The effects of additive solution pH and metabolic rejuvenation on anaerobic storage of red cells

Tatsuro Yoshida, James P. AuBuchon, Larry J. Dumont, James D. Gorham, Sean C. Gifford, Kevin Y. Foster, and Mark W. Bitensky

BACKGROUND: Red cell (RBC) storage can be extended to 9 weeks under anaerobic or alkaline conditions. Simultaneous use of these approaches has not provided additive benefit. Our objective was to determine whether anaerobic storage with acidified additive solution (AS) coupled with metabolic rejuvenation might further improve the benefits of anaerobic storage.

STUDY DESIGN AND METHODS: RBC storage in AS with a pH value of 6.5, 7.4, or 8.3 in aerobic or anaerobic conditions was examined using a panel of in vitro biochemical and RBC markers. RBC rejuvenation during cold storage was also evaluated. A randomized crossover radiolabeled recovery study (eight subjects) evaluated anaerobic RBC storage using AS65 with cold rejuvenation for up to 16 weeks of storage.

RESULTS: Adenosine triphosphate (ATP) and diphosphoglycerate acid (DPG) were better maintained in anaerobic storage than in aerobic storage. Acidic or neutral AS preserved ATP concentration better, while a neutral or basic pH AS favored maintenance of DPG levels at higher levels for a longer period. AS pH had less of an effect on exposure of phosphatidylserine (PS), vesicle protein release, and hemolysis. Rejuvenation of RBCs during cold, anaerobic storage resulted in increases in ATP and DPG levels and a reversal of PS exposure. Anaerobic storage of RBCs in pH 6.5 AS rejuvenated at 7 weeks of storage yielded RBC 24-hour recoveries of 77.3 ± 12.5 percent after 10 weeks' storage time. After a second rejuvenation at Week 11, six subjects' units demonstrated a recovery of 75.9 ± 7.3 percent at 12 weeks of storage.

CONCLUSION: Extended RBC storage may be achieved using anaerobic conditions combined with low-pH AS and rejuvenation during storage.

The potential benefits of extending the shelf life of banked blood beyond 6 weeks are numerous. These include improved logistics of blood banking, such as planning for seasonal shortages and stocking remote locations, reduction of outdates, and improvement of the availability of autologous transfusion for preplanned surgeries. Storage duration is limited by the accumulation of the "red cell storage lesion," reflected in multiple biochemical measures, including progressively increasing hemolysis, progressively decreasing adenosine triphosphate (ATP) and 2,3-diphosphoglycerate acid (2,3-DPG) levels and, ultimately, reduced posttransfusion recovery.[1] Recent reports of negative outcomes associated with transfusion in critically ill patients[2-4] suggest that the storage lesion may be associated with deleterious clinical effects, including possibly increased morbidity and mortality. Identifying storage conditions that ameliorate the storage lesion is an important goal in transfusion science and the focus of this work.

We have previously shown that the acceptable shelf life of refrigerated AS3 additive system red cell (RBC) units can be prolonged from 6 to 9 weeks by first depleting the oxygen content of units before storage and then maintaining anaerobic conditions throughout storage.[5] Units stored anaerobically for 9 weeks demonstrated a 24-hour

From the Biomedical Engineering Department, Boston University College of Engineering, Boston, Massachusetts; the Department of Pathology, Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire; and New Health Sciences, Inc., Bethesda, Maryland.

Address reprint requests to: Tatsuro Yoshida, Biomedical Engineering Department, Boston University College of Engineering, 36 Cummington Street, Boston, MA 02215; e-mail: tatsuro.yoshida@newhealthsciences.com.

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in vivo radiolabeled recovery of 75.8 ± 5.5%. We and others have shown that the pH of the additive solution (AS) can also positively affect storage time duration. We therefore hypothesized that the duration of RBC storage could be further extended by combining anaerobic conditions with an improved additive storage solution. We have tested that hypothesis here. In addition, we have explored whether substrate rejuvenation during cold storage under anaerobic conditions could be a promising avenue for the extension of the duration of RBC storage.

MATERIALS AND METHODS

For each in vitro study, six units of citrate-phosphate-dextrose (CPD)-anticoagulated whole blood were acquired from the Oklahoma Blood Institute (Oklahoma City, OK) from normal volunteers meeting Food and Drug Administration (FDA; 21CFR640) and AABB[6] donation criteria and further subdivided for different storage conditions as indicated below. Units were shipped overnight on wet ice and processed into the final storage configuration within 28 hours of collection. After the blood was received by overnight courier, each CPD unit was leukoreduced via an attached leukoreduction filter (Sepacell RS2000, 4C-4300, Fenwal, Round Lake, IL), and then an RBC unit was prepared by a standard soft-spin method.[7] We established anaerobic conditions by oxygen depletion as previously described.[5] Briefly, oxygen was removed by gas exchange with addition and removal of sterile Ar in the bag (10 min each, gas exchanged six times) and placed in an anaerobic canister filled with 10% H_2-90% Ar with Pd catalyst to prevent oxygen reentry over time through the plastic. In this trial of an early anaerobic storage prototype system, all gas exchanges were conducted using a 0.2-µm filter to reduce the risk of bacterial contamination. Aerobic and anaerobic units in the canister were stored at 1 to 6°C. Aerobic storage was under standard blood banking conditions without altering the gas in the bag. The anaerobic environment in the storage canister was reestablished in test units after each sampling by filling the storage canister with H_2-Ar gas mixture, but the units' contents always remained under anaerobic conditions.

