

# Extended storage of red blood cells under anaerobic conditions

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## Vox Sanguinis

**Background** Red blood cells (RBC) are subject to oxidative stress by reactive oxygen species during refrigerated storage. Near-complete removal of oxygen from red cells during storage should eliminate this contributor to the red cell 'storage lesion'. The *in vitro* effects of storing red cells under oxygen-depleted conditions for extended periods were investigated, and these were correlated with the observed recoveries after reinfusion.

**Study Design and Methods** Units of red cells, obtained after 'soft spin', were placed in a double volume of AS-3 additive solution and subdivided. Oxygen in the test units was depleted by repeated exposure to Ar gas (to O<sub>2</sub> saturation < 4%), and units were stored in anaerobic canisters for up to 15 weeks. Samples were taken weekly to monitor adenosine triphosphate (ATP), 2,3-diphosphoglycerate (2,3-DPG), cell-free haemoglobin, and vesicle production. In a parallel experiment, six units of red cells was depleted of oxygen in a similar manner, stored for 8, 9 and 10 weeks, and reinfused autologously to determine the 24 h post-transfusion recovery via <sup>51</sup>Cr/<sup>99m</sup>Tc radiolabelling. A similar study was also carried out using EAS61 additive solution, which by itself, had shown the ability to support 9-week storage, comparing biochemical profiles and *in vivo* recovery after aerobic vs. anaerobic storage.

**Results** Oxygen-depleted AS-3 units had significantly elevated ATP levels compared to controls. They also had significantly lower cell free haemoglobin and vesicle production when RBCs were stored for more than 9 weeks. An average of over 75% post-transfusion survival was observed after 9 weeks of anaerobic storage with less than 0.43% haemolysis. However, no further extension of storage was achieved with EAS61 additive.

**Conclusion** Anaerobic conditions permit acceptable 9-week storage of RBCs using double-volume AS-3 additive solution. It did not synergize with the alkaline, 9-week additive, EAS61, to further lengthen the acceptable storage time. These studies indicate that anaerobic storage may allow reduction in the effect of the storage lesion, but suggest that other factors contribute to limitations of RBC storage as well.

**Key words:** erythrocytes, anaerobic, storage.

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## Introduction

Changes accumulate in red cells during refrigerated storage (the red cell 'storage lesion' [1]) and limit the time over which

the red cells can be stored but still maintain post-transfusion viability. Among these deteriorations are haemolysis, echinocytosis and membrane area loss, adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) depletions, and phosphatidylserine exposure [2]. These lesions collectively manifest in reduced survival after transfusion and lowered efficacy in terms of reduced O<sub>2</sub> delivery capacity immediately after transfusion.

Improvement in supply logistics and reduction in outdated are obvious benefits of significantly prolonging the allowable storage time of red blood cells. Moreover, recent reports of

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negative outcomes associated with transfusion in critically ill patients [3–5] suggest that the storage lesion may have a clinical impact. If storage-induced deterioration processes are slowed such that the shelf-life of red blood cells (RBC) is extended 50–100%, then units used after 4–6 weeks of storage would be (relatively) ‘fresher’ and would have accumulated less of a storage lesion.

Several investigators have attempted to achieve extension of shelf-life storage by enhancing the glycolytic flux and maintaining the ATP levels through alteration of additive solutions. Examples include Meryman *et al.* [6] who achieved 100 days of storage with a hypotonic solution containing high concentrations of ammonia, and Greenwalt *et al.* [7] who achieved 9-week storage with a similar solution. However, the ammonia content was felt to preclude clinical application. A series of alkaline hypotonic solutions were tested with *in vivo* trials by Hess *et al.* [8–11] and they documented successful storage for 9, 10, 11 and ultimately 12 weeks. Other workers turned their attention to oxidative damages as elements of the storage lesion, since the red cell is uniquely susceptible to oxidative damage because of the high concentration of oxygen being carried in the cell and the presence of reactive Fe species in haemoglobin. Some of the storage lesions, particularly the loss of membrane area, are attributed at least in part to oxidative damage to membrane lipids and cytoskeletal proteins [12,13]. Previous attempts by others workers to reduce oxidative damage to red cells during storage have included storage under carbon monoxide (CO) (to stabilize haemoglobin) and prevention of oxygen diffusion into the bag during storage [14,15]. The former approach did not result in acceptable red cell recovery on reinfusion, however (Lawrence Wolfe, personal communication to M.W.B., 1995). The latter approach reported improved energy metabolism (ATP and adenylate energy charge) as well as reduced rate of red cell fluidity loss, but no *in vivo* studies were included in this report.

In this study, we attempted to reduce the storage lesion linked to oxidative damage by storing red cells under nearly anaerobic conditions. Most of oxygen bound to haemoglobin in the red cells was removed (oxygen saturation of haemoglobin, SO<sub>2</sub>, below 4%) at the onset of storage and kept free of oxygen throughout the storage. Radio-labelled autologous red cell recovery studies documented successful 9-week storage when anaerobic storage was coupled with the AS-3 additive solution. However, anaerobic storage did not synergize and extend storage time when it was coupled with an alkaline additive solution (EAS61) [8], a solution that allows for 9 weeks of storage in aerobic conditions.

