REPORT

Cryopreservation of red blood cell units with a modified method of glycerolization and deglycerolization with the ACP 215 device complies with American and European requirements

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Current red blood cell (RBC) glycerolization with the ACP 215 device is followed by volume reduction of the glycerolized RBCs before freezing. We investigated a modified method of glycerolization and deglycerolization which eliminates the final centrifugation step that reduces glycerolized RBC supernatant. A total of 37 RBC units collected from healthy volunteers were analyzed. After removal of the supernatant, RBCs were glycerolized using the high glycerol method and stored at –80°C. After deglycerolization, RBCs were preserved with either SAG-M or AS3 and stored for at least 10 or 14 days, respectively. Quality of stored RBCs was assessed by measuring osmolarity, blood cell counts, free hemoglobin, adenosine triphosphate (ATP), hemolysis, and glucose. The overall RBC mass recovery after deglycerolization was 86 ± 7.6 percent, and the osmolarity was 336 ± 23 mOsml/kg H_2O. The hemolysis for stored components at the end of their shelf life was 0.21 ± 0.08 percent for AS3-preserved RBCs and 0.25 ± 0.08 percent for RBCs preserved with SAG-M. On expiration, 32 percent of initial ATP values were measured in AS3-preserved RBCs vs. 62 percent in SAG-M–preserved RBCs. This modified method of glycerolization and deglycerolization meets the quality requirements of the European Council and the AABB standards. The prolonged storage of thawed RBCs enables optimized transfusion management for patients with rare blood groups. ***Immunohematology* 2012;28:67–73.**

Key Words: cryopreservation, intracellular purines, hemolysis, high glycerol method, red blood cell units, ACP 215

Cryopreservation of red blood cells (RBCs) still remains an important method for maintaining an inventory of rare RBC units and managing special transfusion circumstances. The influence of RBC distress on the quality of RBCs during the collection process and the extent of storage lesions during liquid storage (4°C ± 2°C) is well known.[1,2] It has been shown previously that the length of centrifugation and especially the gravitational force play an important role in the impairment of RBCs.[1] High gravitational forces or a long exposure time results in accelerated intracellular ATP degradation and consumption of glucose during storage as a result of increased membrane repair processes. Subzero storage of RBC units reduces erythrocyte metabolism and allows a frozen storage period of at least 10 years.[3] However, RBC damage during the collection procedure may affect the quality of deglycerolized RBC units during postthaw storage. A variety of freeze and thaw protocols have evolved so far. The methods differ with regard to cryoprotectants, storage temperature, and thawing and washing procedures.[4] In Europe, predominantly the high-glycerol method (HGM) is used because of the ease of storage in a –80°C freezer without the need for highly technical equipment. The low-glycerol method (LGM) requires a storage temperature of –190°C, which can only be achieved in liquid nitrogen. Currently, RBC units that undergo the HGM are centrifuged at least three times: during the collection process, before glycerolization to achieve a hematocrit (Hct) of at least 75 percent, and before freezing to remove the supernatant glycerol.[5] In this prospective study we investigated a modified freeze and thaw protocol by eliminating the last centrifugation step in conventionally collected and apheresis-derived RBC units. The quality of RBCs after thawing was assessed by analyzing osmolarity, recovery of RBC mass and hemoglobin content, intracellular ATP, hemolysis, and glucose at defined time points.

Donors and Methods

Study Design

The aim of this study was to evaluate a modified method of glycerolization and deglycerolization of RBC units with the automated device ACP 215 (Haemonetics Corp, Braintree, MA), which eliminates the highly manual step of supernatant reduction before freezing. This change in manipulation is compensated by a modification in the deglycerolization process as shown in Table 1. The dilution volume and the

dilution rate are the crucial points. They were calculated by the scientific board of Haemonetics. The objective was to reduce the osmolality of the glycerolized and frozen components to values of conventionally cryopreserved units before deglycerolization and to avoid osmotic shock of RBCs during the deglycerolization process.

