Hu; Funding Acquisition, Zebin Hu.

Ethics statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Henan Red Cross Blood Center (Approval No. 2023008).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

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Declaration of competing interest

The authors declared no conflict of interests.

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