## Materials and methods

The study was conducted in five parts: (I) a preliminary *in vitro* study to determine the extent of oxygen depletion in

anaerobic units; (II) *in vitro* studies using split units in AS-3 additive to explore the effect of anaerobic storage in a standard additive solution; (III) a small *in vitro* study using split units in EAS2 additive to study the effect of CO; (IV) an *in vivo* recovery study with AS-3 additive solution to compare *in vitro* and *in vivo* effects of anaerobic storage; and (V) an *in vivo* recovery study with EAS61 additive solution to determine whether additional benefits accrue using this solution in anaerobic storage. They were conducted with the authorization of the Committee for the Protection of Human Subjects of the Dartmouth-Hitchcock Medical Center. All subjects gave their written informed consent to participate.

## I. Extent of achievement of anaerobic conditions

Six units of whole blood (450 ml ± 10%) in CP2D (Pall Medical, Covina, CA) were held for 1–2 h at room temperature before a soft spin [16] and manual separation. Two 50-ml aliquots were transferred to 150 ml polyvinyl chloride (PVC; PL146) bags (Baxter Healthcare, Round Lake, IL). AS-3 additive solution (Pall Medical) was added to each of the two aliquots at the ratio of 200 ml per unit (33 ml per aliquot) and stored at 4 °C. For the one unit of each pair to be stored anaerobically, oxygen depletion was accomplished by conducting a repetitive gas exchange. The 150-ml bag was filled with ultra-pure Ar (AirGas Inc., Salem, NH) through a sterilely connected 0.22 µm filter (Gelman 4423, Ann Arbor, MI), and gently agitated horizontally at 60 r.p.m. for 10 min in a 4 °C cold room. The gas in the bag was then expressed out through the filter, and the process was repeated five more times. The anaerobic units were then stored in vented anaerobic chambers (Difco BLL, Detroit, MI) that were filled with ~20% H<sub>2</sub> and ~80% Ar in the presence of a Pd catalyst (Difco low temperature catalyst) to prevent re-oxygenation and further deplete oxygen during storage. The chamber was recharged whenever the unit was accessed for sampling. Units were stored in a monitored 4 °C refrigerator for up to 10 weeks.

SO<sub>2</sub> of oxygen-depleted units was estimated from pO<sub>2</sub> that was measured during the gas exchange process using Foxy Fibre Optic Oxygen Sensor System (Ocean Optics, Dunedin, FL) equipped with FOXY-R 1 mm fibre optic probe in the separate experiment. The instrument was calibrated prior to the experiment with deionized water maintained at 22 °C and saturated with gas standards (ambient air: 20.9% O<sub>2</sub>; 3.04% O<sub>2</sub> in N<sub>2</sub>; 2.10% O<sub>2</sub> in N<sub>2</sub>; 1.06% O<sub>2</sub> in N<sub>2</sub>; and 100% N<sub>2</sub>, AirGas Inc.). A calibration curve was obtained by non-linear least square fitting these data with second degree polynomial. The oxygen probe was inserted through septum port into the blood bag, and placed in constant temperature rotator shaker/incubator and agitated 60 r.p.m.

SO<sub>2</sub> and pO<sub>2</sub> for moderate to highly oxygenated blood samples in physiological range were obtained using a clinical CO-Oximeter (Nova Biomedical, Waltham, MA). Non-physiological SO<sub>2</sub> values were estimated based on simulated oxygen binding curve from pO<sub>2</sub> measured by the optical sensor. For SO<sub>2</sub> below pO<sub>2</sub> of 10 torr, an oxygen binding curve of the same blood, where pO<sub>2</sub> was measured during oxygen depletion, was constructed as oxygen was added back and pO<sub>2</sub> and SO<sub>2</sub> values were measured at multiple points within the physiological range by a clinical CO-Oximeter. These data were then fitted to a Hill equation and SO<sub>2</sub> values were calculated for measured pO<sub>2</sub> from the equation:

$$SO_2 = (pO_2)^n / [(p_{50})^n + (pO_2)^n]$$

where  $n = 2.70$  and  $p_{50} = 11.586$  torr.

## II. *In vitro* analyses of anaerobically stored red cells

Twelve units of whole blood were prepared as in study I. Samples for routine biochemical and haematologic studies in the *in vitro* portion of the study were taken weekly during storage. ATP and 2,3-DPG levels were measured using diagnostic kits (#366 and #35-A, Sigma-Aldrich, St Louis, MO), modified and adapted to be used in a COBAS FARA 2 automated analyser (Roche Diagnostics, Alameda, CA). Glucose was measured by Stat-Ultra4 electrolyte analyser (Nova Biomedical). Cell-free haemoglobin was determined from supernatant (10 000 *g*, 10 min) using a Drabkin's reagent (Sigma). Vesicles were collected after 20 000 *g* spin (20 min) from stored blood samples clarified of cells and debris by previously centrifuging 1000 *g* for 5 min. Vesicles were washed once by resuspending them in phosphate-buffered saline (20 000 *g* for 20 min), and protein was measured using Coomassie Plus protein assay (Pierce Biotechnology Inc., Rockford, IL).