Table 1. Comparison of variables using the current and postthaw modified ACP 215 washing procedures

	Current ACP 215 configuration	Study ACP 215 configuration*
Hypertonic solution volume (mL)	50	50
Hypertonic solution flow rate (mL/min)	150	150
Saline dilution volume no. 1 (mL)	340	600
Saline dilution flow rate (mL/min)	60	110

*Modified washing volumes

Each manipulation step bears the risk of bacterial contamination or artificial damage of the component, which may be of particular interest in certain hemoglobin abnormalities.[6,7] A simplified method reduces the risk of component impairment and is also time sparing, which is certainly an advantage in daily routine. Second, glycerolized RBCs have a large volume that is difficult to handle in routine blood banks as the centrifuge buckets are normally not designed for such high volumes.

To evaluate the applicability of this simplified method to different production methods, conventionally collected as well as apheresis-derived RBCs were investigated. A major requirement for managing stockpiling of cryopreserved RBC units is the shelf life after deglycerolization. We included two preservation solutions (SAG-M, AS3) in our postthaw investigation to compare their suitability. In consideration of previous studies, which showed hemolysis above the threshold of 0.8 percent in SAG-M–preserved deglycerolized units within 1 week, the postthaw shelf life was set at 10 days for SAG-M units vs. 14 days for AS3 units.[8,9]

Finally, we assessed the compliance of these modified RBC units with requirements for cryopreserved RBC concentrates, as defined by the Council of Europe (CE) and the AABB standards.[3,10] In brief, hemolysis should stay less than 0.8 percent at the end of shelf life, and the minimal required content of hemoglobin (Hb) is 36 g/unit.[10]

Donors

RBCs were collected from routine donors of the Austrian Red Cross Blood Service by standard whole blood (WB) collection (n = 17), and from volunteer donors of the Department of Blood Group Serology and Transfusion Medicine (Medical University of Vienna) who qualified for multicomponent collection (MCC). RBC double units were collected by two automated collection devices (MCS+, Haemonetics, n = 6) and (Cymbal, Haemonetics, n = 4) according to the technical manual. The study was approved by the local ethics committee, and the donors gave written informed consent.

Red Blood Cell Units

All RBCs (WB collections and apheresis-derived) were leukocyte-depleted, suspended in SAG-M, and stored for 6 days at 4°C ± 2°C before freezing. A prerequisite for further manipulation was the compliance of the RBC units with the requirements of the EC concerning Hb content/unit (40 g/unit) and leukocyte contamination ($\leq 1 \times 10^6$/unit).[11] RBC units were connected to 500-mL transfer bags by a sterile connecting device (SCD, Haemonetics) and centrifuged for 15 minutes at 2000 rpm (2858*g*) in a floor model, cooling centrifuge (Roto Silenta RP, Hettich, Vienna, Austria). Using a plasma extractor, supernatant was extracted to the transfer bag to achieve a Hct of at least 75 percent.[5] Finally, after drawing blood samples to determine the actual Hct, the remaining packed RBC units were connected to the 1800-mL freezing bag (EVA bag reference GSR 8000AU Macopharma, Werken, Belgium) using a sterile connecting device. RBC units were defined as small (RBC mass ≤130 g), typical (RBC mass >130 g and ≤180 g), or large (RBC mass >180 g).

Freezing Procedure

To achieve the final concentration of 40 volume-percent glycerol, the amount of added 57 gram-percent glycerol solution was calculated depending on the actual hematocrit of the units. The RBC units were glycerolized with the ACP 215 using the HGM (Haemonetics) according to protocol. RBC units and the glycerol solution were at room temperature. Calculation of the amount of added glycerol was done automatically by the device and was completed within 10 minutes. Finally, glycerolized RBC units were frozen by placing them into a −80°C freezer (Revco Freezer, Thermo Scientific, Langenselbold, Germany) and stored for at least 10 to 14 days. Glycerolized RBCs had a mean volume of 578 mL (standard deviation, 89 mL; range, 354 to 736 mL).

