

Anaerobic storage of red blood cells

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Red blood cells (RBCs) collected and stored in an additive solution deteriorate progressively throughout the 6 weeks of conventional refrigerated storage. RBCs develop “storage lesions” that include a reduction in ATP, 2,3-DPG and GSH levels, decrease of deformability, loss of membrane, lipid scrambling, formation of senescent antigens, hemolysis and reduction in post-transfusion viability. Infusion of damaged RBCs may result in less-than-expected efficacy of transfusion therapy and lead to adverse clinical outcomes.

RBCs contain high concentrations of highly reactive ferrous iron atoms sequestered in the porphyrin ring of hemoglobin, and free molecular oxygen in the cytosol and bound to hemoglobin. RBCs *in vivo* are protected by their microenvironment but become vulnerable when they are removed from the body and cooled in storage. Under such conditions, reactions between iron and oxygen cause oxidative damage to stored RBCs. Oxidative damage is a major cause of storage lesions.

One way to reduce oxidative injury to RBCs is to strip oxygen from RBCs at the onset of storage and maintain strict anaerobic conditions throughout the duration of storage. This approach has been tested successfully in several preliminary clinical studies that showed higher 24-hr post-transfusion recovery rates *in vivo* for RBCs stored anaerobically. The unique environment of anaerobic storage alleviated oxidative damage (by removing oxygen that fuels oxidative reactions) and enhanced the metabolic status of RBCs (by increasing the rate of glycolysis).

In this article, mechanisms of oxidative storage lesion and their consequences in RBCs, as well as previous attempts to reduce oxidative damage, are summarized. Results from a series of preliminary clinical studies on storage of RBCs under strictly anaerobic conditions and the current efforts on designing, fabricating and testing a blood-bank compatible, disposable anaerobic storage system are discussed.

Keywords: Erythrocyte, storage, anaerobic, transfusion, ROS

Introduction

In 2007, approximately 56 million units of blood were collected in developed countries. In these countries (~25% of the world's population), the common risks associated with blood transfusions are minimized by careful screening of donors, extensive testing of units for infectious agents and routine cross-matching. Well-developed logistics yield low wastage due to outdating of stored units.

Recent clinical studies demonstrating a negative correlation between RBC transfusions and clinical outcomes have focused a significant attention on the quality and efficacy of stored blood used in transfusion therapy¹⁻¹⁶.

In RBCs separated from whole blood and stored in a refrigerator away from the turbulent environment of circulation, the normal senescence processes are suspended. When stored RBCs are returned to circulation, a normal life-span is observed but only for cells not removed by the reticuloendothelial system of the recipient shortly after reinfusion¹⁷. After 6 weeks of storage, however, up to 25% of stored cells are removed within 24 hour of infusion due to the damage inflicted on RBCs during collection, component preparation and refrigerated storage. This damage (“storage lesion”) manifests as changes in biochemical and physical parameters of RBCs as well as in-storage hemolysis and reduced 24-hr recovery post-transfusion *in vivo*.

The clearance of damaged RBCs reduces the therapeutic efficacy of the unit, stresses the reticuloendothelial system of the recipient and adds an excess iron burden to chronically transfused patients. Moreover, transfusion of damaged RBCs is suspected as a possible cause of clinically observed complications of transfusion therapy. Current controversy surrounding the possible causal relationship between the “age” of units and negative outcomes in patients receiving transfusions^{13,15,16,18-41} stems from the fact the most of the storage-related damages increase with storage duration.

In the past, various strategies were suggested and tested to reduce the rate of development of storage lesions. These strategies can be classified into four categories: (i) manipulation of pH; (ii) supplementing metabolic precursors; (iii) manipulating osmotic balance and increasing the volume of the suspending medium; and (iv) reduction of oxidative stress by adding protective molecules. Several studies employing a combination of these strategies have reported maintenance of viability allowing an extension of storage beyond the current limit of 6 weeks⁴²⁻⁴⁷.

Our approach to improving the quality and efficacy of stored RBCs has been focused on reducing oxidative damage by removing oxygen at the beginning of storage and maintaining the anaerobic state throughout the storage period⁴⁸⁻⁵².

In this article, the mechanisms of oxidative damage, its involvement in the development of storage lesion, as well as various attempts to reduce such damage will be summarized. Emphasis is placed on reviewing results of a series of preliminary clinical studies conducted by our group to investigate the effect of anaerobic storage to reduce such damage by storing RBC under oxygen-depleted conditions.

RBC storage lesions and their effect on RBC function

Physiological function of RBCs

One of the primary functions of RBCs is to transport O₂ from the lungs to the metabolizing tissues: O₂ diffuses from the alveoli into the pulmonary capillaries, binds to Hb in RBCs and dissolves in plasma, and is then transported with the flow of blood to the microvascular networks where it is released from Hb, diffuses across the walls of capillaries, pre-capillary arterioles and post-capillary venules to the cells of tissues⁵³⁻⁵⁷. To maintain adequate perfusion of microvascular networks, RBCs must be able to continually deform at physiologically high hematocrits, under a wide range of flow conditions, in vessels ranging from 3-8µm capillaries to 50-100µm arterioles and venules. Therefore, maintaining an appropriate level of “deformability” is crucial for RBC physiological function^{58,59}.

In addition to transporting O₂, RBCs can sense the state of local tissue oxygenation and adjust the rate of O₂ delivery by regulating the local blood flow in microvascular networks⁶⁰⁻⁶² via three possible NO-mediated mechanisms: (i) release of ATP to stimulate production of NO by the endothelial cells lining the walls of the vessels⁶³⁻⁶⁸; (ii) release of NO from S-nitroso- (SNO) Hb upon deoxygenation of Hb⁶⁹⁻⁷²; and (iii) reduction of nitrite (NO²⁻) present in the blood stream to NO by deoxyhemoglobin⁷³⁻⁷⁵. Vessels dilate in response to the release of NO, thus changing their fluidic resistance and consequently the rate of blood flow.