## III. Storage in EAS2 under CO

Whole blood units ( $n = 6$ ) were collected as in study I above but were not processed until the fourth day after collection and the additive solution used was EAS2 storage solution (20 mM NH<sub>4</sub>Cl, 30 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM adenine, 110 mM dextrose, 55 mM mannitol, pH 7.15) made in the laboratory using ingredients purchased from Sigma Aldrich [7]. One subunit was reacted with CO by injecting 100% CO into the storage bag for 10 min at 22 °C before expression of unreacted CO; this was repeated three times prior to storage at 4 °C in an anaerobic chamber filled with ~20% H<sub>2</sub> and ~80% Ar in the presence of a Pd catalyst. The other unit of the pair was used as an aerobic control without any gas exchange and stored in ambient air. These units were held to 75 days of storage with periodic assessment of ATP concentration.

## IV. *In vivo* 24 h recovery using AS-3 additive

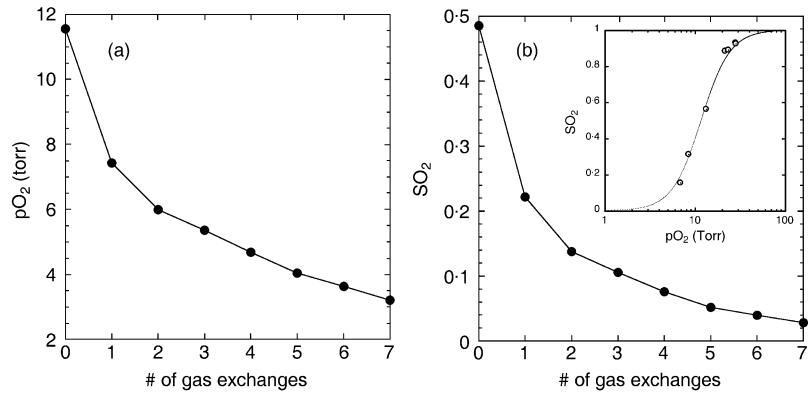
Eight normal, healthy subjects each donated 450 ml whole blood as above that were converted to AS3 additive system units with twice the usual volume (i.e. 200 ml) of AS-3. The units were stored anaerobically after oxygen depletion using the above protocol conducted in 1000 ml polyvinyl chloride bags (Baxter Healthcare). After 8 weeks of storage, an aliquot (20 ml) was used for a double label (<sup>51</sup>Cr/<sup>99m</sup>Tc) radio-labelled autologous red cell recovery [17–19]. Repeat re-infusions (to a maximum of 3) were performed at weekly intervals if acceptable results were noted after each. (Acceptability was defined as haemolysis < 0.8% and 24 h red cell recovery > 75% using the double label technique.) Each subsequent reinfusion used a larger dose of radioactive cells, and pre-existing radioactivity determined in a pre-infusion sample was subtracted from subsequent samples-observed activity. Samples for routine biochemical and haematologic studies were taken before storage and after 8, 9 and 10 weeks of storage. All samplings of the units were through sterile connection with a transfer pack docked via a sterile connecting device (SCD, SCD312 Terumo, Elkton, MD). Units were culture-negative for bacteria at the time of each re-infusion.

Cell counts were performed by automated counters (Advia 120, Bayer, Norwood, MA). Supernatants from the units were spun twice at 2100 *g* (MP4R, International Equipment Company, Needham Heights, MA) for 10 min and then analysed for haemoglobin using a Drabkin's reagent method (Sigma) automated on the COBAS FARA 2 with a turbidity correction. Supernatant electrolyte concentrations were determined by ion-specific electrode (Hitachi 917, Boehringer Mannheim Corp., Indianapolis, IN). Glucose was determined by glucose oxidase (Hitachi 917). Lactate was determined by lactate oxidase/peroxidase end-point reaction (Hitachi 917). The pH was determined on a blood gas analyser (Model 855, Bayer) and read at 37 °C. A red cell perchloric acid extract was neutralized with 3M K<sub>2</sub>CO<sub>3</sub> and analysed for ATP (by measurement of NADH oxidation by glyceraldehyde phosphate dehydrogenase following use of ATP by phosphoglycerate phosphokinase) and DPG (by measurement of NADH oxidation) on the COBAS FARA using adapted reagent kits (Sigma).

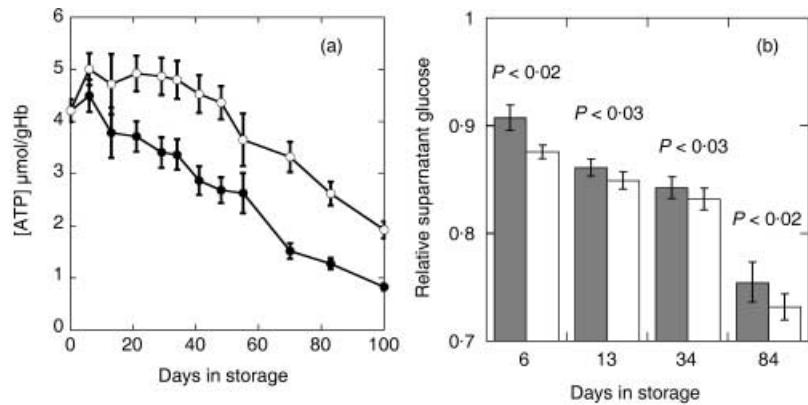
## V. *In vivo* 24 h recovery using EAS61 additive

Eight normal, healthy subjects donated two units of whole blood as above at least 10 weeks apart. The red cells were converted to additive system units as above using EAS61 [8] additive solution (adenine, 2 mM; dextrose, 110 mM; mannitol, 55 mM; NaCl, 26 mM; Na<sub>2</sub>HPO<sub>4</sub>, 12 mM; pH 8.3; compounded and filter-sterilized by Pharmaceutical Development Corp., Charleston, SC). The units were randomized to be stored either in the routine fashion in a monitored 4 °C refrigerator for 9 weeks (that is, aerobically, as 'control' units) or in an

**Fig. 1** Oxygen depletion from RBC suspension by repeated gas exchange. (a)  $pO_2$  after 10 min incubation in Ar filled transfer bag. (b) Estimated  $SO_2$  of RBC after each gas exchange.  $SO_2$  was estimated from a fit to the Hill equation for  $pO_2$  vs.  $SO_2$  data at physiological range as measured by a CO-Oximeter (Insert).