We used AS3, EAS61,[8] and two additional ASs that were identical in composition to EAS61 but with the pH altered with the addition of HCl (Table 1). ASs for in vitro use were made in the laboratory using ingredients purchased from Sigma Aldrich (St Louis, MO) and sterilized by filtration. AS65 used in the in vivo study was compounded and filter-sterilized (Pharmaceutical Development Corp., Charleston, SC). AS was added to RBCs in the ratio of 200 mL AS to 1 unit of RBCs. Some RBC units in AS65 and AS83 were "rejuvenated" with Rejuvesol (EnCyte Systems, Inc., Braintree, MA; 0.55 g sodium pyruvate, 1.34 g inosine, 0.034 g adenine, 0.5 g dibasic sodium phosphate [anhydrous], 0.2 g monobasic sodium phosphate [monohydrate], pH 6.7-7.4, in total volume of 50 mL per 450 mL of RBCs) that was added aseptically through sterilely docked 0.2-µm filter to each aliquot while they were kept on ice and then returned to 1 to 6°C without warming. Although rejuvenating units intended for in vivo reinfusions (see below) usually includes a washing step at the end of rejuvenation, this was omitted because the small aliquots to be reinfused would be washed as part of the radiolabeling procedure.

TABLE 1. ASs used in this report*

AS	AS83†	AS74	AS65	AS3
Adenine	2	2	2	2.2
Dextrose	110	110	110	61
Mannitol	55	55	55	0
NaCl	26	26	26	70
Na_2HPO_4	12	12	12	20
$pH_{22°C}$	8.3	7.4	6.5	5.8‡
Na_3-citrate	0	0	0	20
Citric acid	0	0	0	20
Osmolarity	254[8]	254	254	291‡

* All units are mmol except for pH values.
† Equivalent to EAS61.
‡ Measured value.

To determine the effects of AS pH on anaerobic RBC storage, two aliquots each of AS65-, AS74-, and AS83-suspended RBCs (in 150-mL polyvinylchloride transfer bags [Baxter, Round Lake, IL]) from each of 6 units were prepared with one of each pair stored anaerobically. Bags were stored for up to 100 days with periodic sampling to determine ATP, 2,3-DPG, hemolysis, and phosphatidylserine (PS) exposure.

To study the effects of rejuvenation during cold storage, aliquots were similarly prepared to make AS65 or AS83 units stored aerobically or anaerobically. Rejuvesol (12.5 mL; 50 mL per full unit of blood) was added to subunits at the 9th (63 days) and 14th (98 days) weeks of storage. Bags were stored up to 120 days with periodic sampling to determine ATP, 2,3-DPG, hemolysis, and PS exposure.

The effect of the timing of the rejuvenations was examined using aliquots of 6 units converted to AS65 RBCs and stored anaerobically. Rejuvesol was added (8.3 mL; 50 mL per full unit) to four different subunits at 0, 2 (15 days), 4 (29 days), or 6 (43 days) weeks. At Week 8, the same quantity of Rejuvesol was added to the fifth aliquot, and the first four aliquots received second additions. The sixth subunit was held without Rejuvesol as a control. All bags were held anaerobically for 78 days with periodic sampling to determine 2,3-DPG concentration.

In vivo correlations of these findings were sought through a randomized crossover radiolabeled RBC recovery study conducted at Dartmouth-Hitchcock Medical Center with the authorization of the Committee for the Protection of Human Subjects. Written informed consent was obtained from eight normal, healthy subjects meeting

FDA (21CFR640) and AABB[7] donation criteria. Two units of whole blood (450 mL ± 10%) were collected from each subject into citrate-phosphate-double dextrose (Pall Medical, Covina, CA) at least 10 weeks apart. RBC units were prepared by the standard soft-spin method,[7] and 200 mL of AS65 was added. Units were not leukoreduced. The units were randomized to the control arm (aerobic storage) under routine conditions at 1 to 6°C or the anaerobic test arm. Oxygen depletion in test units was handled as described previously using a 2000-mL transfer pack for gas exchange.[5] Rejuvesol (50 mL) was added to test units on Days 49 (7 weeks) and 77 (11 weeks) of storage as described. Control units were stored for 70 days (10 weeks), and test units were stored up to 16 weeks (112 days).

In vivo 24-hour recovery of ^{51}Cr-labeled RBCs was determined at 10 weeks for each unit using a double labeling method as previously described.[5,9,10] Briefly, the unit was well mixed by hand (1 min), and approximately 15 mL of the RBCs was labeled with 15 (10-20) μCi of ^{51}Cr. The labeling agent, ^{51}Cr sodium chromate, was mixed aseptically with the RBCs at room temperature for 30 minutes. One double-volume saline wash was conducted. An aliquot of the final volume was reserved for assay as a standard, and the remaining labeled cells (approx. 10 mL) were injected into a free-flowing peripheral vein. Samples (5 mL each) were taken from a contralateral vein at 5, 7.5, 10, 12.5, 15, 20, and 25 to 30 minutes as well as at 24 hours. The samples were counted in a gamma counter to determine ^{51}Cr activity. A determination of the subject's blood volume using freshly collected autologous RBCs labeled with ^{99m}Tc was conducted simultaneously with each reinfusion.[11] For the purposes of subject safety, an aliquot from each unit was cultured during the week before each reinfusion via blood agar plates read at 24 and 48 hours after inoculation; all units were negative. Recovery for each control and test unit was determined at 10 weeks. For test units with a 10-week recovery of more than 75 percent, ^{51}Cr RBC labeling and in vivo recovery was repeated at 12 and 14 weeks (4 units) and 14 and 16 weeks (2 units).