The effect of storage on RBC functions

In 4°C liquid storage, the biochemical and mechanical properties of RBCs deteriorate progressively. These “storage lesions” affect all of the key RBC functions^{15,36,71,76}.

Loss of viability

RBCs stored for a period of time at 4°C lose viability. Some may undergo spontaneous hemolysis while in storage; others lose the ability to survive in the recipient’s circulation following transfusion. The survival of transfused RBCs is usually measured by infusing subjects with a sample of stored RBCs tagged with a radioactive label (⁵¹Cr), and then sampling the blood of the recipient periodically to determine the kinetics of RBCs clearance^{51,77-79}. A sizable portion of transfused RBCs is cleared from the circulation of the recipient in the first 24 hours after transfusion, and the number of these non-viable RBCs increases with duration of storage. Transfused RBCs that still remain in circulation after this initial period of rapid clearance will have relatively normal life-spans. Currently, the US

FDA requires that stored RBCs experience less than 1% in-storage hemolysis and that at least 75% of stored RBCs survive in the recipient 24 hours after transfusion^{80,81}.

Biochemical changes

ATP

When RBCs are stored in additive solutions, ATP concentration remains level or even increases early in storage, peaks at about 2 weeks, but then gradually declines to below 50% by week 6 of storage⁸²⁻⁸⁴. Anaerobic oxidation of glucose (i.e., glycolysis) is the only source of energy for RBCs. The initial steps of this process require ATP; it cannot continue when ATP becomes depleted. The fall of ATP levels and of the total adenylate content (ATP+ADP+AMP) is associated with poor *in vivo* survival⁸⁵. This loss of ATP may also diminish the ability of transfused RBCs to effect NO-mediated arteriole vasodilation in response to hypoxia^{64,86,87}.

2,3-DPG

The 2,3-DPG levels decline rapidly over the first week of storage, falling to undetectable levels by the end of week 2^{15,71,83,84}. Because of the loss of 2,3-DPG, stored RBCs release O₂ to the tissues less readily than 2,3-DPG-depleted cells. After transfusion, however, 2,3-DPG is rapidly re-synthesized to 50% of the normal level in as little as 7 hrs, and to 95% of the normal level in 2-3 days^{36,88-91}. This restoration of 2,3-DPG returns the Hb-O₂ dissociation curve of stored RBCs back to its normal position, and thus normalizes the amount of O₂ functionally available to tissues.

SNO-Hb

SNO-Hb is lost precipitously in the first few hours of storage, which may affect the ability of RBCs to regulate the local blood flow in response to low pO₂^{71,92}. SNO-Hb, however, restores within several hours in the body¹⁵. Also, it may be not essential for the RBC-dependent hypoxic vasodilation⁷².

Changes in mechanical properties

During storage, some RBCs change shape from deformable biconcave disks to echinocytes to spherocytocytes^{36,93}. The number of the irreversibly deformed spherocytes in the population of stored RBC gradually increases throughout the duration of storage⁹³. The significantly reduced surface area-to-volume ratio of spherocytes limits their ability to pass through the smallest capillaries⁹⁴; spherocytes are quickly culled from circulation by the body of the recipient.^{84,95}

RBCs gradually lose their “deformability” during storage – specifically, the ability to undergo folding deformations (the kind 8- μ m RBCs need to be able to do to traverse 3-7 μ m capillaries)^{83,93,96-100}, and shear deformations (the kind RBCs will experience in larger vessels, arterioles and venules)¹⁰¹⁻¹⁰⁶. Because of this storage-induced degradation of their mechanical properties, stored RBCs may be unable to maintain adequate blood flow in capillaries and deliver oxygen as effectively as their fresh counterparts. If transfused, stored RBCs may be unable to improve perfusion of the microvascular networks and oxygenation of tissues immediately after transfusion, and this will reduce the clinical efficacy of RBC transfusion^{10,107,108}.

Mechanisms of oxidative damage incurred by stored RBCs

Oxidative damage – lipid and protein oxidation / peroxidation and cross-linking caused by reactive oxygen species, such as hydroxyl, peroxy and alkoxy radicals – is one of the major factors contributing to the development of storage lesions. RBC contains highly reactive mixture of iron (in Hb) and oxygen (dissolved in the cytosol and bound to Hb) at very high concentrations. Iron atoms in Hb must be maintained at ferrous state in order to reversibly bind O₂. *In vivo*, ferrous iron in Hb is protected from oxidation within RBC, but when they are removed from the body, and stored under refrigerated conditions, the RBC’s protection mechanisms lose their efficiency and Hb becomes vulnerable to oxidation.

Hemoglobin oxidation and oxidative damage pathway

Hemoglobin and the products of its denaturation play a central role in the development of oxidative damage to stored RBCs by serving as catalysts of this process. Although the importance of denatured Hb in the context of oxidative damage during refrigerated storage was raised back in 1989 by Wolfe¹⁰⁹, relatively few studies that address this issue directly have been reported since. The involvement of Hb in the development of storage lesions has been reviewed recently¹¹⁰.

Hemoglobin contains four ferrous ions, one in each of its four subunits coordinated in the porphyrin ring. To fulfill its main physiological function, each deoxyHb molecule binds four molecules of O₂ reversibly, without exchanging electrons (**Figure 1A**). RBCs maintain a highly reducing environment in their cytosol to preserve iron in its ferrous form and utilize efficient enzymes to reverse Hb oxidation.

