

Anaerobic storage of red blood cells in a novel additive solution improves in vivo recovery

Larry J. Dumont, Tatsuro Yoshida, and James P. AuBuchon

BACKGROUND: In preliminary studies, anaerobic red blood cell (RBC) storage reduced oxidative damage and phosphatidylserine exposure while maintaining adenosine triphosphate levels. The purpose of this study was to compare the 24-hour recovery and life span of autologous RBCs stored 6 and 9 weeks using OFAS3 additive solution in an anaerobic environment, compared to control RBCs aerobically stored in AS-3 for 6 weeks.

STUDY DESIGN AND METHODS: Eight subjects were entered into a randomized, crossover study. Whole blood was collected from each subject twice separated by 12 weeks or more into CP2D and leukoreduced. Controls were stored in AS-3. Test units in OFAS3 were oxygen depleted with argon then stored 9 weeks in an anaerobic chamber at 1 to 6°C. At the end of each storage period, RBCs were labeled with ^{51}Cr and $^{99\text{m}}\text{Tc}$ and reinfused to the subject following standard methods to determine double-label recovery and life span. Hypotheses tests were conducted using paired, repeated-measures analysis of variance.

RESULTS: Recovery for the anaerobically stored test RBC was significantly better than control at 6 weeks ($p = 0.023$). Test units at 9 weeks were not different than the 6-week control units ($p = 0.73$). Other in vitro measures of RBC characteristics followed the same trend. Two test units at 9 weeks had hemolysis of greater than 1 percent.

CONCLUSION: Anaerobically stored RBCs in OFAS3 have superior recovery at 6 weeks compared to the controls and equivalent recovery at 9 weeks with no change in life span. Anaerobic storage of RBCs may provide improved RBCs for transfusion at 6 weeks of storage and may enable extending storage beyond the current 42-day limit.

Storage under anaerobic conditions to minimize product degradation is a widespread practice in the pharmaceutical and food industries. We investigated this technique for refrigerated red blood cell (RBC) storage based on the RBC's unique characteristics of not requiring oxygen to support essential metabolic processes. In a series of reports, we have shown that anaerobic storage generally enhanced the metabolic status of the RBC as well as increasing the potential storage time using a variety of additive solutions (ASs) without exhibiting significant negative consequences, except for an occasional increase in hemolysis.¹⁻⁵ When we evaluated the putative additional benefit of an alkaline AS⁶ with anaerobic storage, we observed that storage under anaerobic conditions yielded insignificant benefits in terms of 24-hour recovery or adenosine triphosphate (ATP) levels. However, when the RBC additive pH was lowered from 8.1 to 6.5, significant improvement in metabolic variables was observed under anaerobic conditions.² The 24-hour RBC recovery was reasonably maintained in a pilot study even after 12 weeks when RBCs were stored under anaerobic conditions with acidic additive and supplemented with rejuvenation solution (Rejuvesol, Cytosol Laboratories, Braintree, MA) at the 7th and 11th

ABBREVIATIONS: DLR = dual-label calculation method; SLR = single-label calculation method.

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

Address reprint requests to: Tatsuro Yoshida, New Health Sciences, Inc., 6903 Rockledge Drive, Suite 230, Bethesda, MD 20817; e-mail: tatsuro.yoshida@newhealthsciences.com.

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weeks without warming the units. Previous investigations of anaerobic storage have been limited because they relied upon historical controls. Thus, the specific contributions from acidic additive and anaerobic storage to the improved 24-hour recovery could not be determined. The purpose of the present study is to see if anaerobic storage of RBCs in an acidic AS is superior to conventionally, aerobically stored RBCs after 6 weeks as indicated by 24-hour recovery and RBC life span.