Thawing Procedure

After 10 days (SAG-M) and 14 days (AS3), frozen RBCs were thawed with a a 37°C pulsed-air chamber with infrared temperature control of RBC bag content (Sahara Thawer, Transmed Medizintechnik Gmbh, Bad Wünnenberg, Germany). The thawing procedure was completed when a

temperature of 34°C was reached. Deglycerolization was performed with the ACP 215 according to the manufacturer's protocol, using either the regular bowl set (LN235) for small and typical units (130–180 g of RBC mass) or the large bowl set (LN236) for large units with more than 180 g of RBC mass. The deglycerolization process included an initial deglycerolization step with hypertonic saline solution (12%) to facilitate the intracellular glycerol removal and five consecutive washing steps with a saline rinse solution (0.9% saline, 0.2% glucose-buffered solution bags, BIO-Wash, Haemonetics) to achieve a remaining glycerol content of less than 1 gram-percent. For this purpose we compared the color of the final wash fluid with the blocks of a color comparator (Hemolysis Color chart, Haemonetics) and measured the osmolarity of the component, as recommended by the AABB.[3,12] The entire deglycerolization process lasted an average of 69 minutes (standard deviation ± 4.5 min). The main difference between the standard and modified deglycerolization methods is shown in Table 1. Postdeglycerolization RBC units were resuspended either in SAG-M (Cymbal, n = 4; MCS, n = 6; WB, n = 8) or in AS3 (Cymbal, n = 4; MCS, n = 6; WB, n = 9) and stored at 4°C (± 2°C) for another 10 (SAG-M) or 14 (AS3) days.

Assessment of In Vitro Quality

Our aim was to assure that the RBC units treated with the new, modified method of glycerolization, freezing, and deglycerolization still met the required standards of the CE and the AABB.[3,10,12] Storage lesion was assessed by measuring volume (net weight), blood counts (RBC mass), Hct, Hb, glucose, pH, supernatant (free) hemoglobin (fHb), and intracellular ATP content. The samples were taken at defined time points: after collection, before glycerolization, after thawing, after deglycerolization, and during storage. Additionally, supernatant osmolarity was measured after deglycerolization. All samples were drawn under aseptic conditions on days 0 (day of deglycerolization), 3, 5, 7, and 10 (SAG-M), and on days 0, 5, 7, 10, and 14 (AS3) of storage.

Laboratory Analyses

The weight was measured by an electronic scale and calculated by the net weight of the component divided by weight density of the RBCs. The concentration of Hb in the RBC supernatant was analyzed (AU 5430, Olympus Diagnostika, County Clare, Ireland) by the photometric color method. The blood count was measured using an automated cell counter (Cell Dyn 3500CS, Abbott Diagnostics, South Pasadena, CA). Osmolarity of the RBC units was determined (OSMO AKRAY OM-6050, A. Menari Diagnostics, Florence, Italy). Glucose was measured (Olympus AU 5430, Olympus Diagnostika) with the enzymatic, hexokinase method. ATP was measured by high-performance liquid chromatography as described previously,[13] and pH was analyzed at 37°C (ABL 80, Radiometer Gmbh, Brønshøj, Denmark). All analyses were done according to the manufacturer's instructions. Hemolysis (%) was calculated as follows: $([fHb \times \{100-Hct\}]/Hb_{total})/1000$.

Statistical Analyses

Results were expressed as mean and standard deviation for descriptive purposes in the text. Because of the nonnormal distribution of data all comparisons were made by nonparametric statistics. Procedures were split into four subgroups by the nature of the blood collection method (WB or apheresis) and of the preservation solution used to resuspend deglycerolized RBCs (SAG-M or AS3). Asigned-rank test was done with Minitab (Wilcoxin, Minitab®15.1.0.0, Inc., State College, PA). Comparisons between the various collection methods within a group were done using the Kruskal-Wallis test. A probability value of less than 0.05 was considered significant.

Results

Red Blood Cell Collections

All collected RBC units (17 WB collections, 6 MCS+–derived double units [12 single RBC units], and 4 Cymbal-derived double units [8 single RBC units]) met the requirements of the CE and were included in the study.[11] No significant differences were found in component characteristics among the different collection methods. Values are detailed in Table 2. Thirty-three RBC units were defined as typical (130 g < RBC mass < 180 g), and four were defined as large (RBC mass > 180 g).