A small fraction of oxyHb spontaneously auto-oxidizes to form ferric methemoglobin (metHb) and a superoxide anion. In circulation, ferric metHb is reduced back to ferrous Hb by NADH-linked cytochrome b₅ reductase. In refrigerated storage, however, this reaction is retarded while the formation of metHb is enhanced for stored RBC with partially oxygenated Hb (**Figure 1B**). Once formed, metHb is inherently unstable; it denatures readily first to reversible hemichromes, then to irreversible hemichromes, and finally to globin and free heme (hemin; **Figure 1C**). The stability of metHb may be further compromised at storage temperatures because of a lower thermodynamic stability of met-myoglobin at 4°C (cold denaturation)¹¹¹.

Ferric iron in hemichromes, free heme and molecular iron released from heme can all function as a Fenton reagent in Harber-Weiss cycle fed by H₂O₂ to generate highly reactive hydroxyl radicals (**Figure 1D**). Hydroxyl radicals (•OH) attack proteins and (in the presence of oxygen) initiate lipid peroxidation cycle in RBC membrane (**Figure 1E**). Polyunsaturated fatty acids in lipids are attacked by •OH to form lipid radicals, which then form lipid hydroperoxyl radicals with oxygen, which in turn attack polyunsaturated lipid to complete the cycle. This cycle continues in presence of oxygen until two radicals react to terminate the reaction, resulting in cross-linked lipids¹¹².

Hemoglobin auto-oxidation under the conditions of partial oxygen depletion

H₂O₂ is a substrate for Harber-Weiss reaction that produces hydroxyl radicals and itself is a product of superoxide dismutase reacting on superoxide anion, a byproduct of Hb auto-oxidation (**Figure 1B and D**). Because metHb forms when oxyHb auto-oxidizes, the concentration of dissolved O₂ is a critical factor in determining the rate of metHb formation. Balagopalakrishna et al. reported that the rate of metHb formation and superoxide production reached a maximum when Hb was only partially occupied by O₂ in a hypoxic state (SO₂ ~60%) rather than fully oxygenated at a high pO₂¹¹³. In this context, the current practice of collecting and processing venous blood, and placing it in storage at a starting SO₂ of nearly 60% and then allowing the blood to oxygenate further to nearly 100% during the 6 weeks of storage as oxygen slowly traverses the plastic film of the storage bag, likely exacerbates the oxidative damage experienced by stored RBCs.

The effect of oxidative damage on stored RBCs

Obtaining unequivocal direct evidence of oxidative damage to RBCs during refrigerated storage has been difficult prior to application of highly sensitive and specific proteomics techniques^{114,115}. For example, a simple measurement of metHb levels may not accurately reflect the rate of metHb formation or the extent of oxidative reactions during storage due to the transient nature of unstable metHb. A small fraction of RBCs that were nearly senescent at the time of blood collection and damaged RBCs may cause high readings of free Hb, heme and other byproducts of oxidation. Thus, most available evidence regarding the mechanisms of Hb-mediated oxidative damage and their impact on the physiological function of RBCs were obtained from *in vitro* experiments exposing RBCs to oxidative stress^{104,116-121} or from studying RBCs from various hemoglobinopathies (such as sickle cell disease or thalassemia)¹²²⁻¹²⁵ and other conditions (such as Glucose-6-phosphate dehydrogenase deficiency or myelodysplastic syndrome)^{126,127}.

During conventional (aerobic) storage, a progressive increase in overall protein oxidation has been observed¹²⁸⁻¹³⁰. D'Amici et al. demonstrated a significant *reduction* in the rate of increase in number of spots on 2D gel of RBC proteins (an indicator of protein modification and fragmentation) in the first week of storage for anaerobically stored RBCs relative to those stored aerobically (conventionally), suggesting a reduced rate of RBC protein oxidation under anaerobic conditions. By employing standard proteomics techniques, they also identified proteins that were modified early and in an O₂-dependent manner, including band 4.2 (prevalent), band 4.1, band 3 and spectrin (minor)¹¹⁴. Messian et al. observed a loss of oxygen-dependent metabolic modulation in RBCs stored aerobically¹³¹. In addition to these physical and chemical perturbations, a decrease in glutathione (GSH) levels and in activity of GSH-dependent glutathione peroxidase were observed during storage¹³².

After Hb auto-oxidizes to metHb, it denatures to hemichromes, which then precipitate onto the lipids and the cytoskeleton of the RBC membrane. Subsequent clustering of Band 3 caused by this precipitation allows the immune system of the recipient to recognize and cull the transfused RBCs from circulation¹⁰⁹. Precipitated hemichromes also disrupt the interactions between other proteins of the cytoskeleton (spectrin, actin and band 4.1)^{109,133}. Hemichromes undergo further denaturation into heme, globin and free molecular iron, all of which readily partition into lipid bilayers. Free heme is a potent hemolytic agent; it causes RBC hemolysis by collapsing the cationic gradient¹³⁴. Heme and free molecular iron can also function as Fenton reagents to generate hydroxyl radicals, which in turn initiate lipid and protein peroxidation reactions. Peroxidation of the lipids and proteins of RBC membrane results in an increased susceptibility to cation leaks, elevated phosphatidyl serine (PS) exposure, vesiculation and hemolysis. All of these changes prime RBCs for suicidal cell death (eryptosis) in circulation¹³⁵. Excessive vesiculation due to oxidative damage leads to a disproportionately higher loss of membrane area than volume; as the surface area-to-volume ratio decreases, stored RBCs change shape, ultimately approaching a sphere. This change in the surface area-to-volume ratio and the cross-linking of membrane lipid and proteins of the cytoskeleton caused by the hydroxyl radicals contribute to the significant reduction of deformability of stored RBCs^{93,96,109,118,120,124,125}.