MATERIALS AND METHODS

We designed a randomized controlled, crossover study to evaluate the 24-hour recovery and life span of 6- and 9-week-old autologous RBCs stored using OFAS3 AS in an anaerobic environment (test), compared to control RBCs stored using a licensed AS in an aerobic environment after 6 weeks of storage (control; cf. Fig. 1). The study was conducted at Dartmouth-Hitchcock Medical Center with the authorization of the Committee for the Protection of Human Subjects and under an approved Investigational New Drug application. Written informed consent was obtained from eight normal, healthy subjects meeting Food and Drug Administration (FDA; 21CFR640) and AABB⁷ donation criteria. Whole blood (500 ± 50 mL) was collected from each subject on two occasions, separated by a minimum of 12 weeks. Subjects were assigned to the test or control groups at the time of the first whole blood donation to balance the number of first donations in each group. Subjects donated 1 unit of whole blood into a standard, licensed primary collection container with CP2D anticoagulant solution (Pall Medical, Covina, CA). Collected blood was leukoreduced by means of an integral, whole blood leukoreduction filter and processed by centrifugation into RBCs by removing plasma and adding either 110 mL of AS-3 (Nutricel, Pall Medical) or 200 mL OFAS3 (University of Iowa School of Pharmacy, Iowa City, IA; Table 1).

The anaerobic environment was established for the test units as previously described.³ Briefly, the blood was transferred to a 1000-mL polyvinyl chloride transfer bag (Baxter Healthcare, Round Lake, IL), and oxygen was depleted by six cycles of gas exchange with ultrapure argon through a sterilely connected 0.22- μ m filter. The blood was transferred to a 600-mL transfer pack (Baxter) for storage in an anaerobic chamber (Difco BBL, Detroit, MI) that was filled with 10 percent hydrogen and 90 percent argon in the presence of a palladium catalyst to prevent reoxygenation and to further deplete oxygen

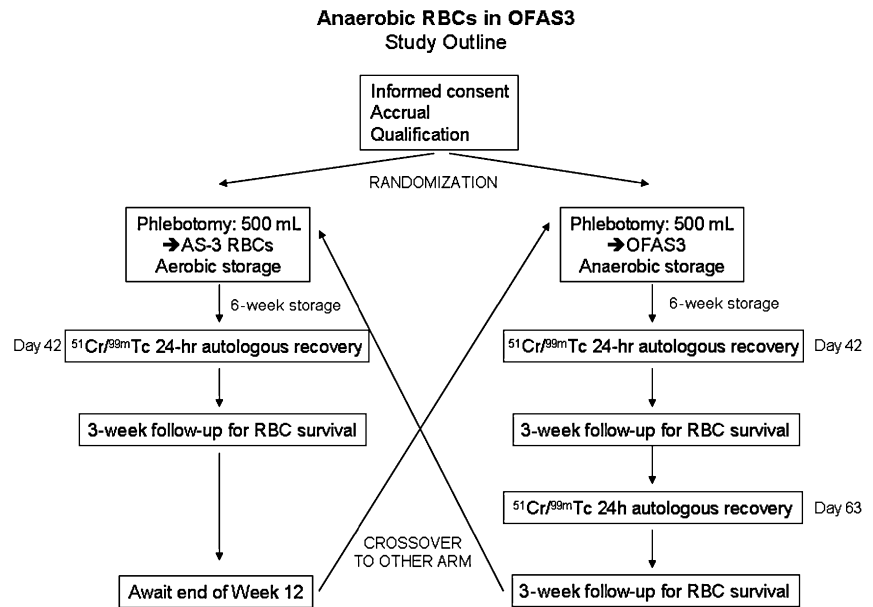


Fig. 1. Study design. Eight study subjects were randomly assigned to test or control study arms. A minimum of 56 days elapsed between RBC collections.

TABLE 1. Composition of RBC ASs (mmol/L)

Component	AS-3	OFAS3
Adenine	2.2	2
Glucose	55.5	110
Mannitol	0	55
NaCl	70.1	26
Na ₂ HPO ₄	20	12
Citric acid	12	
pH	5.8	6.5

during storage. The RBC unit was gently mixed and the chamber was recharged weekly for up to 9 weeks of storage. Control units were leukoreduced, processed into AS-3 RBCs, and stored undisturbed for 6 weeks. All units were stored at 1 to 6°C in a monitored refrigerator.

