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13. ABSTRACT *(Maximum 200 Words)*

The Bionetics Corporation staffed and maintained laboratories to support red blood cell preservation and pathogen inactivation research for the Blood Research Detachment and Department, Walter Reed Army Institute of Research, Building 503, Walter Reed Forest Glen Annex, Silver Spring, MD 20910. Contract staff completed three *in vitro* trials with another three are in progress at the conclusion of the reporting period. The three completed trials evaluated the effects on red cell storage of 1) incremental changes in salt and 2) phosphate content of the storage solution and the 3) incremental pH adjustment of the anticoagulant into which the blood was collected. The results of the three trials indicate potential for optimizing existing storage solutions. Two of three trials in progress are evaluations of separate pathogen inactivation processes. The first is to measure the effects of treatment on red cell storage; the second is to determine the ability of riboflavin to inactivate *Plasmodium falciparum* in red cells. The third trial in progress is designed to provide data on a process improvement for the processing of frozen red cells. Data from our laboratory resulted in four published manuscripts, a fifth has been accepted. The Bionetics Corporation advanced the WRAIR Blood Research mission.

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21 September 2000 - 20 September 2001

INTRODUCTION

Nature of the Problem:

Because combat is synonymous with bloodshed and blood replacement saves lives, the US Army Medical Research and Materiel Command maintains facilities and programs to develop improved blood products.

The Background of the Previous Work:

The US Army has, for decades, conducted research in red blood cell preservation and the production of acellular hemoglobin solutions for use in combat casualty care. From 1974 through 1992, that research took place at the Letterman Army Institute of Research (LAIR) located at the Presidio in California. The LAIR facility was closed as the result of Base Realignment and Closure actions and the Blood Research Detachment was relocated to leased laboratory space at 1413 Research Boulevard, Rockville, Maryland. On 19 September 1994 The Bionetics Corporation (TBC) was awarded a contract to operate and maintain equipment and provide technical support to the Blood Research Detachment (BRD). For the first two years of the contract, the contractors supported the Hemoglobin Production Facility (HPF) and an integral analytical chemistry laboratory that provided quality control and characterization testing. The HPF produced several hundred liters of a high-purity hemoglobin-based blood substitute material for research. The acellular hemoglobin solutions manufactured by the HPF were based on biochemical modification of stroma-free hemoglobin as described in the literature. [1,2,3,4] A detailed description of the hemoglobin solution production process and process improvements is contained in a manuscript, published in October 1997 by the journal "Biologics," the journal of the International Association of Biological Standardization.[5] During the last several months of HPF operation, the contract staff also supported the US Navy lipisome encapsulated hemoglobin research.[6,7] HPF-related activities were terminated on 20 September 1996 and the facility

closed. The production facility equipment was subsequently either turned-in for disposal or transferred to other Institute activities.

Contract staff has continuously operated and maintained the blood cell preservation research of the Blood Storage Laboratory (BSL), a fully equipped red cell research laboratory currently staffed by three full-time and two part-time employees. One of the Blood Banking Specialist members of this team is also the Project Manager of the contract and the contract-designated Principle Investigator holds the specialty certification in Blood Banking. A list of contract staff as of the close of the FY is included in Appendix 1.

The current Food and Drug Administration (FDA) licensed anticoagulant, preservative solutions allow storage of red blood cells at 4°C for 42 days after collection. Work by Meryman *et. al.*[8,9] and Greenwalt, Dumaswala and colleagues 1,11[10,11] indicated potential for extended storage. Greenwalt and colleagues have recently developed an experimental additive solution, which preserves red cells for 56 days with average red cell survival of at least 75%.[12] Subsequent modifications have yielded greater than 75% 24-hour post transfusion survival after 10 weeks of storage.[13] Collaborative research with Greenwalt and colleagues have led to the development of a solution allowing 11 weeks of liquid storage.[14]

The laboratory extended its research from the preservation of red cells stored exclusively at refrigerator temperatures (2 - 6°C) to the preservation of red cells after frozen storage. Red cells destined for frozen storage are initially refrigerated for a few days, ordinarily up to 6 days, before they are mixed with the cryoprotectant glycerol and then stored in a freezer (-65° C or colder) for up to 10 years. Before those red cells can be transfused, they must be thawed and glycerol removed. Currently available systems for the processing of frozen blood limit the post-thaw storage to 24 hours. The 24-hour limit is imposed because the current systems are open as defined by the FDA and therefore vulnerable to bacterial contamination. Storage beyond 24 hours would allow the proliferation of the contaminating bacteria to levels that would harm the recipient. Under development are two systems that would allow post thaw storage beyond 24 hours because the fluid paths are closed to potential bacterial contamination. Therefore, the limitation on the post thaw storage is limited by the survival of the cells after transfusion. Candidate systems are designed to allow two weeks of post-thaw storage, an enhancement that would greatly expand the utility of frozen red cells for military and civilian uses. Our laboratory

was one of collaborating laboratories, which provided data used by the Food and Drug Administration to clear one of those instruments for use in the United States; that device is the Haemonetics ACP215. The data was also published in a peer-reviewed journal.[15]

The Purpose of the Present Work:

The BRD supports the Military Blood Program by providing data that will evaluate the safety, effectiveness, and practicality of new products or procedures related to the collection, processing, safety and distribution of red blood cells. Fiscal year 2001 was a period of transition for the laboratory. The BSL continued to evaluate the effectiveness of candidate red blood cell anticoagulant preservative systems and storage strategies along with their potential for further development. In addition, pathogen inactivation became a core-funded program in FY01; consequently, two pathogen inactivation projects were initiated. The first was a collaborative effort with Cerus Corporation to perform an initial evaluation of a pathogen-inactivating compound, S303, on red cell storage and quality. The second was to evaluate the ability of the Riboflavin Pathogen Eradication technology of Gambro BCT to inactivate malaria in red cells. The later research was performed under the terms of a recently negotiated CRADA.

Organization of Report:

During this reporting period, the BSL has performed research under a phlebotomy protocol, which permits collection of blood from volunteers for *in vitro* research projects. Three of those *in vitro* projects are related to red cell preservation, two are pathogen inactivation related and the sixth to frozen red cell deglycerolization process improvement. The initiation of an already approved clinical trial awaits the completion of the *in vitro* evaluation of a second device for closed system processing of frozen blood.

The BACKGROUND, METHODS, RESULTS, and DISCUSSION / CONCLUSION from each research effort will be described separately in order to maximize continuity. A short description of the status of each project begins the section of each protocol. If a manuscript has either been submitted for publication or published, the abstract from the manuscript is reproduced in the body of the report and the entire manuscript or copy of the published journal article is added to the report as an appendix.

The phlebotomy type protocol activities will be described first, followed by description of a clinical trial, completed in the previous fiscal years, but for which results were published in the current period. The report continues with descriptions and data from the three completed *in vitro* red cell preservation protocols, followed by descriptions of the two in-progress pathogen inactivation and the single frozen blood-processing studies. The report is concluded by abstracts and descriptions of two published manuscripts and two manuscripts accepted for publication.

Description of research conducted:

A. "Phlebotomy Procedures for Use on Human Subjects" WRAIR #776.

BACKGROUND

Aspects of red blood cell physiology critical to blood storage are species specific; therefore, valid *in vitro* studies of the red blood cell storage lesion require freshly collected human blood. The quantities required range from as little as 3.0 mL to as much as a full unit, 450 or 500 mL.

METHODS

Volunteers are recruited from within the Department, other tenants of Building 503, Forest Glen, Walter Reed Army Medical Center (WRAMC) and the surrounding community. Potential volunteers were informed fully as to the risks of donation and screened for anemia, transfusion transmitted diseases (TTD) and medical conditions that would make blood donation unsafe using the criteria of the American Association of Blood Banks[16] and the Food and Drug Administration 21CFR640.[17] The total amount of blood collected in an 8-week period is limited to 550 mL. A physician certified in Advance Cardiac Life Support was present at all full unit phlebotomies. Volunteers are compensated for their blood donations IAW 24 USC 30 and AR 40-2.

Three protocol addenda were approved, two in support of investigators outside the Department of Blood Research and a third addendum to add another investigator from the Department of Blood Research to receive blood for a specific project not specified in the original protocol. The first addendum allows the monitoring of physiological data from up to 200 volunteers with the LSTAT. This addendum adds Dr. Fred Pearce as a co-investigator. The blood, collected while the volunteer's blood pressure, pulse, etc. is being monitored, is used for the purposes specified in the WRAIR #776 protocol; Dr. Pearce only obtains physiological data.

The second addendum expanded the purpose of blood drawing to include up to 200 more volunteers for the integrated product team pyridostygmine project of the biochemical defense program. The modification added Drs. Shawn Feaster, Gregory Garcia, and Richard K. Gordon as co-investigators for the purpose of receiving the blood. The third addendum added Dr. Irina Balkaltcheva as co-investigator for the purpose of collecting blood for the Blood Research freeze-dried blood research program.

A personal computer database is used to maintain documentation of all volunteer-related transactions and assure compliance with donation volume and interval limitations. Phlebotomies were performed by trained contract staff and selected, trained active duty personnel.

RESULTS:

During fiscal year 2001, 96 volunteers made 278 blood donations in amounts as described in the following table.

Table: Blood Collections for FY 2001

a.	1 – 50 mL	122
b.	51 – 100 mL	27
c.	101 – 200 mL	5
d.	201 – 400 mL	0
e.	Units (450 or 500 mL)	134
	Total	278

Sixty-eight volunteers were enrolled into the protocol in fiscal year 2001. Twenty-one volunteers were placed on inactive status from the predecessor protocol, WRAIR #514, and four additional volunteers were permanently deferred because of a disqualifying condition. Volunteers were placed on inactive status either because they had moved from the area and were no longer available or are medically disqualified. One hundred eleven volunteers remain active in the protocol.

One confirmed positive transfusion-transmitted disease marker test was identified, a positive for anti-hepatitis B core antibody. This individual has been excluded from further blood

donation and is one of the four identified in the previous paragraph as being permanently deferred.

Physiological monitoring using the LSTAT was conducted simultaneously during collection of unit donations from 13 volunteers. Twenty-one donations from eight volunteers were in support of the Military Casualty Research freeze-dried blood program. Those 21 donations are included in the total donations detailed in the preceding table.

No side effects or reactions were observed other than the commonly encountered donor reactions, i.e., lightheadedness, syncope, tachycardia or bruising at the phlebotomy site, which occur occasionally during blood donation. The staff employed the procedures specified in the protocol to deal with the reactions; there were no sequelae.

DISCUSSION / CONCLUSION

The requirements of Blood Research for human blood were safely provided from a pool of healthy, screened volunteers.

B. "Evaluation of *in vitro* Storage of Human Red Blood Cells after Sterile Frozen Storage, Processing on the Haemonetics 215 and Two Weeks of Liquid Storage in AS-3" WRAIR # 714.

and

C. "Evaluation of *in vivo* viability of human red blood cells after sterile frozen storage and processing on the Haemonetics 215 and 2-week liquid storage in AS-3". WRAIR #715, Log # A-8654.

STATUS:

These two protocols were completed and fulfilled our obligations, which are part of a Memorandum of Understanding between the Office of Naval Research and WRAIR. WRAIR was but a part of a multi-center study to provide data for the eventual licensure of this experimental system. Four other sites are performing *in vitro* evaluations similar to the protocol #714 and one other site is performing red cell survival studies on Haemonetics 215 processed red cells, similar to protocol #715.

All testing except the 24-hour post-transfusion red cell survival measurements was performed at the Naval Blood Research Laboratory (NBRL) in Boston, MA. Locally, volunteers were recruited, blood drawn and processed with the Haemonetics instrument, stored either in the frozen or liquid state for the prescribed time, and samples collected for shipment to NBRL. A manuscript describing the results of the evaluation from all participating sites was published in the journal TRANSFUSION.

The Haemonetics Corporation has prepared and submitted a 510K application for Food and Drug Administration clearance for use. The FDA granted clearance for use the summer 2001.

ABSTRACT

Background: The FDA has approved the storage of frozen RBCs at -80°C for 10 years. After deglycerolization, the RBCs can be stored at 4°C for no more than 24 hours, because open systems are currently being used. Five laboratories have been evaluating an automated functionally closed system (ACP 215, Haemonetics) for both the glycerolization and deglycerolization processes.

Study Design and Methods: Studies were performed at three military sites and two civilian sites. Each site performed *in vitro* testing of 20 units of RBCs. In addition, on military and two civilian sites conducted autologous transfusion studies on then units of previously frozen, deglycerolized RBCs that had been stored at 4°C in AS-3 for 15 days after deglycerolization and once with liquid-preserved RBCs that had been stored at 4°C in AS-1 for 42 days. At one of the civilian sites, 10 volunteers received autologous transfusions on two occasions in a randomized manner, once with previously frozen RBCs that had been stored at 4°C in AS-3 for 15 days after deglycerolization and once with liquid-preserved RBCs that had been stored at 4°C in AS-1 for 42 days.

Results: The mean ± SD *in vitro* freeze-thaw-wash recovery value was 87 ± 5 percent; the mean ± SD supernatant osmolality on the day of deglycerolization was 297 ± 5 mOsm ker kg of H_20 and the mean ± SD percentage of hemolysis after storage at 4°C in AS-3 for 15 days was 0.60 ± 0.2 percent. The paired data from the study of 10 persons at the civilian site showed a mean ± SD 24-hour posttransfusion survival of 76 ± b percent for RBCs stored at 4°C for 15 days after deglycerolization and 72 ± 5 percent for RBCs stored at 4°C in AS-1 for 42 days. At the three

sites at which 24-hour posttransfusion survival values were measured by three double-label procedures, a mean ± SD 24-hour posttransfusion survival of 77 ± 9 percent was observed for 36 autologous transfusions to 12 females and 24 males of previously frozen RBCs that had been stored at 4°C in AS-3 for 15 days after deglycerolization.

Conclusion: The multicenter study showed the acceptable quality of RBCs that were glycerolized and deglycerolized in the automated ACP 215 instrument and stored in AS-3 at 4°C for 15 days.

BACKGROUND / METHODS / RESULTS / DISCUSSION / CONCLUSION

A copy of the published manuscript describing the background, methods, results, discussion and conclusion is attached to this report as Appendix 2. The data from our laboratory is not listed separately but included in the aggregate data from the other four sites for the *in vitro* studies and the other site performing the red cell survival studies.

D. "Evaluation of *in vivo* viability of Human Red Blood Cells After Sterile Storage, Processing on the Mission Medical FBPS and 2 Weeks of Liquid Storage in AS-3" WRAIR # 817, HSRRB Log #A - 10159.

STATUS

The Frozen Blood Processing System (FBPS) has been developed by Mission Medical Incorporated (MMI) under an MRMC SBIR initiative. Prototype instruments and disposables have been manufactured and tested extensively at MMI, onboard US Navy vessels and at the Institute. The FBPS is designed to perform the same essential functions as the Haemonetics 215 (ACP 215), evaluated previously and described in a preceding section of this report.

This protocol is still pending. Before the red cell survival studies can be initiated, the *in vitro* evaluation must be completed. That evaluation will begin after MMI has validated the software controlling instrument function and final details of the pre and post-glycerolization blood processing are established.

Since the initial writing of the protocol, we have been instructed by the Armed Service Blood Program Office (ASBPO) to switch the blood collected from AS-3 to AS-5 collection systems

and change the collection volume from 450 to 500mL. The rational for switching collection systems is based on the relative costs of the two systems, AS-5 is less expensive, and the general preference among phlebotomists at the military donor centers for the phlebotomy needles incorporated into the AS-5 collection sets. The FDA licenses both systems for 42 days of liquid storage. The change in volume is made in order to compensate for loss of red cells resulting from leukoreduction filtration and glycerolization, deglycerolization processing. The goal is to provide a frozen red cell product with the same red cell mass as would be provided with a liquid stored unit of blood. Both 450 and 500 mL collections are allowed in FDA, American Association of Blood Banks and DoD guidelines.

BACKGROUND

The preparation of a unit of blood for frozen storage and eventual transfusion involves a series of related processes. Red cells destined for frozen storage are initially refrigerated for a few days, ordinarily up to 6 days, before they are mixed with the cryoprotectant glycerol and then stored in a freezer -65°C or colder for up to 10 years. Before those red cells can be transfused, they must be thawed and glycerol removed. Currently available systems for the processing of frozen blood limit the post thaw storage to 24 hours. The 24-hour limit is imposed because the current systems are open as defined by the FDA and therefore vulnerable to bacterial contamination. Storage beyond 24 hours would allow the proliferation of the contaminating bacteria to levels, which would harm the recipient. The FBPS would allow post thaw storage beyond 24 hours because the fluid paths are closed to potential bacterial contamination. Therefore, the limitation on the post thaw storage is limited by the survival of the cells after transfusion. Hess and colleagues have demonstrated successful three-week storage followed by greater than 75% survival of the red cells 24 hours after transfusion into autologous recipients. Two weeks of post thaw storage was chosen for the evaluation of the FBPS, which is the same end point for the MDL 215 evaluation.

The FBPS differs from the MDL 215 in two significant ways. First, the FBPS provides a higher level of automation that should serve to allow the operation of more instruments, thereby extending the ability of a technician to process more units of blood faster when needed. The FBPS also utilizes a different technology to remove the glycerol. The MDL 215 utilizes

centrifugal separation of the liquid glycerol and the red cells for glycerol removal. The FBPS utilizes dialysis by incorporating hollow fiber filters similar to those used for renal hemodialysis. The hollow fiber filters retain the red cells; the glycerol and wash solution are discarded in the filter effluent. The primary advantage of the dialysis-based separation is the elimination of the internal motor required to drive the centrifuge required for centrifugal separation. The enhanced level of automation, simplicity of design combined with two weeks of shelf life for the processed blood would greatly expand the utility of frozen red cells for military and civilian uses.

RESEARCH PLAN

We will recruit approximately 15 volunteers (permission to enroll up to 20 is requested) with the expectation of getting a minimum of 10 of them through the protocol. Each volunteer's participation will last a minimum of ten weeks in order to complete two red cell survival studies. Recruited volunteers will be screened and, if acceptable, will donate a full unit of blood as described in WRAIR Protocol #776, "The Repeated Use of Small Volumes of Purchased Human Blood in Laboratory Tests, Instrument Calibration and In Vitro Experiments." The blood will be glycerolized using the automated procedure on the Mission Medical FBPS. The glycerolized blood will be frozen at -65°C or colder for at least a month. Two weeks before the scheduled day of reinfusion the unit will be thawed and deglycerolized using the automated procedure on the Mission Medical FBPS. The cells will then be stored at 4°C for 14 days in a conventional blood bank refrigerator. On the fifteenth day of liquid storage, the stored cells along with 6 mL of fresh cells drawn from the same volunteer will be radio-labeled and reinfused to measure the recovery and survival of autologous human erythrocytes *in vivo*.

Blood units will be stored as above before *in vivo* survival testing.[18] Three to five days before a reinfusion, the blood will be inspected and cultured for bacterial contamination. On the day of the reinfusion, the bacterial cultures will be confirmed to be negative before the unit is gently mixed and the post-storage sample taken for *in vitro* testing and radioisotope labeling. This procedure has two basic parts that are performed simultaneously. In the first, fresh red cells are radio labeled with ^{99m}Tc, which has a half-life of only 6 hours. These are injected followed by rapid, timed sampling and radiation counting to accurately measure the blood volume. Gamma emissions from ^{99m}Tc labeled cells are essentially undetectable 24 hours after injection.

In the second part, autologous stored red cells are radio labeled with ^{51}Cr, which has a half-life of 27.7 days. The ^{51}Cr labeled red cells are injected and their survival is tracked for two weeks by analyzing timed blood samples for gamma emissions. The mean 24-hour red cell survival for all the volunteers must exceed 75% for the storage system to be considered effective by the FDA. The FDA also requires the red cell survival studies be repeated at another laboratory with another set of volunteers. Therefore, the same evaluation will be conducted at another laboratory chosen by MMI. The FDA will combine the results for a 510k submission and eventual clearance for clinical use.

The clinical trial will be preceded by an *in vitro* evaluation involving 20 units of blood, half of which will have the white cells removed by leukoreduction filtration (LR). The *in vitro* phase of the evaluation is designed to assess the quality of the red cells as assessed with an array of biochemical and hematological testing. The more important parameters being assessed are maintenance of red cell ATP and level of hemolysis. The effect of LR will be measured by comparing the results of the biochemical and hematological testing when blood is collected with and without filtration. Based on previous research from our laboratory, hemolysis is expected to be lower in the filtered cells. [19]

It is important to evaluate the effect of leukoreduction because current practice is mixed. Furthermore, white blood cells and cell fragments are retained during processing with the FBPS; whereas, white blood cells are removed with the supernatant during processing with the centrifugal-based systems. If as expected the LR product will have lower hemolysis, it is important to have data determine if processing with the FBPS is sufficiently robust to tolerate processing with and without LR.

E. "Red Cell Storage in EAS-76 after Collection in Unmodified CPD, pH 5.5, and pH Modified CPD: pH 6.5, 7.5 and 8.5 " Short Title: pH Modified CPD

STATUS

This protocol has been completed.

BACKGROUND

Data from previous *in vitro* protocols completed in our laboratory have shown the importance of pH in red cell preservation. All but one of those protocols have attempted to examine the role of pH in the additive storage solutions. Furthermore, data from two of those studies indicate the pH of the solution into which the blood is collected is crucial and that pH changes can occur with relative quickness in the interval between collection and addition of the additive storage solution to the red cells. In order to examine the relative effects of pH during the interval between collection of the blood and addition of the additive solution, it was necessary to create CPD variants with graduated pH and sample more frequently early in that interval. Second, in order to retain the discrimination power of the pooled studies, it was necessary to initially collect the blood in a pH "neutral" anticoagulant (heparin) before pooling.

Data from another completed protocol, in which blood was collected into both unmodified (pH 5.5) and a pH modified (pH 8.6) variant of CPD, gave somewhat surprising ATP results. Ordinarily, the ATP levels rise during the first 1-3 weeks after the addition of the adenine-containing additive solution. In that experiment, the ATP levels never rose, and in the pH modified CPD, the ATP dropped by nearly one half by day 7. Because the DPG levels were maintained for more than two weeks, one interpretation of the data is that the DPG was maintained at the expense of ATP production. It is likely that phosphate is the limiting ingredient if the pH is favorable because phosphate is known to traverse the red cell membrane slowly.