Prevention of oxidative damage during RBC storage

Previous attempts to reduce oxidative damage

There are two possible approaches to reducing the rate of oxidative damage during RBC storage: adding anti-oxidants to the storage solution, or removing oxidants from RBC suspension.

Reducing oxidative damage by addition of anti-oxidants or precursor molecules to augment the RBC's own anti-oxidative mechanisms is an attractive approach that has shown its effectiveness in improving RBC biochemical parameters measured *in vitro*^{116,136,137}. For example, Dumaswala et al. proposed supplementing additive storage solution with GSH precursor amino acids (glutamine, glycine and N-acetyl-L-cysteine) to prevent the gradual decline in GSH levels during storage, and to maintain a reducing environment in RBC cytosol. Although an enhancement in GSH levels and various other biochemical parameters was observed, the actual impact of these changes on *in vivo* recoveries is not yet known¹³⁸. Any modification of currently used storage solutions (especially by adding *new* ingredients), however, will require exhaustive studies of safety before gaining an approval for routine use from regulatory authorities (such as US FDA).

An alternative approach to reducing oxidative damage is to remove O₂ from RBC suspensions in the beginning and throughout storage – thus shutting down the hemoglobin denaturation pathway and arresting all oxidative reactions fueled by O₂ (**Figure 1**). This approach is feasible due to a unique feature of RBCs: unlike normal eukaryotic cells, RBCs rely solely on anaerobic glycolysis (rather than oxidative phosphorylation carried out by mitochondria) for their energy metabolism.

Högman's group reported on *in vitro* characterization of RBCs stored in a canister filled with N₂ to prevent oxygenation of venous blood during a 6-week storage¹³⁹. In that study, RBCs were prepared for storage conventionally (i.e., *not* de-oxygenated). By preventing oxygenation of Hb through the wall of the polyvinyl chloride (PVC) blood bag (which happens slowly during conventional storage), and allowing gradual equilibration of O₂ and N₂ in the canister, they observed a decrease of SO₂ from

~60% to 32% over the 6 week period, compared to an increase in SO₂ to nearly 100% for RBCs stored conventionally¹³⁹. These authors reported a higher ATP level and an increased RBC deformability, although no *in vivo* data were collected. However, since the rate of Hb auto-oxidation reaches maximum at SO₂ around 60%¹¹³, it is doubtful that the procedure used by Högman et al. is an optimal approach to reducing oxidative damage.

