Red blood cell preservation by droplet freezing with polyvinylpyrrolidone or sucrose-dextrose and by bulk freezing with glycerol

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BACKGROUND: Red blood cell (RBC) preservation is essential to transfusion medicine. Many blood group reference laboratories need a method to preserve rare blood samples for serologic testing at a later date. This study offers a comparison of three common cryoprotective agents and protocols used today: bulk preservation with glycerol and droplet freezing with sucrose-dextrose (S+D) or polyvinylpyrrolidone (PVP).

STUDY DESIGN AND METHODS: Human blood from 14 volunteers was collected and frozen at set intervals over 2 weeks with PVP, S+D, or glycerol. The frozen RBCs were later thawed and the percentage of surviving RBCs was determined. Detailed protocols and an instructional video are supplied.

RESULTS: Over a 2-week period, RBCs preserved with glycerol and thawed with a widely used protocol showed a recovery of 41 ± 16% (mean ± standard deviation) while those thawed with a modified glycerol protocol showed a recovery of 76 ± 8%. RBCs preserved by droplet freezing with S+D showed a recovery of 56 ± 11% while those preserved by droplet freezing with PVP showed a recovery of 85 ± 6%. Recovery values were similar with ethylenediaminetetraacetic acid or heparin anticoagulants, differing freezing rates, and varying droplet volumes.

CONCLUSION: Droplet freezing with PVP offered the greatest recovery. While bulk freezing with glycerol can also be effective, droplet freezing may be a more convenient method overall. It requires less effort to thaw, needs much less storage room, and allows blood group laboratories to be frugal with thawing rare samples.

Red blood cell (RBC) preservation of small volumes (i.e., <1 mL) is an important tool in modern transfusion medicine for serologic testing. Many blood group reference laboratories need to have the ability to freeze, reconstitute, and then analyze rare RBCs that were collected months or years earlier. RBC preservation methods offer a reliable long-term supply of rare RBCs that have been previously collected. Several cryopreservation methods are used for maintaining ex vivo RBC structure.

One common method is glycerol preservation.[1,2] Glycerol is a permeant and relatively nontoxic cryoprotective agent (CPA) that protects RBCs from injury during slow cooling. It works by entering RBCs, shifting their osmotic balance, and stopping excessive volume reduction due to extracellular ice formation.[3-7] By halting volume reduction, it is thought that RBCs avoid reaching a

ABBREVIATIONS: CPA(s) = cryoprotective agent(s); PVP = polyvinylpyrrolidone; S+D = sucrose-dextrose.

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critically damaging electrolyte concentration.[3-7] Glycerol also depresses the system's freezing point, which may help mitigate the formation of extracellular ice.[7-9] Although this method is effective, glycerol preservation is typically used with large volumes and deglycerolization after thawing is time-consuming.[10]

Freezing RBCs with liquid nitrogen, notably by droplet freezing, was developed later and may be an underutilized alternative.[11-13] Droplet freezing is a method that preserves RBC samples in small, individual droplets. Minute quantities of RBCs can then be recovered to obtain the minimal amount of sample needed to perform a serologic test. This method uses nonpermeable CPAs, such as sucrose and dextrose (S+D) or polyvinylpyrrolidone (PVP), to protect the RBCs. Nonpermeable CPAs may work by dehydrating the RBCs and minimizing intracellular ice formation.[5,14] They may also stabilize the RBC membranes and help macromolecules retain their native conformations.[15-17]

What is unknown about droplet freezing, however, is which CPA works best and whether droplet freezing can surpass the effectiveness of freezing with glycerol. We surveyed blood banks and blood group reference laboratories and determined that glycerol, S+D, and PVP were the most common CPAs used in small-volume blood storage. In this study we compared the recovery of RBCs frozen with glycerol and RBCs droplet frozen with S+D or PVP.