This protocol was built on the hypothesis that the intracellular phosphate becomes the limiting factor when intracellular pH favors both ATP production and DPG maintenance. Furthermore, the intracellular pH determines which predominates; pH above 7.2 favors 2,3 DPG synthesis over lactate production. Therefore, intracellular phosphate and pH determinations have been added to the testing array for this protocol. The degree to which ATP production or DPG maintenance is favored are expected to be revealed, as the pH of CPD in the initial collection is varied step-wise from 5.5 to 8.5 in approximate 1.0 pH unit increments.

METHODS

Volunteers were recruited under the provisions of WRAIR protocol #776, "The Repeated Use of Small Volumes of Purchased Human Blood in Laboratory Tests, Instrument Calibration and *In Vitro* Experiments." Each volunteer donated one unit. This protocol utilized the previously performed pooling and aliquoting scheme but with one significant variation. The major change was in the initial collection. Instead of collecting the blood directly into CPD or a CPD variant, the blood was initially collected into heparin. Only later, after the whole blood had been leukodepleted, pooled and aliquoted, was CPD or the CPD variants added to the red cells. Heparin is a pH "neutral" anticoagulant and is not expected to influence the pH for the period between collection and addition of the CPD or CPD variants.

Twenty-four units of blood were collected into heparin, sampled for pre-leukodepletion white cell counts, and the white cells depleted using the Fenwal Sepacell® leukoreduction filter and pooled into a 2 L transfer bag. After sampling, four aliquots were created from the pool and 63 mL of either unmodified CPD or one of the three variants added to one of the aliquots from each pool. The units were held at room temperature for 2 hours before packing to a 75% hematocrit. Each aliquot was sampled, 300 mL of EAS-76 added to all aliquots, the contents mixed and sampled again immediately. The cells with EAS-76 added remained at room temperature for two hours and sampled again before placing them in the refrigerator. Thereafter, all aliquots were sampled weekly for 12 weeks.

Samples were analyzed for CBC, blood gas analysis (BGA), centrifuged hematocrit, red cell morphology, supernatant hemoglobin, sodium, potassium, and phosphate, in addition to whole blood ATP, glucose and lactate. Percent hemolysis was calculated for the supernatant and total hemoglobin content. Two additional tests, not part of the routine repertoire, were performed on selected samples. 1) In addition to the phosphate testing of the supernatant, a phosphate was performed on a sample of deproteinized intracellular fluid (deproteinized packed cells). 2) An intracellular pH determination on supernatant from a freeze-thawed hemolysate of washed cells. The calculated bicarbonate from the BGA was recorded and included in the data analysis.

The composition of the standard CPD and the alkalinized CPD pH 8.5 - 8.7 was as follows.

Composition of Unmodified and Alkalinized CPD

Constituent	CPD (g/L)	PH 5.5 MM	CPD (g/L)	PH 8.5 MM
Trisodium Citrate $2H_2O$	26.349	11	29.333	123
Citric Acid·H_2O	3.27	123	0	0
Dextrose·H_2O	25.556	130	25.556	130
Monosodium Phosphate·H_2O	2.222	16	0	0
Disodium Phosphate·$7H_2O$	0	0	4.25	16

Mixing the unmodified and alkaline CPD creates the pH 6.5 and pH 7.5 CPD solutions. The pH 6.5 solution consisted of 300 mL unmodified and 600 mL of alkaline CPD; the pH 7.5 solution consisted of 50 mL unmodified and 950 mL of alkaline CPD.

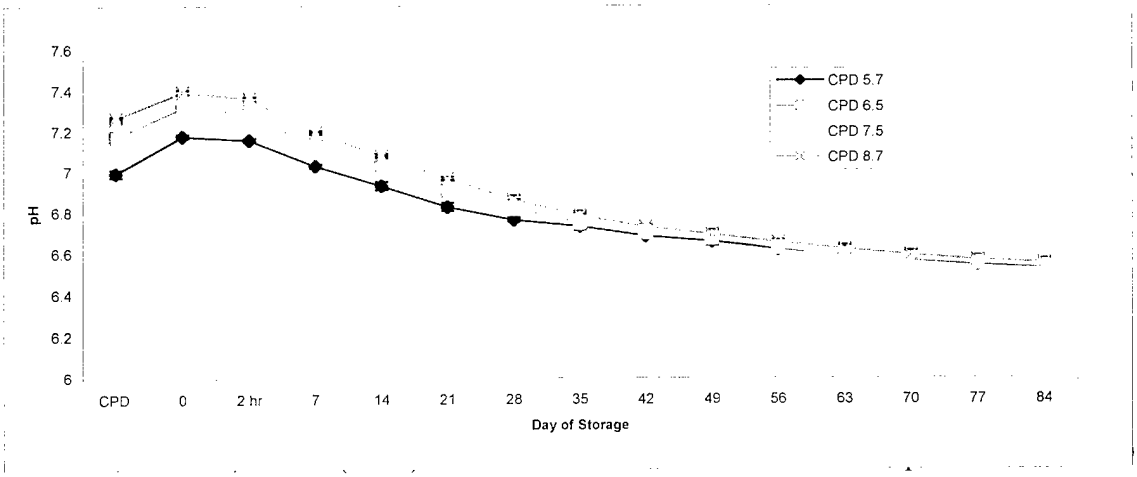
Composition of EAS-76:

Chemical	mM	g/L
NaCl	45	2.63
Adenine	2	0.27
Dextrose	50	9.008
Mannitol	30	5.465
Na_2HPO_4	9	2.413
$NaHCO_3$	30	2.52

RESULTS:

The pH of the CPD collected blood and the blood after the addition of the EAS-76 and through the first 1 - 2 weeks of storage was lower in the unmodified CPD (pH 5.7) and was higher in the pH modified CPD collected units as expected. However, the data from the blood collected in the modified CPD pH 7.5 and 8.7 were nearly identical for both the initially collected blood and after the addition of EAS-76. After 14 days of storage the pH results from the blood collected into any of pH modified CPD was indistinguishable and by day 35 of storage the pH of all the blood was nearly identical. The blood pH data are presented below in Figure 1. The intracellular pH data was unremarkable data is not shown.

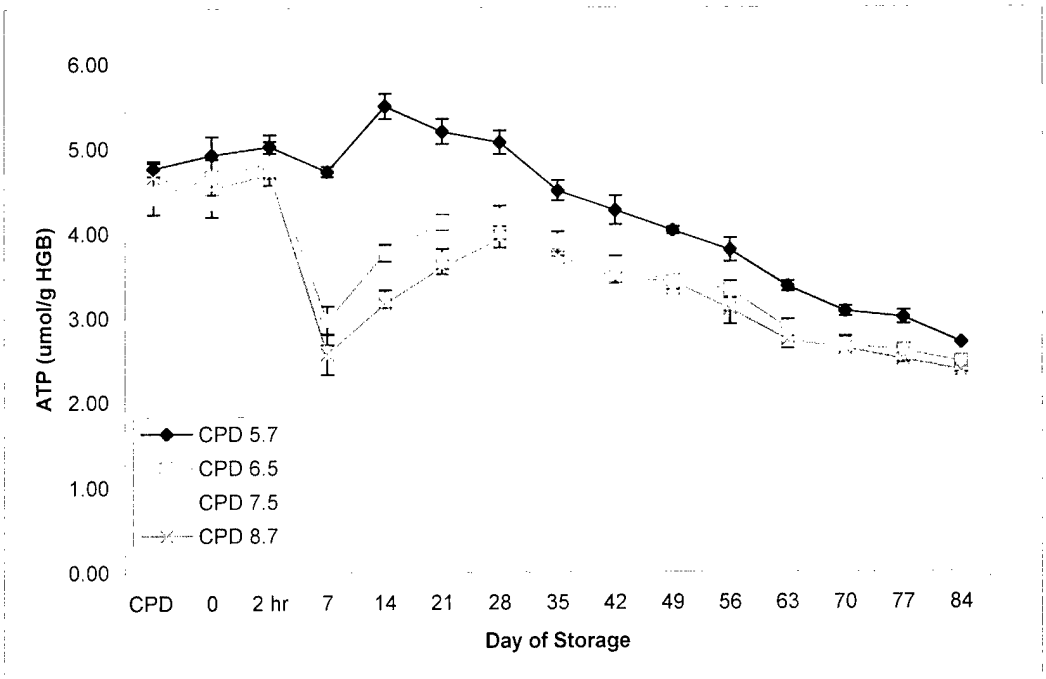
Figure 1:



diverged from those of the unmodified CPD after one week of storage. ATP levels fell sharply after more than 2 hours of storage rather than rising gradually during the first two weeks, as was the case with the unmodified CPD blood. Conversely, DPG levels were maintained and even increased through fourteen days of storage in the pH modified CPD blood, thereafter falling dramatically. The DPG of the unmodified CPD blood, which began falling after two hours of storage, fell to nearly half of the original by one week and to approximately one third of original by two weeks of storage. By five weeks of storage, the DPG levels on all blood were nearly identical. The ATP and DPG data are presented graphically in Figures 2 and 3. It is important to note DPG was assayed for only six weeks of storage whereas ATP was assayed for the entire twelve weeks; DPG testing was terminated when the values in all treatment groups approached undetectable levels.

By two hours of storage, the intracellular phosphate levels of the unmodified CPD blood rose to higher levels than those for the pH modified CPD blood and remained so until three weeks of storage. As a group, the intracellular phosphates from the pH modified CPD were indistinguishable throughout ten weeks of storage. The intracellular phosphate data is presented graphically in Figure 4.

Figure 2:



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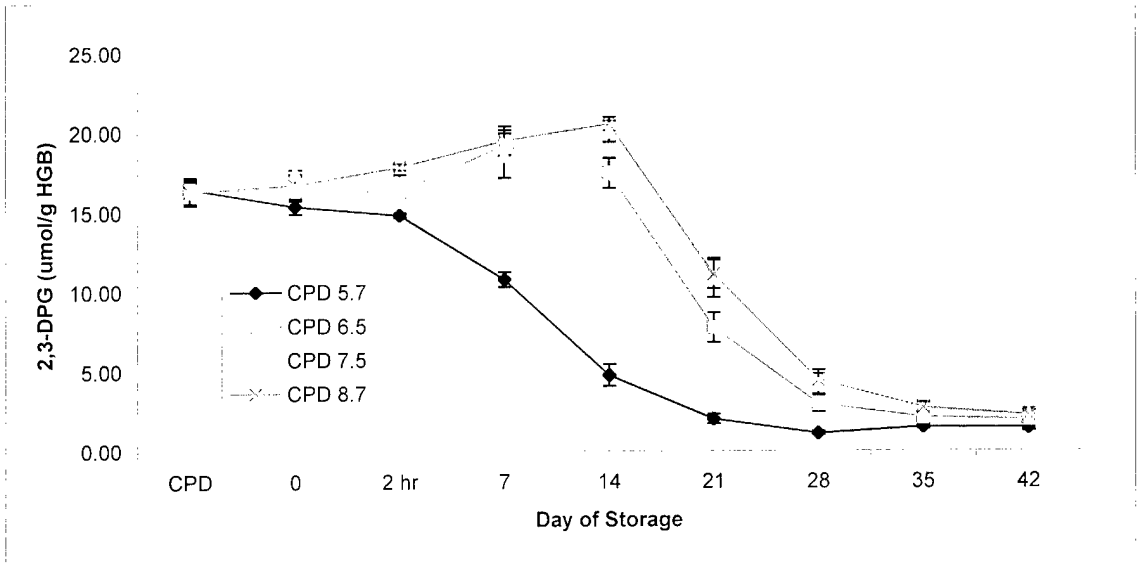
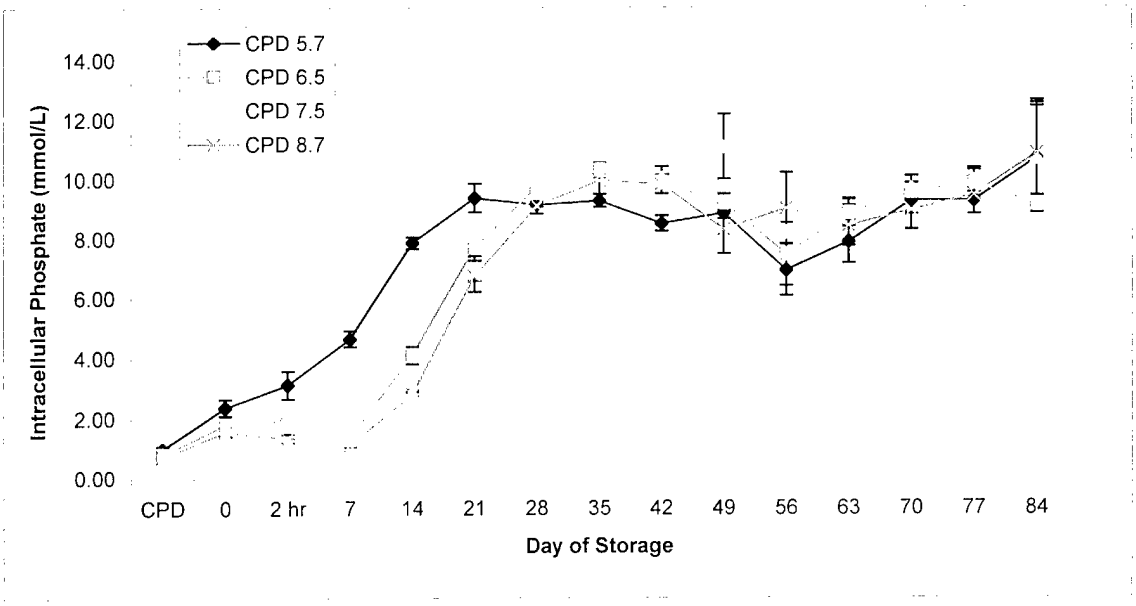


Figure 4:



DISCUSSION:

The single most remarkable observation from the pH data is the degree to which the results from the blood collected in either the pH 7.5 and 8.7 modified CPD correspond and remain nearly identical throughout the twelve-week storage period. This is a curious observation for which there is no ready explanation. However, a similar scheme emerged with both the ATP and DPG results with three relatively distinct patterns, one for the blood collected into unmodified CPD, a second for the CPD pH 6.5, and a third for the CPD pH 75 and 8.7 combined.

The divergence of the ATP and DPG values as the pH increases confirms the observations of others. The metabolism of phosphate during glycolysis proceeds in one of two directions; they are the production of DPG and conversion of ADP to ATP. Higher pH favors DPG production. The practical implication of these results is the ability to retain the more normal oxygen-hemoglobin dissociation for a longer period with the use of the appropriate anticoagulant and additive solutions. The degree in which the post-transfusion survival is compromised, presumably because of lower ATP production, is unanswered.

The divergent intracellular phosphate results between the blood collected in the unmodified CPD versus blood collected in the pH modified CPD during the first three weeks of

storage are explained by one of two general mechanisms. Either the influx of phosphate is favored by a lower pH or the accumulation of phosphate from the net synthesis versus breakdown of ATP and DPG is proceeding more rapidly in the higher pH. It is generally accepted that phosphate traverses the intact red cell membrane very slowly. The accumulated results provide no indication of which process predominates.

F. "Twelve-Week Red Blood Cell Storage in 300 mL of Salt Variants of Bicarbonate Containing EAS-76 after Collection in Unmodified CPD," Short Title: EAS-76 Salt Variants.

BACKGROUND:

Results from previous *in vitro* studies in which blood was stored in EAS-76 have shown increasing hemolysis as the storage time progresses beyond eight or nine weeks. All other measurements have given results that would indicate twelve-week storage is possible. Results from previous studies have indicated that as the tonicity of a solution drops, and as a consequence the mean cell volume (MCV) increases, the hemolysis decreases and, when studied, the amount of microvesicle formation drops. This relationship between salt content and hemolysis has been tested with the nine-week storage solution EAS-61, but not with the bicarbonate containing EAS-76. This protocol is specifically designed to determine if the same salt content to hemolysis relationship exists for EAS-76 and to test the limits to which salt content can be restricted without adversely affecting other measures of the storage lesion, particularly ATP content. The hypothesis for this study is that reduction of NaCl content in the additive solution EAS-76 will reduce hemolysis. The objective of the research was to determine the degree to which hemolysis is affected during twelve weeks of storage as the NaCl content is incrementally reduced in variants of the EAS-76 additive solution. The overall goal was to further develop a system, which will allow refrigerated red blood cells to be stored for 12 weeks or longer.

METHODS: Twenty-four units of blood were collected into unmodified CPD. The whole blood was white cell depleted using the Fenwal Sepacell® leukoreduction filter and packed to a target hematocrit of 75%. Four units with identical ABO were pooled and pool sampled. Three

hundred mL EAS-76 or one of the EAS-76 variants was added to all cells and stored at 1-6°C. Each of the aliquots was sampled on the day of preparation and weekly thereafter for 12 weeks. Samples were analyzed for CBC, blood gas analysis (BGA), centrifuged hematocrit, red cell morphology, supernatant hemoglobin, sodium, potassium, and phosphate, in addition to whole blood ATP, glucose and lactate. Percent hemolysis was calculated for the supernatant and total hemoglobin content. After 12 weeks of storage and the final sampling, microvesicles were harvested from the plasma for measurement of microvesicle protein.

The composition of the unmodified EAS-76 is as follows:

Chemical	**mM**	**g/L**
NaCl	45	2.63
Adenine	2	0.27
Dextrose	50	9.008
Mannitol	30	5.465
Na_2HPO_4	9	2.413
$NaHCO_3$	30	2.52

For each of the following three EAS-76 variants, NaCl was incrementally reduced; all other components were unchanged.

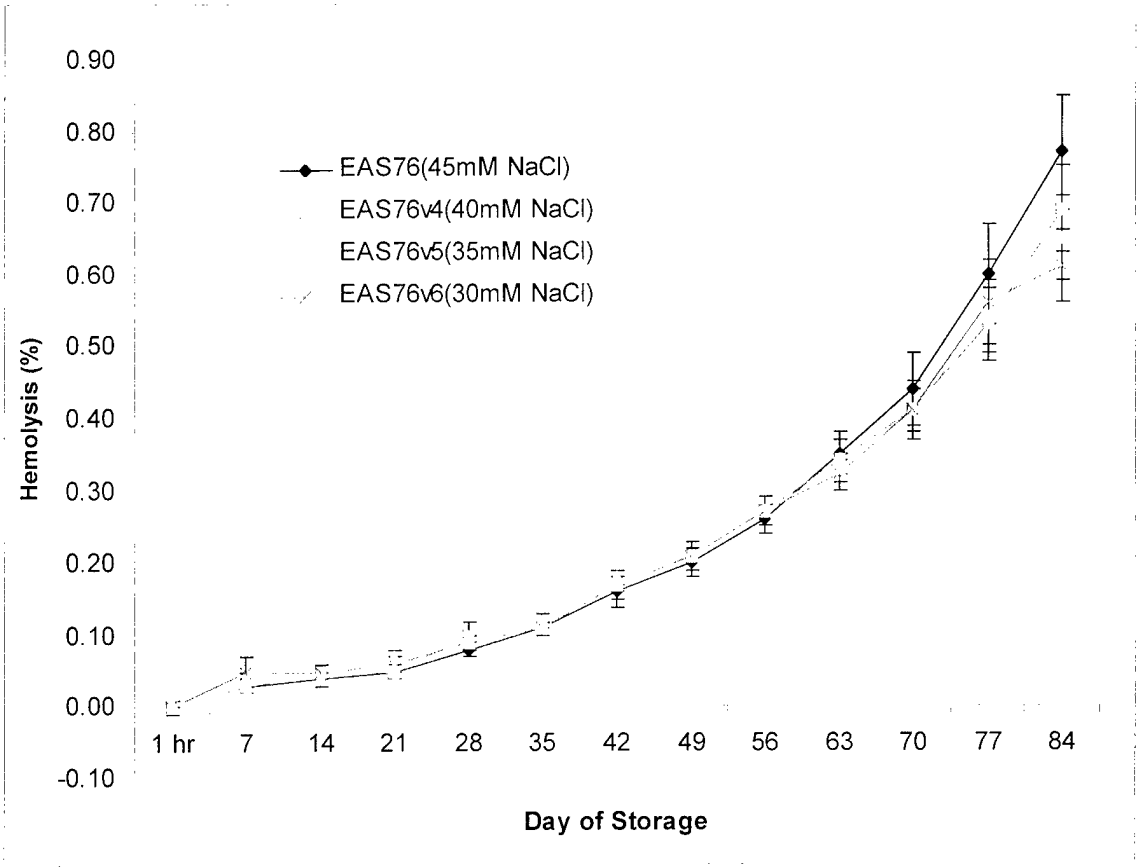
	Variant #4	**Variant #5**	**Variant #6**
Chemical	**mM**	**mM**	**mM**
NaCl	40	35	30
Adenine	2	2	2
Dextrose	50	50	50
Mannitol	30	30	30
Na_2HPO_4	9	9	9
$NaHCO_3$	30	30	30

RESULTS:

There were no measurable differences in hemolysis through nine weeks of storage. By twelve weeks perceptible, but not statistically significant, differences were apparent as shown on

the following figure. The solutions with the lower salt content produce less hemolysis in a step-wise fashion.

Figure 5:



Red cell vesiculation, measured as vesicle protein content at the end of the storage period, ranged from 5.93 +/- 0.63 (n=5), 6.45 +/- 1.17 (n=5), 7.25 +/- 0.99 (n=4) and 9.56 +/- 1.32 (n=5) mg/mL of the EAS-76-V5, V6, V4 and unmodified EAS-76 respectively. The ATP and 2,3-DPG levels were nearly identical throughout 12 weeks of storage (data not shown). The expected inverse relationship between MCV and salt content, were retained throughout the storage period (data not shown).

DISCUSSION:

As predicted by the hypothesis, total hemolysis at the end of storage was directly proportional to the salt content of the storage solution. Granted, the differences were subtle and not apparent until after at least ten weeks of storage. On the other hand, the amount of red cell vesiculation did not vary with salt content. Though not significantly different, the trend was for

greater vesiculation at the highest and lowest salt concentrations tested. The vesiculation data must be interpreted cautiously because of the inherent variability in the assay and the small number of samples.

CONCLUSION:

The formulation of the bicarbonate containing experimental additive solutions can be further optimized to reduce long-term hemolysis by reducing the sodium chloride content.