Red Blood Cells Deglycerolized

The volume of all deglycerolized RBC units was 305 ± 13 mL and ranged from 277 to 357 mL. The RBC mass in all units was greater than 140 mL, and Hb content/unit was greater than 36 g (Table 3). The overall Hb recovery in the 37 tested RBC units after deglycerolization was 75 ± 6.5 percent, and the overall RBC mass recovery was 86 ± 7.6 percent. Recovery of Hb and RBC mass was significantly lower in MCS+ and Cymbal than in WB-derived components. We measured a recovery of 72 percent Hb and 85 percent RBC mass for MCS+ and Cymbal components vs. 78 percent Hb and 90 percent RBC mass for WB-derived components ($p < 0.05$ for Hb), respectively. The overall osmolarity was 336 ± 23 mOsm/kg

H_2O after deglycerolization, which was well below the AABB requirements of 400 mOsm/kg,[8,12] and MCV returned from a mean of 134 femtoliters (fL; ± 4.3) during frozen storage to 99 fL (± 4.3), which represents 108 percent of initial values. The color comparator indicated a residual glycerol of less than 1 percent. Detailed values for both additive solutions are given in Table 4.

Red Blood Cells Stored in Either SAG-M or AS3

Hemolysis

The mean overall hemolysis for all stored components was 0.23 ± 0.08 percent at the end of shelf life, set by the study design. It increased during storage as expected, but remained less than the threshold of 0.8 percent in all units. Although it was slightly higher in SAG-M–preserved units, there was no significant difference between the preservation solutions (SAG-M, AS3) for Cymbal- and WB-derived RBC units. RBCs collected with the MCS+ showed significantly higher hemolysis when stored in SAG-M ($p < 0.05$). The significantly lowest hemolysis was measured in WB-derived RBCs irrespective of the preservation solution (Fig. 1).

Intracellular Adenosine Triphosphate Content and Glucose

The overall intracellular ATP was 48 ± 19 percent of initial values at the end of shelf life, which is considerably greater than the threshold of 10 percent residual ATP content.[14] During storage, ATP values decreased continuously to 32 percent (Day 14) of initial values for AS3-preserved units and to 62 percent (Day 10) for units stored in SAG-M, respectively ($p < 0.05$; Table 4, Fig. 2). On day 10, 40 percent of initial ATP values were measured in AS3-preserved units. Initial intracellular ATP content was significantly higher in WB collections than in apheresis-derived units (Table 2, Fig. 2; $p < 0.05$). The lowest intracellular ATP values and the highest purine degradation were measured in AS3-preserved, MCS+–derived RBCs ($p < 0.05$; Fig. 2A). The preservation solution had almost no impact on the course of ATP degradation in RBCs produced with Cymbal (Fig 2). Consumption of glucose was comparable irrespective of the collection method and preservation solution (Table 2).

Discussion

In this study we evaluated a modified RBC freeze and thaw method that eliminated the third centrifugation step. This procedure is not only simpler to perform, but it also may reduce RBC lesions by shortening exposure to centrifuge force. The impact of shear stress caused by centrifugation is

Table 2. Manipulation characteristics of RBC units collected with MCS+, with Cymbal, or conventionally (whole blood)*

Variable	MCS+ (n = 12)	Cymbal (n = 8)	WB (n = 17)
Volume (mL)	276 ± 20	256 ± 9	292 ± 14
Hct (%)	60 ± 2	63 ± 6	58 ± 5
Hb (g/dL)	19.8 ± 0.7	20.6 ± 2.0	19.3 ± 1.6
ATP (pg 10^6 erythrocytes)	118 ± 15	101 ± 17	146 ± 16†
Glucose (mg/dL)	457 ± 19	421 ± 18	488 ± 24
Hb/unit (g/unit)	55 ± 6	53 ± 5	56 ± 6
RBC mass (mL)	165 ± 17	161 ± 14	171 ± 19

*Values are expressed as mean ± standard deviation.
†$p < 0.05$.
ATP = adenosine triphosphate; Hb = hemoglobin; Hct = hematocrit; RBC mass = red blood cell volume; WB = whole blood.