**Fig. 2** Effects of haemoglobin status during red cell storage in AS3. (a) Mean ATP levels of control (filled circles) and oxygen-depleted blood (open circles). For each data pairs at given time, paired student *t*-test was carried out. Every storage date had  $P < 0.00001$  except for day 6 ( $P < 0.17$ ) and day 55 ( $P < 0.003$ );  $n = 12$ . (b) Relative glucose levels in the storage medium during storage. Control RBC (shaded) and oxygen-depleted RBC (empty). Day 0 values were not available and estimated from a separate experiment. However, units were split to 'test' and 'control' after AS-3 addition, assuring the identical concentrations of glucose at the onset of the storage. *P*-values for each observation date are given;  $n = 12$ .



oxygen-depleted environment for up to 10 weeks ('test' units). Oxygen was depleted to less than 4%  $SO_2$ , and double label radio-labelled autologous red cell recovery studies were performed as above. Biochemical and haematologic analyses were performed as in study IV above.

## Statistics

Descriptive statistics are presented as arithmetic mean  $\pm$  1 standard deviation. Paired student *t*-tests were carried out on as indicated in figure legends with  $P < 0.05$  used to reject the null hypothesis.

## Results

### I. Extent of achievement of anaerobic conditions

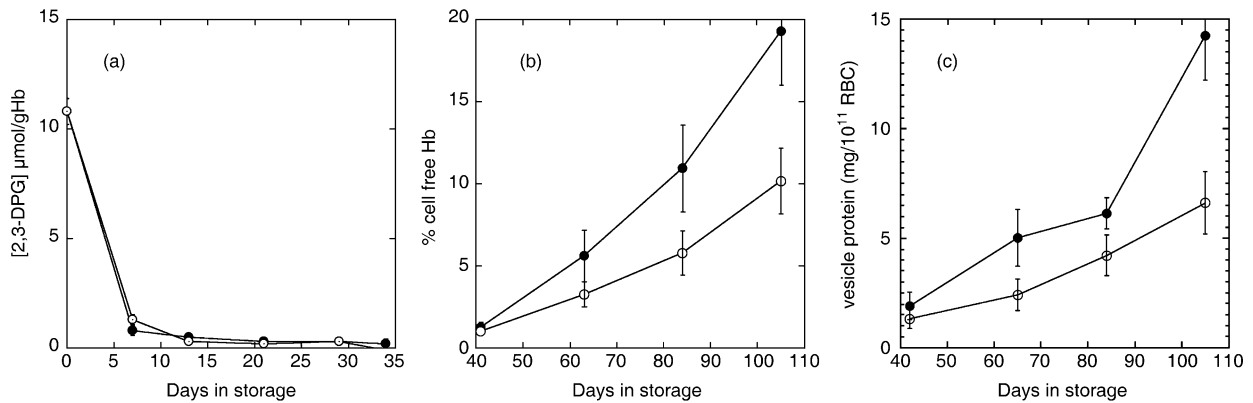
The *in vitro* studies documented a progressive decline in the  $pO_2$  through the gas exchange protocol (Fig. 1a). An oxygen-binding curve was constructed by re-oxygenating the same blood sample with pure  $O_2$  while measuring both  $pO_2$  and  $SO_2$  in physiological range and non-linearly fitting a Hill equation through these data (Fig. 1b inset). The estimated  $SO_2$

from  $pO_2$  measurement for a representative unit of blood during gas exchange is shown in Fig. 1(b). For a typical unit of blood used in this study,  $SO_2$  was reduced from  $\sim 50\%$  to  $< 4\%$  before it was placed in the storage canister.

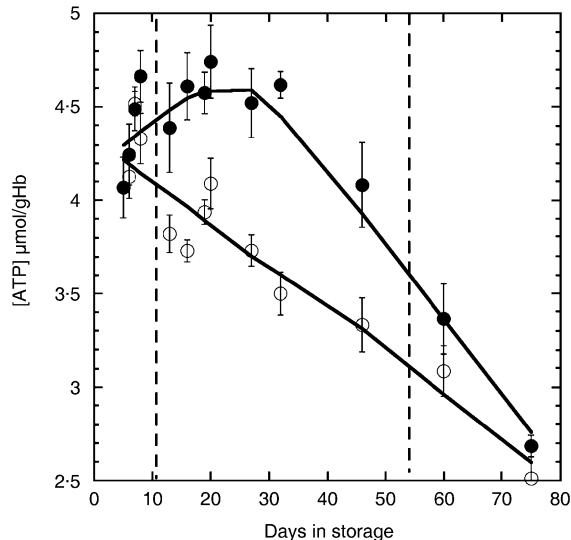
### II. *In vitro* analyses of anaerobically stored red cells