G. "Twelve-Week Red Blood Cell Storage in 300 mL of Phosphate Variants of Bicarbonate Containing EAS-76 after Collection in Alkaline CPD," Short Title: EAS-76 Phosphate Variants

BACKGROUND:

Preliminary results from the study described above, in which cells were stored in EAS-76 and the pH variants of CPD (approximate pH 5.5, 6.5, 7.5 and 8.5), indicate a profound shift from ATP production to sustained 2,3-DPG production. ATP concentration fell to half of initial levels by day seven of storage. Conversely, DPG levels were sustained for several weeks when the cells were initially stored in CPD 8.7, packed and stored in EAS-76, rather than falling to negligible levels within two weeks when cells are collected in standard formula CPD (pH 5.5) and stored in EAS-76 or any other additive solution. These observations raise a set of interrelated questions. 1) Is the intracellular supply of phosphate a limiting factor? 2) Will supplementing the additive with more phosphate change the kinetics of ATP production? 4) 2,3-DPG production? 5) Or, can both ATP and 2,3-DPG production be maintained if more phosphate is available in the additive solution?

This protocol was based on the hypothesis that by adding phosphate to the additive solution net production of ATP can be maintained and DPG synthesis can also be sustained. Adding phosphate will drive intracellular phosphate higher, making it available to support both. The objective was twofold. The first was to determine if ATP content at conclusion of storage in the phosphate variants of EAS-76 will be maintained at levels near or nearer those observed when the blood collected into unmodified CPD and stored in unmodified EAS-76. The second

was to simultaneously sustain 2,3-DPG production for 2 weeks or longer. The goal was to maintain 2,3-DPG levels without sacrificing ATP production in liquid stored red cells.

METHODS and MATERIALS:

Volunteers were recruited under the provisions of WRAIR protocol #776, "The Repeated Use of Small Volumes of Purchased Human Blood in Laboratory Tests, Instrument Calibration and In Vitro Experiments." Each volunteer donated one unit. Sixteen units of blood were collected into alkalinized CPD with an approximate pH 8.5. The composition of the modified CPD is detailed below. The whole blood was white cell depleted using the Fenwal Sepacell leukoreduction filter and packed to a target hematocrit of 75%. Four units with identical ABO were pooled and each pool sampled. Three hundred mL of EAS-76 or one of the EAS-76 variants was added to all cells and stored at 1 - 6°C. Each of the aliquots was sampled on the day of preparation and weekly thereafter for 12 weeks. Samples were analyzed for CBC, blood gas analysis (BGA), centrifuged hematocrit, red cell morphology, supernatant hemoglobin, sodium, potassium, and phosphate, in addition to whole blood ATP, glucose and lactate. Percent hemolysis was calculated for the supernatant and total hemoglobin content.

The composition of the standard CPD and the alkalinized CPD is as follows.

	CPD pH 5.5		**CPD pH 8.5**	
Constituent	(g/L)	mM	(g/L)	mM
Trisodium Citrate $2H_2O$	26.349	11	29.333	123
Citric Acid·H_2O	3.27	123	0	0
Dextrose·H_2O	25.556	130	25.556	130
Monosodium Phosphate·H_2O	2.222	16	0	0
Disodium Phosphate·$7H_2O$	0	0	4.25	16

The composition of the unmodified EAS-76 is as follows:

Chemical	**mM**	**g/L**
NaCl	45	2.63
Adenine	2	0.27
Dextrose	50	9.008
Mannitol	30	5.465
Na_2HPO_4	9	2.413
$NaHCO_3$	30	2.52

For each of the three variants, Na_2HPO_4 is either increased or eliminated and the mM equivalents of sodium are maintained by adjusting the NaCl content. Therefore, this set of phosphate variants of EAS-76 increases in phosphate content in the following order: Variant #1, unmodified EAS-76, Variant #2 and Variant #3.

Chemical	Variant #1 mM	EAS-76 mM	Variant #2 mM	Variant #3 mM
NaCl	63	45	27	9
Adenine	2	2	2	2
Dextrose	50	50	50	50
Mannitol	30	30	30	30
Na_2HPO_4	9	9	9	9
$NaHCO_3$	30	30	30	30

RESULTS:

Both ATP and 2,3-DPG content were better maintained as storage progressed when the phosphate content was increased. The data is shown graphically in the following figures 6 and 7 that follow. In both instances the difference among the variants was not apparent until two weeks of storage. However, once established, the differences remained through the remainder of the twelve weeks of storage.

Hemolysis differed little among the pools at each of the sampling points. The hemolysis in any of the samples was <1% through eleven weeks and did not exceed 1.25% in any sample at

twelve weeks of storage. Average hemolysis at twelve weeks was 0.74, 0.75, 0.69 and 0.67 % for the unmodified EAS-76, Variant #1, Variant #2 and Variant #3 respectively. A graph of hemolysis results is included at Figure 8.

Figure 6:

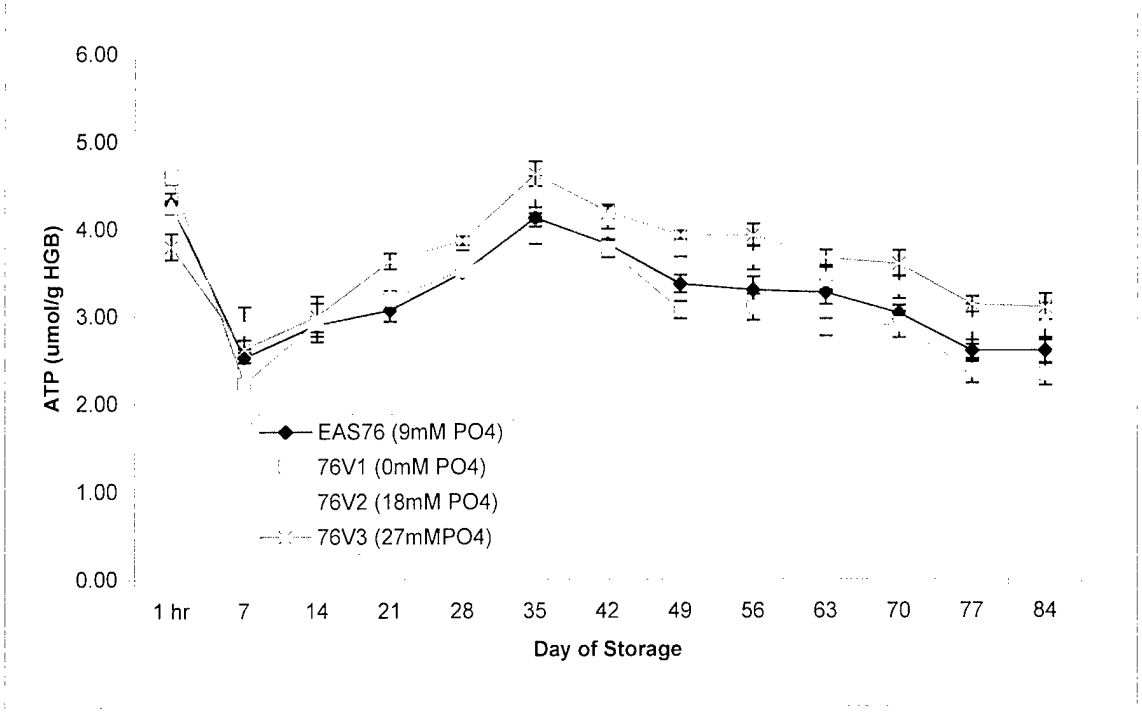


Figure 7:

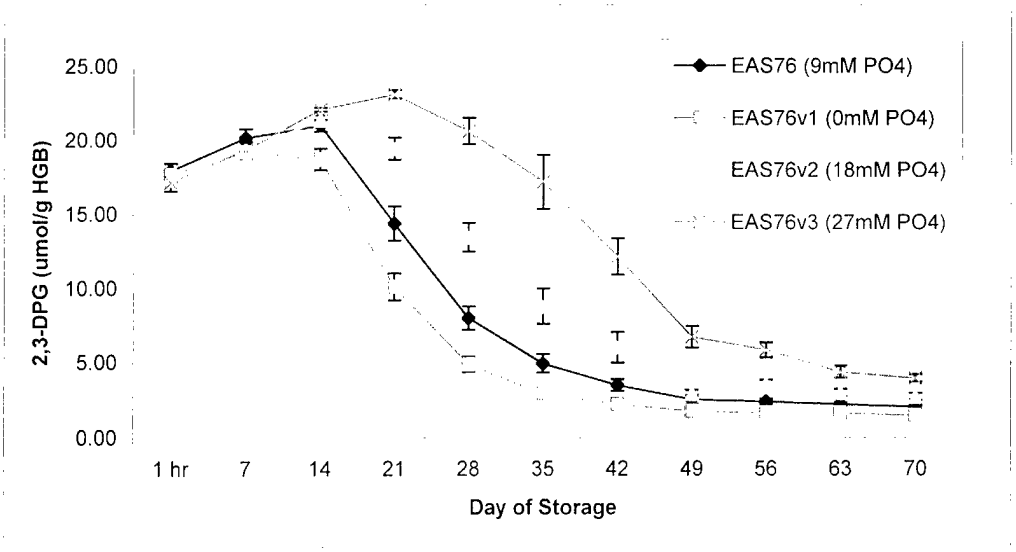
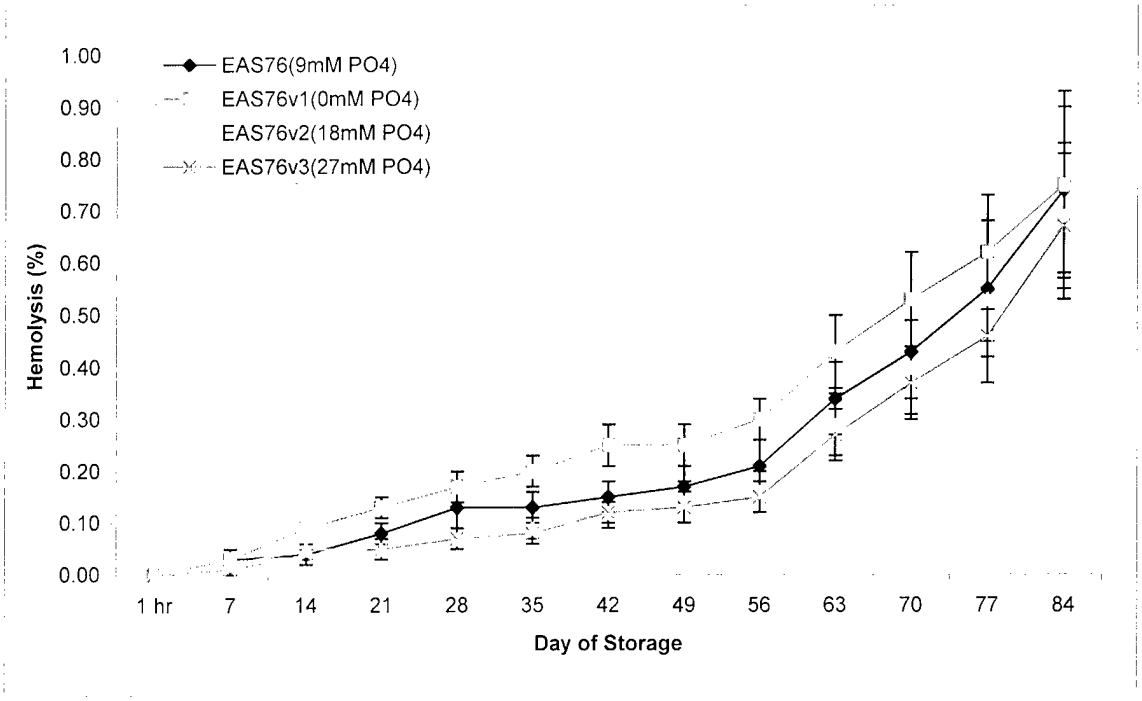


Figure 8:



For those variants with higher ATP and 2,3-DPG levels, glucose consumption and lactate production was higher after 2 weeks of storage (data not shown).

DISCUSSION:

DPG levels were sustained for four weeks and longer when phosphate levels were increased while simultaneously maintaining ATP levels. In the previous quarterly report, we reported the sustained DPG production at the apparent expense of ATP production when blood was collected into an alkalinized CPD but stored in unmodified EAS-76. The added phosphate allowed both to be maintained simultaneously though the differences were not apparent until at least 2 weeks of storage. The delay may, in part, be the result of relatively slow passage across the red cell membrane. No phosphate transport mechanisms have been described for red cell membranes, leaving a slow diffusion following a concentration gradient as the most plausible mechanism.

The increased glucose consumption and lactate production, which accompanied the higher ATP and DPG levels, indicate a more metabolically active red cell. It appears that both pH and phosphate play a role in DPG production as storage progress beyond 2 weeks.

CONCLUSION:

ATP and DPG can be maintained beyond 2 weeks of storage when the pH of the anticoagulant solution is increased and phosphate levels of the storage solution are maintained.

H. " New Techniques to Improve Safety and Availability of Blood in the Armed Services: in *vitro* Evaluation of Red Cells Treated With S303"

STATUS:

At the conclusion of the report period all 36 units to be evaluated had been received, and sampling through ten weeks of storage had been completed on 24. Sampling on the remaining 12 was completed on 10 October 2001. Completion of testing was pending.

BACKGROUND:

This protocol is a collaborative project with the Cerus Corporation of Concord California, and as outlined in Cooperative Agreement DAMD17-01-2-0007, titled "New Techniques to Improve Safety and Availability of Blood in the Armed Services, Project 2." This is but one of several blood safety initiatives being pursued jointly by WRAIR and Cerus Corporation, as specified in the cooperative agreement.

METHODS and MATERIALS:

Briefly, sets of seven units are collected by a Sacramento, California-based blood center and delivered to Cerus Corporation in Concord, CA for treatment with the S303 pathogen-inactivating agent. After leukoreduction filtration, the seven units are pooled and divided into six aliquots of 500 mL each. Each of the six aliquots was centrifuged to prepare packed cells and additive solution added. Two received Erythrosol; two received 100 mL of EAS-61 and the final two received 200 mL of EAS-61. One of each pair was treated with the S303 inactivation process, the test units, and the other of the pair received a sham treatment, the control units. The treatment consisted of a transfer to a reaction container, incubation at room temperature for 20 hours and then refrigerated. The units from each pool were then shipped to our laboratory to arrive some time before the seventh day following the day of collection along with samples collected pre and post treatment. After the units were received, they have been sampled weekly for ten weeks. Each of the samples is being assayed with the battery of tests used in previous red cell preservation studies.

RESULTS, DISCUSSION and CONCLUSION:

Completion of testing is pending.

I. "Inactivation of Pathogens with the Riboflavin-Based Pathogen Eradication Technology"

STATUS:

This is a report of research in progress.

BACKGROUND:

A cooperative research and development agreement (CRADA) has been negotiated between WRAIR and Gambro BCT, Inc. (Gambro). Blood safety is one of the Institute-funded core programs and Gambro is developing a pathogen inactivation process that is of interest to the Institute. The CRADA will provide access to the technology.

The initial research tests the pathogen inactivation process using whole blood deliberately infected with Plasmodium falciparum. The initial research will involve two additional WRAIR Divisions. A collaborator in Communicable Diseases and Immunology (CD&I), LTC Jack Williams, provides the parasites. Experimental Therapeutics (ET) Division provides access to their internally developed malaria viability assay.

METHODS AND MATERIALS:

Parasitized red cells are provided by the CD&I collaborator as a 6% hematocrit culture in RPMI 1640 media; at least 10% of the cells are infected with *Plasmodium falciparum.* The parasitized inoculum is diluted with donor red cells to a hematocrit of 38% or treated directly without dilution in donor red cells. The test specimens were treated with 500-micromolar riboflavin (RF) and either 45 or 60 minutes of illumination with agitation in the specially designed Pathogen Eradication Technology (PET) illuminator, which followed one-hour pre illumination incubation at room temperature. The controls consisted of an aliquot, which received no RF and no illumination; an aliquot, which received RF but was not illuminated; and a third, which received no RF but was illuminated. All specimens were then cultured in a modified RPMI with antibiotics and sampled every 3 –5 days for a total of two weeks. The samples were examined microscopically for morphological evidence of the parasite and an aliquot tested for parasitic lactate dehydrogenase (pLDH).

RESULTS (PRELIMINARY):

The treatment with 500 μM RF dramatically suppressed parasitic growth in the culture. The results from microscopic examination and pLDH were concordant. The no RF/no illumination control cultures showed evidence of parasitic growth through the two weeks of observation. The RF with no illumination control exhibited growth of parasite early in the observation period, a suppression of growth at approximately one week later and recovery of growth at the end of the observation period. The illumination with no RF became bacterially contaminated and, therefore, could not be interpreted.

RESULTS, DISCUSSION and CONCLUSION:

Final results from this set of experiments and their analysis are pending the completion of the research.

J. "The Effect of Two Additive Solutions on the Postthaw Storage of RBCs"

BACKGROUND:

This manuscript, published during the current reporting period, incorporates data from research completed in the previous FY.

ABSTRACT:

BACKGROUND: Sterile systems for freezing and for washing thawed blood will allow the storage of RBCs for more than 24 hours after removal of the cryoprotectant glycerol. This study assessed the effect of two ASs in maintaining deglycerolized RBCs.

STUDY DESIGN AND METHODS: Twenty-four RBC units were stored for 6 days, pooled in groups of 4, realiquoted, sterilely glycerolized, and frozen. One month later, the units were thawed, sterilely deglycerolized by using an automated system (H215; Haemonetics), and stored for 5 weeks in either 100 or 200 mL of AS-3 or an experimental AS (EAS-61). Sterile samples were taken weekly for chemical and morphometirc analysis.

RESULTS: The glycerolization and deglycerolization process produced highly comparable RBC units, but it caused a marked reduction of RBC pH, to about 6.4 at the beginning of storage. The addition of acidic AS-3 further reduced the pH, which in turn reduced glucose consumption, lactate formation, and RBC ATP concentration. Alkaline EAS-61 increased these measures. Hypotonic EAS-61 increased these measures. Hypotonic EAS-61 caused increased cell swelling and hemolysis, despite better RBC morphology.

CONCLUSIONS: Automation of sterile glycerolization and deglycerolization with the H215 works well, but the solutions should be reformulated for extended postthaw storage. This would best be accomplished by raising the pH of the wash solutions by addition of disodium phosphate or sodium bicarbonate or both, by using alkaline ASs, and by matching the osmolality of the wash solution and ASs.

METHODS, RESULTS, DISCUSSION and CONCLUSION:

The Methods, Results, Discussion and Conclusions are contained in copy of the published manuscript, which is attached to this report as Appendix 3.

K . "The Role of Electrolytes and pH in RBC ASs "

BACKGROUND:

This published manuscript incorporates data from a series of three *in vitro* studies completed in our laboratory and the laboratory of a collaborator, Dr. Tibor Greenwalt. The goal of these experiments was to optimize the performance of the experimental additive solution under study by sequentially examining variations in volume, NaCl content and the addition bicarbonate buffer. The experimentation was completed in the previous FY and results published during the current report period.

ABSTRACT:

Background: Experimental additive solutions (EASs) containing saline, adenine, glucose, mannitol and disodium phosphate can support RBCs for 9 or 10 weeks if used in 200- or 300-mL

volumes. The effects of variations in the electrolyte composition and volume of EASs were explored.

Study Design And Methods: In three four-arm studies, 24 RBC units were pooled in groups of 4 and realiquoted as test units to ensure that all donors were equally represented in each study arm. In Study 1, units were stored for 11 weeks in EAS containing 1, 10, 20, or 30 mmol per L of sodium bicarbonate. In Study 2, units were stored for 9 weeks in EAS containing 26, 50, 100, or 150 mmol per L of sodium chloride. In Study 3, units were stored in 100 or 200 mL of AS-3 or EAS-61. RBC ATP concentrations and hemolysis were measured weekly.

Results: Increasing the sodium bicarbonate content of EASs increased the pH throughout storage and increased RBC ATP concentrations in the later phases of storage, but it had no effect on hemolysis. Increased sodium chloride content of EASs led to lower RBC ATP concentrations and increased hemolysis. In EAS-61, RBC ATP concentrations were increased throughout storage, and hemolysis was lower than that of RBCs stored in AS-3.

Conclusion: RBC ATP synthesis is highly dependent on the pH of the AS. Hemolysis is affected by the salt content and volume of the AS.

METHODS, RESULTS, DISCUSSION and CONCLUSION:

The Methods, Results, Discussion and Conclusions are contained in copy of the published manuscript, which is attached to this report as Appendix 4.

L. *In vitro* comparison of Red Blood Cells Stored in EAS-61 in Polyolefin versus Polyvinyl Chloride Storage Bags.

STATUS:

This protocol was completed during the previous FY. A manuscript submitted and accepted by the journal Vox Sanguinis during this report period, this is included as Appendix 5.

ABSTRACT:

Background: Red blood cells (RBCs) must be stored in polyvinyl chloride (PVC) bags plasticized with di-2-ethylhexyl phthalate or a similar plasticizer to achieve their full storage life with conventional storage solutions. Improved storage solutions might remove this requirement and allow blood storage in other plastics. Experimental Additive Solution - 61 (EAS-61), which maintains RBCs for 9 weeks with reduced hemolysis and satisfactory ^{51}Cr 24-hour recovery, is an appropriate candidate improved RBC storage solution.

Study Design and Methods: Twenty four units of packed RBCs were pooled in groups of 4 units, each pool was realiquoted into 4 units, and stored, six pooled units per arm, in 1) 100 mL of EAS-61 in PVC, 2) 200 mL of EAS-61 in PVC, 3) 100 ml of EAS-61 in polyolefin (PO), and 4) 200 mL of EAS-61 in PO. Hemolysis, RBC morphology indices, RBC ATP concentrations, and other measures of RBC metabolism and function were measured weekly.

Results: RBC hemolysis exceeded 1% by 7 weeks in PO bags containing 100 or 200 mL of EAS-61. In PVC bags, hemolysis was less than 1% at 11 weeks. RBC ATP concentrations were 1 μmol/g Hb higher at 2 weeks in the PVC-stored units.

Conclusions: RBCs stored in PVC had markedly less hemolysis and higher RBC ATP concentrations than those stored in PO. Hemolysis would limit RBC storage in PO bags to 5 weeks even with EAS

BACKGROUND: The impetus for this study was threefold. First, the polyvinyl chloride (PVC) plastic used in blood collection system bags, as well as most of the IV solution bags and dialysis equipment, retains its pliability through the use of a plasticizer diethylhexylpthalate (DEHP). DEHP is a known health hazard in laboratory animals and a perceived health risk in humans. Second, the manufacture of PVC plastic requires the use of vinyl chloride, a carcinogen. Third, the incineration of PVC, the customary method of medical waste disposal, produces a variable amount of dioxins, another health and environmental hazard.