Table 3. Component characteristics and recovery of deglycerolized RBC units collected with MCS+, with Cymbal, or conventionally (whole blood)*

Variable	MCS+ (n = 12)	Cymbal (n = 8)	WB (n = 17)
Recovered Hb content/unit (%)	73.5 ± 3.8	69.0 ± 7.7	78.0 ± 7.2
Hb (g/dL)	12.3 ± 0.6	12.5 ± 0.9	14.1 ± 1.4†
Recovered RBC mass (%)	85.5 ± 4.2	83.0 ± 8.4	90.0 ± 10.2†
Hct (%)	43.6 ± 3.08	45.2 ± 3.1	49.1 ± 5.06†
MCV (fL)	96.5 ± 2.4	102 ± 7.6	98 ± 3.1
Osmolarity (mOsm/kg H_2O)	336 ± 25	345 ± 23	332 ± 23

*Values are expressed as mean ± standard deviation.
†$p < 0.05$.
Hb = hemoglobin; Hct = hematocrit; MCV = mean cell volume; RBC = red blood cell; WB = whole blood.

Table 4. Osmolarity after deglycerolization on Day 0 and the course of hemolysis and adenosine triphosphate percent in SAG-M– and AS3-preserved deglycerolized red blood cells after deglycerolization and during storage until end of shelf life*

Day 0	SAG-M (n = 18) Osmolarity mOsm/kg H_2O 358 ± 12		AS3 (n = 19) Osmolarity mOsm/kg H_2O 316 ± 8	
	Hemolysis (%)	ATP (%)	Hemolysis (%)	ATP (%)
Day 0	0.14 ± 0.04	100 ± 12	0.14 ± 0.03	93 ± 18
Day 3	0.17 ± 0.06	86 ± 27	—	—
Day 5	0.17 ± 0.04	83 ± 15†	0.14 ± 0.03	73 ± 14†
Day 7	0.18 ± 0.05	72 ± 15†	0.16 ± 0.07	59 ± 14†
Day 10	0.25 ± 0.08	62 ± 12†	0.17 ± 0.05	40 ± 12†
Day 14	—	—	0.21 ± 0.08	32 ± 10

*Adenosine triphosphate (ATP) results are calculated in percent of the initial (preglycerolization) values; for comparison day 10 (expiration for SAG-M–preserved units) is highlighted.
†Statistical significance between SAG-M and AS3 with $p < 0.05$.

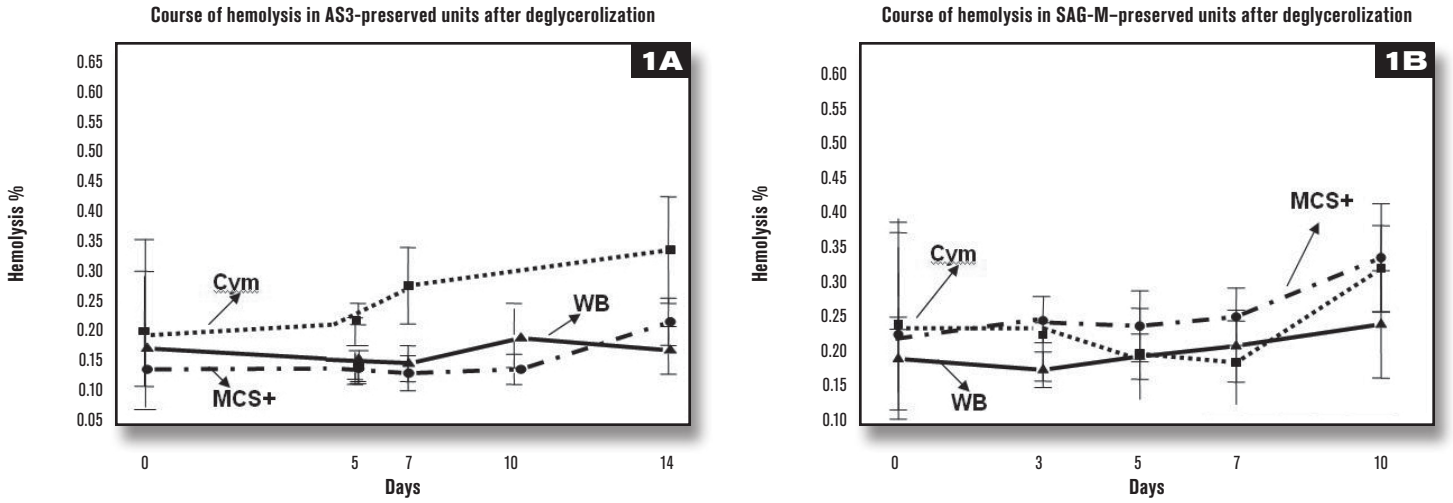

Fig. 1. Course of hemolysis during 14 days' postthaw storage for AS3-preserved (**A**) and SAG-M–preserved (**B**) components of deglycerolized red blood cell units collected with MCS+ (circles, dash-dotted line), with Cymbal (Cym; squares, dotted line), or conventionally (whole blood [WB]; triangles, solid line). Values are given in mean and standard deviation on Days 0 (immediately after deglycerolization), 3, 5, 7, and 10 and on expiration (Day 10 for SAG-M; Day 14 for AS3).