The in vitro and in vivo portions of this study were conducted concurrently. An evaluation of RBC function was conducted on the day of donation and the day of infusion at Weeks 6 and/or 9 using a standard panel of in vitro assessments. Automated hematology testing was performed (Advia 120, Bayer Corp., Diagnostic Division, Norwood, MA). Spun hematocrit levels were tested using a microhematocrit centrifuge (Hettich Mikro 20, Hettich, Tuttlingen, Germany). Postfiltration white blood cell enumeration was performed via flow cytometry (Leuko-Count, Becton-Dickinson, San Jose, CA). Supernatants from the units were prepared for analysis by double centrifugation at 2898 × *g* for 10 minutes (Rotanta 460RS, Hettich). Supernatant sodium and potassium concentrations were determined by ion-specific electrodes and glucose was determined by glucose oxidase (PPE, Roche Diagnostics, Indianapolis, IN). Lactate was analyzed via a

lactate oxidase/peroxidase endpoint reaction on a chemistry analyzer (Cobas Integra, Roche Diagnostics). The pH, pCO₂, and pO₂ were measured at 37°C on a blood gas analyzer (Model 855 or 248, Bayer Corp., Diagnostic Division). Supernatant samples were analyzed for RBC hemoglobin using a Drabkin's reagent method (Sigma, St Louis, MO) automated on a centrifugal analyzer (Cobas Fara, Roche Diagnostics) with a turbidity correction. A RBC perchloric acid extract was neutralized with 3 mol per L K₂CO₃ and analyzed for ATP using the NADP⁺ reduction method of Beutler automated for the Cobas Fara and 2,3-DPG (Roche Diagnostics). RBC morphology was determined after the method of Usry and colleagues.⁸ Microbiologic screening was performed by injecting 4 to 5 mL of RBCs 1 week before infusion into aerobic and anaerobic culture bottles (BTA FA, BTA FN) and testing in the BacT/ALERT 3D system (bioMérieux, Durham, NC).

The radiolabeled RBC recovery on test and control units was conducted following standard techniques as previously described.^{9,10} At the end of the specified storage interval (6 and/or 9 weeks), the unit was well mixed by hand and approximately 15 mL of the RBCs were labeled with ⁵¹Cr using standard techniques. The labeling agent, ⁵¹Cr sodium chromate, was mixed aseptically with the RBCs at room temperature for 30 minutes. A 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 0, 5, 7.5, 10, 12.5, 20, and 25 minutes as well as two samples at 24 hours through a butterfly needle. Additional samples for determination of RBC survival were taken at 1 day, 2 days, 3 days, 1 week, 2 weeks, and 3 weeks after reinfusion. The samples were counted in a gamma counter (1282 Compugamma, Wallac, Turku, Finland) to determine ⁵¹Cr activity. The exact volume of the injectate was determined by its specific activity. The intended subject dose for the first infusion was 5 to 10 μCi. The intended dose for the second infusion was 15 to 20 μCi.

A fresh sample of heparinized blood (10 mL) was collected from the subject before the reinfusion of ⁵¹Cr-labeled RBCs. The RBCs were separated and treated with a sterile solution containing approximately 2.0 μg tin in sodium citrate, dextrose, and sodium chloride. After 5 minutes of incubation at room temperature, the RBCs were washed once with 40 mL of cold saline. Then 20 μCi ^{99m}Tc pertechnetate was added for a 10-minute room temperature incubation. The cells were again washed with 40 mL of cold saline. The exact volume of the injectate was determined by its specific activity, a value that depends on the amount of radionuclide used and the labeling efficiency. The volume was selected to yield an injectate of 10 to 20 μCi of ^{99m}Tc. The ^{99m}Tc-labeled cells were drawn up

into the same syringe used to inject the ⁵¹Cr cells. The same samples taken for the ⁵¹Cr RBC recovery was analyzed for ^{99m}Tc activity.

An a priori assessment of sample size requirements estimated from preliminary data indicated that we could expect to distinguish between the test mean and a 75 percent target 24-hour recovery when the test mean is either less than 70.5 or greater than 79.5 percent with six evaluable subjects at an alpha level of 0.05 and power of 80 percent. Preliminary data presented here are reported as summary statistics. A mixed-effects, repeated-measures analysis of variance model was used for hypotheses tests and estimates of the 95 percent confidence intervals for recovery and survival (SAS, Version 9.1, SAS Institute, Cary, NC).