Samples were taken before and after storage for cell counts, supernatant hemoglobin (Hb), glucose, electrolyte, lactate, pH, ATP, and 2,3-DPG were determined as described previously.[5] The RBC morphology score was determined by the method of Usry and colleagues.[12]

All in vitro assays were performed using standard, published methods. ATP and 2,3-DPG levels and microvesicle production were measured as described previously.[5] PS exposure was quantified using a flow cytometer (FACSCalibur, Becton-Dickinson, Palo Alto, CA) as described previously using a single-label annexin V-FITC (R&D Systems, Minneapolis, MN).[13]

Descriptive statistics are presented as arithmetic mean ± 1 standard deviation (SD) unless noted specifically to be given with the standard error of the mean (SEM). A random-effects ANOVA model, which accounts for intraunit correlation and repeated measures, was fit to the data and used for all hypotheses tests at an alpha level of 0.05 (Proc Mixed, SAS, Version 9.1, SAS Institute, Cary, NC).

RESULTS

Effects of AS pH on RBC storage under aerobic and anaerobic conditions

We tested whether the pH of the AS influenced the storage lesion of RBCs stored under aerobic or anaerobic conditions, using ATP and 2,3-DPG concentrations as readouts.

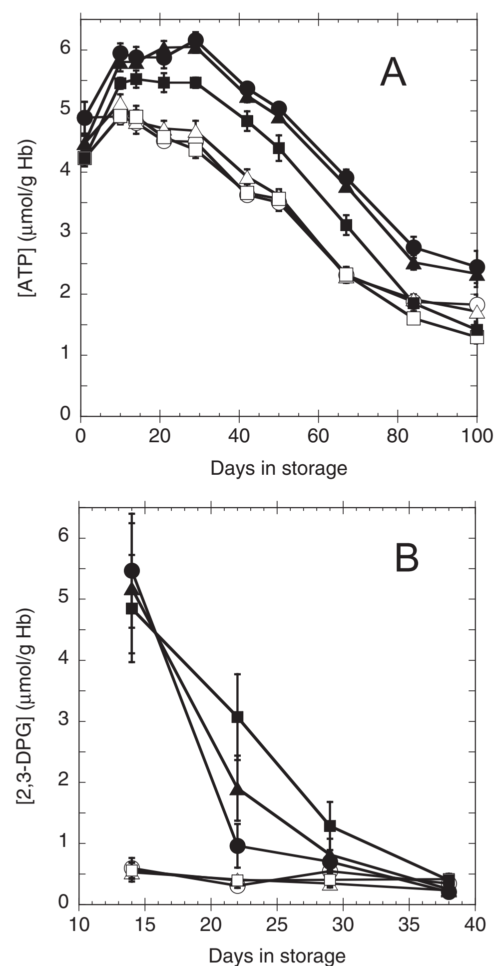

Fig. 1. Effects of the AS pH and anaerobic storage on in vitro biochemical measures. (A) Mean ATP levels. Anaerobic storage acts synergistically with AS pH for maintenance of ATP over 100-days storage with lower pH AS65 and AS74 anaerobic conditions superior to all other conditions ($p < 0.0001$). (B) Mean 2,3-DPG levels. 2,3-DPG is better maintained in anaerobic conditions ($p < 0.0001$). (○) AS65 aerobic; (●) AS65 anaerobic; (△) AS74 aerobic; (▲) AS74 anaerobic; (□) AS83 aerobic; (■) AS83 anaerobic. Error bars indicate SEM.

TABLE 2. Effects of additive pH*

Day	AS	Storage condition	Hemolysis (%)	Supernatant vesicle protein (mg/10^{12} cells)	Proportion of RBCs exposing PS (%)
41/42	AS65	Anaerobic	0.50 ± 0.09 (0.34-0.62)	7.77 ± 1.57 (5.56-10.16)	0.88 ± 0.16 (0.70-1.16)
		Aerobic	0.45 ± 0.14 (0.28-0.68)	9.48 ± 2.59 (7.07-13.52)	1.10 ± 0.29 (0.87-1.61)
	AS74	Anaerobic	0.40 ± 0.06 (0.3-0.46)	10.09 ± 4.16 (4.97-16.48)	1.06 ± 0.27 (0.82-1.57)
		Aerobic	0.44 ± 0.12 (0.31-0.62)	11.16 ± 5.48 (5.62-20.30)	1.10 ± 0.24 (0.78-1.41)
	AS83	Anaerobic	0.44 ± 0.15 (0.30-0.71)	10.17 ± 5.55 (4.4417.96)	2.43 ± 1.30 (1.64-5.04)
		Aerobic	0.35 ± 0.07 (0.26-0.42)	11.37 ± 6.16 (5.67-19.08)	2.15 ± 0.75 (0.96-2.92)
58	AS65	Anaerobic			1.26 ± 0.28 (0.86-1.65)
		Aerobic			1.73 ± 0.45 (1.24-2.50)
	AS74	Anaerobic			1.39 ± 0.37 (1.02-1.99)
		Aerobic			1.42 ± 0.34 (1.10-2.07)
	AS83	Anaerobic			1.32 ± 0.24 (1.16-1.79)
		Aerobic			1.77 ± 0.47 (1.29-2.42)
62/63	AS65	Anaerobic	1.02 ± 0.33 (0.61-1.56)	16.68 ± 3.55 (9.84-19.23)	1.47 ± 0.24 (1.09-1.78)
		Aerobic	0.91 ± 0.33 (0.41-1.36)	19.55 ± 4.42 (14.62-25.85)	2.00 ± 0.69 (1.05-3.03)
	AS74	Anaerobic	0.89 ± 0.27 (0.50-1.30)	18.93 ± 8.53 (10.26-38.58)	1.47 ± 0.21 (1.17-1.74)
		Aerobic	0.81 ± 0.23 (0.42-1.11)	23.11 ± 10.53 (11.81-39.94)	1.95 ± 0.57 (1.34-2.87)
	AS83	Anaerobic	1.06 ± 0.44 (0.46-1.80)	19.95 ± 11.72 (7.35-34.85)	1.48 ± 0.41 (0.99-2.03)
		Aerobic	0.78 ± 0.25 (0.36-1.05)	22.30 ± 10.14 (11.94-35.29)	1.78 ± 0.48 (1.20-2.41)

* Data are reported as mean ± SD (range).