Adenosine triphosphate (ATP) levels initially increased and then declined through 100 d of storage in AS-3 (Fig. 2a). The initial increase in ATP was seen only to the day 6 sample for units stored aerobically but continued to around 3 weeks in the anaerobic units. Thereafter, the rates of decline in aerobically and anaerobically stored units appeared similar. Of note, the ATP level was  $79 \pm 24\%$  of day 0 levels at 10 weeks of storage in the anaerobic units, but only  $36 \pm 12\%$  in the aerobic units ( $P < 0.05$ ). The increased ATP levels were accompanied by an increase in glucose consumption (Fig. 2b).<sup>1</sup>

<sup>1</sup> Glucose levels at day 0 were not determined but were estimated from other experiments under similar condition. However, anaerobic and aerobic units were expected to have identical glucose levels, because red cells were suspended in AS-3 additive before units were split.



**Fig. 3** Effect of oxygen-depleted storage in AS-3 on red cell metabolism. (a) Mean 2,3-DPG levels of control (filled circles) and oxygen-depleted blood (open circles). No significant differences were observed. (b) Mean haemolysis of control (filled circles) and oxygen-depleted blood (open circles). *P*-values are: day 41 ( $P < 0.085$ ); day 63 ( $P < 0.02$ ); days 84 and 105 ( $P < 0.003$ ). (c) Mean vesicle protein per  $10^{11}$  RBC of control (filled circles) and oxygen-depleted red cells (open circles).  $P < 0.003$  except for day 42 ( $P < 0.014$ ).



**Fig. 4** Effect of CO on ATP levels during anaerobic storage. Control (closed circles); CO-exposed (open circles). Data pairs between day 13 and day 46 (as shown between dashed lines) had *P*-values less than 0.05.

By the end of week 1 of storage, 2,3-DPG levels were near zero whether the units were stored aerobically or anaerobically (Fig. 3a). Storage under oxygen depletion was associated with a significant reduction in haemolysis at and beyond 63 days of storage (Fig. 3b). Note that, in general, haemolysis values were higher for these *in vitro* units, compared to units stored for *in vivo* trials (*vide infra*) and that these '*in vitro*' units were undergoing weekly agitation as part of the sampling process. Effects of oxygen depletion on preservation of red cell membranes were observed indirectly as a significant reduction of the rate of vesicle production at six or more weeks of storage (Fig. 3c).

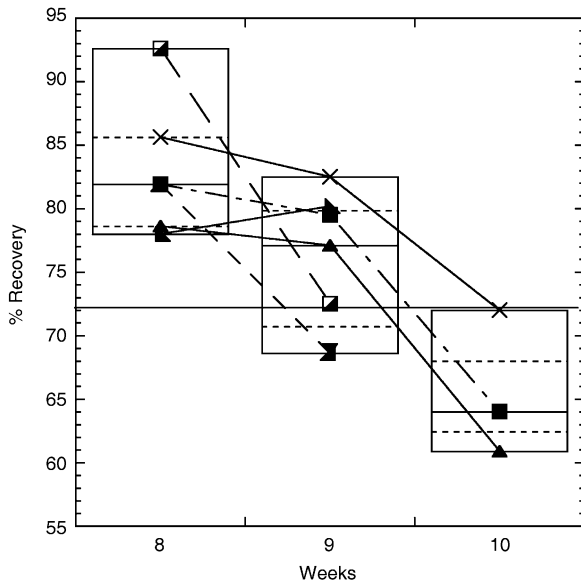
### III. Storage in EAS2 under CO

In order to further examine a possible mechanism behind ATP increase and enhanced glycolytic rate observed with anaerobic units, we examined the ATP levels of oxygen-depleted RBC in which CO was used in place of Ar for oxygen removal, with haemoglobin locked in stable CO-haemoglobin. Here, we used the EAS2 additive solution, which had shown previously to increase ATP levels [7] under normal storage conditions. As shown in Fig. 4, expected increases in ATP levels were observed with aerobic units. However, when these red cells were stored under CO, the effect of EAS2 to elevate ATP levels was suppressed.

### IV. *In vivo* 24 h recovery using AS-3 additive

One of the eight subjects decided to withdraw from the study for personal reasons unrelated to the study following donation of the unit; the unit was retained for biochemical analyses. Another subject was unavailable for re-infusion after 8 weeks of red cell storage; the subject resumed participation with the week 9 re-infusion based on good recoveries at week 8 with other units and biochemical results similar to those in other units. The final subject slated to undergo re-infusion at week 10 was removed from the study because all week 10 recoveries already performed had results  $< 75\%$ .