RESULTS

Eight subjects were entered into the study. RBC unit characteristics just before storage on Day 0 and then at the end of the 6- and 9-week storage periods are shown in Table 2. The starting volume was larger and the Hb concentration was lower in the test units because a larger volume of additive is used compared to control. Sodium concentration was lower and glucose concentration higher because of the OFAS3 formulation. At 6 weeks of storage, test units had significantly higher ATP content than control units ($p = 0.0037$), and by 9 weeks the test units were not different in ATP content than the 6-week control ($p = 0.30$). No significant difference in in vitro hemolysis was noted between arms at 6 weeks ($p = 0.31$), although by 9 weeks the test units had 2 of 8 units with hemolysis of more than 1 percent and 3 of 8 units with hemolysis of more than 0.8 percent ($p = 0.017$ compared to 6-week control). Hemolysis over storage shows a strong relationship with the individual subject. Four of eight subjects had very low hemolysis even up to 9 weeks of storage in OFAS3. The other four had distinctly higher hemolysis for all conditions (Fig. 2).

The principal purpose of this study is to see if anaerobic storage of RBC in an acidic AS is superior to conventionally, aerobically stored RBC after 6 weeks as indicated by 24-hour recovery and RBC lifespan. The primary outcome of 24-hour recovery of radiolabeled cells is shown in Table 2 for both the single-label calculation method (SLR) and the dual-label calculation method (DLR). Recovery for the anaerobically stored test RBCs had 7.9 ± 2.7 percent higher 24-hour DLR than the standard control at 6 weeks ($p = 0.023$). Test at 9 weeks was not different than the 6-week control ($p = 0.41$). There was no difference in the secondary outcome of life span across the three arms in a global test of hypothesis ($ndf = 2$, $ddf = 7$, $p = 0.73$). The individual DLR and life span results by subject are plotted in Figs. 3 and 4.

TABLE 2. Initial conditions and summary outcomes of RBCs at Weeks 6 and 9

Variable	Control AS-3 Aerobic		Test OFAS-3 Anaerobic	
	Day 0	6 week	Day 0	6 week
SLR (%)		77.8 ± 5.4 (83.5-67.3)	82.3 ± 4.6 (87.5-71.9)	73.5 ± 8.5 (83.5-61.0)
DLR (%)		75.1 ± 7.7 (83.5-62.0)	83.0 ± 5.0† (87.7-72.2)	72.6 ± 10.5‡ (85.7-54.1)
Life span (days)		84 ± 16§ (112-58)	82 ± 12§ (97-67)	86 ± 7§ (98-74)
Volume (mL)	393 ± 62 (493-334)	378 ± 63 (482-322)	444 ± 25 (493-414)	376 ± 28 (425-348)
pH (37°C)	6.83 ± 0.04 (6.90-6.79)	6.44 ± 0.07 (6.56-6.36)	6.89 ± 0.03 (6.96-6.86)	6.25 ± 0.05 (6.32-6.20)
Hb (mg/dL)	15.3 ± 2.2 (17.9-11.9)	15.0 ± 2.0 (16.8-11.6)	13.1 ± 1.2 (14.8-11.0)	13.1 ± 1.2 (14.8-10.9)
MCV (fL)	92 ± 4 (99-84)	97 ± 5 (104-88)	98 ± 5 (107-92)	101 ± 6 (109-89)
RDW (%)	14.2 ± 1.9 (17.7-12.1)	14.2 ± 2.2 (18.5-12.3)	14.2 ± 2.0 (17.5-12.6)	15.7 ± 2.6 (19.8-13.1)
Na (mmol/L)	165 ± 2 (168-164)	136 ± 6 (145-128)	98 ± 5 (108-91)	75 ± 6 (88-69)
K (mmol/L)	2.1 ± 0.3 (2.4-1.7)	37.1 ± 6.8 (47.1-26.2)	1.6 ± 0.2 (2.1-1.4)	36.3 ± 3.7 (42.2-31.3)
Glucose (mg/dL)	721 ± 19 (748-695)	547 ± 37 (594-490)	1209 ± 76 (1307-1096)	900 ± 34 (962-853)
Lactate (mmol/L)	3 ± 4 (13-1)	22 ± 6 (29-14)	2 ± 0 (2-1)	31 ± 2 (34-29)
Morphology (%)	100 ± 1 (100-99)	51 ± 18 (73-24)	98 ± 4 (100-87)	54 ± 15 (75-25)
ATP (µmol/gHb)	3.59 ± 0.91 (4.46-1.67)	2.93 ± 0.80 (4.28-1.95)	3.48 ± 0.86 (4.17-1.50)	2.71 ± 0.69 (3.75-1.86)
Hemolysis (%)	0.06 ± 0.03 (0.10-0.03)	0.4 ± 0.2 (0.7-0.2)	0.06 ± 0.03 (0.13-0.03)	0.8 ± 0.7 (2.1-0.2)