We stored units for 100 days either aerobically or anaerobically in ASs in which the pH was the only known variable (Table 1). Regardless of AS pH, units stored under anaerobic conditions maintained both ATP and 2,3-DPG concentrations better than units stored under aerobic conditions (Fig. 1), as we have previously reported.[5] Examining the effects of pH on ATP concentrations, anaerobically stored units maintained ATP concentrations throughout the storage period better when acidic (pH 6.5) or neutral (pH 7.4) ASs were used than when a basic (pH 8.3) AS was used ($p = 0.002$). No such effects of pH were seen in units stored aerobically, in which ATP concentrations decreased equivalently regardless of AS pH. The effects of pH on 2,3-DPG concentrations in anaerobically stored units were different than the effects of pH on ATP concentrations in these units. After Day 14, the concentration of 2,3-DPG declined precipitously in units stored in the acidic AS, but the decline was slower in units stored in neutral or basic ASs ($p = 0.005$).

Next, we measured RBC hemolysis, the release of vesicle protein, and the presence of PS on RBCs, all of which are associated with development of the RBC storage lesion. Under all storage conditions, all three measures were greater on Day 62 than on Days 41 and 42 ($p < 0.0001$). Anaerobically stored units exhibited moderately greater hemolysis than aerobically stored units ($p = 0.016$; Table 2), but no effect of AS pH on hemolysis was observed ($p = 0.2$). Anaerobically stored units had lower vesicle protein release than aerobically stored units. Here, AS pH was found to have a modest effect, with lower pH yielding lower vesicle protein release. Anaerobically stored units had lower PS exposure than did aerobically stored units ($p < 0.0001$), but there was little discernible effect of pH (Table 2).

To summarize these data, with prolonged storage, units stored anaerobically fared considerably better than units stored aerobically in terms of ATP, 2,3-DPG, vesicle protein release, and PS exposure, but aerobically stored units fared better in terms of hemolysis. For units stored anaerobically, storage solution pH had the following effects: pH 6.5 storage was associated with augmented ATP levels and reduced vesicle protein release, whereas pH 8.3 storage was associated with improved maintenance of 2,3-DPG; for RBC hemolysis or PS exposure, pH had little effect. For units stored aerobically, storage solution pH had little effect on any of the five readouts measured.

Addition of Rejuvesol during anaerobic storage restores ATP and 2,3-DPG concentrations

Despite early increases in ATP and 2,3-DPG concentrations during anaerobic storage, 2,3-DPG declined nearly to 0 by 4 to 5 weeks, and ATP started to decline after approximately 5 weeks (Fig. 1). In additional experiments (data not shown), we ruled out such causes as acidification of storage medium due to lactate accumulation; depletion of glucose, phosphate, or adenine; and accumulation of toxic diffusible metabolites in the storage medium, because washing and exchange of ASs at 6 to 8 weeks of storage did not prevent the decline in ATP and 2,3-DPG.

We therefore examined whether supplementation of metabolic substrates in the units during refrigerated storage could maintain ATP and 2,3-DPG concentrations. The addition of Rejuvesol on Day 63 resulted in increases in both ATP and 2,3-DPG concentrations regardless of additive pH or oxygen status (Fig. 2). Interestingly, a second addition of Rejuvesol on Day 98 resulted in

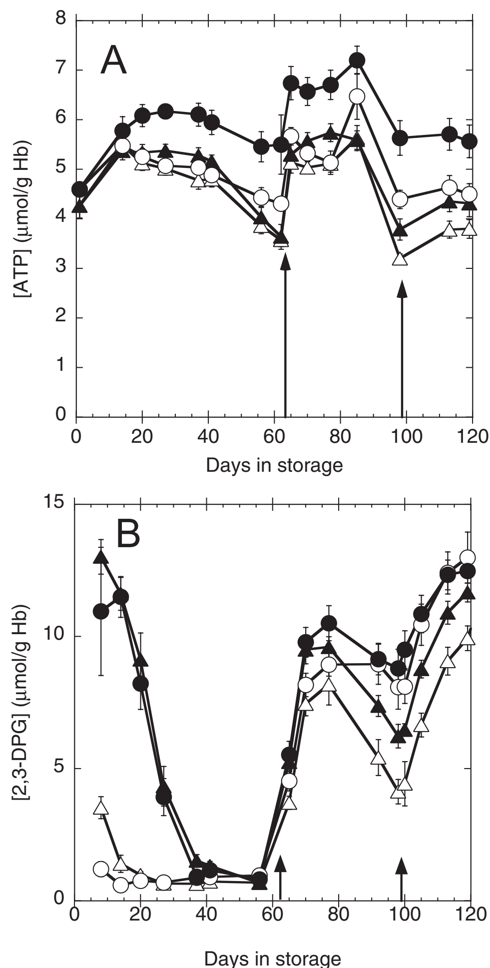

Fig. 2. Effects of additive pH and metabolic supplement addition during blood storage on ATP and DPG levels. (A) Mean ATP levels. Oxygen-depleted AS65 units had higher ATP compared to AS83 and aerobic AS65 units. ATP level increased after addition of Rejuvesol on Day 63. Anaerobically stored AS65 units maintained higher ATP levels beyond Day 63 ($p < 0.0001$). (B) Mean 2,3-DPG levels. 2,3-DPG levels were higher in anaerobically stored units over the first 28 days of storage. Rejuvesol boosted 2,3-DPG in all units. Anaerobically stored AS65 maintained the highest levels after Rejuvesol addition. (△) AS83 aerobic; (▲) AS83 anaerobic; (○) AS65 aerobic; (●) AS65 anaerobic. Rejuvesol additions are indicated by the arrows. Error bars indicate SEM.