MATERIALS AND METHODS

Survey method

Forty-seven invitations for an online survey (http://www.surveymonkey.com) were sent to contacts at reference laboratories through the International Society of Blood Transfusion Rare Donor Working Party; International Society of Blood Transfusion Red Cell Immunogenetics and Terminology Working Party; and Serum, Cells and Rare Fluids Exchange member lists. Twenty-five responses were received.

RBC preparation and processing

Human blood (30 mL) from 14 healthy volunteers was collected into ethylenediaminetetraacetic acid (EDTA) or heparin anticoagulant after obtaining informed consent. This blood was then aliquoted (Vacutainer 5-mL Red Top (glass) tubes, Becton Dickinson, Franklin Lakes, NJ) and stored at 4°C. Over the next 2 weeks, aliquots from each donor were removed from storage and frozen on Days 1, 4, 7, 10, and 14.

When the blood was removed from 4°C storage, it was centrifuged at $1000 \times g$ for 5 minutes to isolate the RBCs. The RBCs were then repeatedly, for instance three to five times, washed with 0.9% saline and centrifuged at $1000 \times g$ for 5 minutes until no hemolysis was present in the supernatant.

Bulk freezing with glycerol

Freezing

For each sample, 2 vol of glycerol freezing solution (Glycerolyte 57 freezing solution, Fenwal, Lake Zurich, IL) was used for every 1 vol of RBCs. First, 20% of the total glycerol freezing solution volume was added dropwise to the RBCs and allowed to incubate with mixing for 10 minutes. Second, the remaining glycerol freezing solution was added dropwise to the RBCs with gentle mixing. Third, the glycerol-RBC mixture was incubated for 10 minutes with gentle mixing. Finally, the RBCs were transferred directly to cryovials and placed at –80°C or the cryovials were cooled slowly at 1°C/min by incubating in a controlled-rate freezing tray at –80°C (Nalgene Cryo 1°C freezing container, Thermo Fisher Scientific, Pittsburgh, PA). A quantity of 1.8 mL of RBCs mixed with glycerol was in each cryovial.

Thawing

A standard deglycerolization protocol[18] and a modified protocol were used. Samples recovered with the standard deglycerolization protocol were first thawed at room temperature. Next, 1 mL of thawed RBCs was transferred to a 5-mL test tube (Becton Dickinson), 0.5 mL of 12% saline solution was added dropwise with gentle mixing, and this mixture was left standing for 3 minutes at room temperature. After 3 minutes, a 1.6% saline solution was added until the test tube was at half volume, and then 0.9% saline was added until the test tube was full. The samples were repeatedly centrifuged at $1000 \times g$ for 1 minute and washed with 0.9% saline until the supernatants showed no signs of hemolysis.

For samples recovered with the modified protocol it was necessary to gradually lower the hypertonic intracellular environment with multiple wash steps. First, the cryovials were thawed at room temperature and the desired volume of RBCs was transferred to a 5-mL test tube (Becton Dickinson) and centrifuged at $1000 \times g$ for 2 minutes. Second, the glycerol freezing solution was aspirated. Third, 0.16 mL of 12% saline for every 0.5 mL of RBCs was added dropwise and with gentle shaking over a period of 5 minutes. The RBCs were then left standing for 3 minutes at room temperature. Next, 0.5 mL of 0.2%-0.9% dextrose-saline was added slowly and dropwise with gentle shaking. The RBCs were then left standing for 2 minutes at room temperature. This process—adding 0.5 mL of 0.2%-0.9% dextrose-saline and letting the RBCs stand undisturbed at room temperature—was repeated until the total volume in the test tube was 4 mL.

At this point, the RBCs were centrifuged for $1000 \times g$ for 1 minute, and then 0.5 mL of supernatant was aspirated. The RBCs were then resuspended in the test tube by repeated inversion. Next, 0.5 mL of 0.2%-0.9% dextrose-saline was added slowly and dropwise with gentle shaking.