For these reasons, it has been proposed to replace PVC with other non-PVC plastics. There is some evidence in the literature, however, that DEHP has a beneficial effect on red cell storage,[17,18,19,20,21] and the storage lesion, i.e., the deterioration of the red cells as measured by loss of ATP, disk to sphere morphological changes and hemolysis, is accelerated when red cells are stored in non-PVC containers. This study was designed to determine the extent of the

detriment created by storage in polyolefin plastic (PO), a non-PVC plastic, and to determine if the superior red cell preservation qualities of the experimental additive solution EAS-61 was sufficient to overcome the deficit created by depriving the red cells of exposure to DEHP during storage.

METHODS, RESULTS, DISCUSSION AND CONCLUSION:

A copy of the accepted manuscript describing the methods, results, discussion and conclusion for this research is attached to this report as Appendix 5.

M. "Eleven Week Red Cell Storage"

STATUS:

This manuscript was accepted for publication by the journal Transfusion during the current report period. The data included in this report was generated from research completed previously.

BACKGROUND:

This manuscript is the third in a series detailing the development of the EASs. The previous publications describe a nine-week storage solution,[28] followed by successful demonstration of ten weeks of storage of red cells at 4^0C. The data for this manuscript resulted from a collaboration with Dr. Tibor Greenwalt of the University of Cincinnati Hoxworth Blood Center.

ABSTRACT:

Background: Increasing the duration of red blood cell (RBC) storage can increase both RBC availability and quality. This work addresses 11-week RBC storage in experimental additive solutions (EAS).

Study Design and Methods: Three studies were performed. In the first, 24-hour *in vivo* recovery of ^{51}Cr-labeled autologous RBC was measured in 9 volunteers after storage of their RBC for 11

weeks in EAS 67. In the second study, four units of blood were divided, stored in aliquots with an EAS containing 0, 15, 30, or 45 mmol/L of mannitol, and hemolysis, RBC morphology, and microvesicle protein measured. In the third study, six full units were stored for 12 weeks in the EAS containing 30 mmol/L of mannitol with weekly sampling for morphologic and biochemical measures of RBC quality.

Results: RBC stored for 11 weeks in EAS-67 had a 24 hr *in vivo* recovery of 79 ± 5%, but the hemolysis was 1.35 ± 0.68 %. Increasing mannitol content of the EAS reduced hemolysis but increased microvesiculation. EAS-76, with 30 mmol/L of mannitol allowed 11 week storage with 0.48 ± 0.10 % hemolysis at 11 weeks and 0.62 ± 0.14 % hemolysis at 12 weeks.

Conclusions: It is possible to store RBC for 11 weeks in EAS with greater than 75% recovery and less than 1% hemolysis.

METHODS, RESULTS, DISCUSSION AND CONCLUSION:

A copy of the accepted manuscript describing the methods, results, discussion and conclusion for this research is attached to this report as Appendix 6.

GENERAL AND ADMINISTRATIVE:

There have been two significant administrative changes in contract operation since the previous annual report. The first is the retirement of COL John R. Hess, the Commander of the Blood Research Detachment and the COR. The second was the closure the Detachment and subsequent incorporation of its assets into a newly created Blood Research Department under the directorship of COL Thomas J. Reid. COL Reid also became the COR. Blood Research is one of the departments within the division of Military Casualty Research.

Manuscripts of research results from contract-supported research have been submitted for publication with some published during the report period. The status of each manuscript submission is detailed in previous sections of this report.

Last fiscal year the Institute Director announced the intended relocation of WRAIR-based combat casualty care research activities, including Blood Research, to the Institute of Surgical Research in San Antonio Texas. Early this fiscal year it was announced those plans were abandoned.

SUMMARY

The contract staff has supported the BRD by operating and maintaining the Blood Storage Laboratory to support both existing and new requirements. The staff is trained and systems are in place which support red cell survival protocols. The Laboratory has been successful in completing protocols and having its work accepted for publication in peer-reviewed scientific journals during fiscal year 2001. The BRD mission has been supported.

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Appendix 1:

Contract Employees as of end of Fiscal Year 2001:

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A multicenter study of in vitro and in vivo values in human RBCs frozen with 40-percent (wt/vol) glycerol and stored after deglycerolization for 15 days at 4°C in AS-3: assessment of RBC processing in the ACP 215

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BACKGROUND: The FDA has approved the storage of frozen RBCs at –80°C for 10 years. After deglycerolization, the RBCs can be stored at 4°C for no more than 24 hours, because open systems are currently being used. Five laboratoris have been evaluating an automated, functionally closed system (ACP 215, Haemonetics) for both the glycerolization and deglycerolization processes.

STUDY DESIGN AND METHODS: Studies were performed at three military sites and two civilian sites. Each site performed in vitro testing of 20 units of RBCs. In addition, one military site and two civilian sites conducted autologous transfusion studies on ten units of previously frozen, deglycerolized RBCs that had been stored at 4°C in AS-3 for 15 days. At one of the civilian sites, 10 volunteers received autologous transfusions on two occasions in a randomized manner, once with previously frozen RBCs that had been stored at 4°C in AS-3 for 15 days after deglycerolization and once with liquid-preserved RBCs that had been stored at 4°C in AS-1 for 42 days.

RESULTS: The mean ± SD in vitro freeze-thaw-wash recovery value was 87 ± 5 percent; the mean ± SD supernatant osmolality on the day of deglycerolization was 297 ± 5 mOsm per kg of H_2O, and the mean ± SD percentage of hemolysis after storage at 4°C in AS-3 for 15 days was 0.60 ± 0.2 percent. The paired data from the study of 10 persons at the civilian site showed a mean ± SD 24-hour posttransfusion survival of 76 ± 6 percent for RBCs that had been stored at 4°C for 15 days after deglycerolization and 72 ± 5 percent for RBCs stored at 4°C in AS-1 for 42 days. At the three sites at which 24-hour posttransfusion survival values were measured by three double-label procedures, a mean ± SD 24-hour posttransfusion survival of 77 ± 9 percent was observed for 36 autologous transfusions to 12 females and 24 males of previously frozen RBCs that had been stored at 4°C in AS-3 for 15 days after deglycerolization.

CONCLUSION: The multicenter study showed the acceptable quality of RBCs that were glycerolized and deglycerolized in the automated ACP 215 instrument and stored in AS-3 at 4°C for 15 days.

Previously frozen RBCs are FDA-approved for storage at –80°C for 10 years. After deglycerolization, the RBCs can be stored at 4°C for no more than 24 hours, because the current methods for glycerolization and deglycerolization are open systems with the potential for bacteriologic contamination. The instrument used in this study is an automated, functionally closed system for the glycerolization and deglycerolization of human RBCs (ACP 215, Haemonetics Corp., Braintree, MA) that uses a sterile connector device (SCD, Haemonetics), inline 0.22-µ filters to deliver solutions, a disposable polycarbonate bowl with an external seal, and an integrally attached shaker and a printer to record the glycerolization and deglycerolization processes. The quality of the RBCs is monitored by an optical system that measures the Hb concentration in the waste solution during the deglycerolization

ABBREVIATIONS: FT = freeze-thaw; FTW = freeze-thaw-wash; NBRL = Naval Blood Research Laboratory; NMC = Naval Medical Center; SCD = sterile connector device; UMASS = University of Massachusetts Memorial Health Care; WRAIR = Walter Reed Army Institute of Research.

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ues of 87.0 ± 5 percent (Tables 1 and 2). The previously frozen RBCs stored in AS-3 at 4°C for 15 days after deglycerolization showed a mean hemolysis of 0.6 ± 0.2 percent (Tables 1 and 2) in 141 units.

Aerobic and anaerobic cultures showed no bacteriologic growth in 141 of the 142 units of previously frozen RBCs cultured after postwash storage at 4°C in AS-3 solution for 15 days. One in vitro unit grew the skin contaminant *Propionibacterium acnes* on Day 15 and on Day 42. One of the 10 liquid-preserved CPD RBC units that had been stored in AS-1 at 4°C for 42 days also grew *P. acnes* after 3 days in culture (Table 3), although no growth was observed on a prior culture of this unit on Day 39 or on culture 30 days after storage at 22°C. It is our opinion that contamination of the previously frozen RBCs occurred at the time of collection and that contamination of the liquid-preserved RBCs occurred when the culture sample was collected on Day 42.

When the double-label procedure was used to measure the 24-hour posttransfusion survival of previously frozen, autologous RBCs stored in AS-3 for 15 days after deglycerolization and transfused to the 14 volunteers in the study at the NBRL, the mean ± SD value was 75 ± 7 percent; in 13 of these volunteers, the $^{51}Cr\ T_{50}$ value was 29 ± 4 days (Table 4). In 10 of these 14 subjects in whom liquid-preserved, autologous RBCs stored in AS-1 for 42 days at 4°C were transfused, the 24-hour posttrans-fusion survival value measured by the double-label procedure was 72 ± 5 percent, and the $^{51}Cr\ T_{50}$ value was 27 ± 3 days (Tables 4 and 5). Five of the 10 units of previously frozen RBCs stored at 4°C for 15 days had 24-hour posttransfusion survival values of <75 percent, while 8 of the 10 units stored for 42 days at 4°C in AS-1 had 24-hour posttransfusion survival values of <75 percent (Table 5).

At WRAIR, where deglycerolized autologous RBCs stored at 4°C in AS-3 solution for 15 days were returned to their eight volunteers, ^{51}Cr was used to label the deglycerolized RBCs, and fresh autologous RBCs were labeled with ^{99m}Tc to measure the RBC volume. The mean ± SD 24-hour posttranfusion survival value was 78 ± 9 percent, and, for seven of the eight subjects, the $^{51}Cr\ T_{50}$ value was 24 ± 5 days.

At UMASS, the double ^{51}Cr procedure was used for measurements in 14 autologous transfusions of previously frozen RBCs that had been stored in AS-3 for 15 days after deglycerolization. The mean ± SD 24-hour posttransfusion survival value was 78 ± 10 percent, and the $^{51}Cr\ T_{50}$ value was 29 ± 5 days (Table 4).

In the 36 autologous transfusions of previously frozen RBCs performed at three of the study sites, the mean ± SD ITE was 68 ± 8 percent. At the one site where 10 autologous transfusions of liquid-preserved CPD RBCs stored in AS-1 at 4°C for 42 days were performed, the Mean ± SD ITE was 72 ± 5 percent.

In Nageotte WBC counts measured both on the day of deglycerolization and

TABLE 1. RBCs processed in the ACP 215 for in vitro analyses: combined data from five test sites

		Postwash storage at 4°C		
	After thaw	Day 0	Day 1	Day 15
FT Recovery (%)				
Mean	98.7	—	—	—
SD	1.1	—	—	—
N	106	—	—	—
FTW Recovery (%)				
Mean	—	86.8	—	—
SD	—	5	—	—
N	—	106	—	—
Hct (%)				
Mean	61	53	53	51
SD	5	3	3	3
N	106	106	106	106
Hb (g/dL)				
Mean	17.6	14.4	14.5	14.4
SD	1.6	1.1	1.1	1.2
N	106	106	106	106
RBC count (× 10^9/mL)				
Mean	5.9	4.8	4.8	4.8
SD	0.6	0.4	0.4	0.4
N	105	106	106	106
Total WBCs (× 10^6/unit)				
Mean	540	—	9.7	9.4
SD	210	—	7.9	7.5
N	99	—	64	64
Total platelet count (× 10^8/unit)				
Mean	175.8	5.6	1.8	1.8
SD	161.8	21.4	2.5	2.1
N	94	106	105	89
Blood pH at 22°C				
Mean	6.9	6.4	6.4	6.3
SD	0.1	0.1	0.1	0.1
N	75	96	100	106
Supernatant osmolality (mOsm/kg H_2O)				
Mean	4579	296	297	300
SD	295	4	5	4
N	106	106	106	106
Supernatant K+ (mEq/L)				
Mean	14.3	1.3	6.9	24.5
SD	4.0	0.9	3.0	4.9
N	94	106	106	106
Supernatant Hb (mg/dL)				
Mean	607	40	91	174
SD	495	13	31	51
N	105	106	106	106
Percentage of hemolysis				
Mean	—	0.1	0.3	0.6
SD	—	0	0.1	0.2
N	—	106	106	106

TABLE 2. RBCs processed in the ACP 215 for in vivo analyses: combined data from three test sites

		Postwash storage at 4°C	
	After	Day 0	Day 15
FT Recovery (%)			
Mean	98.1	—	—
SD	1.8	—	—
N	36	—	—
FTW recovery (%)			
Mean	—	87.9	—
SD	—	3.6	—
N	—	36	—
Hct (%)			
Mean	60	50	48
SD	7	4	4
N	36	36	35
Hb (g/dL)			
Mean	17.8	13.6	13.4
SD	2.2	1.4	1.3
N	36	36	35
RBC count (× 10^9/mL)			
Mean	6.1	4.6	4.5
SD	0.6	0.4	0.4
N	36	36	34
Total WBCs (× 10^6/unit)			
Mean	513	—	3.7
SD	261	—	3.8
N	36	—	9
Total platelet count (× 10^8/unit)			
Mean	130.4	3.8	2.9
SD	8.1	8.8	4.7
N	35	35	29
Blood pH at 22°C			
Mean	6.9	6.4	6.3
SD	0.1	0.1	0.1
N	27	33	33
Supernatant osmolality (mOsm/kg H_2O)			
Mean	4662	299	302
SD	267	6	5
N	36	36	33
Supernatant K+ (mEq/L)			
Mean	16.3	1.1	22.8
SD	4.5	0.7	5.7
N	36	36	35
Supernatant Hb (mg/dL)			
Mean	883	41	143
SD	793	24	61
N	36	36	35
Percentage of hemolysis			
Mean	—	0.2	0.5
SD	—	0.1	0.2
N	—	36	35

after storage at 4°C in AS-3 solution for 15 days, the total number of WBCs was 1×10^7 per unit, with a range from 1×10^6 to 4×10^7 for 73 units (Tables 1 and 2).

DISCUSSION

The ACP 215 is an automated, functionally closed instrument for the glycerolization and deglycerolization of human RBCs. This instrument uses a disposable 275-mL blow-molded bowl with a diverter, an external rotating seal, and a capacity to process 180 mL of RBCs. If the volume exceeds

TABLE 3. CPD RBCs stored in AS-1 at 4°C for 42 days at NBRL

	Day 42
Hct (%)	
Mean	53
SD	2
N	10
Hb (g/dL)	
Mean	17.0
SD	0.9
N	10
RBC count (× 10^9/mL)	
Mean	5.7
SD	0.2
N	10
Total WBCs (× 10^9/per unit)	
Mean	1.1
SD	0.5
N	10
Total platelet count (× 10^8/unit)	
Mean	180.1
SD	106.2
N	10
Blood pH at 22°C	
Mean	6.5
SD	0.1
N	9
Supernatant Hb (mg/dL)	
Mean	203
SD	67
N	8
Percentage of hemolysis	
Mean	0.6
SD	0.2
N	8

180 mL, the RBCs will spill over into the waste solution during the deglycerolization procedure. A mean ± SD in vitro recovery of 87 ± 5 percent of the RBCs was observed in this study; this value is similar to that previously reported for studies in which another instrument (115, Haemonetics) was used.[1,11] Previously frozen RBCs stored at 4°C in AS-3 solution for 15 days after deglycerolization were shown to have a mean ± SD 24-hour posttransfusion survival value of 77 ± 9 percent and 0.6 ± 0.2 percent hemolysis. In a study of human RBCs deglycerolized in the 115 instrument, Moore et al.[12] concentrated the deglycerolized RBCs resuspended in a sodium chloride-glucose solution to remove the supernatant solution on the day of washing and then diluted the RBC concentrates with a Hct of 80 percent with 100 mL of AS-3 before storage at 4°C for 21 days. These authors reported that, after 21 days of postwash storage at 4°C, hemolysis was 0.57 percent and the mean 24-hour posttransfusion survival value was 77 percent when measured by the single-isotope method.[12]

In our study, the ITE for the deglycerolized RBCs processed in the ACP 215 and stored at 4°C in AS-3 for 15 days was 68 percent. When deglycerolized RBCs processed in the 115 were stored at 4°C in sodium chloride-glucose solution for only 24 hours, the ITE ranged from 75 to 80 percent.[1,11] The reason for the difference between the reduced ITE for

TABLE 4. 24-hour posttranfsusion survival, lifespan, and ITE of RBCs deglycerolized in the ACP 215 and stored at 4°C in AS-3 for 15 days

Test site	Label method		24-hour posttransfusion survival value (Double-label procedure)*	Lifespan (T_{50}, days)	ITE* (%)
Combined		Mean	77	28	68
		SD	9	4	8
		N	36	34	36
NBRL	$^{51}CR/^{125}I$ Albumin	Mean	75	29	65
		SD	7	4	6
		N	14	13	14
WRAIR	$^{51}CR/^{99m}TC$	Mean	78	24	68
		SD	9	5	8
		N	8	7	8
UMASS	Double ^{51}CR	Mean	78	29	71
		SD	10	5	10
		N	14	14	14
NBRL†		Mean	72	27	72
		SD	5	3	5
		N	10	10	10

* Product of in vitro FTW recovery and 24-hour posttransfusion survival value.
† Control: CPD RBCs stored in AS-1 at 4°C for 42 days.

TABLE 5. Comparison of 24-hour posttransfusion survival using the $^{51}CR/^{125}I$ albumin double-label procedure in autologous RBCs processed in the ACP 215 and stored at 4°C in AS-3 for 15 days after deglycerolization and autologous CPD RBCs stored in AS-1 at 4°C for 42 days in the same donor at NBRL*

Donor	24-hour posttransfusion survival (%) ACP 215	CPD RBCs in AS-1
1	81	68
2	70	71
3	72	70
4	79	73
5	73	68
6	83	78
7	84	72
8	68	67
9	79	74
10	66	82
Mean	76	72
SD	6	5
N	10	10

* Paired *t* test, 1.3; p = NS.

these two groups of deglycerolized human RBCs is the longer postwash storage period of 15 days for those RBCs processed in the ACP 215.

Ten units of liquid-preserved CPD RBCs stored in AS-1 at 4°C for 42 days exhibited a mean ± SD 24-hour posttransfusion survival value of 72 ± 5 percent when measured by the $^{51}Cr/^{125}I$ albumin double-label procedure, a finding similar to that observed in a study at NBRL in 1988[7] and not significantly lower than the value of 77 ± 9 percent observed in the present study for the 36 autologous transfusions of deglycerolized RBCs stored at 4°C in AS-3 for 15 days and measured by the $^{51}Cr/^{125}I$ albumin, $^{51}Cr/^{99m}Tc$, and double ^{51}Cr procedures. The ITE of 68 percent for deglycerolized RBCs processed in the ACP 215 instrument and stored at 4°C in AS-3 for 15 days was comparable to that of 72 percent for liquid-preserved CPD RBCs stored at 4°C in AS-1 for 42 days.

One in vitro unit of deglycerolized RBCs grew *P. acnes* after storage at 4°C in AS-3 for 15 days. One CPD unit from a separate donor, which was stored in the liquid state in AS-1 at 4°C for 42 days, tested positive for *P. acnes* on Day 42 but showed no growth in a sample tested on Day 39 or after storage at 22°C for 30 days. These findings suggest that the skin contaminant seen in the deglycerolized RBCs most likely was introduced at the time of blood collection and that contamination of the liquid-preserved RBC occurred during sample collection on Day 42. Information from the ongoing bacterial contamination study of blood by the AABB, ARC, CDC, and Department of Defense (BaCon Study) indicates that this bacterium has not been associated with posttransfusion sepsis.

Concern about the transmission of disease from allogeneic RBCs has led to a call for the quarantine of donor RBCs and subsequent retesting of the donor. Frozen RBCs can be stored for at least 10 years. If the blood donor continues to test negative for infectious disease markers over a 6-month period, the frozen RBCs can be considered safe for allogeneic transfusion. The ability to store the deglycerolized RBCs at 4°C for at least 2 weeks makes this practice even more valuable.

In previous studies, the processes of glycerolizing and deglycerolizing RBCs in the presence and absence of freezing was shown to reduce the total number of WBCs in the deglycerolized unit.[13-17] In the study reported here, in which measurements were made by using the Nageotte chamber with Turk's solution in 73 units on the day of deglycerolization and after postwash storage at 4°C in AS-3 for 15 days, it was shown that the FTW process reduced the total number of WBCs to a mean of 1×10^7 per unit, with a range from 1×10^6 to 4×10^7.

Washing previously frozen RBC to remove the cryoprotectant glycerol reduces not only the number of WBCs in the unit but also the number of biologically active substances that some investigators believe may contribute to the immunomodulation effects in recipients.[18-20]

Several factors affecting the safety and therapeutic effectiveness of an RBC transfusion include the quantity of RBCs in the unit; the survival and function of RBCs, the residual hemolysis and sterility; the residual number and function of WBCs; and the number of residual biologically active plasma and nonplasma substances.

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The effect of two additive solutions on the postthaw storage of RBCs

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BACKGROUND: Sterile systems for freezing and for washing thawed blood will allow the storage of RBCs for more than 24 hours after removal of the cryoprotectant glycerol. This study assessed the effect of two ASs in maintaining deglycerolized RBCs.

STUDY DESIGN AND METHODS: Twenty-four RBC units were stored for 6 days, pooled in groups of 4, realiquoted, sterilely glycerolized, and frozen. One month later, the units were thawed, sterilely deglycerolized by using an automated system (H215; Haemonetics), and stored for 5 weeks in either 100 or 200 mL of AS-3 or an experimental AS (EAS-61). Sterile samples were taken weekly for chemical and morphometric analysis.

RESULTS: The glycerolization and deglycerolization process produced highly comparable RBC units, but it caused a marked reduction of RBC pH, to about 6.4 at the beginning of storage. The addition of acidic AS-3 further reduced the pH, which in turn reduced glucose consumption, lactate formation, and RBC ATP concentrations. Alkaline EAS-61 increased these measures. Hypotonic EAS-61 caused increased cell swelling and hemolysis, despite better RBC morphology.

CONCLUSIONS: Automation of sterile glycerolization and deglycerolization with the H215 works well, but the solutions should be reformulated for extended postthaw storage. This would best be accomplished by raising the pH of the wash solutions by the addition of disodium phosphate or sodium bicarbonate or both, by using alkaline ASs, and by matching the osmolality of the wash solution and ASs.

Frozen RBC storage has been practiced for 50 years.[1] However, it is not commonly used, because it is labor-intensive and is performed in open systems with the potential for bacterial contamination, and because unit quality is difficult to control. The potential for bacterial contamination with the washing of RBCs in open centrifugal systems led the FDA to direct that deglycerolized RBCs be administered within 24 hours of thawing or be destroyed.[2] These shortcomings led to the design and development of closed, automated, and performance-monitored systems for RBC glycerolization and deglycerolization. The system used in this study (H215, Haemonetics Corp., Braintree, MA) is the first such system available for evaluation.