increases in 2,3-DPG, but mainly arrested the decline in ATP (Fig. 2). Nevertheless, beyond Day 63, anaerobic AS65 units had significantly higher ATP levels than units stored in the other conditions ($p < 0.0001$). After the first addition of Rejuvesol on Day 63, 2,3-DPG concentrations increased rapidly, reaching a peak 13 days later, followed by a slow decline (Fig. 2B). After the second addition of Rejuvesol on Day 98, 2,3-DPG concentrations increased, but more slowly than after the first addition, reaching a peak 4 weeks later. After the first addition of Rejuvesol, the highest concentrations of 2,3-DPG were observed in RBC units stored anaerobically in pH 6.5 additive ($p < 0.0001$). All units showed a mean hemolysis of approximately 1 percent on Day 84, which increased to 2 to 2.7 percent on Day 101 (Table 3). As before, hemolysis after extended storage (84 days or longer) was marginally higher in the anaerobic storage environment (Table 3).

RBC surface PS exposure increased with storage time, although this was somewhat delayed in anaerobic storage (Fig. 3A). For all storage conditions, the first addition of Rejuvesol on Day 63 sharply reversed the rapid increase in PS exposure, returning within 6 days to nearly baseline levels (Fig. 3B). Low PS levels were sustained for approximately 2 weeks, after which the increase in PS exposure resumed. The second addition of Rejuvesol also reversed PS exposure, albeit less robustly than the first addition. Comparing storage conditions, units stored anaerobically at pH 6.5 had the lowest PS exposure after Rejuvesol addition ($p = 0.02$).

Timing of metabolic supplement addition to maintain high level of 2,3-DPG

Next, we examined how the timing of addition of Rejuvesol would affect 2,3-DPG concentrations during storage. For this experiment, we used RBC units stored anaerobically at pH 6.5. Rejuvesol was first added at Weeks 0, 2, 4, or 6; for all units, Rejuvesol was then added again at Week 8. Controls included units to which Rejuvesol was not added and units to which Rejuvesol was added only at 8 weeks. Regardless of the time of addition, Rejuvesol rapidly restored 2,3-DPG concentrations (Fig. 4). The addition of Rejuvesol at Weeks 2 and 8 was the most effective combination, with 2,3-DPG concentrations near or above the value expected for fresh units maintained for the duration of the 12-week storage period.

In vivo trial

In a preliminary clinical study, we examined whether units stored anaerobically for extended periods could return adequate 24-hour recovery after transfusion. For this study, we collected 2 units from eight normal healthy volunteers. One unit was stored aerobically for 10 weeks (control) and the other unit was stored anaerobically for up to 16 weeks. Because pH 6.5 storage was associated with augmented ATP levels and reduced vesicle protein release for anaerobic conditions, we stored all units in an AS of pH 6.5. For units stored anaerobically, Rejuvesol was added at 7 weeks and again at 11 weeks. Figure 5 shows 24-hour recoveries for the eight subjects. Recovery of control RBCs (10 weeks, aerobic) varied between 49.7 and 79.7 percent (mean, 68.1 ± 10.0%). Recovery of RBCs stored anaerobically for the same time period (10 weeks, anaerobic) was somewhat

TABLE 3. Effects of additive pH on hemolysis*

Storage period† (days)	AS	Storage condition	Hemolysis (%)
55	AS65	Anaerobic	0.5 ± 0.1 (0.3-0.5)
		Aerobic	0.4 ± 0.1 (0.3-0.6)
	AS83	Anaerobic	0.4 ± 0.1 (0.3-0.5)
		Aerobic	0.4 ± 0.0 (0.3-0.4)
61	AS65	Anaerobic	0.4 ± 0.1 (0.3-0.6)
		Aerobic	0.4 ± 0.1 (0.3-0.5)
	AS83	Anaerobic	0.4 ± 0.1 (0.3-0.6)
		Aerobic	0.3 ± 0.3 (0.2-0.4)
84	AS65	Anaerobic	1.3 ± 0.4 (1.0-2.0)
		Aerobic	1.1 ± 0.3 (0.8-1.5)
	AS83	Anaerobic	1.3 ± 0.3 (0.9-1.7)
		Aerobic	0.9 ± 0.9 (0.7-1.2)
101	AS65	Anaerobic	2.4 ± 0.6 (1.7-3.5)
		Aerobic	2.0 ± 0.4 (1.5-2.7)
	AS83	Anaerobic	2.7 ± 0.6 (1.9-3.6)
		Aerobic	2.0 ± 0.4 (1.3-2.5)

* Data are reported as mean (range).
† Rejuvesol added on Day 63.

higher, varying between 56.7 and 96.0 percent (mean, 77.3 ± 12.5%); the difference between the two groups approached but did not reach significance ($p = 0.08$). Of the eight subjects, six showed improved recovery with RBCs stored in anaerobic conditions, one (Subject 3) showed no difference, and one (Subject 6) showed decreased recovery (Fig. 5). For the six subjects showing improved recovery in anaerobic conditions, we tested recoveries upon subsequent transfusions with the anaerobically stored units. For three subjects (Subjects 1, 2, and 4), improved recoveries were not sustained by 14 weeks, at which point recoveries were below that of the respective control units (10 weeks, aerobic). For three subjects (Subjects 5, 7, and 8), recoveries were sustained at 14 weeks, with recoveries at or above that of the respective control units. Indeed, in two of these three subjects, recoveries tested after 16 weeks of anaerobic storage were as good or better than those of the respective control units. Thus, in this initial pilot study, anaerobic storage of RBC in pH 6.5 storage solution and periodic addition of Rejuvesol allows for respectable in vivo recoveries even as late as 16 weeks of storage. Corresponding biochemical measures for units used in the in vivo trial are shown in Table 4.