The six units re-infused at 8 weeks all had survivals above 75% with a mean recovery of  $83.1 \pm 5.4\%$  (Fig. 5 and Table 1). At 9 weeks, mean survival was  $75.8 \pm 5.5\%$ , with four out of seven units registering over 75%. Samples from three of these four units were then infused again at 10 weeks, with all showing a recovery  $< 75\%$  and a mean recovery of  $65.7 \pm 5.8\%$  ATP levels (Table 1) were comparable to the parallel experiments for the *in vitro* studies (Fig. 2a). Hemolysis rates were significantly

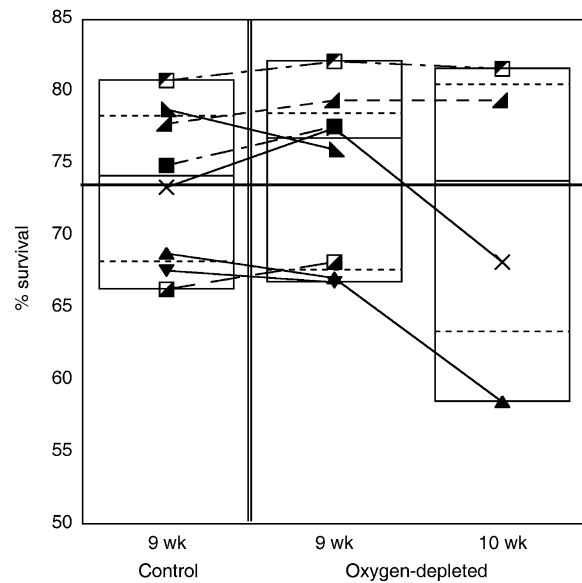


**Fig. 5** Twenty-four h autologous recovery trial with AS-3 additive. Individual data are superimposed on percentile plot. The bottom and top of each box represent 5% and 95% of the data. The middle line represents the median value, while the lower and upper dashed lines represent 25% and 75% of the data, respectively.

lower in these units that had been left relatively undisturbed during storage compared to weekly assayed units for *in vitro* study II.

#### V. *In vivo* 24 h recovery using EAS61 additive

A paired *in vivo* recovery study of test and control units from eight subjects in EAS61 failed to show a synergistic effect of combining the experimental additive solution with the anaerobic storage of red cells (Fig. 6 and Table 2). After 9 weeks of storage, the 24 h (double label) recovery was  $73.9 \pm 4.6\%$



**Fig. 6** Twenty-four h autologous recovery trial with EAS61 additive. Individual data are superimposed on percentile plot. The bottom and top of each box represent 5% and 95% of the data. The middle line represents the median value, while the lower and upper dashed lines represent 25% and 75% of the data, respectively.

for the units stored anaerobically and  $74.9 \pm 5.4\%$  for the control units. These results were not statistically distinguishable, and, although the control (aerobic) value was slightly below the Food and Drug Administration requirement of 75%, it is statistically similar to data reported earlier [8]. Although five out of eight anaerobic units had slightly higher recoveries at 9 weeks compared to the controls, statistically there was no significant difference in recoveries between the two storage conditions. Furthermore, there were no statistically significant differences in any of the biochemical or haematologic parameters between test and control after 9 weeks

**Table 1** Effects of anaerobic storage on AS-3 red cell units used in *in vivo* studies

	<i>n</i>	Day 0	<i>n</i>	week 8	<i>n</i>	week 9	<i>n</i>	week 10
Calculated MCV (fL) <sup>a</sup>	8	100.1 ± 5.6	8	92.3 ± 4.1	8	95.9 ± 9.0	8	92.9 ± 5.4
pH <sub>37°C</sub>	8	6.72 ± 0.04	8	6.37 ± 0.05	8	6.35 ± 0.04	8	6.32 ± 0.04
Supernatant sodium (mEq/l)	8	168 ± 7	8	140 ± 6	8	138 ± 2	8	133 ± 7
Supernatant potassium (mEq/l)	8	1.50 ± 0.0	8	31.9 ± 1.9	8	34.4 ± 2.9	8	36.4 ± 2.7
Supernatant glucose (mg/dl)	8	822 ± 50	8	633 ± 22	8	632 ± 26	8	595 ± 42
Supernatant lactate (mm)	8	1.20 ± 0.40	8	21.3 ± 1.5	8	22.7 ± 1.9	8	24.0 ± 1.4
ATP (μmol/gHb)	8	3.96 ± 0.74	8	3.71 ± 0.85	8	3.17 ± 0.54	8	2.84 ± 0.52
2,3-DPG (μmol/gHb)	8	12.5 ± 2.5	8	0.30 ± 0.05	8	0.45 ± 0.10	8	0.37 ± 0.10
Haemolysis (%)	8	0.03 ± 0.01	8	0.24 ± 0.1	8	0.34 ± 0.09	8	0.43 ± 0.15
24 h recovery (%)			6	83.1 ± 5.4	7	75.8 ± 5.5	3	65.7 ± 5.8

<sup>a</sup>MCV: significant differences ( $0.05 < P$ ) only between day 0 and weeks 8–10.