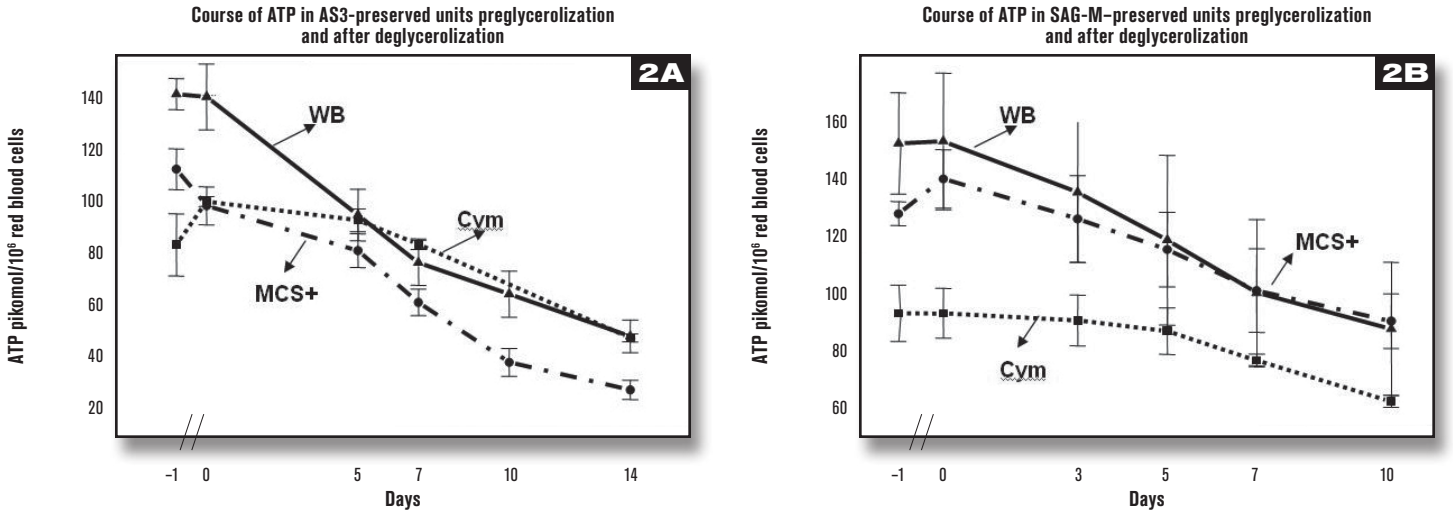

Fig. 2. Course of intracellular adenosine triphosphate (ATP) values during 14 days' postthaw storage for AS3-preserved (**A**) and SAG-M–preserved (**B**) components of deglycerolized red blood cell units collected with MCS+ (circles, dash-dotted line), with Cymbal (Cym; squares, dotted line), or conventionally (whole blood [WB]; triangles, solid line). Values are given in mean and standard deviation on Days minus (–) 1, which represents the initial value after collection and before glycerolization, on Day 0 (immediately after deglycerolization), on Days 3, 5, 7, and 10, and on expiration (Day 10 for SAG-M; Day 14 for AS3).

well known.[1,2,6,15] A major drawback of our study is the lack of a conventionally treated control group, but this study was conducted to provide evidence that the modified method is an alternative for all centers lacking the appropriate equipment, as we do. However, the *Technical Manual* of the AABB and the European guidelines provide clear requirements for frozen and thawed RBC units. Thus, we verified our results with the requirements of the international authorities and compared them with previous publications.