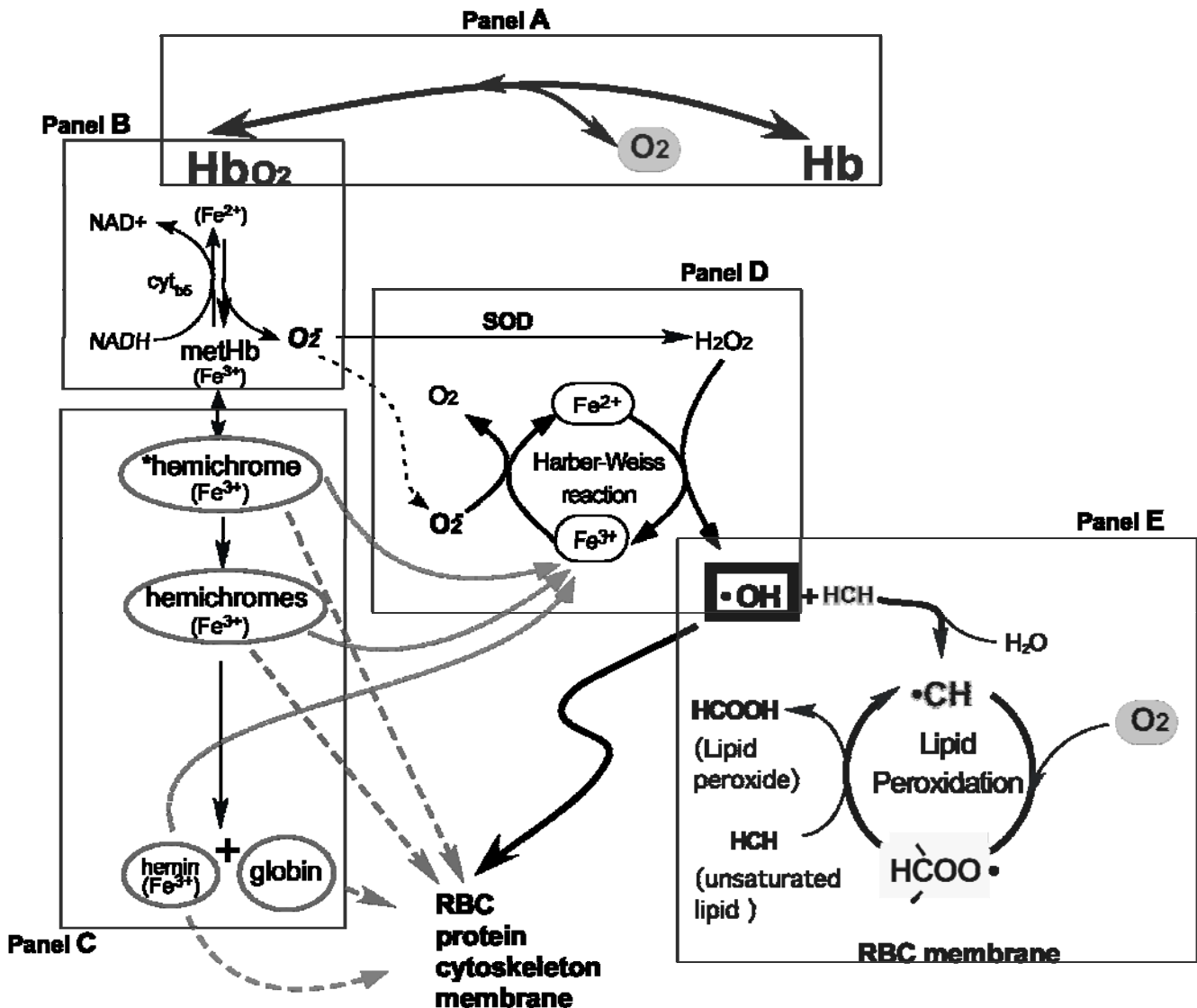


Figure 1 Hemoglobin and pathways of oxidative damage in RBC. **Panel A:** Normal function of Hb—reversible binding of O₂ to reduced (ferrous) hemes in Hb. **Panel B:** Auto-oxidation of oxyHb to methemoglobin (metHb; ferric) with production of superoxide anion. In a steady state, 1-2% of Hb exists as metHb in the circulation; metHb is readily reduced back to ferrous Hb by NADH-linked cytochrome b₅ metHb reductase. **Panel C:** Denaturation of metHb. MetHb denatures first to ‘reversible hemichromes’, in which conformational distortions are minor and can still be reversed. Reversible hemichromes further denature to ‘irreversible hemichromes’, which subsequently dissociate to globins and heme moiety. **Panel D:** Harber-Weiss reaction produces hydroxyl radicals, •OH. Superoxide anions generated in production of metHb are converted into H₂O₂ by superoxide dismutase. Hydroxyl radicals are produced with H₂O₂ and ferrous iron from denatured metHb products functioning as Fenton reagents. Ferric iron is reduced by superoxide anions. Hydroxyl radicals oxidize and cross-link RBC proteins in their vicinity. **Panel E:** Lipid peroxidation cycle. Hydroxyl radicals in the membrane attack unsaturated lipids to form lipid radicals, then combine with molecular oxygen to form lipid peroxyl radicals, which in turn attack unsaturated lipid to complete the cycle.

RBC storage under strictly anaerobic conditions

Apart from our own work⁴⁸⁻⁵¹, there has been neither systematic nor *in vivo* studies reported in the literature on storage of RBCs in which O₂ is stripped from Hb at the *beginning* of storage and maintained at that level (e.g., in an anaerobic canister) throughout the whole duration of storage.

In our efforts to reduce oxidative damage during RBC storage, we attempted to reduce SO₂ at the beginning of storage to the level as low as practical, and then maintained (and further reduced) SO₂ during the entire storage duration. This was accomplished by first equilibrating the RBC suspension with Ar gas repeatedly over a period of 1 hr prior to refrigerated storage (SO₂ was reduced to less than 3.6%), then storing RBCs in a standard blood bag inside an anaerobic canister filled with Ar and H₂ (9:1) with a palladium catalyst (to further deplete O₂ during the 6 to 9 week of storage)⁵¹.

Impact of oxygen removal on development of oxidative damage

Reducing SO₂ to a very low level early in storage may alleviate the extent of oxidative damage being accumulated by RBCs via the following possible mechanisms:

- i) Reducing the concentration of oxyHb and thus reducing the concentration of metHb produced by auto-oxidation of oxyHb (Figure 1, Panel A-B);
- ii) Preventing disruption of the membrane and cytoskeleton caused by hemichromes, hemin and globin (the products of metHb denaturation) and thus reducing hemolysis and eryptosis (Panel C);
- iii) Preventing production of free heme and iron (Fenton reagents in Harber-Weiss reaction for hydroxyl radical production);
- iv) Reducing production of superoxide anion (superoxide anion feeds the Harber-Weiss reaction in two ways: as a substrate to reduce ferric iron and as a precursor to H₂O₂, Panel D);
- v) Reducing production of hydroxyl radicals and subsequent cross-linking of cytoskeletal proteins to prevent reduction in deformability (Panel D);
- vi) Curtailing lipid peroxidation reactions (at SO₂=4%, free oxygen concentration is reduced to less than 1% of air-saturated blood at 4°C, Panel E) to reduce hemolysis and eryptosis.

Metabolic consequences of oxygen removal

An overwhelming fraction of protein contained in the cytosol of RBCs is hemoglobin. Consequently, the biochemistry of Hb determines the rates of oxidative reactions and influences the energy metabolism of RBCs, and thus Hb plays a primary role in the development of metabolic storage lesions.

Hb is a tetrameric protein that may exist in two conformations with different affinities for O₂ – the high affinity R-state (oxygenated) and the low affinity T-state (deoxygenated). In addition to O₂, Hb binds other ligands – *heterotropic effectors* – with affinities that depend on its conformation. Hb exerts control of metabolic reactions by modulating the concentration of free ligands through its own conformational transition. H⁺, 2,3-DPG and (to a lesser extent) ATP are the heterotropic effectors of Hb that directly affect the production of ATP by the glycolytic pathway and of 2,3-DPG by the Rapoport-Luebering shunt. As illustrated in **Figure 2A**, ATP and 2,3-DPG function as end-product inhibitors of phosphofructokinase (PFK) and pyruvate kinase (PK). PFK is also inhibited by low pH resulting from lactate production. In addition, Hb modulates the activity of glycolytic enzymes, namely PFK, glyceraldehyde-3-phosphate dehydrogenase, aldolase and lactate dehydrogenase by oxygen-dependent metabolic modulation¹⁴⁰. When these enzymes bind to the cytoplasmic binding domain of Band 3, their activities are inhibited¹⁴¹. DeoxyHb (Hb in T-state) competitively binds the same binding domain of Band 3, thus releasing PFK and other enzymes, disinhibiting their activity and increasing the glycolytic flux.