**Table 2** Effects of anaerobic storage on EAS61 red cell units used in *in vivo* studies

	Test-day 0	Control-day 0	Test-week 9	Control-week 9	Test-week 10
Calculated MCV	102.4 ± 10.5	101.0 ± 6.1	92.7 ± 8.4	93.5 ± 7.8	91.1 ± 8.1
pH <sub>37°C</sub>	6.92 ± 0.02	6.92 ± 0.02	6.14 ± 0.09	6.17 ± 0.05	6.11 ± 0.07
Supernatant sodium (mEq/l)	78.5 ± 4.9	77.0 ± 2.8	57.6 ± 4.5	59.3 ± 1.8	57.0 ± 6.3
Supernatant potassium (mEq/l)	1.5 ± 0.0	1.5 ± 0.0	42.5 ± 2.7	41.4 ± 3.0	44.3 ± 1.8
Supernatant glucose (mg/dl)	1537 ± 69	1541 ± 118	1048 ± 32	1092 ± 25	1055 ± 57
Supernatant lactate (mM)	1.03 ± 0.28	0.91 ± 0.34	34.7 ± 1.6	31.7 ± 4.2	31.8 ± 3.8
ATP (μmol/gHb)	3.92 ± 0.55	4.09 ± 0.32	2.35 ± 0.38	2.17 ± 0.69	2.09 ± 0.19
Haemolysis (%)	0.05 ± 0.02	0.04 ± 0.03	0.33 ± 0.07	0.31 ± 0.11	0.42 ± 0.09
Red cell morphology score	99.9 ± 0.1	100.0 ± 0.1	65.1 ± 6.7 <sup>a</sup>	70.7 ± 9.3 <sup>a</sup>	64.4 ± 4.8
24 h recovery (%)		73.9 ± 4.6	74.9 ± 5.4	68.0 ± 10.9	

Number of observations = 8 at all time points except at Week 10 where *n* = 4.

<sup>a</sup>*P* < 0.05 by paired *t*-test (one-tail).

of storage for these units except for a small difference in morphology score (data not shown). The criteria for repeat re-infusion after 10 weeks were satisfied for four of eight test units, and the mean 24 h recovery rate declined to 68.0 ± 10.9%.

## Discussion

Oxidative damages resulting from reactions with denatured haemoglobin and oxygen in red cells are well known [20–26]. Red cells maintain their structure and function despite the oxidizing environment with a variety of reducing mechanisms. Placing red cells under refrigeration reduces the overall reaction rates of the oxidative process. However, this advantage is counteracted by three effects: (I) the rate of methemoglobin reduction by cytochrome b5 reductase is reduced; (II) methemoglobin may be more prone to denaturation as suggested by lower thermodynamic stability of metmyoglobin at 4 °C [27]; and (III) the solubility of oxygen is doubled at 4 °C. As a result, oxidative damage can accumulate with refrigerated red cell storage.

Although polyvinyl chloride-based blood storage bags currently in use cannot satisfy the oxygen permeability requirement for extended platelet storage, they are nevertheless permeable to oxygen. A previous study examined the effect of reducing SO<sub>2</sub> gradually over 6-week storage from 60% to 32% [15]. In the current study, a more stringently anaerobic environment was achieved at the outset, with the pO<sub>2</sub> of units reduced to 3.5 torr and with an estimated SO<sub>2</sub> of 3.6%. This translates to a 50-fold reduction in free oxygen concentrations compared to oxygen-saturated unit.

As might be predicted from the less-oxidizing environment, anaerobic storage was associated with increased membrane stability. The *in vitro* study in AS-3 showed reduced haemolysis and membrane vesiculation rates for oxygen-depleted AS-3 units compared to aerobic controls. An

unexpected benefit of oxygen-depleted storage was a significant increase in [ATP], peaking around the third week and then declining in a parallel fashion to the control. Thus, even after 7 weeks, [ATP] was comparable to that of the fresh unit while the [ATP] in the control was 64% of initial levels; the anaerobic units' [ATP] was at 85% at 10 weeks compared to 36% for the control. The increased levels of ATP under anaerobic conditions may be mainly attributed to the increase in glycolytic flux at 4 °C as shown by the increased rate of glucose consumption in the first week of storage (Fig. 2b). Beyond the first week, glucose consumption rates were similar for both units, although the [ATP] increase was observed for up to 3–4 weeks for anaerobic units. With a given glucose consumption rate, this higher ATP concentrations may be attributable to reduction in flux through the pentose pathway as a consequence of reduced rate of NADPH oxidation in anaerobic units.

To prevent prolonged ATP production from exhausting the supply of glucose, red cells were stored in double the usual volume of additive solution. This reduction in relative red cell concentration has, in itself, been associated with improved *in vitro* biochemical results and improved post-infusion recovery (Meryman and AuBuchon, unpublished observations). However, the *in vitro* portion of this study showed an additional effect of anaerobic storage, and previous work suggests mechanisms for these observations. An increased glycolytic rate in red cells under anaerobic conditions at 37 °C was originally reported in 1966 by Asakura *et al.* [28]. With the subsequent discovery of 2,3-DPG as a major heterotrophic ligand of haemoglobin [29,30], the observation of an increased glycolytic rate of anaerobically stored red cells can be explained as a result of oxygen-dependent modulation of free cytosolic [2,3-DPG] and [ATP] resulting from changes in affinity for these molecules depending on oxygenation status of haemoglobin. The glycolytic rate is modulated by cytosolic concentrations of 2,3-DPG and ATP,

because they are potent product inhibitors of enzymes involved in ATP production. Deoxygenated haemoglobin (Hb) (T-state) has more than 100-fold higher affinity towards 2,3-DPG and 30-fold higher for ATP compared to oxygenated Hb (R-state) [31]. Thus, under nearly 0% SO<sub>2</sub>, cytosolic 2,3-DPG is bound to deoxygenated Hb, and later, when 2,3-DPG is depleted, a significant portion of free ATP is bound to deoxygenated Hb [32]. This greatly reduces free [ATP] and prevents product inhibition at phosphofructokinase (ATP) and pyruvate kinase (ATP and 2,3-DPG) enzymatic steps, thereby increasing the flux through glycolytic pathway. In addition, Hogman *et al.* [15] suggested release of inhibition of ribose-phosphate pyrophosphokinase by 2,3-DPG (through binding to deoxyhemoglobin), as well as a reduction in pentose pathway flux due to reduced oxidative stress, as potential explanations for prolonged ATP maintenance during oxygen-depleted storage. With achievement of much greater oxygen depletion in this work than that of Hogman *et al.*, this effect was even more pronounced.