* Control and test are paired with the order randomly assigned to first or second collection (n = 8). Test units were stored anaerobically as described under Materials and Methods. Data are shown as mean ± SD (range).

† p = 0.023 compared to control.

‡ p = 0.41 compared to control.

§ No difference in study arms, p = 0.73.

MCV = mean cell volume; RDW = RBC distribution width.

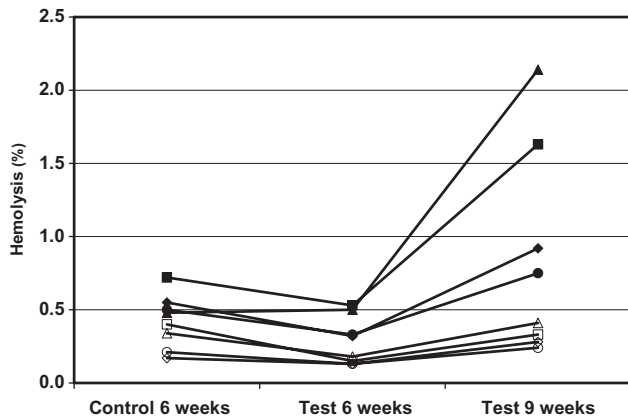


Fig. 2. Hemolysis over storage. Percent hemolysis at the end of storage for aerobically stored RBCs in AS-3 and anaerobically stored RBCs in OFAS3 for 6 and 9 weeks from the same subject are shown. Four subjects (solid symbols) had distinctly higher hemolysis for all conditions.

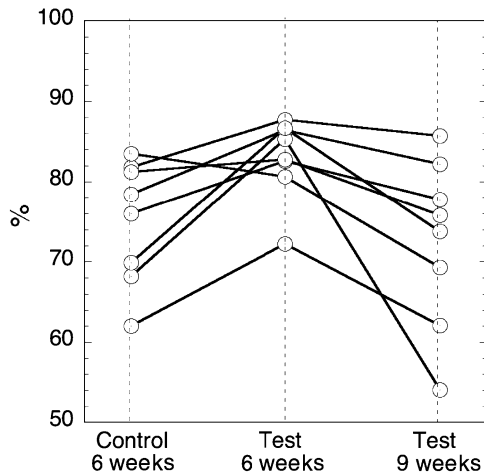


Fig. 3. Twenty-four-hour recovery (DLR) by subject. Control at 6 weeks, test at 6 weeks, and test at 9 weeks. There is no difference demonstrated between control 6 weeks and test 9 weeks ($p = 0.41$). Test at 6 weeks is superior to control at 6 weeks ($p = 0.023$).

DISCUSSION

RBC storage under anaerobic condition in OFAS3 had superior 24-hour recovery at 6 weeks of storage with no compromise in life span compared to standard control RBCs stored under aerobic conditions. By 9 weeks, the 24-hour recovery was diminished to approximately that of control units stored 6 weeks. Other in vitro RBC characteristics of ATP content and morphology were also similar between the 9-week test and 6-week control units. Although test units had lower hemolysis compared to control after 6 weeks, it was greater in the test units after 9 weeks. Previous anaerobic storage studies with various additives did not show high hemolysis accompanying

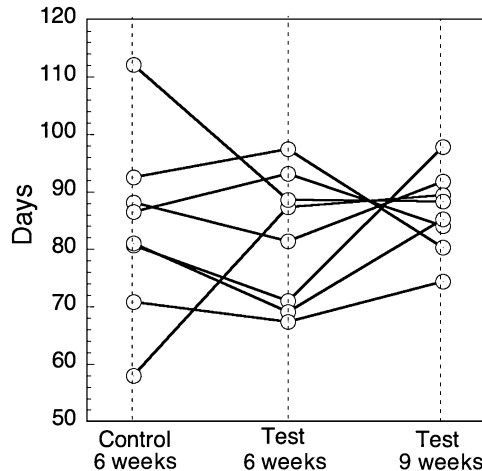


Fig. 4. Life span by subject. Control at 6 weeks, test at 6 weeks, and test at 9 weeks. There is no difference between the three study arms ($p = 0.73$).