A practical consideration in the design of such a system is the composition of the postthaw RBC storage solutions. Several investigators have demonstrated 2-week postthaw storage of deglycerolized RBCs, and several groups of researchers[3-7] showed that RBCs frozen on the day of collection could be stored for 3 weeks after deglycerolization in AS-3 (Nutricel, Pall Corp., Covina, CA). However, RBCs are rarely frozen on the day of collection. They are usually frozen only after the return of virus testing results or even longer, out to the FDA limit of 6 days.

ABBREVIATION: EAS-61 = an experimental AS.

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To explore the potential for longer postthaw storage at 1 to 6°C, we compared the chemical and morphologic characteristics of RBCs stored in liquid form for 6 days; then frozen, stored 1 month, thawed, and deglycerolized; and finally stored in liquid form for 5 weeks in either 100 or 200 mL of AS-3 or an experimental AS (EAS-61). EAS-61 was chosen as an alternative AS, because it allows the storage of RBCs for 9 weeks at 1 to 6°C.[8] The results of this study show that the deglycerolization process leads to a marked reduction in storage pH at the beginning of postthaw liquid storage. This reduction in pH and subsequent suppression of glycolysis was worse with the use of acidic AS-3. On the other hand, the use of hypotonic EAS-61 led to increased hemolysis. On the whole, the study suggests that better wash and storage solutions can be formulated.

MATERIALS AND METHODS

Volunteers

Twenty-four volunteers donated full units of blood under a protocol approved by the institutional review board of the Walter Reed Army Institute of Research. The donors met standard blood donor criteria.

Storage solutions

The compositions of AS-3 and EAS-61 are compared in Table 1. The significant differences in the solutions are the absence of citrate, the reduced salt content, and the presence of mannitol and disodium phosphate in the EAS-61. The presence of disodium phosphate raises the pH of the EAS-61 to about 8.4 at room temperature, while that of AS-3 is 5.8.

The EAS-61 was made in the laboratory from USP adenine, sugars, and salts and was sterilely filtered into 1-L storage bags. The bags were held at 37°C for 2 weeks. The solutions were then cultured and the cultures incubated for another 2 weeks. When sterility was confirmed by the absence of bacteria growth for 7 to 14 days, the solutions were aliquoted by weight into 150- or 300-mL bags. All connections were made with a sterile connecting device (SCD 312, Terumo Medical Corp., Elkton, MD).

TABLE 1. Composition of the additive solutions used*

	AS-3	EAS-61
Volume (mL)	100, 200	100, 200
NaCl	70	26
NaH_2PO_4	23	0
Na_2HPO_4	0	12
Adenine	2	2
Dextrose	55	110
Mannitol	0	50
Citric acid	2	0
Na_3 citrate	20	0
pH	5.8	8.4

* All concentrations are given in mmol per L.

Study design

We conducted a "pooling" study to evaluate RBC metabolism and physiology over the course of storage. Pooling restricts the largest source of variability in conventional blood storage studies, the differences between the RBCs from different donors, by placing some of the cells from every donor in every arm of the study while maintaining conventional unit size and geometry. In this study, the RBC units were grouped into sets of 4 ABO-matched units. Each set was then pooled, mixed, and realiquoted into identical pooled units.

Specifically, 24 units of whole blood were drawn into 800-mL CPDA-1 bags (Code 4R3420, Baxter Healthcare Corp., Deerfield, IL) and stored for 6 days at 1 to 6°C. On the sixth day, they were pooled in groups of 4, realiquoted, sterilely glycerolized by the automated procedure on the H215, and frozen at –80°C. A month later, the units were thawed, sterilely deglycerolized by the automated procedure on the H215, and stored in 100 or 200 mL of AS-3 or EAS-61. The automated procedure for deglycerolization is a modification of the standard US military procedure of Valeri.[9-11] It uses 12-percent saline for initial cell shrinkage to facilitate glycerol removal and then five washes in 0.9-percent saline and 0.2-percent glucose, and it ends with a final wash in the AS. When 200 mL of AS was used, the second 100 mL was added to the product by sterile tubing connection. Units were then sampled, placed in a 1 to 6°C refrigerator, and sampled weekly for 5 weeks.

In vitro measurements

Samples from stored units were collected, after gentle mixing by inversion, by a sterile sampling procedure in a biologic safety cabinet. A battery of in vitro tests was performed on all units at the beginning of storage and weekly thereafter.

The total Hb concentration, WBC counts, and RBC counts were measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9000, Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically by the modified Drabkins assay.[12] The percentage of hemolysis was determined by the ratio of free Hb to total Hb. The results are expressed as percentage of hemolysis to compensate for the differences in Hct and Hb concentrations in samples. Centrifuged microhematocrits (Clay Adams, Becton Dickinson, Rutherford, NJ) were performed to avoid the readjustment of cell volume and subsequent errors caused by the isotonic diluent used in the blood analyzers. The MCV was calculated from the machine RBC concentrations and the microhematocrit.

RBC ATP concentrations were measured in deproteinized supernatants. Whole-blood or packed cell aliquots were mixed with cold 10-percent trichloroacetic acid to precipitate blood proteins and then centrifuged at 2700 × *g* for 10 minutes, and the protein-free supernatant was frozen at –80°C until testing. ATP was assayed enzymatically by using a commercially available test kit (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO).

Blood gases and pH were measured on a blood gas analyzer (Corning 855, Ithaca, NY). Thus, pH was measured at 37°C. Phosphate and glucose were measured on a programmable chemical analyzer (Hitachi 902, Boehringer Mannheim, Indianapolis, IN).

The average degree of RBC shape change from discocyte to echinocyte to spherocyte was graded by the RBC morphology index on samples of 400 cells, determined according to the method of Usry et al.[13]

Statistical analysis

Comparisons of means of measured values at given times within the individual crossover trials were evaluated with ANOVA using statistical software (Numerical Algorithms Group Statistical Inserts for Microsoft Excel, NAG, Downers Grove, IL; Microsoft Corp., Redmond WA). Probabilities <0.05 were considered significant.

RESULTS

The measured pH at the beginning of postthaw storage was low in all groups. The pH of the units stored in 100 mL or 200 mL of AS-3 averaged 6.301 ± 0.010 or 6.213 ± 0.010, respectively, and that of units stored in 100 mL or 200 mL of EAS-61 averaged 6.529 ± 0.005 or 6.578 ± 0.005, respectively (Fig. 1A). These low pH measurements were associated with extremely low concentrations of plasma bicarbonate (<1 mEq/L) in all groups.

RBC ATP concentrations averaged 5.6 ± 0.2 µM per g of Hb in all groups at the beginning of storage but differed thereafter, with the EAS-61-stored cells maintaining higher concentrations (Fig. 1B). By 2 weeks, the AS-3-stored cells averaged RBC ATP concentrations of about 2 µM per g of Hb, while the concentration in the EAS-61-stored cells was 4.2 ± 0.2.

Glucose concentrations were higher in the EAS-61-stored cells (Fig. 1C). Moreover, the rate of decrease in the EAS-61-stored cells was greater. This difference in the rate of glycolysis is confirmed by the greater rate of lactate production in the cells stored in EAS-61 (Fig. 1D).

RBCs had a greater initial MCV (about 118 fL) when stored in EAS-61 than did the cells stored in AS-3 (about 110 fL) (Fig. 2A). RBCs had good morphology in all groups at the beginning of postthaw liquid storage, with an average morphology index of 89 ± 1. The morphology was initially identical but was better maintained in EAS-61 at both storage volumes (Fig. 2B). Nevertheless, hemolysis was higher in the EAS-61-stored cells at all time points, including the initial measurement (Fig. 2C).

DISCUSSION

Freezing RBCs has proven valuable for the storage of units of rare blood types, for the storage of autologous units, and for the supplementation of inventory in certain high-use

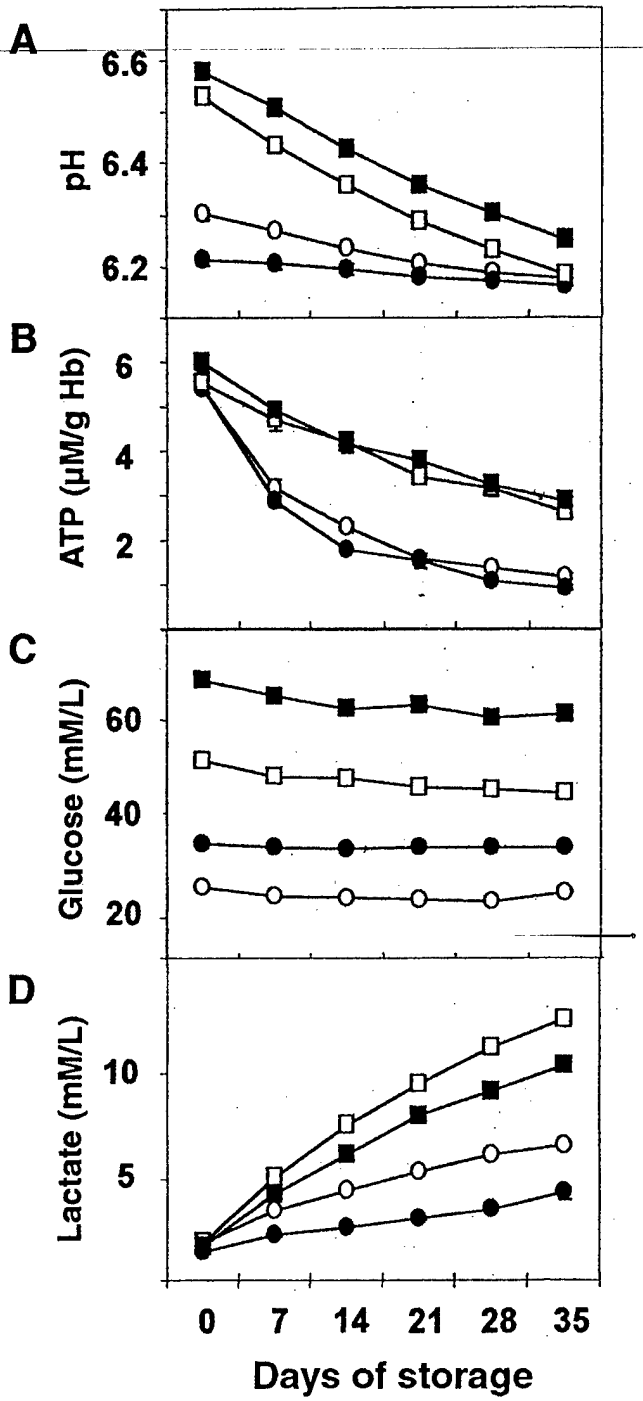

Fig. 1. A comparison of the effects of postthaw storage in two ASs at two volumes on extracellular pH (A), RBC ATP content (B), extracellular glucose concentration (C), and whole-blood lactate content (D). The individual solutions and volumes were: AS-3 at 100 mL (○), AS-3 at 200 mL (●), EAS-61 at 100 mL (□), and EAS-61 at 200 mL (■). The difference in ATP concentrations in RBCs stored in the different solutions at 14 days is about 2 µ*M* per g of Hb or about 120 µ*M* per unit. This is about one-eighth of the ATP produced, as determined from the production of lactate.

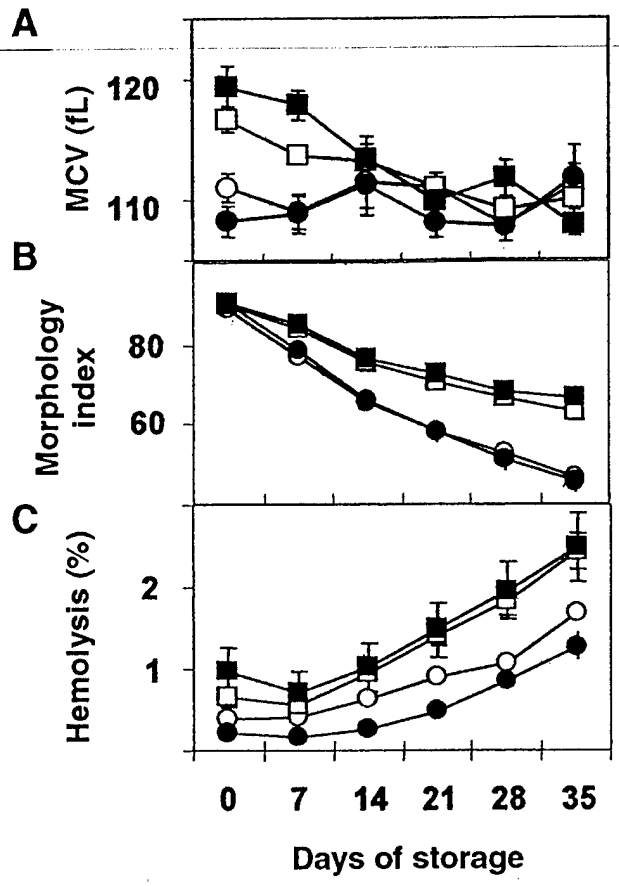

Fig. 2. A comparison of the effects of postthaw storage in two ASs at two volumes on MCV (A), morphology index (B), and hemolysis (C). The individual solutions and volumes were: AS-3 at 100 mL (○), AS-3 at 200 mL (●), EAS-61 at 100 mL (□), and EAS-61 at 200 mL (■). The smaller content of nonpenetrating ions in EAS-61 leads to greater RBC swelling initially, and, despite better preservation of RBC morphology with EAS-61, there was greater hemolysis.

situations where partial automation (IBM 2991, IBM, Armonk, NY) can be used. The technology has proven much less useful in remote locations, where high cost and low levels of training combine to make fully manual systems problematic.

With the development of sterile blood bag connection methods, it became possible to design closed systems for deglycerolizing RBCs. The first such system was built in prototype using a hollow-fiber dialysis cartridge at the former Letterman Army Institute of Research in San Francisco in 1986. The Letterman group went on to demonstrate that liquid storage for as long as 3 weeks after deglycerolization was possible.[6] They then solicited the manufacture of fully automated devices for the sterile processing of frozen RBCs.

The first of those devices is now undergoing preclinical testing. The H215 achieves sterile glycerolization by the addition of the glycerol to the primary blood bag through a sterilely connected 0.22-µm filter. It achieves sterile deglycerolization of RBCs by sterilely connecting the frozen-storage bag to the washing plastic ware, by washing the cells in a special centrifuge bowl with protected rotating seals, and by 0.22-µm filtration of all added solutions. The device is automated, requires approximately 50 minutes to process each unit, and is designed for use with AS-3 as the final wash and storage solution.

We recently developed EAS-61 as a 9-week storage solution and were interested in whether this solution could improve the storage of frozen RBCs as well. As EAS-61 is used in a 200-mL volume with liquid-stored cells, we tested both solutions in 100- and 200-mL volumes.

The pooling design of this investigation appears to have worked well. It produced RBCs with equivalent RBC ATP concentrations, lactate concentrations, and RBC morphology in all four groups at the beginning of the study. It also produced excellent statistical resolution of the differences between the groups that appeared with the addition of the solutions and storage.

The cell-washing process reduced the pH of the washed cells to about 6.4. The addition of acidic AS-3 further reduced the pH to 6.3 or below, which markedly reduced glycolysis. In the absence of significant RBC ATP production, RBC ATP concentrations decreased to values below those associated with successful in vivo RBC recovery at about 2 weeks. Alkaline EAS-61 raised the pH to 6.5 or above and supported two to three times the rate of glycolysis, but it caused increased hemolysis. This increased hemolysis, which was present at the beginning of storage, is probably related to the hypotonic nature of EAS-61, with increased swelling of these fragile cells and the poor match of the tonicity of the EAS-61 and the saline-dextrose wash solution.

The pH of venous blood is normally about 7.35 when measured at body temperature. The addition of the 63 mL of citric acid and monosodium phosphate present in CPD or CPDA-1 lowers the pH of whole blood in a primary collection bag to about 7.2. Further addition of 100 mL of AS-3, which contains 23 mEq per L of monosodium phosphate at pH 5.5, will reduce the pH of AS-3-stored RBCs to 7.0 at the beginning of storage. During the first week of storage, the pH will decline by about 0.2 pH units. These values are above the value of about 6.65 at which rates of RBC ATP synthesis and consumption are balanced. Thus, during conventional liquid storage at 1 to 6°C, RBCs stored in AS-3 show a rise in RBC ATP concentrations during the first 2 weeks of storage and a decline thereafter, as the products of metabolism accumulate in the bag, reduce the pH, and inhibit glycolysis. In this study, ATP consumption exceeded synthesis at all times. This is probably entirely a function of the pH, as adenine, glucose, and phosphate concentrations were adequate.

The data suggest that modification of the cell-washing procedure by the addition of disodium phosphate or so-

dium bicarbonate to the wash solution to raise storage pH will be useful. We have done this, adding 10 mEq per L of disodium phosphate to the washing solution of a single unit of frozen RBCs, and we were able to raise the initial pH to 6.9. Matching the tonicity of the wash and storage solutions may prevent osmotic RBC lysis, which appears to have occurred when a hypotonic storage solution added after completion of the wash caused marked cell swelling. Otherwise, a high pH to maintain glycolysis and a low final tonicity of approximately 290 mOsm to preserve morphology are probably useful.

Automated frozen blood-processing systems that will allow 2 weeks of postthaw liquid storage will be available in the near future. Improving the wash and storage solutions for even longer postthaw storage should be possible.

ACKNOWLEDGMENTS

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The role of electrolytes and pH in RBC ASs

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BACKGROUND: Experimental additive solutions (EASs) containing saline, adenine, glucose, mannitol and disodium phosphate can support RBCs for 9 or 10 weeks if used in 200- or 300-mL volumes. The effects of variations in the electrolyte composition and volume of EASs were explored.

STUDY DESIGN AND METHODS: In three four-arm studies, 24 RBC units were pooled in groups of 4 and realiquoted as test units to ensure that all donors were equally represented in each study arm. In Study 1, units were stored for 11 weeks in EAS containing 0, 10, 20, or 30 mmol per L of sodium bicarbonate. In Study 2, units were stored for 9 weeks in EAS containing 26, 50, 100, or 150 mmol per L of sodium chloride. In Study 3, units were stored in 100 or 200 mL of AS-3 or EAS-61. RBC ATP concentrations and hemolysis were measured weekly.

RESULTS: Increasing the sodium bicarbonate content of EASs increased the pH throughout storage and increased RBC ATP concentrations in the later phases of storage, but it had no effect on hemolysis. Increased sodium chloride content of EASs led to lower RBC ATP concentrations and increased hemolysis. In EAS-61, RBC ATP concentrations were increased throughout storage, and hemolysis was lower than that of RBCs stored in AS-3.

CONCLUSION: RBC ATP synthesis is highly dependent on the pH of the AS. Hemolysis is affected by the salt content and volume of the AS.

Better RBC storage solutions will improve RBC availability and safety.[1] With the current 5- and 6-week liquid storage systems, 600,000 RBC units outdate each year across the United States. As many as half of these outdating units probably could find a recipient if storage times were 2 weeks longer. Remote centers and hospitals would find it easier to maintain blood inventories to meet emergency needs if longer storage times allowed stock rotation with regional blood centers. Autologous blood storage would also be more useful if liquid storage times were longer. This would allow such donors both to give multiple units and to enter the operating room with a normal Hb. Greater use of autologous blood would free allogeneic RBCs for other uses.

Conventionally stored RBCs show a storage lesion that includes the loss of membrane, ATP, 2,3 DPG, and viability.[2] These RBC changes may injure the transfusion recipient.[3] Storage-related loss of membrane makes RBCs rigid, reducing microvascular flow.[4] Shed RBC membrane microvesicles in the stored RBC supernatant are procoagulant, and lysophospholipids, such as platelet-activating factor, shed from RBC membranes during storage, are proinflamatory.[5-7] Loss of RBC ATP during storage reduces the amount available for secretion, a mechanism by which RBCs control microvas-

ABBREVIATION: EAS(s) = experimental AS(s).

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cular tone.[8,9] Loss of 2,3 DPG increases RBC oxygen affinity and can reduce peripheral oxygen delivery.[10] The administration of nonviable RBCs can lead to a partial blockade of macrophage clearance mechanisms and can reduce the clearance of bacteria in patients with injury or infection.[11] All of these problems may be diminished with better RBC ASs.

We have developed 9- and 10-week RBC storage systems that appear to decrease most of the above-mentioned components of the storage lesion.[12,13] The systems are based on storing packed RBCs in 200 or 300 mL of experimental ASs (EASs) composed of saline, adenine, glucose, mannitol, and disodium phosphate at pH 8.4. They work best in combination with WBC reduction.

This work describes three pooling studies that further explored the effects of the pH and the electrolyte composition of these EASs on storage metabolism, shape change, and hemolysis of the RBCs. In each study, 24 units of packed RBCs were pooled in groups of 4 units and aliquoted into mixed units of conventional size. Within each study, 1 unit from each pool was allocated to each of the four arms. Thus, blood from each of the 24 donors was equally represented in each arm of each particular study. In the first study, the effect of adding sodium bicarbonate to the storage media in concentrations of 0, 10, 20, and 30 mmol per L was examined. In the second study, the effect of varying the sodium chloride concentration was explored with concentrations of 26, 50, 100, and 150 mmol per L. Finally, the combined effects of saline, phosphate, and pH were explored in a comparison of the storage of whole units of packed RBCs in 100 or 200 mL of AS-3 and ~~that in~~ 100 or 200 mL of EAS-61. The results suggest that a high pH is important for ATP synthesis throughout storage and that a low salt concentration reduces hemolysis by decreasing microvesiculation. Phosphate, reported to drive ATP synthesis, had a much smaller effect than pH in the ranges found in these solutions.

MATERIALS AND METHODS

Volunteers

Three groups of 24 healthy volunteers met standard blood donor criteria and gave informed consent to participate in these studies. The first two groups donated full units of blood under protocols approved by the Hoxworth Blood Center and the University of Cincinnati Institutional Review Board, while the third group donated in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research.

Storage solutions

The compositions of the various EASs and AS-3 (Nutricel, Pall Medical, Covina, CA) are compared in Table 1. The EASs were made in the laboratory from high-purity adenine, sugars, and salts and sterilely filtered into 1-L storage bags (Code 4R2032, Baxter Healthcare, Deerfield, IL). The solutions were then cultured and the cultures incubated for 2 weeks. When sterility was confirmed by the absence of bacterial and fungal growth for 14 days, the solutions were aliquoted by weight into 600-mL bags (Code 4R2023, Baxter). All connections were made by using a sterile connecting device (SCD 312, Terumo Medical Corp., Elkton, MD).