When O₂ dissociates from oxyHb, the following events take place: (i) Hb conformation is forced from the R- to T-state; (ii) Hb binds 2,3-DPG at a near 1:1 stoichiometric ratio because Hb's affinity for 2,3-DPG is higher in T-state; (iii) deoxyHb binds H⁺ (Bohr proton); and (iv) deoxyHb preferentially binds to the cytoplasmic binding domain of Band 3, thereby releasing phosphofructokinase (PFK) and other glycolytic enzymes (**Figure 2B**). The increased affinity of deoxyHb to 2,3-DPG and ATP reduces their cytosolic concentrations thus removing inhibition from PFK and pyruvate kinase (PK); uptake of Bohr proton increases cytosolic pH, up-regulating PFK even further. The binding of deoxyHb to the

binding domain of Band 3 and the consequent displacement of PFK and other glycolytic enzymes also up-regulates glycolytic activity^{131,140}. These changes result in an increase in glycolytic flux and activity of 1,3-DPG mutase as indicated by the elevated levels of lactate and ATP (**Table I**) as well as 2,3-DPG levels in the first two weeks of storage⁵⁰.

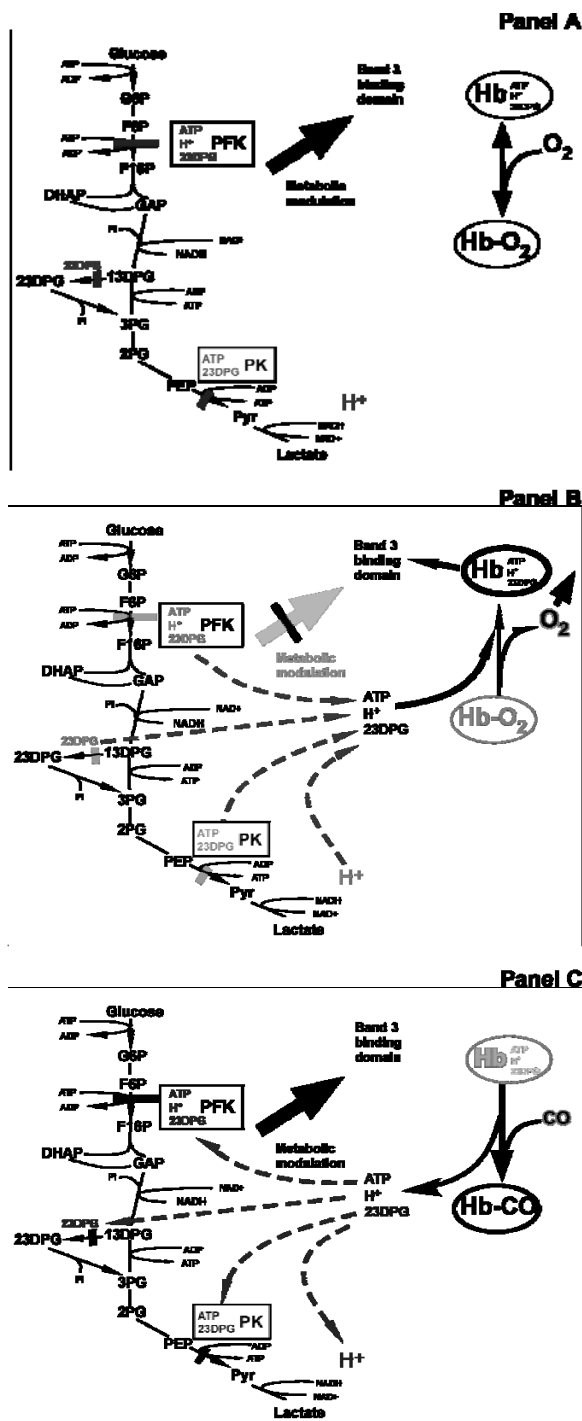


Figure 2 Oxygenation state of hemoglobin and RBC energy metabolism. **Panel A:** Hb in oxygenated state. Bars at PFK, PK and 1,3-DPG mutase steps indicate end product inhibitions by ATP, 2,3-DPG and low pH. Large arrow indicates binding of PFK and other glycolytic enzymes to cytosolic binding domain of Band 3 (and thus reducing their enzymatic activity). **Panel B:** RBC in anaerobic conditions. Hb in T-state binds 2,3-DPG, H⁺ and ATP (to a limited extent) thereby releasing the inhibitory steps (grayed bars). DeoxyHb has higher affinity toward cytoplasmic binding domain of Band 3; upon binding to Band 3, deoxyHb displaces and thus activates PFK and other metabolic enzymes. **Panel C:** When CO is used in place of Ar to deplete O₂ in gas exchange, CO binds (nearly) irreversibly to Hb, fixing it in R-state and releasing Hb from the binding domain of Band 3. This enables PFK and other enzymes to bind to Band 3, which effectively removes them from glycolytic reactions. Due to a lower affinity of R-state, Hb releases ATP, H⁺ and 2,3-DPG, elevating their free cytosolic concentrations, and inhibiting key enzymes resulting in reduction of glycolytic flux.