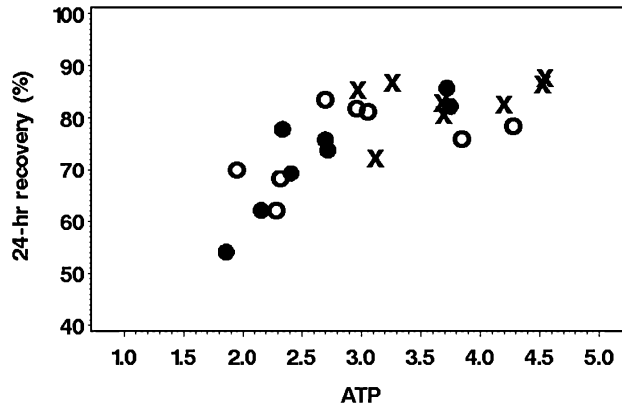


Fig. 5. RBC ATP content affects 24-hour RBC recovery. ATP ($\mu\text{mol/g Hb}$) content in the RBCs on the day of study is shown against the 24-hour RBC in vivo recovery. AS-3 6-week (\circ), OFAS3 6-week (\times), and OFAS2 9-week (\bullet).

acceptable 24-hour recovery.¹⁻⁵ The cause of the present observations is unknown. The data in Fig. 2 suggest that hemolysis may be a subject-dependent phenomenon. Additional work is indicated to understand and prevent this hemolysis in RBCs of more susceptible individuals over storage.

RBC 24-hour recoveries were related to the RBC ATP concentration (Fig. 5) as previously reported by several investigators.¹¹ Although it is tempting to assign a causal relationship to the ATP level or to other in vitro characteristics of the RBC such as morphology or hemolysis, all these variables are highly correlated with each other, making true cause-and-effect deductions difficult.

Of particular interest in the study are the 24-hour recoveries when examined in light of the most recent FDA performance criteria.¹² The SLR observed here for control RBCs at 6 weeks ($77.8 \pm 5.4\%$ with 2 of 8 less than 75%) has

only a 11.5 percent chance of passing the current FDA criteria of twenty-one 24-hour recoveries of 75 percent or more of 24 tested.¹³ The 6-week anaerobically stored test units (SLR, $82.3 \pm 4.6\%$ with 1 of 8 less than 75%) would stand a better chance (64.8%) of passing these criteria with a reasonable number of study subjects, although this chance is still disappointingly low even though superiority to control units at 6 weeks has been demonstrated.

The observed 8 percent increase in recovery rate translates to approximately a one-third reduction in the quantity of nonviable RBCs transfused, representing a potentially significant benefit to patients who are chronically or massively transfused, since nonviable cells will not only be nonfunctional, they will also increase the iron overload and potentially stress the reticuloendothelial system. Currently, only 3.2 percent of collected units get discarded due to outdating.¹⁴ Thus, there are no immediate needs for significant extension of storage time, at least in countries with a well-developed infrastructure. On the other hand, there have been numerous reports that suggested the negative consequences of transfusing cells stored for extended time.¹⁵⁻¹⁹ However, due to small sample sizes or inadequate controls, those studies have not been conclusive. This, coupled with numerous reports highlighting negative consequences of transfusion (positive correlations between number of units transfused and negative outcomes),²⁰⁻²⁴ suggests possible clinical advantages that might accrue with improving the storage conditions for RBCs.

Anaerobically stored RBCs may offer attractive alternatives to the current practice if the development of storage lesions can be significantly delayed, thereby providing RBCs with improved in vitro characteristics and in vivo recoveries within the current distribution systems. The methods used to demonstrate feasibility in study are not amenable to implementation within the current component preparation methods and practices. Development of improved methods for a simple and cost-effective method to deplete oxygen from collected blood without modifying the current blood banking infrastructure is indicated.

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