Study design

We conducted three pooling studies to evaluate RBC metabolism, morphology, and hemolysis over the course of storage. Pooling reduces the largest source of variability in conventional blood-storage studies, the differences between RBCs from different donors, by placing some of the cells from every donor in each arm of the study while maintaining conventional unit size and geometry. In each of the three studies, the RBC ~~units~~ were grouped into sets of 4 ABO-matched units, and each set was then pooled, mixed, and realiquoted into identical pooled units. Six pooled units, 1 from each set, were used in each arm of each study.

In the first study, units were stored in variants of EAS-64 with increasing sodium bicarbonate concentrations but a constant total sodium concentration and a constant 300-mL AS volume. In the second study, units were stored in variants of EAS-61 with increasing sodium chloride concentrations but at a constant 200-mL volume. In the third study, units were stored in 100 and 200 mL of AS-3 and in 100 and 200 mL of EAS-61. Electrolytes, pH, metabolites, RBC morphology

TABLE 1. Composition of the ASs*

	Study 1				Study 2				Study 3	
	EAS-64	EAS-65	EAS-66	EAS-67	EAS-61	EAS-71	EAS-72	EAS-73	AS-3	EAS-61
Volume (mL)	300	300	300	300	200	200	200	200	100 and 200	100 and 200
NaCl	75	65	55	45	26	50	100	150	70	26
$NaHCO_3$	0	10	20	30	0	0	0	0	0	0
NaH_2PO_4	0	0	0	0	0	0	0	0	23	0
Na_2HPO_4	9	9	9	9	12	12	12	12	0	12
Adenine	2	2	2	2	2	2	2	2	2	2
Dextrose	50	50	50	50	110	110	110	110	55	110
Mannitol	20	20	20	20	50	50	50	50	0	50
Citric acid									2	
Na_3Citrate									23	
pH	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	5.8	8.4

* All concentrations in mmol per L.

indices, and RBC ATP concentrations were measured weekly during storage by sterile sampling techniques.

RBC unit preparation

Study 1: EAS-64 variant units. Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD in the primary PVC bag of an AS-1 triple-bag collection set (Code 4R1436, Baxter). Packed RBCs were prepared by centrifugation at 5,000 × *g* for 5 minutes at room temperature, which was followed by the removal of sufficient plasma to achieve a Hct of 75 to 80 percent. ABO-matched RBCs were then pooled in groups of 4 units in 1-L PVC bags (4R2032), mixed thoroughly, and distributed equally by weight into four 600-mL PVC transfer bags (4R2023) by use of the sterile connecting device. AS (300 mL) was then added to the RBCs. After mixing, each unit was kept at room temperature (approx. 22°C) for 1 hour.

Study 2: EAS-61 variant units. Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD in the primary PVC bag of a collection set with a WBC-reduction filter included (RS-2000, Code 4R3303, Baxter). After WBC reduction, packed RBCs were prepared by centrifugation at 5000 × *g* for 5 minutes at room temperature, which was followed by the removal of sufficient plasma to achieve a Hct of 75 to 80 percent. ABO-matched RBCs were then pooled in groups of 4 units in 1-L sterile bags (4R2032), mixed thoroughly, and distributed equally by weight as described above. EAS (200 mL) was then added to the packed RBC aliquots. The units were mixed and kept at room temperature as in Study 1.

Study 3: AS-3 and EAS-61 units. Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CP2D in the primary PVC bag of an AS-3 double-bag collection set (Code 762-54, Pall Corp., Covina, CA). Packed cells were prepared by centrifugation at 5000 × *g* for 5 minutes at room temperature, which was followed by the removal of sufficient plasma to achieve a Hct of 75 to 80 percent. Four units of identical ABO type were then pooled and realiquoted as described above, and either 100 or 200 mL of AS-3 or 100 or 200 mL of EAS-61 was added.

All units were gently mixed by inversion approximately 30 times, sampled aseptically for in vitro testing, and placed upright in refrigerated storage (1-6°C) 4 hours or less after processing.

In vitro measurements

After gentle mixing by inversion, samples from stored units were collected by a sterile sampling procedure in a biologic safety cabinet. A battery of in vitro tests was performed on all units at the beginning of storage and weekly thereafter.

For Studies 1 and 2, performed at the Hoxworth Blood Center, the total Hb concentration, WBC counts, and RBC counts were measured with a clinical hematology analyzer (MaxM Coulter Counter, Coulter Electronics, Hialeah, FL). Supernatant Hb was measured by using 3,3′,5,5′-tetramethylbenzidine (Procedure No. 527, Sigma Diagnostics, St. Louis, MO). The average degree of RBC shape change from discocytes to echinocytes to spherocytes was measured as RBC morphology scores on 200 cells, determined by a modification of the method of Usry et al.[14] The percentage of hemolysis was determined by the ratio of free Hb to total Hb. The results are expressed as a percentage of hemolysis to compensate for the differences in storage Hct and Hb concentrations in samples. Centrifuged microhematocrits (Clay Adams, Becton Dickinson, Rutherford, NJ) were performed to prevent osmotic changes in cell volume caused by the isotonic diluent used in the blood analyzer. MCV was calculated from the centrifuged microhematocrit and the RBC count. Microvesicle protein was measured as previously described.[15]

RBC ATP concentrations and whole-blood lactate concentrations were measured in deproteinized supernatants. Whole-blood or packed cell aliquots were mixed with cold 12-percent trichloroacetic acid to precipitate blood proteins and centrifuged at 2700 × *g* for 10 minutes, and the protein-free supernatant was frozen at –80°C until it was tested. ATP was assayed enzymatically with a commercially available test kit (Procedure 366-UV, Sigma).

In Study 1, intracellular and extracellular pH was measured with a benchtop pH meter (Orion 900A, Orion Research, Boston, MA) at 22°C. Blood pH has a temperature coefficient of –0.015 pH units per °C, so pH measured at 22°C will be about 0.22 pH units higher than that measured at 37°C. Blood gases and pH were measured in the second study on a blood gas analyzer (855, Corning, Ithaca, NY). Thus, pH was measured at 37°C.

In Studies 1 and 2, supernatant potassium, glucose, lactate, and inorganic phosphorus testing was performed by standard methods at a licensed central laboratory (Health Alliance Laboratories, Cincinnati, OH).

For Study 3, performed at Walter Reed Army Institute of Research, the total Hb concentration, WBC counts, and RBC counts were measured with a different clinical hematology analyzer (Hematology Cell Counter System Series 9110+, Baker, Allentown, PA), but the centrifuged microhematocrit was used to correct for cell swelling. Supernatant Hb was measured spectrophotometrically by using a modified Drabkin assay.[16]

RBC ATP and lactate concentrations were measured in deproteinized packed cells and whole blood, respectively. Whole-blood or packed cell aliquots were mixed with cold 10-percent trichloroacetic acid to precipitate blood proteins and centrifuged at 2700 × *g* for 10 minutes, and the protein-free supernatant was frozen at –80°C until it was tested. ATP was assayed enzymatically with a commercially available test kit (Procedure 366-UV, Sigma).

Blood gases and pH were measured on a blood gas analyzer (855, Corning). The pH was measured at 37°C. Phos-

phate, lactate, and glucose were measured on a programmable chemical analyzer (Hitachi 902, Boehringer-Mannheim Corp., Indianapolis, IN).

Statistical analysis

Comparisons of means of measured values at given times within the trials were evaluated with the ANOVA using procedures that take account of the weekly repeated measures. Probabilities less likely than 0.05 were considered significant.

RESULTS

In Study 1, the replacement of sodium chloride by sodium bicarbonate was associated, in a linear and dose-dependent manner, with increased RBC ATP concentrations at the end of storage (Fig. 1A). The increases in RBC ATP concentrations were temporally associated with increased glucose consumption (data not shown) and increased lactate production (Fig. 1B). This increased rate of glycolysis occurred in cells stored in solutions with a higher extracellular pH (Fig. 1C), which in turn resulted in a higher intracellular pH (Fig. 1D). The concentration of bicarbonate in the supernatant was increased in proportion to the amount added, and it declined linearly with the length of storage (Fig. 1E). As bicarbonate was broken down, CO_2 was released, and the PCO_2 in the bag initially increased and then declined as the CO_2 diffused from the bags (Fig. 1F). There was no significant differences in RBC morphology or hemolysis with storage in the different solutions (Figs. 1G and 1H). Hemolysis at 10 and 11 weeks was over 2 percent in this study, in which RBCs were stored without WBC reduction.

In Study 2, successive increases in sodium chloride concentration were associated with decreases in MCV and the RBC morphologic index and increases in the shed microvesicle protein in the storage supernatant (Table 2). The decrease in RBC ATP concentration was statistically significant only for the highest dose (Table 2 and Fig. 2A). This change in RBC ATP concentration was not associated with any change in extracellular pH (Fig. 2B) or lactate production (Fig. 2C). At the highest sodium concentration, there was increased hemolysis in the last 2 weeks of storage (Fig. 2D). WBC-reduced RBCs stored in 200 mL of EAS-61 had 0.4-percent hemolysis at 9 weeks and 0.7-percent hemolysis at 11 weeks.

In Study 3, EAS-61, an alkaline AS, caused RBC ATP concentrations to increase during the first 3 weeks of storage (Fig. 3A). This increase was less with 100 mL of AS-3 and did not occur with 200 mL of AS-3 (Fig. 3A). These increased concentrations of RBC ATP were temporally associated with increased supernatant pH (Fig. 3B) and increased lactate production (Fig. 3C). After the pH of the suspending solutions became equivalent at 4 weeks of storage, the rates of lactate production, indicated by the slopes of the lactate concentration plots in Fig. 3C, are only slightly greater in EAS-61. However, storage in EAS-61 was associated with better RBC morphology, and 200 mL of this additive was better than 100 mL (Fig. 3D). Hemolysis was lower in EAS-61 than in AS-3, and the 200-mL volume of each additive solution was better than the 100-mL volume in this regard (Fig. 3E). RBCs stored for 9 weeks in EAS-61 without WBC reduction had 0.4-percent hemolysis.

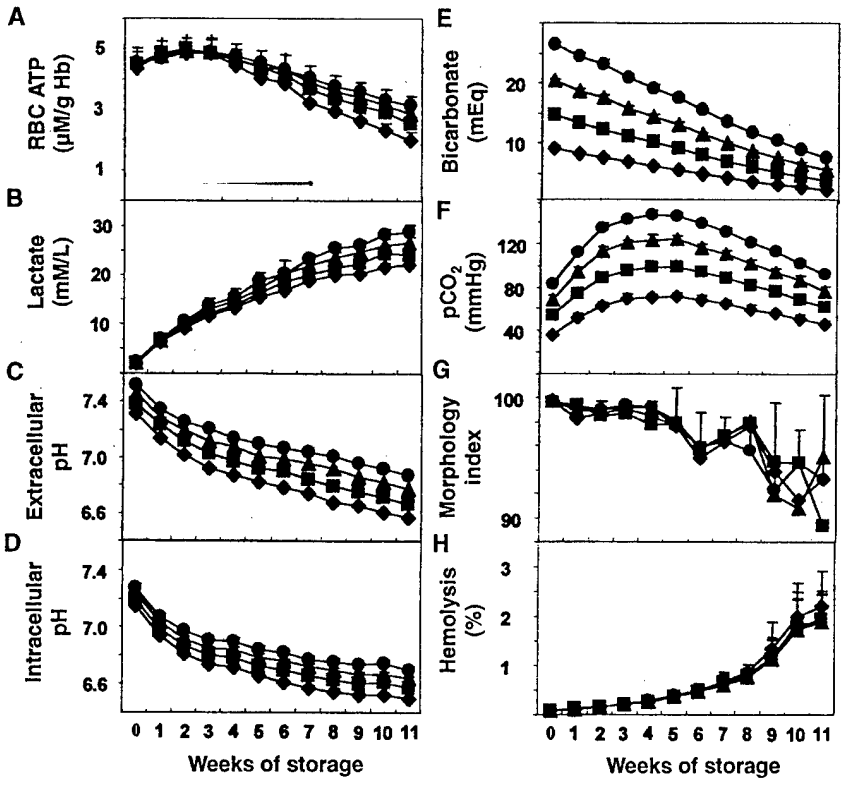

Fig. 1. The effect of increasing concentrations of sodium bicarbonate on A) RBC ATP content, B) whole-blood lactate concentration, C) extracellular pH, D) intracellular pH, E) supernatant bicarbonate concentration, F) supernatant pCO_2, G) RBC morphology index, and H) percentage of hemolysis. The individual solutions contain 0 (◆), 10 (■), 20 (▲), and 30 (●) mEq per L of sodium bicarbonate. Data are presented as mean ± SD; pH was measured at 22°C.

DISCUSSION

We have previously shown that it is possible to make ASs for 9- and 10-week RBC storage.[12,13] These solutions are mixtures of saline, adenine, glucose, mannitol, and disodium phosphate. The solutions are mildly hypotonic to induce cell swelling

TABLE 2. Outcomes of Study 2 after 9-week storage*

	EAS-61 variants with increasing NaCl content			
Measures	EAS-61 (26 mEq)	EAS-71 (50 mEq)	EAS-72 (100 mEq)	EAS-73 (150 mEq)
MCV (fL)	105.5 ± 3.2	100.4 ± 2.1	93.4 ± 2.1	88.5 ± 2.1
Morphology index	81.1 ± 7.8	72.1 ± 9.4	61.2 ± 10.6	53.8 ± 12.3
Vesicle protein (mg/dL RBCs)	8.7 ± 1.7	13.6 ± 1.9	26.7 ± 5.1	40.4 ± 7.8
Hemolysis (%)	0.78 ± 0.11	0.73 ± 0.10	0.84 ± 0.18	1.28 ± 0.33
ATP (% of initial)	53 ± 5	54 ± 5	50 ± 6	45 ± 4

* Values expressed as mean ±SD (N = 6).

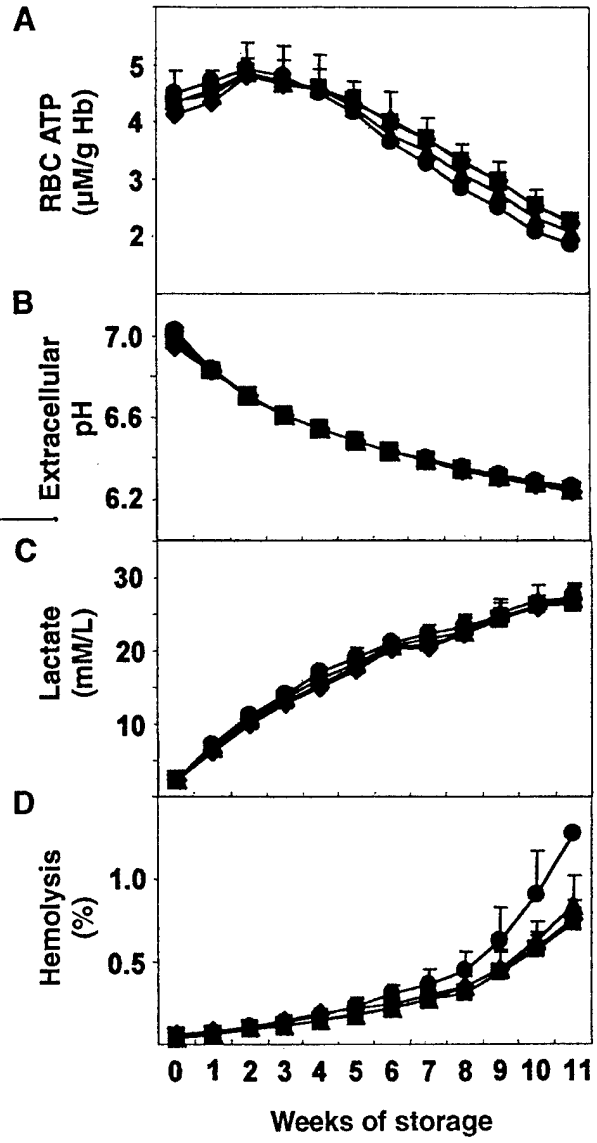

Fig. 2. The effect of increasing concentrations of sodium chloride on A) RBC ATP content, B) extracellular pH, C) whole-blood lactate content, and D) percentage of hemolysis. The individual solutions contain 26 (◆), 50 (■), 100 (▲), and 150 (●) mEq per L of sodium chloride. Data are presented as mean ± SD; pH was measured at 37°C.

and have a pH of about 8.4 as determined by the pK of the disodium phosphate. The solutions appear to work, because they drive RBC ATP synthesis in the first 2 to 3 weeks of storage and because increasing volumes of the ASs are associated with reduced hemolysis. WBC reduction independently reduces hemolysis and is usually necessary when these solutions are used beyond 9 weeks.[17]

To further our understanding and advance the development of these ASs, we have conducted a series of studies to measure the effects of specific changes in solution composition on RBC ATP content and RBC hemolysis. Further, we have tried to improve our understanding of the determinants of altered RBC ATP content by measuring the rates of lactate production and pH decline and of the determinants of hemolysis by measuring changes in RBC morphology and supernatant microvesicle protein.

In Study 1, EAS-64, a 300-mL 10-week AS, was modified by the replacement of sodium chloride with sodium bicarbonate in amounts of 0, 10, 20, and 30 mmol per L, as suggested by the earlier work of Beutler and West.[18] The addition of this salt of a strong base and weak acid makes the solutions more basic initially. The removal of protons in the carbonic anhydrase reaction and subsequent loss of carbon dioxide by diffusion through the plastic bag keep the solutions relatively more basic throughout storage. Lactic acid was produced continuously during storage but at a decreasing rate with time in all the variants of the solution (Fig. 1B). With increasing amounts of bicarbonate, the rate of lactic acid production was better maintained during the course of storage, so that, at the end of storage, the solution with 30 mEq per L of bicarbonate contained 25 percent more lactate. This increased glucolytic activity was associated with increased RBC ATP concentrations after the third week of storage. RBC ATP content is the sum of ATP synthesis and ATP use. The rising ATP concentrations in the first 2 weeks of storage indicate a uniform excess of synthesis in all the solutions. The decreasing concentrations after the third week indicate a net ATP breakdown. If the rate of ATP synthesis is tied to the rate of glycolysis and the rate of breakdown is relatively constant, then the data show how solution pH can be manipulated to maintain RBC energetics, especially at the end of storage. In Study 1, the test units were not WBC reduced, and the hemolysis averaged 2 percent at 10 weeks, as compared to 0.38 percent at 10 weeks in a previous study that included WBC reduction.[13]

In Study 2, EAS-61, a 200-mL 9-week AS, was modified by the addition of sodium chloride so that the resulting solutions had final concentrations of 26, 50, 100, and 150 mmol per L. The addition of sodium chloride had no effect on supernatant pH or the rate of lactate formation, but it did abolish

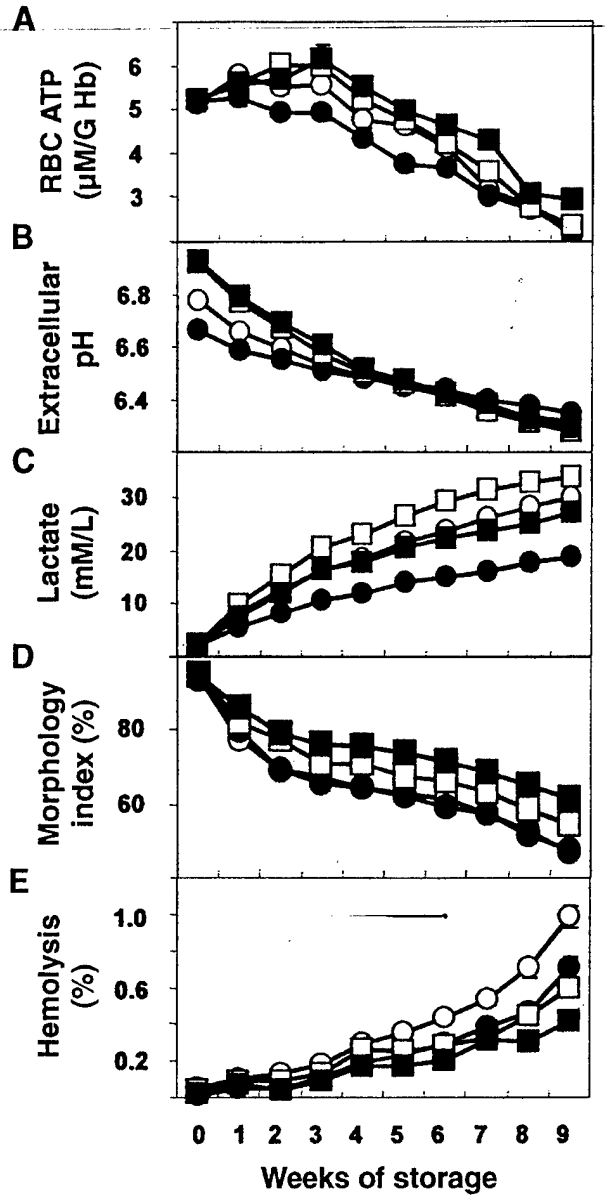

Fig. 3. A comparison of the effects of storage in two different ASs at 2 volumes on A) RBC ATP content, B) extracellular pH, C) whole-blood lactate content, D) morphology index, and E) percentage of hemolysis. The individual solutions and volumes were AS-3 at 100 mL (○), AS-3 at 200 mL (●), EAS-61 at 100 mL (□), and EAS-61 at 200 mL (■). Data are presented as mean ± SEM; pH was measured at 37°C.

cell swelling and led to greater morphologic change, greater loss of membrane microvesicles, and increased overall hemolysis. At the highest salt concentration, the RBC ATP contents were less at the end of storage, which suggests that the shape change and membrane loss were energy-consuming processes.

In Study 3, EAS-61 was compared directly with AS-3 at 100- and 200-mL volumes. EAS-61 has higher pH, but AS-3 has a higher phosphate concentration, which gives it greater buffer capacity. Increasing amounts of AS-3 drove the storage solution pH down, reducing lactate production and preventing the initial rise in RBC ATP concentration. By 4 weeks of storage, the supernatant pH in all the groups was equal and the rate of lactate production became essentially equal. Nevertheless, RBCs stored in the hypotonic EAS-61 had better morphology, and a volume of 200 mL was better than 100 mL in this regard. Both solutions showed reduced hemolysis with increasing storage solution volume, but, at each volume, hemolysis in EAS-61 was approximately half that observed in AS-3.