TABLE I - Clinical studies of anaerobically stored RBCs

Additive solution	Weeks in storage	n	SLR (%)	DLR (%)	Glucose (mg/dL)	Lactate (mmol/L)	Morphology (%)	ATP (mmol/gHb)	Hemolysis (%)	DPG (umol/gHb)	Life span (days) [#]
Study I OFAS1	8	5	79.2±3.5 (83.7-74.8)	70.6±2.4 (71.4-68.2)				4.40±0.37	0.24±0.08		
	9	4	78.5±3.9 (80.9-72.6)	71.5±2.8 (73.4-67.4)				3.76±0.51	0.29±0.08		
	10	5	74.3±4.0 (79.9-69.8)	67.0±3.38 (71.2-63.3)				4.1±0.45	0.17±0.06		
	11	6	66.3±6.5 (72-53.8)	57.3±6.1 (61.9-45.5)				3.24±0.68	0.31±0.11		
Study II AS-3	0	8			822±50	1.2±0.4		3.96±0.74	0.03±0.01	12.5±2.5	
	8	6	82.1±5.8 (92.3-76.4)	83.1±5.4 (92.6-78.0)	633±22	21.3±1.5		3.71±0.85	0.24±0.1	0.3±0.05	
	9	7	72.9±8 (79.9-56)	75.8±5.6(82.5-68.6)	632±26	22.7±1.9		3.17±0.54	0.34±0.09	0.45±0.1	
	10	6	67.3±2.1 69-64.9)	65.6±5.6(72-60.9)	595±42	24±1.4		2.84±0.52	0.43±0.15	0.37±0.1	
Study III EAS-61	0 C	8			1541±118	0.91±0.34	100.0±0.1	4.09±0.32	0.04±0.03		
	0	8			1537±69	1.03±0.28	99.9±0.1	3.92±0.55	0.05±0.02		
	9 C	8	73.5±5.5 (80.8-66.3)	77.4±5.4 (82.1-66.4)	1092±25	31.7±4.2	70.7±9.3*	2.17±0.69	0.31±0.11		
	9	8	74.2±5.8 (80.7-66.8)	74.0±4.7 (83.1-69.4)	1048±32	34.7±1.6	65.1±6.7*	2.35±0.38	0.33±0.07		
	10	4	71.3±10.2 (81.6-58.5)	68.0±10.9 (79.6-54.5)	1055±57	31.8±3.8	64.4±4.8	2.09±0.19	0.42±0.09		
Study IV OFAS3 with PIPA supplement additions	0 C	8			1317±80	1.0±0.4	100	3.52±0.24	0.05±0.03 (0.09-0.01)	8.50±1.12	
	0	8			1308±75	1.1±0.3	99.7±0.6	3.61±0.40	0.08±0.03 (0.11-0.04)	9.14±0.97	
	10 C	8	69.7±9.5 (78.5-54.8)	68.1±10.0 (79.7-49.7)	1014±665	26.6±2.5	60.8±13.3	2.12±0.49	0.59±0.30 (1.2-0.2)	0.88±0.13	
	10	8	73.0±10.1 (90.3-57.3)	77.3±12.5 (96.0-56.7)	887±34	37.7±10.1	80.6±15.0	5.00±0.65	0.35±0.11 (0.7-0.2)	9.32±1.04	
	12	4 [®]	70.7±3.1 (74.3-66.9)	72.2±5.4 (78.4-67.1)	755±67	42.4±2.0	82.1±7.1	5.03±0.61	0.47±0.13 (0.9-0.3)	9.72±0.71	
	14	6 [®]	69.7±9.1 (79.6-58.3)	70.0±9.4 (79.6-57.1)	790±22	47.9±1.5	77.4±9.0	4.41±0.68	0.61±0.16 (1.2-0.4)	11.61±0.95	
16	2	72.4±3.8 (75.1,69.7)	76.7±3.5 (79.2, 74.2)	735±21	55.0 ±0		3.56±1.07	1.15±0.9 (1.8-0.5)	10.8±1.4		

Additive solution	Weeks in storage	n	SLR (%)	DLR (%)	Glucose (mg/dL)	Lactate (mmol/L)	Morphology (%)	ATP (mmol/gHb)	Hemolysis (%)	DPG (umol/gHb)	Life span (days) [#]
Study V OFAS3											
	0 C	8			721 ± 19 (748-695)	3 ± 4 (13-1)	100 ± 1 (100-99)	3.59 ± 0.91 (4.46-1.67)	0.06 ± 0.03 (0.10-0.03)		
	0	8			1209 ± 76 (1307-1096)	2 ± 0 (2-1)	98 ± 4 (100-87)	3.48 ± 0.86 (4.17-1.50)	0.06 ± 0.03 (0.13-0.03)		
	6 C	8	77.8 ± 5.4 (83.5-67.3)	75.1 ± 7.7 (83.5-62.0)	547 ± 37 (594-490)	22 ± 6 (29-14)	51 ± 18 (73-24)	2.93 ± 0.80 (4.28-1.95)	0.4 ± 0.2 (0.7-0.2)		84 ± 16 (112-58)
	6	8	82.3 ± 4.6 (87.5-71.9)	83.0 ± 5.0 [†] (87.7-72.2)	920 ± 39 (971-864)	25 ± 4 (30-21)	63 ± 17 (87-43)	3.76 ± 0.62 (4.55-2.98)	0.3 ± 0.2 (0.5-0.2)		82 ± 12 (97-67)
	9	8	73.5 ± 8.5 (83.5-61.0)	72.6 ± 10.5 [‡] (85.7-54.1)	900 ± 34 (962-853)	31 ± 2 (34-29)	54 ± 15 (75-25)	2.71 ± 0.69 (3.75-1.86)	0.8 ± 0.7 (2.1-0.2)		86 ± 7 (98-74)

[†] 'C' with bold letters— aerobic control; SLR—single ⁵¹Cr label; DLR—double label: ⁵¹Cr and ^{99m}Tc.
 Data are reported as mean ± SD (range)
 * PIPA supplements were added at 7th and 11th week without warming the units.
 # No difference in study arms, p=0.73
 @ Out of 6 subjects with 75% or more at 10 weeks of storage, 4 were tested at week 12 and week 14; 2 subjects with highest values at week 10 were tested at week 14 and week 16.