The RBCs were then left standing for 2 minutes at room temperature. This process—centrifuging, removing the supernatant, resuspending the RBC pellet, adding a volume of 0.2%-0.9% dextrose-saline solution equal to what was aspirated, and then standing for 1 minute—was repeated with the following volumes: 1, 1.5, 2, and 4 mL. Finally, the RBCs were centrifuged at $1000 \times g$ for 1 minute, and all of the supernatant was aspirated. The RBCs were then washed with 0.9% saline until no hemolysis was present.

Both protocols are available in detail in the supplemental materials (available as supporting information in the online version of this paper). The main difference between them is the rate with which the RBCs are brought into an isotonic environment, which is greatly decreased in the modified protocol relative to the standard protocol.

Droplet freezing with PVP or S+D

Freezing

For each sample, 1 vol of PVP freezing solution (27 mL of 30% bovine serum albumin [BSA] with 23 mL of 30% PVP in deionized water; Sigma-Aldrich, St Louis, MO) or S+D freezing solution (15.4 g sucrose, 5.4 g dextrose, and 0.29 g NaCl in 100 mL of deionized water; Sigma-Aldrich) was added for every 1 vol of RBCs. The RBC-freezing solution mixture was allowed to incubate for 1 hour. Next, a fine-tip transfer pipette (PCG Labs, Dahlonega, GA) was used to release individual droplets (approx. 20 µL) of RBCs into liquid nitrogen. This pipette was chosen based on preliminary tests with six pipette types yielding various droplet volumes. The pipette was held vertically approximately 15 cm above the liquid nitrogen, which was being stirred to produce a weak whirlpool. These steps help prevent the droplets from aggregating. The liquid nitrogen containing the frozen droplets was then poured over a metal sieve to isolate the droplets. To transfer the droplets to a cryovial for storage, the cryovial cap was first punctured two or three times to prevent the vial from bursting due to pressure. This cryovial and a funnel were then chilled in liquid nitrogen to prevent the droplets from sticking and thawing on contact. The funnel was used to channel the droplets into the chilled cryovial, which was then placed in –180°C storage.

Thawing

To thaw the droplets, approximately four to six droplets (approx. 80-120 µL) were placed into 1.5 mL of 0.9% saline at 37°C with gentle shaking. After the droplets were completely suspended in warm saline, the total volume was diluted to 2 mL for sampling and then washed until no hemolysis was apparent. The freezing and thawing methods are explained in detail in the supplement and shown in the supplementary video (available as supporting information in the online version of this paper).

Percent recovery calculation

The recovery was evaluated using hemoglobin (Hb) measurements before (total Hb) and after (RBC Hb) the washing procedure. Hb was determined with the cyanmethemoglobin method.[19] Samples were incubated in 1.5 mL of Drabkin's reagent (Sigma-Aldrich) for 15 minutes before absorbance measurement at 540 nm (DU530 spectrophotometer, Beckman, Brea, CA). Absolute Hb values in mg/mL were calculated using a standard curve. The method was validated against the routine Hb measurements in NIH Clinical Center Department of Laboratory Medicine.

Specific volumes were sampled from the RBC solutions to keep the Hb values within the standard curve range. These specific volumes were also dependent on the solution volume during the thawing procedure so that we sampled 10 µL of 1.5 mL for the standard glycerol, 25 µL of 4.0 mL for the modified glycerol, and 120 µL of 2 mL for both droplet freezing protocols. The volumes sampled for total Hb and RBC Hb were controlled to be identical. Because the sample for total Hb was up to 6% of the solution's total volume, we took this loss of volume and RBCs into account in our calculation so that

$$\text{Recovery}\,(\%) = \frac{\text{RBC Hb}\,(\text{mg/mL})}{\text{total Hb}\,(\text{mg/mL})} \cdot \frac{\text{volume}\,(\text{mL})}{\text{volume}\,(\text{mL}) - \text{sample}\,(\text{mL})} \cdot 100 \quad (1)$$

Statistical analysis

Data are shown as mean ± standard deviation (SD). Data analysis was done with computer software (Excel, Microsoft Corp., Redmond, WA; and MedCalc, MedCalc Software, Mariakerke, Belgium).