The EASs, EAS-61 and EAS-64, appear to work to improve storage in several ways. First, they raise the pH in the storage solution and therefore in the intracellular space. The higher intracellular pH appears to drive glycolysis and therefore ATP synthesis in the early weeks of storage, so that the later phase of declining RBC ATP content starts both at a higher maximum and later in the course of storage. Second, relative osmotic hypotonia causes RBC swelling, which in turn improves the morphology and reduces the membrane loss by microvesiculation. The shape change and microvesiculation appear to be energy-consuming processes, and minimizing them appears to conserve ATP for other uses. Finally, as observed previously, increasing the volume of AS reduces the hemolysis in all the solutions we have tested.

The experiments described here suggest several ways to improve these solutions. Replacing some of the sodium chloride with sodium bicarbonate is an obvious first step. Exactly how much bicarbonate to use is less clear, in that more appears to be better from the point of storing the cells, but higher doses may cause clinical problems. Optimizing the tonicity of the solutions relative to the planned AS volume to minimize hemolysis appears to be straightforward also, as the second experiment suggests that optimal tonicity is close to the present formulation of EAS-61 for a 200-mL AS. Finally, other changes in the storage system that increase the initial intracellular pH of the stored cells are expected to be beneficial.[19,20] Obviously, in vitro biochemical measures are only a partial surrogate for measured in vivo recovery and survival of RBCs.[21]

The potentially more difficult question is how best to exploit the success of these solutions. They appear to have the ability to extend to at least 10 weeks and probably beyond the period during which RBCs can be stored with greater than 75-percent recovery and less than 1-percent hemolysis. At the same time, they can reduce the physiologic burden on transfusion recipients due to the consequences of transfusing RBCs with the storage lesion. The exact time of extended storage determines the ratio of these two benefits. The US Army, which paid for this research, would like the additional storage time to improve blood supply logistics. Rational health care economy would suggest that the cost savings would be greater if most of the benefit of these solu-

tions were taken in transfusing cells with less storage damage. Perhaps the lessons learned from these studies can guide further development on a path toward achieving both of these goals.

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Appendix 5:

Vox Sanguinis (2001) 81, 161–166

ORIGINAL PAPER

The effects of polyvinyl chloride and polyolefin blood bags on red blood cells stored in a new additive solution

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Vox Sanguinis

Background and Objectives Red blood cells (RBCs) must be stored in polyvinyl chloride (PVC) bags plasticized with di-2-ethylhexyl phthalate or a similar plasticizer to achieve their full storage life with conventional storage solutions. Improved storage solutions might remove this requirement and allow blood storage in other plastics. Experimental Additive Solution-61 (EAS-61), which maintains RBCs for 9 weeks with reduced haemolysis and satisfactory ^{51}Cr 24-h recovery, is an appropriate candidate improved RBC storage solution.

Materials and Methods Twenty-four units of packed RBCs were pooled in groups of four units, each pool was realiquoted into four units and stored, six pooled units per arm, in one of the following: 100 ml of EAS-61 in PVC; 200 ml of EAS-61 in PVC; 100 ml of EAS-61 in polyolefin (PO); and 200 ml of EAS-61 in PO. Haemolysis, RBC morphology indices, RBC ATP concentrations, and other measures of RBC metabolism and function were measured weekly.

Results RBC haemolysis exceeded 1% by 7 weeks in PO bags containing 100 ml or 200 ml of EAS-61. In PVC bags, haemolysis was less than 1% at 11 weeks. RBC ATP concentrations were 1 mol/g of haemoglobin (Hb) higher at 2 weeks in the PVC-stored units.

Conclusions RBCs stored in PVC had markedly less haemolysis and higher RBC ATP concentrations than those stored in PO. Haemolysis would limit RBC storage in PO bags to a duration of 6 weeks, even with EAS-61.

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Key words: blood storage, di-2-ethylhexyl phthalate, humans, plastics, polyolefin, polyvinyl chloride, RBC ATP concentrations, RBC haemolysis, RBC storage.

Introduction

Plastic blood bags make transfusion safer for patients, make blood handling safer for health care workers, and reduce the burden of medical waste on the environment [1]. However, plasticizers can leach from the plastic bags into the contained fluids [2], bag makers can be exposed to carcinogenic vinyl chloride monomer [3], and the ozone layer can be degraded by incineration of medical wastes containing chlorinated hydrocarbons [4]. Alternatives to the presently used blood bag plastics might retain the healthcare benefits of plastic blood bags while avoiding some of their environmental consequences.

Polyvinyl chloride (PVC) plasticized with di-2-ethylhexyl phthalate (DEHP) is the standard material used for red blood cell (RBC) storage bags. This plastic was chosen initially for its manufacturing and handling qualities [5]. It was subsequently shown that the DEHP improves RBC storage by reducing haemolysis and membrane loss by microvesiculation [6,7]. Other plastics, such as polyolefin (PO), which is used in platelet storage bags because of its high gas permeability, were

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Appendix 6:

associated with greater RBC haemolysis and microvesiculation when used in conjunction with standard storage solutions, but performed better when DEHP or similar plasticizers were added to the stored RBCs [8–10]. Without the protection afforded by the plasticizer, RBC storage would be limited to 3 weeks with conventional storage solutions [11,12].

Recently, we have developed an additive storage solution, Experimental Additive Solution-61 (EAS-61), in which RBCs can be stored for 9 weeks in PVC bags [13]. EAS-61 works, in part, by suppressing RBC microvesiculation and haemolysis. The increased volume of additive solution also seemed to be important for raising RBC ATP concentrations, improving RBC morphology and reducing haemolysis [14]. To evaluate whether EAS-61 might suppress RBC haemolysis during storage in a different plastic and to learn more about cellular mechanisms important for blood storage, we conducted a study comparing RBCs stored in PVC and PO containers in 100- and 200-ml volumes of EAS-61. Although haemolysis of RBCs stored in EAS-61 was below conventional limits for at least 6 weeks, RBCs stored in PO bags had four times more haemolysis and lower RBC ATP concentrations than those stored in PVC.

Materials and methods

Volunteers

Twenty-four healthy volunteers, who were donating full units of blood for the collection of anthrax-immune plasma, gave consent for their RBCs to be used in storage studies. The donors met standard blood donor criteria and gave informed consent in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research and the U.S. Army's Human Subjects Research Review Board.

Storage solution

The EAS-61 solution (composition: NaCl 26 mM, adenine 2 mM, glucose 110 mM, mannitol 55 mM, Na_2HPO_4 12 mM) was prepared in the laboratory from high-purity adenine, sugars, salts and water, and sterile-filtered into 1-l PO storage bags (code 4R2238; Baxter Healthcare Corp., Roundlake, IL). The bags were stored at 37 °C for 2 weeks. On the eighth day, a sample was sent to a commercial reference laboratory (The Associates of Cape Cod, Falmouth, MA) for endotoxin testing using the Limulus Amoebocyte Lysate assay. At the end of 2 weeks, the solutions were cultured. The cultures were incubated for a further 2 weeks. Sterility was confirmed by the absence of bacterial and fungal growth in the cultures after 14 days.

Study design

We conducted a 'pooling' study to evaluate RBC morphology, metabolism and integrity over the course of 11 weeks of storage in PVC and PO bags. Pooling reduces the largest source of variability in conventional blood storage studies, i.e. the differences between RBCs from different donors [15], by placing some of the cells from each donor in every treatment group of the study while maintaining conventional unit size. Twenty-four RBC units, unreactive in the indirect antiglobulin test (IAT), were grouped into sets of four ABO-matched units. Each set was then pooled, mixed and aliquoted by weight to make four pooled units. Blood was handled in a manner intended to minimize contact with PVC plastic until the pooled RBCs were aliquoted.

The four units from each pool were aliquoted as follows: two units into PO bags and two units into PVC bags. The two units in each pair were then diluted, one with 100 ml of EAS-61 and the other with 200 ml of EAS-61. The units were stored at 1–6 °C for 11 weeks. Electrolytes, pH, metabolites, blood gases, RBC morphology indices, haemolysis, and RBC ATP concentrations were measured weekly.

RBC unit preparation

Standard units (450 ± 45 ml) of blood were collected from each donor into 63 ml of citrate–phosphate–dextrose (CPD) anticoagulant in 1-l PO bags (4R2238, Baxter Healthcare Corp.). The bags had been modified by sterile transfer of the CPD and the needle with a short length of the PVC tubing from a commercial triple bag collection set (BB*AGD456A; Terumo Medical Corp, Elkton, MD) using a sterile connecting device (SCD 312; Terumo Medical Corp.). Twenty of the units were drawn on a mobile blood drive and refrigerated at 4 °C for 1–6 h before transportation in a shipping container with wet ice 40 miles to the laboratory. Four units were drawn in the laboratory and processed within 4 h. Packed cells were prepared by centrifugation (at 5000 *g*) for 5 min at 4 °C followed by the removal of sufficient plasma to achieve a haematocrit of 85%. ABO/Rh and IAT testing were performed using tube methods and commercial reagents (Immucor, Inc., Norcross, GA). ABO-matched, IAT non-reactive RBCs were then pooled separately, in groups of four, in six one-litre PO bags, mixed thoroughly, and aliquoted into the study units by weight using sterile tubing connection for all transfers. The aliquots were transferred to either 600-ml PVC transfer bags (code 4R2023; Baxter Healthcare Corp.) or 1-l PO storage bags (code 4R2238, Baxter Healthcare Corp). Either 100 ml or 200 ml of EAS-61 was added to the RBC aliquot prior to storage.

In vitro measurements

Samples were collected, after gentle mixing by inversion, by a sterile sampling procedure in a biological safety cabinet. A battery of *in vitro* tests was performed on all units at the beginning of storage and weekly thereafter.

The total haemoglobin (Hb) concentration was measured by using a clinical haematology analyser (Hematology Cell Counter System Series 9110+; Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically using the modified Drabkin assay [16]. Percentage haemolysis was determined by the ratio of free to total haemoglobin. The results are expressed as percentage haemolysis to compensate for the differences in haematocrit and Hb concentrations between samples. Centrifuged microhaematocrits (Clay Adams; Becton-Dickinson, Rutherford, NJ) were performed to avoid the readjustment of cell volume and subsequent errors caused by the isotonic diluent used in the blood analysers.

RBC ATP, glucose and lactate concentrations were measured in supernatants of deproteinized RBCs. Cell aliquots were mixed with cold 10% trichloroacetic acid to precipitate blood proteins, centrifuged at 2700 *g* for 10 min, and the protein-free supernatant was frozen at −80 °C until tested. ATP was assayed enzymatically using a commercially available test kit (Procedure 366-UV; Sigma Diagnostics, St Louis, MO).

The concentration of supernatant chloride, pH and blood gases were measured using a blood gas analyser (Corning 855; Corning, Ithaca, NY). Thus, pH was measured at 37 °C. Sodium, potassium, phosphate, lactate and glucose were measured on a programmable chemical analyser (Hitachi 902 Analyser; Boehringer Mannheim Corporation, Indianapolis, IN). The average degree of RBC shape change, from discocytes to echinocytes to spherocytes, was measured according to the method of Usry *et al.* [17].

Statistical analysis

Comparisons of means of measured values at given times between the arms of the trial were evaluated by analysis of variance. *P*-values of $< 0{\cdot}05$ were considered statistically significant.

Results

Significant differences were observed between the PVC- and PO-stored cells in RBC ATP concentrations, RBC morphology and haemolysis. Figure 1(a) and Table 1 show that in the early phases of storage, RBC ATP concentrations were, on average, 1 μmol/g of Hb higher in the PVC-stored cells. Initially, the RBC ATP concentration increased in the PVC-stored cells, but not in the PO-stored RBC. At the end of storage, this difference diminished as the RBC ATP concentration decreased more slowly in the PO-stored cells. This difference was not related to differences in the rate of glycolysis (data not shown) or increasing lactate concentrations (Fig. 1b), which were the same for the equivalent volumes of EAS-61. RBC morphology indices were worse in PO-stored cells after the first week of storage (Fig. 1c). The rate of decline in the morphology indices was twice as great in PO than in PVC.

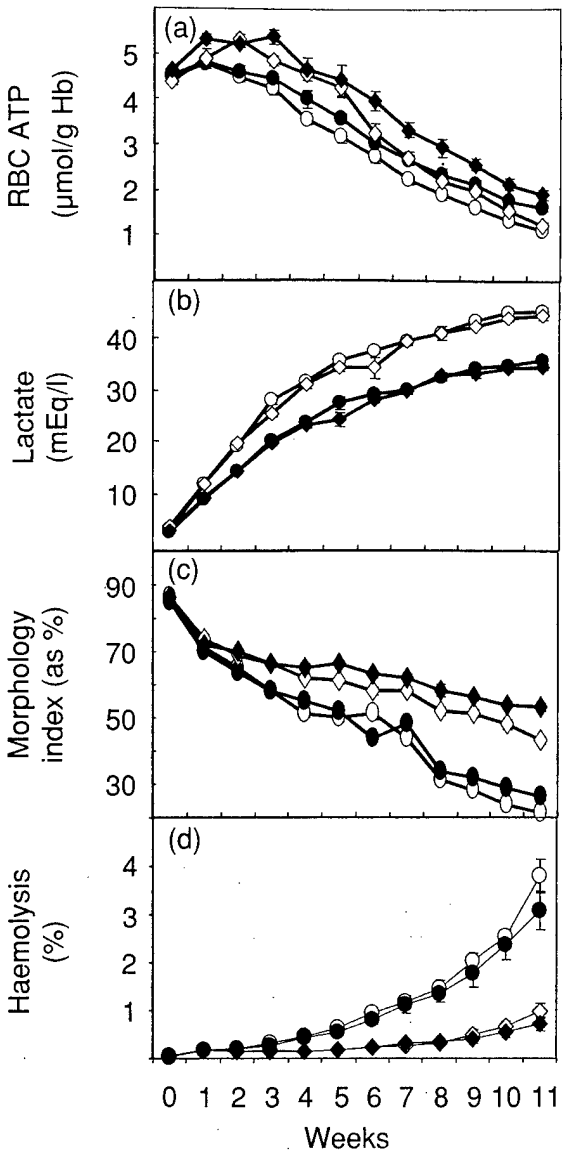

Fig. 1 Weekly measurements of (a) red blood cell (RBC) ATP concentration; (b) RBC lactate concentration; (c) RBC morphologic index; and (d) fractional RBC haemolysis for the RBCs stored in: 100 ml of Experimental Additive Solution-61 (EAS-61) in polyvinyl chloride (PVC) (◇); 200 ml of EAS-61 in PVC (◆); 100 ml of EAS-61 in polyolefin (PO) (○); and 200 ml of EAS-61 in PO (●). RBC ATP concentrations were higher in the early phases of storage in PVC bags, but the amount of lactate produced did not differ. RBCs stored in PO bags showed greater morphological change and haemolysis. Data are presented as mean ± standard error of the mean (n = 6 pooled units per group).

There was a small (but not statistically significant) effect of increased storage volume, leading to slightly better morphology (Table 1). Finally, RBC stored in PO had four times the haemolysis of cells stored in PVC (Fig. 1d). Increasing the volume of EAS-61 from 100 ml to 200 ml had a small (but

Table 1 Selected values of red blood cell (RBC) ATP concentration, haemolysis and morphology during storage

Plastic and volume of EAS-61	RBC ATP (mol/g of Hb)		Haemolysis (%)			Morphology index	
	2 weeks	9 weeks	6 weeks	7 weeks	9 weeks	7 weeks	9 weeks
PVC with 100 ml	5·32 ± 0·11	1·97 ± 0·10	0·24 ± 0·03	0·25 ± 0·02	0·49 ± 0·11	58 ± 1·2	51 ± 1·3
PVC with 200 ml	5·22 ± 0·10	2·58 ± 0·13	0·24 ± 0·02	0·31 ± 0·04	0·39 ± 0·08	62 ± 1·0	56 ± 1·0
PO with 100 ml	4·47 ± 0·08	1·62 ± 0·06	0·93 ± 0·09	1·16 ± 0·12	2·03 ± 0·17	44 ± 1·4	28 ± 0·36
PO with 200 ml	4·60 ± 0·14	2·15 ± 0·08	0·80 ± 0·08	1·12 ± 0·19	1·76 ± 0·27	48 ± 1·4	32 ± 0·41

EAS-61, Experimental Additive Solution-61; Hb, haemoglobin; PO, polyolefin; PVC, polyvinyl chloride.

again not statistically significant) effect on reducing haemolysis in this system (Table 1). RBC haemolysis exceeded 0·8% by 6 weeks and 1% by 7 weeks in PO bags containing 100 ml or 200 ml of EAS-61.

Gas transport was also different in the two kinds of bags, but it did not appreciably affect the storage solution pH (Fig. 2a). The partial pressure of CO_2 (Fig. 2b) and the concentration of bicarbonate (Fig. 2c) were higher in the PVC bags after the first week. The oxygen partial pressure in the PO bags was also higher at all time-points after the initial measurement (Fig. 2d). The shoulder of the oxygen saturation curve was reached at 3 weeks in PO and at 10 weeks in PVC.

Electrolytes were not affected by storage bag composition. Supernatant sodium concentration decreased (Fig. 3a), and potassium concentration increased (Fig. 3b), with storage. The chloride concentration of the suspending fluid (Fig. 3c) increased for the first 2 weeks and then slowly decreased. Phosphate concentration in the suspending solution (Fig. 3d) decreased in the first 2 weeks of storage and slowly increased thereafter. The sum of the concentrations of the cations, sodium and potassium, and the anions, chloride, bicarbonate, phosphate and lactate, were approximately equal when corrected for valence.

Discussion

This study showed that EAS-61, a new RBC storage solution, could potentially increase the shelf-life of RBC stored in PO bags to 5 or 6 weeks. However, RBC stored in PO bags had four times the amount of haemolysis at each weekly measurement as when the same cohort of cells were stored in PVC bags. RBCs that had a clinically acceptable fractional haemolysis of less than 0·8% after 10 weeks when stored in PVC bags, had greater than 0·8% haemolysis at 6 weeks and greater than 1% haemolysis at 7 weeks when stored in PO bags in EAS-61.

The increased haemolysis was probably related to the absence of DEHP in the PO bags. Greenwalt and his colleagues have shown that RBCs stored in CPD in PO bags have more rapid shape change, greater membrane loss and increased haemolysis [10]. This haemolysis was mostly in the form of shed microvesicles. When they added DEHP to RBC stored in the PO bags, the shape change, microvesiculation and haemolysis were reduced. In the present study, exposure of warm blood to PO bags for just a few hours in the course of pooling caused a rapid initial shape change which persisted but progressed at a slower rate after the RBCs were transferred to PVC bags. We have previously reported that RBCs stored continuously in PVC bags in 100 ml or 200 ml of EAS-61 had a more discoid morphology throughout storage than was observed in this study, yet very similar rates of haemolysis with time [14]. Therefore, it appears that DEHP can suppress further shape change and haemolysis, even after the shape change process has been initiated.

RBC ATP concentrations were lower in the PO-stored cells. However, the rates of glucose consumption and lactate formation were the same for RBC stored in both types of plastic bags. This means that overall rates of glycolysis were the same. In the Embden-Meyerhof pathway, 1 mole of lactate is produced for every mole of ATP produced, so the rates of ATP synthesis might be expected to be similar. However, the RBC ATP concentration differences, about 0·8 μmol/g of Hb at 2 weeks, represent the product of only 1% of total glycolysis based on the amount of lactate produced by that time, and thus no useful relationship can be elucidated. The concentrations of RBC ATP are a function of the rates of synthesis and the rates of utilization. RBC ATP was either made less efficiently or was consumed at a greater rate in a process that was temporally related to the RBC shape changes.

The PO bags have greater gas permeability than the PVC bags. As a result, CO_2 partial pressures were lower and O_2 partial pressures were higher in the PO bags throughout the storage interval. These differences in gas tension may have contributed to the RBC ATP differences in two different ways. First, the loss of CO_2 might have affected the intracellular pH through the bicarbonate buffer system. Second, the higher partial pressure of O_2 may have led to oxidative stress. Significant consequences of either of these effects seem unlikely based on the work of Greenwalt and his colleagues cited above, which showed that adding DEHP to RBC in PO bags improved RBC ATP concentrations and reduced haemolysis.

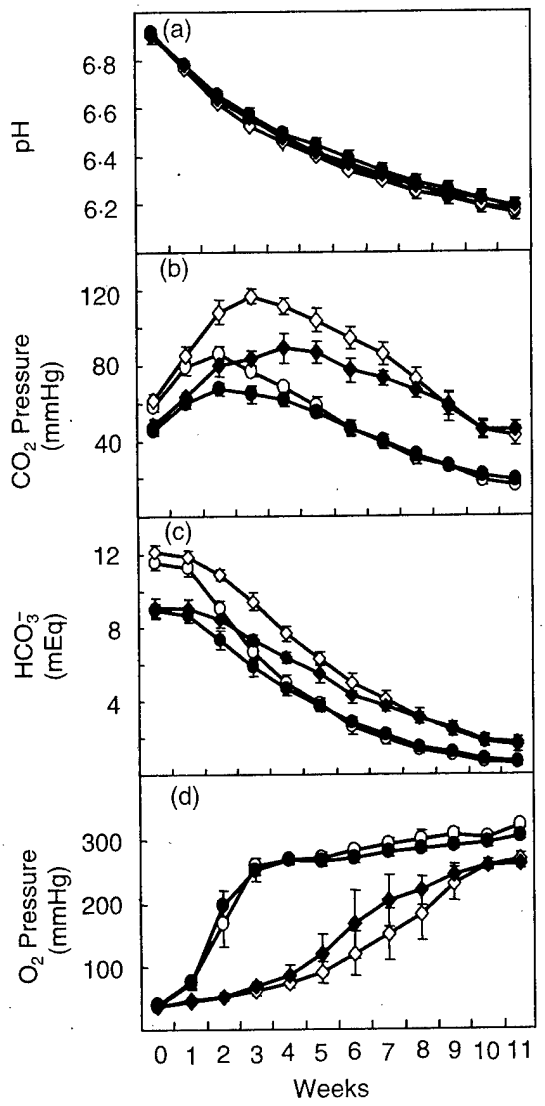

Fig. 2 Weekly measurements of (a) supernatant pH; (b) CO_2 partial pressure; (c) bicarbonate concentration; and (d) oxygen partial pressure for the red blood cells (RBCs) stored in: 100 ml of Experimental Additive Solution-61 (EAS-61) in polyvinyl chloride (PVC) (◇); 200 ml of EAS-61 in PVC (◆); 100 ml of EAS-61 in polyolefin (PO) (○); and 200 ml of EAS-61 in PO (●). Diffusion of CO_2 out of and of O_2 into the PO bags was ≈ three times more rapid than observed with PVC bags, but there was no significant effect on pH. Data are presented as mean ± standard error of the mean (n = 6 pooled units per group).