DISCUSSION

This study documented the synergistic effects of anaerobic storage and reduced-pH AS in reducing the magnitude of several biochemical aspects of the RBC storage lesion and in extending the period of storage time over which RBCs maintained good in vivo recovery. We also documented biochemical benefits of the addition of rejuvenation solution during anaerobic storage at 4°C.

The optimal pH of the glycolytic pathway is higher than that of the commonly used ASs,[14,15] mainly due to the pH-dependent phosphofructokinase.[16] Use of alkaline AS EAS61 at pH 8.3 in *aerobic* storage achieved elevated levels of ATP and allowed 9-week storage, in part by increasing the intracellular pH.[8] However, our results indicated that, under *anaerobic* storage conditions, higher ATP levels were achieved in vitro with a more acidic pH (Fig. 1A). This result may be attributed to an overalkalinization of RBCs in alkaline AS as a consequence of oxygen depletion, which also removed CO_2 and thereby increased intracellular pH.[5]

2,3-DPG levels are depleted by 1 to 2 weeks of routine refrigerated storage. Although this storage lesion is transient,[1,17] it may be critical in the case of recipients with restricted coronary flow.[18] Manipulations of AS composition and pH to maintain 2,3-DPG levels during storage have been described previously.[19-21] As we have reported previously,[5] we noted here a significant reduction in the rate of 2,3-DPG depletion when cells were stored under anaerobic conditions (Fig. 1B). This effect may be attributed to protection of cytosolic 2,3-DPG from phosphatase through binding to deoxyhemoglobin, as well as the increased cytosolic pH resulting from CO_2 removal.[5] The possibility that this effect is attributable to just alkalinization by CO_2 removal during oxygen depletion process alone, and not the anaerobic storage condition, has been raised.[22] We believe that anaerobic storage plays the significant role, however, because CO_2 removal with CO, which binds Hb and releases DPG, depressed the ATP increase normally observed when cells were placed in anaerobic storage.[5,23,24] DPG retention was more pronounced with a higher-pH additive (e.g., AS83 on Day 22; Fig. 1B), although anaerobic storage in all the ASs examined resulted in concentrations below 20 percent of the Day 0 value by the fourth week of storage. We note that anaerobic storage of RBCs produced moderately increased hemolysis compared with standard storage, and acidification did not improve this result. We also note that acidification of media did not substantially reduce PS exposure in anaerobic-stored units.

Rejuvesol is an FDA-approved rejuvenation solution containing pyruvate, inosine, phosphate, and adenine that can be used to replenish depleted ATP and 2,3-DPG before transfusion or subsequent placement in long-term cryopreservation. In normal use for immediate transfusion, a blood unit is incubated for 60 minutes at 37°C after addition of Rejuvesol and then washed to remove excess inosine (to prevent recipient toxicity). This process restores ATP and 2,3-DPG levels to near those of fresh RBCs. In this study, we examined the effect of adding Rejuvesol as a metabolic supplement during refrigerated,

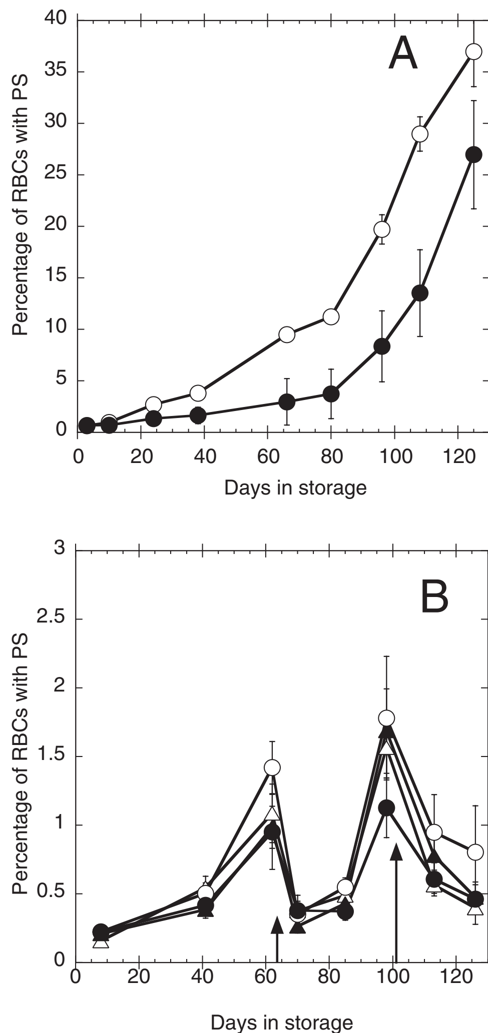

Fig. 3. Effects of pH and metabolic supplement addition during blood storage on PS exposure. (A) PS exposure without Rejuvesol addition. Data from an experiment under similar storage conditions using AS65 anaerobic (●) and AS3 aerobic (○). (B) Rejuvesol was added to refrigerated RBCs on Days 63 and 101 (indicated by the arrows). Anaerobic AS65 had significantly lower PS exposure compared to other treatment arms ($p < 0.0154$). (△) AS83 aerobic; (▲) AS83 anaerobic; (○) AS65 aerobic; (●) AS65 anaerobic. Error bars indicate SEM.