To remove glycerol almost completely after thawing, the washing procedure was modified (Table 1). The mean osmolarity of 336 mOsm/kg H_2O (threshold, 400 mOsm/kg H_2O), the mean MCV of 108 percent of initial values, and the color comparator (degree of hemolysis) indicated sufficient removal of glycerol.[8] In accordance with previous observations, we also found significantly higher osmolarity in SAG-M–preserved units than in AS3 RBCs ($p < 0.05$; Table 4), although no differences were seen among the three collection methods

(Table 3). This can be explained by the mannitol and sodium content in SAG-M causing higher osmolarity of the solution itself.[8] Interestingly, the highest recovery of RBC mass (90%) and total Hb (78%) after deglycerolization was measured in conventionally collected (WB) RBCs. Moreover, during further storage the lowest hemolysis was also measured in WB-derived RBCs, irrespective of the nature of preservation solution (Fig. 1). This can be best explained by the high ATP content found in WB RBCs before freezing (Table 2). ATP is known to power all energy-requiring processes, such as the Na+/K+ pump and membrane repair.[13,14,16] Thus, RBC membrane stability during collection and manipulation could obviously be best maintained in WB-derived RBCs, which may have led to less ATP degradation than in apheresis-derived components.

The composition of the additive solution also influences the extent of hemolysis and degradation of intracellular ATP during storage and treatment.[16–19] As described previously, SAG-M is superior to AS3 in maintaining ATP content and inferior to AS3 in avoiding hemolysis.[18] In our study, unlike in previous investigations, the choice of additive solution had a minor impact on hemolysis in our study (Table 4). Although it was higher in almost all SAG-M–preserved components, a significant difference between SAG-M and AS3 was only seen in MCS+–derived RBCs ($p < 0.05$; Fig. 1). Intracellular ATP was better maintained in SAG-M–preserved WB and MCS+–derived RBCs ($p < 0.05$) than in AS3 units throughout the entire storage period. ATP values in Cymbal RBCs were almost equal for both solutions (Fig. 2). Thus, we assume that the differences in hemolysis, ATP content, and ATP degradation of deglycerolized and stored RBCs cannot be ascribed to the additive solution alone but also to the initial production device.[20–22] As mentioned earlier, each manipulation of RBC units has a negative impact on RBC quality, resulting in increased hemolysis and enhanced loss of intracellular ATP as a result of increased membrane repair processes.[1,2,13] Despite all differences observed between the RBC manipulation devices and preservation solutions after deglycerolization, hemolysis remained less than 0.8 percent in all units. The highest hemolysis of 0.34 percent (± 0.09%; Fig. 1A) was measured in AS3-preserved Cymbal RBCs after 14 days of storage. In all other units hemolysis remained between 0.20 percent and 0.30 percent at the end of shelf life (10 days for SAG-M–preserved units and 14 days for AS3-preserved units; Fig. 1). ATP did not drop below the critical threshold of 10 percent of initial values in any of the RBC units.[14] These observations are in contrast to those in previous investigations that described a rise of hemolysis above the threshold of 0.8 percent in SAG-M–stored deglycerolized RBC units within 1 week.[8] Likewise, hemolysis in AS3-preserved units remained less than values described in the literature.[23–25] These findings may be attributable to the omitted centrifugation step.

Glycerol is known to lead to reduction of ATP and 2,3-diphosphoglycerate levels in glycerolized RBCs.[26] Although a repletion of ATP occurs after thawing and deglycerolization, a possible negative impact of ATP loss during subzero storage cannot be dismissed. However, we were able to show that the omission of the third centrifugation step had no negative effect on SAG-M– and AS3-preserved units, but rather may allow a prolonged storage of deglycerolized RBCs even in SAG-M–preserved components. This is important for the transfusion management of frozen and thawed components and minimizes the waste of RBCs.

In summary, all RBC units treated with the new method complied with the CE and AABB standards for cryopreserved blood components regarding hemolysis and ATP at expiration. The RBCs collected conventionally (WB) showed the best results irrespective of the additive solution. Thus, we conclude that this simplified method is a feasible, time-sparing, and user-friendly alternative to the established method. Additionally, based on the results of this evaluation, the modified procedure as well as the medical device equipment has been CE approved (Declaration of Conformity of Haemonetics Products 93/432EG). To what extent the length of subzero storage influences the quality of deglycerolized RBCs treated with this modified method has yet to be investigated in future studies.

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