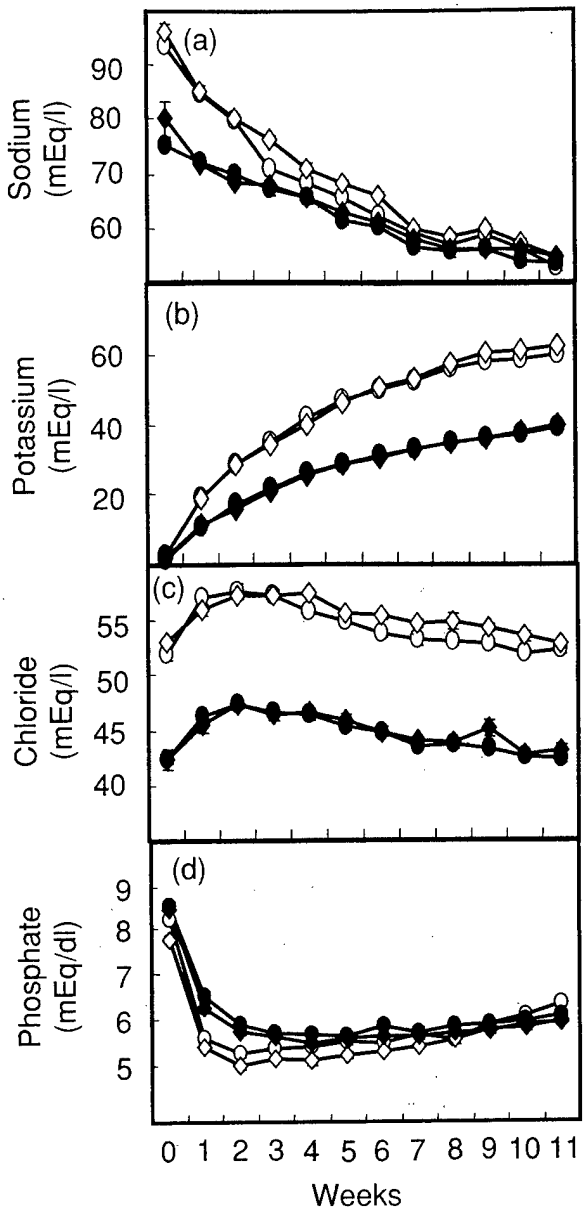

Fig. 3 Weekly measurements of (a) supernatant sodium concentration; (b) supernatant potassium concentration; (c) supernatant chloride concentration; and (d) supernatant inorganic phosphate concentration for the red blood cells (RBCs) stored in: 100 ml of Experimental Additive Solution-61 (EAS-61) in polyvinyl chloride (PVC) (◇); 200 ml of EAS-61 in PVC (◆); 100 ml of EAS-61 in polyolefin (PO) (○); and 200 ml of EAS-61 in PO (●). EAS-61 volume, but not bag composition, affected the electrolyte composition. Data are presented as mean ± standard error of the mean (n = 6 pooled units per group).

Hogman and his colleagues suggested that the lack of DEHP may also contribute to the increased microvesiculation and haemolyis that occurs when RBC are stored tightly packed and unmixed in PVC bags [18]. Reduced RBC ATP concentrations were also observed in these densely packed and unmixed cells, but were attributed to locally low pH.

Recently, efforts to understand the signal transduction of microvesiculation or 'bleb' formation in apoptotic cells has led to the identification of a signal transduction pathway wherein caspase-3 cleaves the ROCK-1 kinase to produce a truncated molecule with constitutive myosin light-chain kinase activity [19,20]. Activation of the mechanism leads to cytoskeletal contraction and bleb formation. Knock-out

deletions of ROCK-1 prevent bleb formation without preventing other aspects of apoptosis. Such a mechanism could tie together the major findings of this study: reduced RBC ATP and increased shape change and haemolysis. It may provide a basis for understanding the effects of DEHP and for understanding significant events in the development of the RBC storage lesion.

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ELEVEN WEEK RED BLOOD CELL STORAGE

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Running Head: 11-week RBC storage

Abstract:

Background: Increasing the duration of red blood cell (RBC) storage can increase both RBC availability and quality. This work addresses 11-week RBC storage in experimental additive solutions (EAS).

Study Design and Methods: Three studies were performed. In the first, 24-hour *in vivo* recovery of ^{51}Cr-labeled autologous RBC was measured in 9 volunteers after storage of their RBC for 11 weeks in EAS 67. In the second study, four units of blood were divided, stored in aliquots with an EAS containing 0, 15, 30, or 45 mmol/L of mannitol, and hemolysis, RBC morphology, and microvesicle protein measured. In the third study, six full units were stored for 12 weeks in the EAS containing 30 mmol/L of mannitol with weekly sampling for morphologic and biochemical measures of RBC quality.

Results: RBC stored for 11 weeks in EAS-67 had a 24 hr *in vivo* recovery of 79 ± 5%, but the hemolysis was 1.35 ± 0.68 %. Increasing mannitol content of the EAS reduced hemolysis but increased microvesiculation. EAS-76, with 30 mmol/L of mannitol allowed 11 week storage with 0.48 ± 0.10 % hemolysis at 11 weeks and 0.62 ± 0.14 % hemolysis at 12 weeks.

Conclusions: It is possible to store RBC for 11 weeks in EAS with greater than 75% recovery and less than 1% hemolysis.

Key Words: Blood storage, RBC storage, Humans, RBC Hemolysis, RBC ATP concentrations,

Introduction:

Longer red blood cell (RBC) storage will require better RBC storage systems[1,2]. The practical limits on RBC storage, hemolysis and the loss of viability, are cumulative events that decrease the quality and utility of the cells. The effects of excessive hemolysis and loss of viability can be partially offset by additional procedures such as cell-washing or metabolic rejuvenation. The patients who receive these RBCs, and the transfusion medicine specialists who manage them can all be better served by building improved quality into the next generation of RBC storage solutions and systems.

The authors have described 9- and 10-week RBC storage systems using leukoreduction and storage in 200 or 300 mL volumes of hypotonic alkaline experimental additive solutions (EASs) containing saline, adenine, glucose, mannitol, and disodium phosphate (SAGMdP)[3,4]. These systems seem to work by supporting an initial increase of RBC adenosine 5'-triphosphate (ATP) synthesis that provides metabolic energy for cell-sustaining processes during longer storage and by suppressing the RBC shape changes and membrane microvesiculation that contribute substantially to hemolysis. The authors have also shown that it is possible to achieve greater rates of ATP synthesis during prolonged storage by replacing some of the sodium chloride with sodium bicarbonate[5].

In an attempt to further increase the time of allowable storage with these SAGMdP EASs, the authors conducted three studies. First, we conducted a human trial measuring the RBC recovery after 11 weeks using bicarbonate-containing EAS-67. This study showed a mean 24-hour RBC recovery of 79% but 1.35 % hemolysis. Second, we measured the effect of increasing the concentrations of mannitol to reduce hemolysis during 11 weeks of storage. Finally, we

measured hemolysis during 12 weeks of RBC storage in one of the solutions containing a higher mannitol concentration. This paper describes these studies.

Materials and Methods:

Volunteers

Three groups of healthy volunteers met standard blood donor criteria for allogeneic blood transfusion and individually gave informed consent to participate in these studies[6]. The first (n=10) and second (n=4) groups donated full units of blood under protocols approved by the Hoxworth Blood Center and the University of Cincinnati's Institutional Review Board, while the third (n=6) group donated in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research.

Storage Solutions

The compositions of the various EASs are compared in Table 1. The EASs were made in the laboratory from USP adenine, sugars and salts (Sigma, St. Louis, MO) and sterilely filtered into one-liter storage bags (Code 4R2032, Baxter Healthcare, Deerfield, IL). The bags were held at room temperature for two weeks. The solutions were then cultured and the cultures incubated for another two weeks. When sterility was confirmed by the absence of bacterial and fungal growth for 7-14 days, the solutions were aliquoted by weight into 600 mL PVC bags (Code 4R2023, Baxter Healthcare Corp., Deerfield, IL) All connections were made using a sterile connecting device (SCD 312, Terumo Medical Corp. Elkton, MD).

Study Designs

Each of the three studies reported in this work had a different design. The different designs were necessitated by the different natures of the questions asked during each phase of the development process.

In the first study, ten volunteers each donated a unit of blood. This was then processed into packed RBCs and mixed with 300 mL of an EAS containing 30 mEq/L of bicarbonate (EAS-67). The units were sampled and then stored at 1-6°C for 11 weeks. At the end of that time the units were checked for sterility, sampled, and aliquots were radiolabeled with ^{51}Cr and reinfused into the original donors for the determination of the 24-hour RBC recovery. Samples were analyzed for RBC ATP content and hemolysis.

In the second study, four volunteers each donated a unit of blood. The units were split into four fractions and stored in variants of EAS 67. The variants were designated EAS-74, 75, 76, and 77 and contained 0, 15, 30 or 45 mmol/L of mannitol. The units were stored at 1-6°C for 11 weeks and sampled at 1 hour and 6 and 11 weeks for bio-chemical and morphologic measurements.

In the third study, six volunteers each donated a unit of blood which was stored as packed RBCs in EAS-76 containing 30 mmol/L of mannitol. The units were stored at 1-6°C for 12 weeks with weekly sampling for biochemical and morphologic measurements.

RBC Unit Preparation

In all studies standard units of blood (450 ± 45 mL) were collected in 63mL of CPD solution in a triple-bag collection system (Code 4R1402, Baxter Healthcare Corp.). Units were

leukoreduced by filtration (Sepacell RS-2000 filters, Code 4R3303, Baxter Healthcare Corp.). All samples were obtained using a sterile connecting device.

First study: Packed RBCs were prepared after centrifugation for 10 min as previously described [7]. Three-hundred milliliters of EAS-67 was added to the units of packed cells using a sterile connecting device. Units were stored undisturbed at 1-6°C for 11 weeks except for sample removal at one week prior to the recovery studies for fungal and bacterial culture [7].

Second study: RBCs were prepared by centrifugation for 10 min and divided into four aliquots in 150 mL transfer packs (Code 4C2405, Baxter Healthcare Corp.). Seventy-five milliliter aliquots of EASs 74, 75, 76, or 77 were added, one each to the RBC aliquots from each original blood donation. Units were stored upright at 1-6°C for 11 weeks except for mixing and sample removal at 1 hour, and 6 and 11 weeks.

Third study: Packed RBCs were prepared by centrifugation for 5 minutes and 300 mL of EAS-76 was added sterilely. Units were stored upright at 1-6°C for 11 weeks except for weekly mixing and sample removal.

In Vitro Measurements at the Hoxworth Blood Center

The methods used for the measurement of hemoglobin and MCV, ATP, pH, osmolality and percent hemolysis have been described previously.[7] Post-leukoreduction leukocyte counts were performed with a Nagaotte chanber. The RBC morphology index was measured by a modification of the method of Usry, Moore, and Manalo[8]. The quantity of total protein shed in microvesicles was determined as described previously[9].

In Vitro Measurements at the Walter Reed Army Institute of Research

Leukoreduction was confirmed by flow cytometry. The total hemoglobin (Hb) concentrations were measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9000, Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically using the modified Drabkin assay.[10] Percent hemolysis was determined by the ratio of free to total hemoglobin. The results are expressed as percent hemolysis to compensate for the differences in hematocrit and Hgb concentrations between samples.

RBC ATP concentrations were measured in the supernatants of deproteinized RBCs. Packed cell aliquots were mixed with cold 10% trichloroacetic acid to precipitate blood proteins, centrifuged at 2700 x g for 10 minutes, and the protein-free supernatant frozen at -80°C until tested. ATP was assayed enzymatically using a commercially available test kit (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO).

Measurement of Autologous 24-hour In Vivo RBC Recovery

On the last day of storage a 10 mL sample was labeled with approximately 15μCi of ^{51}Cr as recommended by the International Committee on Standardization in Hematology [11] and Moroff et al. [12] and reinfused into the donor. Blood samples were drawn at 5, 7.5, 10, 12.5, 15, 20, and 60 minutes and 24 hours postinfusion for gamma-counting (Cobra II, Meriden, CT).

Statistics

Means and standard deviations were calculated as descriptive statistics within the 8- and 9-week storage groups. Box plots and line graphs were used to display the data[13].

Results:

Ten units were collected for the 11-week recovery measurements. One broke in handling. The remaining nine were stored for 11 weeks, showed no bacterial or fungal growth, and were labeled and reinfused into the original donors. The mean 24 hour recovery of RBCs, stored for 11 weeks in 300 mL of EAS-67 and reinfused into the original donors, was 79%, when measured by the ^{51}Cr single-label technique (Figure 1). However, the hemolysis in these units was unacceptably high at 1.35 ± 0.68 %, well above the historic standard of 1% for product licensure for routine transfusion. Survival of the cells beyond 24 hours was not measured.

When RBC were stored in variants of EAS-67 containing increasing concentrations of mannitol, the total hemolysis at the end of 11 weeks of storage declined as the concentration of mannitol increased (Table 2). This suppression of hemolysis with addition of mannitol occurred in spite of the increasing osmolality, which in turn was associated with less RBC swelling, greater morphologic changes, and greater production of microvesicles. Most of the benefit of adding mannitol for reducing hemolysis was obtained at a concentration of 30 mmol/L.

When six further whole units were stored in the variant with 30 mmol/L of mannitol, EAS-76, hemolysis was 0.48 ± 0.10 % at 11 weeks and 0.62 ± 0.14 % at 12 weeks (Figure 2). The RBC ATP concentrations were maintained above 2.5 μmol/g Hb throughout the course of 12-week storage.

Discussion:

We have developed solutions that allow the liquid storage of refrigerated RBCs for 10 weeks. In these solutions, the physical stability of the cells, measured as their fractional

hemolysis, and the viability of the cells, measured as their recovery 24 hours after reinfusion into the original donor, are as good as those of RBCs from the same donors stored for 6 weeks in presently licensed conventional AS[4].

It appears possible to improve the performance of these RBC additive solutions yet further with simple modifications of the solution composition. Thus, we have shown that it is possible to increase the RBC ATP content by 1 μmol/g Hb at the end of 11 weeks of storage by replacing 30 mmol/L of sodium chloride with 30 mmol/L of sodium bicarbonate[5]. RBC ATP content is a correlate of RBC viability, suggesting that the viability of RBCs stored in the new solution, EAS-67, might be greater or that such RBCs could be stored longer.

We tested this hypothesis in the first study reported here. Leukoreduced RBCs stored in 300 mL of EAS-67 had a mean recovery of 79 ± 5%. However, the hemolysis, which had averaged 0.7% in the initial *in vitro* study, averaged 1.4% at the end of this *in vivo* trial.[5]

To reduce this rate of hemolysis, we added more mannitol to the solution. We did this first in a stepwise manner (Study 2), which showed that increasing amounts of mannitol reduced hemolysis. This occurred despite the simultaneous finding that adding more mannitol increased the osmolality of the solution, which in turn was associated with greater RBC shape change and more membrane loss by microvesiculation. Most of the benefit of adding more mannitol appeared to be achieved at a concentration of 30 mmol/L.

In the final study, a variant of EAS-67 with 30 rather than 20 mmol/L of mannitol, EAS-76, was tested during 12 weeks of *in vitro* storage. Mean hemolysis was 0.48 ± 0.10 % at 11 weeks and 0.62 ± 0.14 % at 12 weeks.

These studies suggest that it is possible to make an 11-week RBC storage solution that

would meet the usual FDA criteria of greater than 75% RBC recovery and less than 1% hemolysis. EAS-76 appears to be such a solution. This solution can probably be improved even further by reducing the osmolality while maintaining the high mannitol concentration. This modification would be expected to have the effect of reducing the increased shape change and microvesiculation to the levels observed with EAS-74. Since the microvesiculation is both an energy-requiring process and a component of the total hemolysis, suppressing it would be expected to increase the RBC ATP content and reduce total hemolysis further[14,15,16].

Maintenance of RBC ATP concentrations is a necessary but not always sufficient condition of for high RBC recoveries after long-term storage. The correlation of ATP content and recovery is often weak. Thus, additional *in vivo* studies using larger numbers of volunteers, using second radioactive labels for measures of red cell mass independent of stored-cell dilution, and multiple populations would all be helpful. Multiple populations are especially important because interpersonal differences in the "storeability" of RBCs are the largest source of variation in most RBC recovery studies. Not every group will necessarily have the characteristics of the group of Caucasian women blood center workers who were our study population. Also, studies of the *in vivo* survival of such cells after the first 24 hours and of their ability to regenerate 2,3-diphosphoglycerate *in vitro* would provide additional reassurance that RBC stored for these long periods might behave normally when reinfused.

The utility of increasing liquid RBC storage from 10 to 11 or perhaps 12 weeks is probably small in most clinical settings. However, for the military which maintains blood in remote locations, for certain autologous uses, and for avoiding seasonal RBC shortages, the benefit may be higher. Moreover, if these storage systems work by improving the overall quality

of RBC throughout their storage life, then most RBC transfusion recipients may benefit.

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Table 1. EAS Characteristics (mmol/L)

Name	EAS-67	EAS-74	EAS-75	EAS-76	EAS-77
NaCl	75	45	45	45	45
$NaHCO_3$	30	30	30	30	30
Na_2HPO_4	9	9	9	9	9
Adenine	2	2	2	2	2
Dextrose	50	50	50	50	50
Mannitol	20	0	15	30	45
pH	8.4	8.4	8.4	8.4	8.4

All solutions used in 300 mL volume.

Table 2. Second Study, Solutions and Results

Additive Solution	**Mannitol Content** (mmol/L)	**Osmolality** (mOsm/kg H_2O)	**MCV** (fL)	**Morphology Index**	**Vesicle Protein** (mg/dL RBCs)	**Hemolysis** (%)	**RBC ATP** (μmol/g Hb)
EAS-74	0	242±11	112±6	75±6	1.4±0.5	6.69±1.68	4.52±1.54
EAS-75	15	255±12	110±6	68±9	2.6±1.3	1.23±0.39	4.23±0.72
EAS-76	30	271±11	104±4	69±9	3.2±1.8	0.76±0.31	4.48±1.45
EAS-77	45	284±8	98±3	56±8	3.8±1.5	0.71±0.34	4.45±1.40

Mean ± SD; n=4.

Figure Legends

Figure 1. Twenty-four hour post-transfusion recovery of RBC stored for 11 weeks in EAS-67 and labeled with ^{51}Cr (Mean ± SD, n = 9).

Figure 2. Weekly measures of RBC ATP concentration and hemolysis for the RBCs stored in 300 mL of EAS-76 (Mean ± SEM, n = 6).

Figure 1.

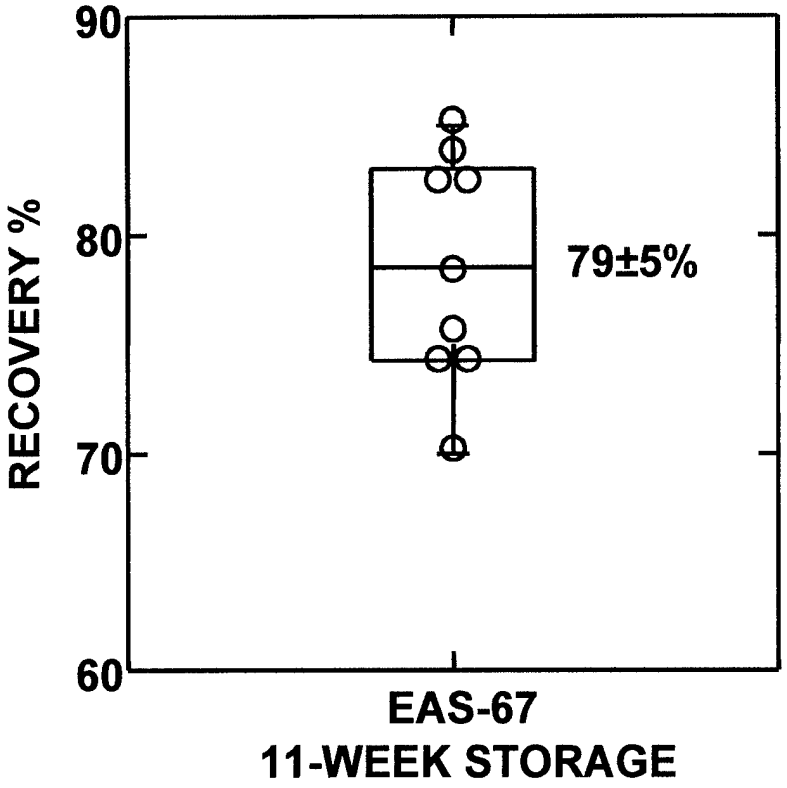
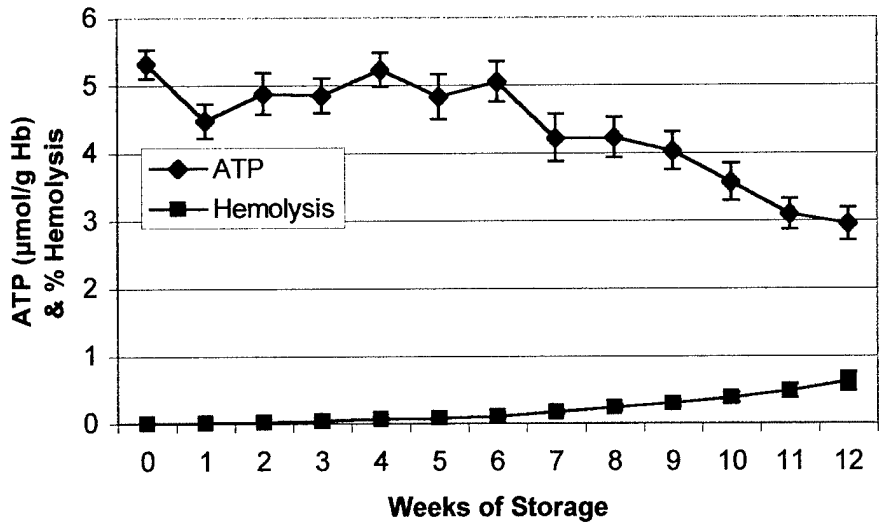


Figure 2.


ATP (µmol/g Hb) & % Hemolysis
6
5
4
3
2
1
0
ATP
Hemolysis
0 1 2 3 4 5 6 7 8 9 10 11 12
Weeks of Storage

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