The effects of CO on ATP levels during anaerobic storage further suggest the link between haemoglobin oxygenation status and ATP metabolism. CO binds Hb with 210 times the affinity of oxygen [33] and protects against oxidation and denaturation of haemoglobin. However, as shown in Fig. 4, storing CO-exposed red cells under oxygen depleted-conditions *reduced* [ATP] compared to red cells stored aerobically without CO exposure. (Note here that we used EAS2, an additive developed by Greenwalt's group that has the ability to enhance [ATP] under normal storage conditions [7], as shown in Fig. 4.) This observation can be explained by the fact that CO stabilizes haemoglobin's quaternary conformation to the R-state, similar to oxy-haemoglobin, with reduced affinities towards DPG and ATP. Release of DPG and ATP from CO-Hb raises cytosolic concentrations and reduces the glycolytic flux by product inhibition.<sup>2,3</sup>

Alternative explanations for enhanced ATP levels may be provided by alkalization of cytosol, as a consequence of CO<sub>2</sub> removal during the oxygen depletion process, and uptake of the Böhr protons by deoxy-Hb. However, these effects may be considered secondary to the 2,3-DPG effect in our case, because in the CO gas exchange experiment, which also effectively removed CO<sub>2</sub>, still suppressed the expected ATP boost (Fig. 4). Alkalization of cytosol by CO<sub>2</sub> removal with CO may be counteracted in part by release of the Böhr

protons by Hb-CO. Again, we consider this secondary effect since CO induced oxygen-depletion not only prevented ATP increase compared to the aerobic control as with AS-3 or EAS61 (data not shown), it *suppressed* EAS2's ability to boost ATP. Additional effects that may have caused enhanced ATP levels include binding of deoxyhemoglobin to a Band 3 site in place of phosphofructokinase resulting in its activation [34]; and the reduction of ATP precursors being siphoned off to other pathways in anaerobic conditions [35].

Biochemical analyses showed significant improvements for oxygen-depleted units over controls in all measured parameters except for 2,3-DPG which was nearly depleted by the end of week 1 in either situation. These data from the *in vitro* portion of the study were reflected in 24 h post-transfusion recoveries, with a better mean recovery (83%) after week 8 than reported with routine storage at 6 weeks [36], and an acceptable mean recovery at week 9. Although haemolysis was less than 0.5% and 72% of initial ATP was present in the test units after 10 weeks of storage, none of three units that were re-infused had an acceptable recovery.

Although *in vivo* and *in vitro* units had different area/volume configurations during storage, extents of *initial* oxygen depletion was similar, and pO<sub>2</sub> was too low (SO<sub>2</sub> < 3%) to reliably differentiate the further oxygen depletion *during* storage (data not shown). The ATP levels were comparable after 8 and 10 weeks of storage between both configurations. Cell free haemoglobin levels were higher for *in vitro* units (study II) due to thorough mixing necessitated by weekly sampling.

Anaerobic storage of red cells in double-volume AS-3 resulted in acceptable *in vivo* recovery after 9 weeks of storage, 3 weeks beyond the FDA-approved limit of 6 weeks with aerobic storage. Surprisingly, anaerobic storage in the EAS61 additive, which yielded viable red cells after 9 weeks of (aerobic) storage [8], did not lead to a further extension of the acceptable storage period. This result suggests that the oxidative damage caused by denatured haemoglobin in the presence of oxygen apparently was not a major determinant of *in vivo* recovery after 9 weeks of storage so long as the metabolic status of the red cells was maintained adequately by additive solution.

Taken together, these data suggest that storing blood under oxygen-depleted conditions may have reduced membrane damage, as represented in reduced haemolysis and vesicle production, and kept ATP levels high throughout storage, but other factors became determinative after 9–10 weeks. These factors may include depletion of unknown metabolites or oxidative damage caused by residual low concentrations of oxygen combined with ~1% methHb normally present at the beginning of the storage.

The gas exchange for initial oxygen depletion and storage in anaerobic canister used in this report are impractical in routine blood banking. However, a self-contained, anaerobic

<sup>2</sup> The observation of CO-storage of red cells failing to extend their acceptable storage time beyond 6 weeks in human trials (Wolf, personal communication to M.W.B.) may be explained by the reduced [ATP] negating any benefits obtained by reducing oxidative damages.

<sup>3</sup> The effect of CO on NADH cytochrome b5 reductase system on erythrocyte is not known. However, NADH metabolism should not be affected by CO, because even if methemoglobin reduction pathway is inhibited by CO, methemoglobin production is reduced by oxygen depletion.

storage system that would function within current infrastructure can be designed. A commonly used non-toxic oxygen sorbant could be enclosed within the storage bag to remove oxygen, and an oxygen barrier film can be laminated over the storage bag to prevent re-oxygenation. Suitable materials for storage bag and oxygen-depletion have been identified and preliminary studies are currently under way [37]. These may allow benefits of anaerobic storage using routine additive solutions, such as AS-3, to be studied further and perhaps introduced into routine use.

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