RESULTS

Laboratory survey

Various RBC preservation additives were used in 24 reference laboratories for either bulk or droplet freezing (Table 1). One laboratory replied and indicated that no RBC freezing is done due to costs, complexity, and lack of adequate instructions. We concluded from this nonrepre-

TABLE 1. Survey of RBC preservation additives in 24 reference laboratories

CPA	Usage*
Glycerol, various concentrations and freezing speeds	12
Sucrose, alone or with dextrose, albumin or plasma	10
PVP, alone	2
Other†	5

* Multiple answers were possible.
† Including a glycerol-gelatin mix (Glycigel),[27] the MAPI system (CryoBioSystem, Paris, France), and direct storage in liquid nitrogen.

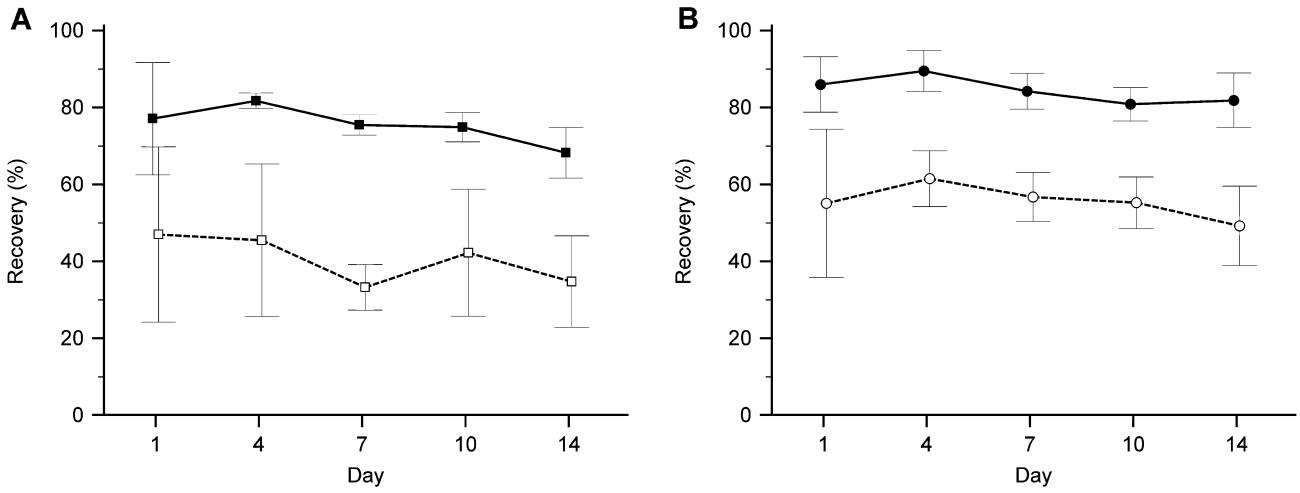

Fig. 1. Recovery of RBCs preserved by various methods over a period of 2 weeks (mean ± SD). (A) Bulk glycerol freezing with standard thawing protocol (n = 4; □) and modified thawing protocol (n = 8; ■). (B) Droplet freezing with PVP (n = 8; ●) or S+D (n = 8; ○).

sentative set of replies that glycerol and S+D are often used, while use of PVP may be uncommon.

Comparison of freezing methods

RBCs from 14 healthy volunteers were collected and preserved with glycerol, S+D, or PVP at set intervals over a period of 2 weeks. Across all intervals, the mean ± SS recovery of RBCs preserved by bulk freezing with glycerol and thawed with the standard protocol was 41 ± 16% (n = 4) and with the modified protocol was 76 ± 8% (n = 8; Fig. 1A). Across all intervals, the mean ± SD recovery of RBCs preserved by droplet freezing with S+D was 56 ± 11% (n = 8) and with PVP was 85 ± 6% (n = 8; Fig. 1B).