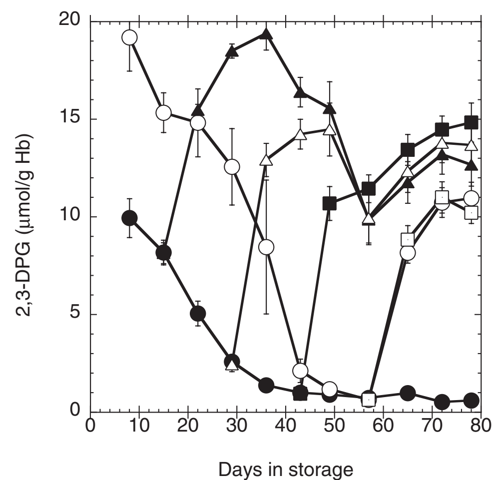

Fig. 4. Effect of the timing of metabolic supplement addition on 2,3-DPG levels. Rejuvesol was added at varying times after RBCs were stored under anaerobic conditions in AS65 AS. (●) No addition; (○) Days 1 and 57; (▲) Days 15 and 57; (△) Days 29 and 57; (■) Days 43 and 57; (□) Day 57 only. Error bars indicate SEM.

anaerobic storage of RBCs in EAS61 AS (AS83) and its low-pH variant (AS65). Significant boosts in ATP and 2,3-DPG levels were observed within 1 week after the addition of the rejuvenating solution. By adjusting the timing of supplement addition, 2,3-DPG levels could be kept at or above the levels of fresh blood throughout storage (Fig. 5). Although 37°C incubation is the only currently approved procedure, we combined anaerobic blood storage with rejuvenation at 4°C[25,26] to achieve good maintenance of or even increased ATP and 2,3-DPG levels. This protocol also was associated with good posttransfusion viability of the cells with six of the eight rejuvenated, anaerobically stored units showing increased 24-hour recovery at 10 weeks compared to their paired controls (Fig. 5).

The contribution of the lower pH to prolongation of the acceptable anaerobic storage time may be independent of the Rejuvesol addition for several reasons. Anaerobic storage with acidic AS3 additive produced better biochemical and in vivo recovery results compared to alkaline AS.[5] Similar success was obtained with anaerobic storage in OFAS1 AS (pH 7.2) as well,[27] exhibiting better in vitro variables, such as significantly higher ATP levels and reduced rates of microvesicle production and PS exposure. Although it appears that anaerobic storage may be optimized with AS more acidic than what maximally benefits aerobic storage, further studies are needed to understand the true effect of Rejuvesol compared with acidification on the storage properties of blood.

A notable effect of rejuvenation during cold storage is a reduction in the fraction of RBCs exposing PS. PS exposure on the RBC surface has been reported to be a signal for senescent RBC removal in vivo.[28] Although PS exposure is relatively small after 6 weeks of (aerobic or anaerobic) storage, it increases steeply beyond 9 weeks (Fig. 3A). Once Rejuvesol was added, these increases were reversed within 1 week, and low levels were maintained for an additional 2 weeks before PS started to increase again. Moving PS back from the outer to the inner bilayer is carried out by an ATP-linked aminophospholipid translocase.[29] Because Rejuvesol's addition caused a dramatic increase in ATP levels, one might speculate that the increased PS exposure, particularly later in storage, may be related to inadequate ATP levels to maintain normal or sufficient

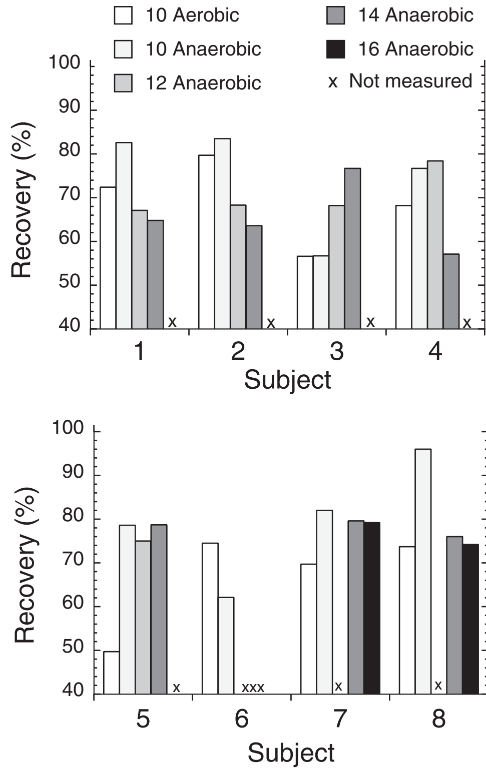

Fig. 5. In vivo 24-hour recoveries shown by subject. Controls at 10 weeks are matched with test RBCs. Test RBC units were sampled and tested sequentially through Week 16 until recovery was less than 65 percent. Rejuvesol was added to test units at 7 and 11 weeks.

translocase activity. Our observations are consistent with the partial restoration of the translocase activity reported by Verhoeven and colleagues[30] on stored cells after 37°C rejuvenation. Examination of data from Table 2 and Fig. 3, as well as similar data from units not treated with Rejuvesol (data not shown), however, did not reveal any threshold ATP level below which PS exposure rate increased significantly. More detailed examinations, including shorter time intervals between data collection, are needed to address this issue. Because of different timing in the Rejuvesol additions between in vivo and in vitro experiments, this study cannot make a definitive correlation between PS exposure and 24-hour recovery. However, the data are consistent with the view that cells with exposed PS are removed after transfusion.

In this small-scale pilot trial, the in vivo reinfusion studies did not identify any biochemical variables that would predict the posttransfusion outcome. The correlation between ATP and 24-hour recovery was weak, as might be expected from previous work.[31] The differences in the biochemical environments between the cells of different donors require additional investigation to understand which metabolic processes were more