Lack of impact by freezing rate, anticoagulants, and droplet volume

Differences in freezing rate, that is, slow or fast freezing, had minimal impact on the recovery of RBCs (Fig. 2). EDTA and heparin as anticoagulants differed little in regard to the recovery of RBCs (Fig. 3). Preliminary tests with six different pipette types creating droplet volumes between 15 and 50 μL revealed no correlation between droplet volumes and recovery rates, which showed little variation (SD, 3.9 and 4.3% for S+D and PVP, respectively). A fine-tip transfer pipette, which yielded a mean droplet volume of approximately 20 μL, was selected for this study due to its practicality.

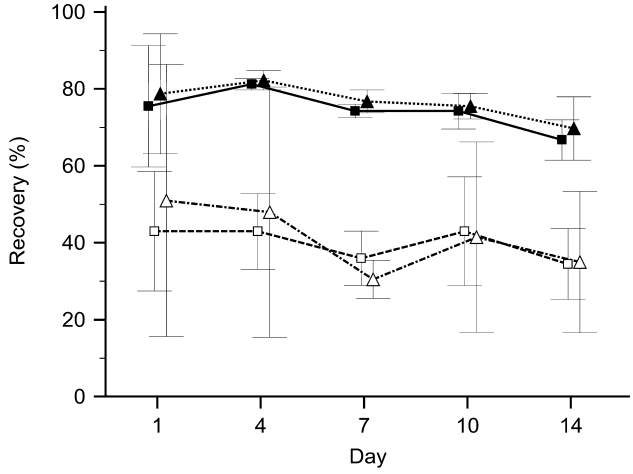

Fig. 2. Effect of controlled freezing rate on bulk glycerol frozen samples. Recovery of RBCs preserved by various glycerol freezing and thawing methods over a period of 2 weeks (mean ± SD). Controlled freezing rate (n = 2; △) compared to regular bulk glycerol freezing (n = 2; □) with standard thawing protocol, and controlled freezing rate (n = 4; ▲) compared to regular bulk glycerol freezing (n = 4; ■) with the modified thawing protocol.

DISCUSSION

The aim of this study was to compare RBC preservation methods which might offer good approaches to preserving small volumes of blood for serologic tests. We propose that determining the best method involves weighing two factors: first, the effectiveness of the CPA, and second, the convenience of the protocol.

A CPA is effective insofar as it prevents hemolysis during freezing and thawing. One of the oldest CPAs is glycerol, a simple compound that remains widely used for freezing whole blood units. AABB Standard 5.7.5.4 stipulates that the mean recovery of a glycerol-preserved unit intended for transfusion should be at least 80%,[20] but no minimum recovery has been identified for RBCs intended

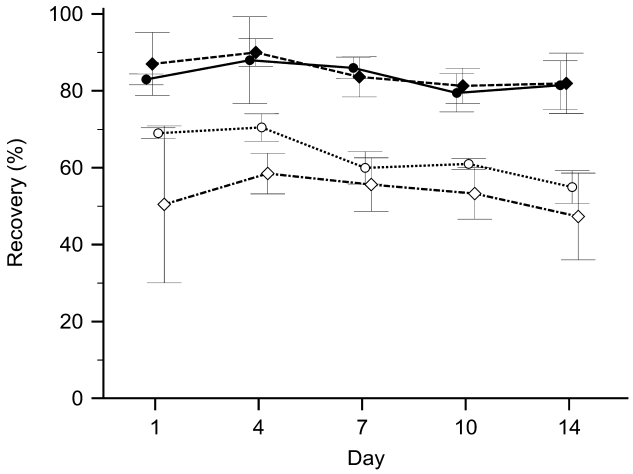

Fig. 3. Effects of the anticoagulant on droplet freezing with S+D or PVP. Recovery of preserved RBCs over a period of 2 weeks (mean ± SD). Droplet freezing of PVP-treated RBCs obtained from blood anticoagulated with EDTA (n = 2; ●) compared to heparin (n = 6; ◆), and droplet freezing of S+D-treated RBCs obtained from blood anticoagulated with EDTA (n = 2; ○) compared to heparin (n = 6; ◇).

only for serologic testing. The standard protocol for thawing small volumes of RBCs preserved with glycerol,[18] which is simpler and much faster than the protocol for whole units, was unable to provide such a recovery (Fig. 1A). This may be attributed to the fact that the small-volume protocol was designed to be convenient for serologic testing. A yield of 55% or less can easily accommodate multiple serologic tests. Nevertheless, a modified protocol enabled a recovery of 80% (Fig. 1A). We found that the freezing rate had little impact on the recovery (Fig. 2); however, a slow transition from a hypertonic to an isotonic environment during thawing was essential. Unfortunately, the modified protocol takes approximately twice as long as the standard protocol. Since the standard protocol already yields enough RBCs for serologic tests, the modified protocol may not offer much advantage in many laboratories.

Droplet freezing requires less thawing time and produces impressive recoveries. Our survey indicated that S+D was the most common CPA used for droplet freezing (Table 1), but S+D only achieved a recovery of 55% (Fig. 1B). PVP offers the same benefits of S+D and better recoveries. PVP is a water-soluble polymer that was initially used as a blood plasma substitute.[21] We used 30% BSA to create the PVP freezing solution in our experiments. For routine application, laboratories are using 22% BSA. Hence, BSA concentrations in this range are suitable and can be chosen based on practicability. There are no published recovery rates of human RBCs preserved by droplet freezing with PVP. Our results are comparable with previously reported recovery rates of trypsin-treated RBCs protected with dextran (82%) or hydroxyethyl starch (40%).[22] Droplet freezing with PVP is more effective than standard bulk glycerol preservation. EDTA and heparin as anticoagulants were tested, but they had little impact on the recovery of S+D or PVP droplets (Fig. 3).

Overall, droplet freezing may be more convenient than bulk glycerol preservation. We propose that droplet freezing holds primarily two advantages over glycerol preservation: one advantage is thawing ease. Droplet freezing only requires warm saline to thaw. The CPAs are nonpermeable, and therefore the RBCs require no additional wash step. Another advantage is prudence. Droplet freezing allows one to thaw the very minimum amount of rare sample needed for a serologic test, which is often a critical consideration for very rare RBC samples.

Our study has several limitations because we focused on simulating the conditions a technologist in a blood group reference laboratory would often encounter. Therefore, we opted to produce the droplets with a commonly used fine-tip transfer pipette rather than a standardized 100-µL pipette, which may produce even more consistent droplet volumes. We also recognize that liquid nitrogen may not be available in all laboratories because it is expensive and requires training to handle. In this study, we focused on quantifying RBC recovery by measuring ratios of Hb concentration rather than the serologic properties of the reconstituted RBCs. Nevertheless, RBCs have been shown to maintain their antigen pattern and usability for serologic testing even after freezing in liquid nitrogen.[23-25] Finally, our comparison of short-term freezing cannot address any potential differences among the three methods after long-term freezing, which may often exceed 10 years.

It should be noted that thawed RBCs are generally a poor source of DNA and RNA. Washing reduces the content of white blood cells and reticulocytes in the sample. Mature RBCs contain spurious RNA and no genomic DNA. Special procedures for leukoreduced blood are required to extract the minimal amounts of residual DNA and RNA.[26]

In conclusion, the recovery of frozen RBCs treated with glycerol, S+D, or PVP was measured and the effectiveness of the CPAs was determined. Droplet freezing with PVP was the most effective method. Glycerol was effective too, but it may be less convenient than droplet freezing, a method that offers many advantages over bulk glycerol preservation.

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CONFLICT OF INTEREST

None.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Protocol for red blood cell preservation by bulk glycerol freezing.

Protocol for red blood cell preservation by droplet freezing with polyvinyl pyrrolidone or sucrose/dextrose.

Instructional video on droplet freezing with polyvinyl pyrrolidone or sucrose/dextrose.

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