TABLE 4. Biochemical measures for units in the in vivo trial*

Storage condition	Sample point (week)	Na^+ (mmol/L)	K^+ (mmol/L)	Glucose (mg/dL)	Lactate (mmol/L)	pH	ATP (μmol/g Hb)	2,3-DPG (μmol/g Hb)	Supernatant Hb (mg/dL)	Hemolysis (%)
Aerobic	0	92.4 ± 7.7	1.7 ± 0.2	1317 ± 80	1.0 ± 0.4	6.87 ± 0.05	3.52 ± 0.24	8.50 ± 1.12	12.2 ± 7.9	0.05 ± 0.03 (0.01-0.09)
	10	72.4 ± 7.8	34.8 ± 2.8	1014 ± 665	26.6 ± 2.5	6.20 ± 0.06	2.12 ± 0.49	0.88 ± 0.13	140.5 ± 69	0.59 ± 0.29 (0.2-1.2)
Anaerobic	0	89.3 ± 8.5	1.7 ± 0.2	1308 ± 75	1.1 ± 0.3	6.86 ± 0.03	3.61 ± 0.40	9.14 ± 0.97	20.5 ± 7.7	0.08 ± 0.03 (0.35 ± 0.11)
	10	93.3 ± 3.8	32.1 ± 1.9	887 ± 34	37.7 ± 10.1	6.10 ± 0.03	5.00 ± 0.65	9.32 ± 1.04	70.8 ± 25.6	0.35 ± 0.11 (0.2-0.7)
	12	114 ± 7.2	29.1 ± 1.1	755 ± 67	42.4 ± 2.0	6.20 ± 0.05	5.03 ± 0.61	9.72 ± 0.71	76.1 ± 20.4	0.47 ± 0.13 (0.3-0.9)
	14	110 ± 2.8	31.8 ± 1.2	790 ± 22	47.9 ± 1.5	6.09 ± 0.03	4.41 ± 0.68	11.61 ± 0.95	101.9 ± 30.8	0.61 ± 0.16 (0.4-1.2)
	16	111 ± 1.4	34.7 ± 0.1	735 ± 21	55.0 ± 0	6.03 ± 0.02	3.56 ± 1.07	10.8 ± 1.4	206 ± 146	1.15 ± 0.9 (0.5-1.8)

* Data are reported as mean ± SD (range).

affected by changes in the storage environment. Clearly, not all units benefited from improvements in the biochemical markers of the RBC storage lesion that were being followed, and insufficient data exist at this point to determine the length of time over which these storage system manipulations will allow RBCs to be stored and meet regulatory requirements for licensure.

Although the advantages of anaerobic storage with supplemental additions are significant, they do require additional handling which diminishes their utility. The protocol used in this study with addition of Rejuvesol during storage did not specifically include a wash step before radiolabeling (and its washing step). In clinical settings, excess inosine, as well as its metabolic byproducts (hypoxanthine, xanthene, etc.), would need to be removed before transfusion to prevent potential uremia. In light of an increasing awareness of the possibly detrimental effects of transfusion of stored blood in critical care, as well as potential negative outcomes of transfusing RBCs stored for longer periods (reviewed by Tinmouth et al.[32]), these extra (or other) steps may be justified in situations such as massively transfused, septic, and chronically anemic patients if clinical improvements can be documented. Improved logistics in dealing with periodic blood shortage, remote locations, and autologous transfusion would also benefit from this process. Based on the success of this and our previous studies with anaerobic storage, a modified RBC storage system is being designed that utilizes a common nontoxic oxygen sorbent enveloped into the storage bag to deplete oxygen initially and maintain a nearly anaerobic state during extended refrigerated storage. This could replace the cumbersome series of gas exchanges and anaerobic canister storage used for this study in further investigations of this improvement in RBC storage.

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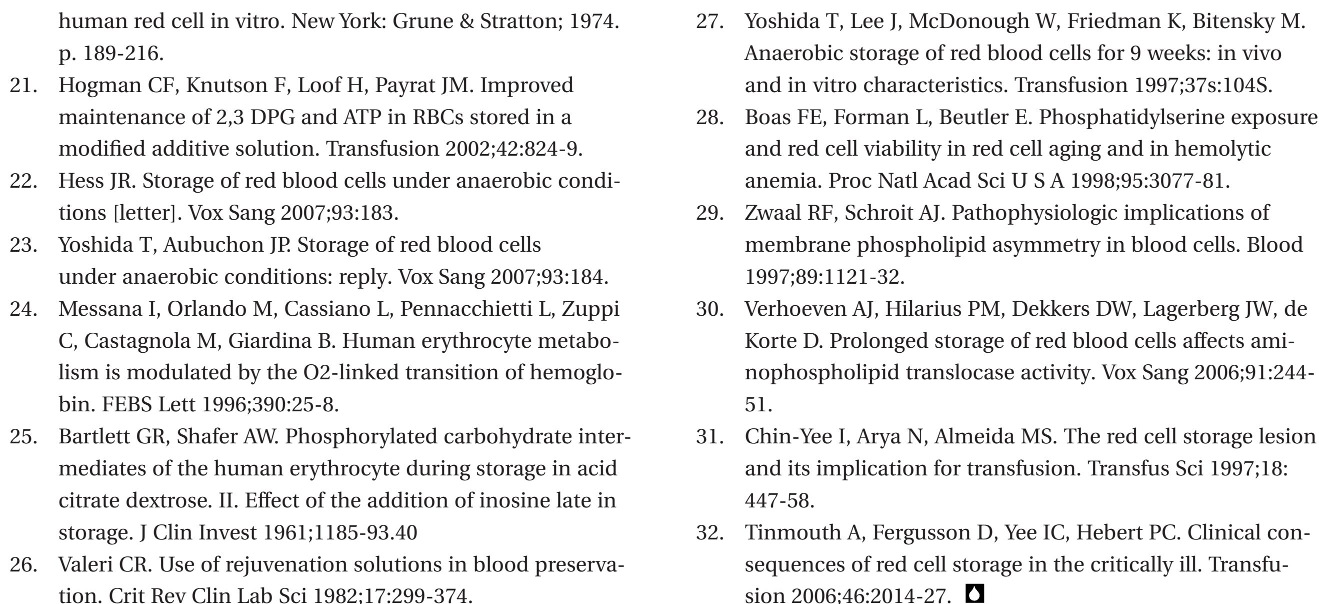

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