Clinical studies with anaerobically stored RBC

In the past 12 years, we conducted five clinical studies with anaerobically stored RBCs to assess the utility of anaerobic storage using an *in vivo* metric of quality of stored RBCs – autologous 24-hr recovery of ⁵¹Cr-labeled RBCs in human subjects. Studies III-V (described below) were designed as dual-arm trials in which RBCs from the same subject was stored following conventional aerobic procedures (Control) and under anaerobic conditions (Test). We tested several experimental additive solutions as well as AS-3 (Pall Corp., Port Washington, NY, USA) in combination with the anaerobic conditions in these studies, the initial goal of which was to achieve the longest possible storage shelf-life of RBC units (**Table II**).

In all five studies, SO₂ of Hb was reduced to below 4% by an experimental gas exchange protocol⁵¹. The gas exchange was conducted by transferring packed RBCs into 1 L transfer bags, filling the bags sterilely with Ar, gently agitating the bags for 10 min, and finally expressing Ar/O₂. After repeating the gas exchange six times, the RBC suspension was transferred to the original PVC storage bag, and placed in an anaerobic canister filled with 90% Ar / 10% H₂; a palladium catalyst was included in the canister to prevent O₂ re-entry and to further deplete O₂ during storage. The results of these studies are summarized below.

	Study			
(mM)	I	II	III	IV*, V
	OFAS1	AS-3	EAS61	OFAS3
Adenine	2	2.2	2	2
Dextrose	110	61	110	110
Mannitol	65		55	55
NaCl		70	26	26
Na citrate	20	20		
Citric acid		2		
Na ₂ HPO ₄			12	12
NaH ₂ PO ₄	20	20		
pH	7.2	5.8	8.3	6.5
mL added	250	200	200	200
* In Study IV, PIPA supplement was also added at 7 th and 11 th week of storage for Test (anaerobic) units. Rejuvesol (0.55g sodium pyruvate, 1.34g inosine, 0.034g adenine, 0.5g dibasic sodium phosphate monohydrate, ph6.7-7.4; 50mL per addition, EnCyte Systems, MA).				

Metabolic and biochemical parameters

Under conventional (aerobic) storage conditions, ATP concentration peaks at 10-20% above starting level around 3 weeks of storage and then gradually declines. Under anaerobic conditions, depending on the additive used, ATP peaks at higher levels (50-70% above the initial concentration), and this phase of ATP boost is sustained for a longer period (5-7 weeks). After the level of ATP reaches its peak, the rate of decline is similar for aerobic and anaerobic storage. Because ATP peaks later and at a higher level, however, ATP levels of anaerobic RBCs remain above the fresh level even after 6 weeks of storage.

In anaerobic storage, 2,3-DPG levels are sustained significantly longer than in conventional storage: 2,3-DPG increases to over 100% of the initial level within the first week and then declines to below the initial concentration only in week 3. The levels of hemolysis are similar for the first 6 weeks for both types of storage, but then diverge significantly beyond 8 weeks when rate of hemolysis of aerobically stored RBCs begins to increase. PS exposure increases slowly in the first 6 weeks in both aerobic and anaerobic conditions; at week 6, aerobic RBCs show an exponential increase in PS exposure, while for

anaerobic RBCs the rate of increase is more gradual and the exponential phase is delayed for about 3 weeks.

Prior to initiating Study IV, we had examined the effect of pH of the experimental additive solutions on *in vitro* parameters (ATP, 2,3DPG and hemolysis) and found that good results could be obtained by acidifying an alkaline experimental additive solution (EAS61⁴⁶). In Study IV, in addition to using this adjusted additive with low pH (OFAS3), we added metabolic precursors (PIPA: phosphate, inosine, pyruvate, adenine; Rejuvesol, EnCyte System Inc., Braintree, MA, USA) to the storage solution to ensure adequate supply of nutrients during a storage period of 12 weeks and beyond. In this study, we demonstrated that⁵⁰:

- i) PIPA supplementation at 4°C (as opposed to incubation at 37°C) boosted ATP and 2,3-DPG levels within one week.
- ii) PIPA addition reversed the scrambling of membrane lipid by allowing the translocation of previously exposed PS back to the inner leaflet of the membrane.
- iii) Addition of PIPA to the blood bag two times during anaerobic storage maintained ATP and 2,3-DPG above the levels of fresh RBCs and suppressed PS exposure for more than 17 weeks.

24-hr recovery of RBCs stored anaerobically

Study I

Our first study utilized a hypotonic experimental additive solution (250 mL, 125 mOsm/L effective, **Table II**). In this small, pilot trial 24-hr recovery of more than 75% were measured for RBCs stored in this additive solution anaerobically for 8 (n=6) and 9 weeks (n=4). This study provided the first evidence of the utility of anaerobic storage⁴⁸.

Study II

Our second study examined an existing additive solution, AS-3, in a serial protocol in which one unit collected from a donor was tested for 24-hr recovery three times consecutively, at weeks 8, 9 and 10 of anaerobic storage. A double volume of AS3 additive was used to prevent the possible exhaustion of glucose under the conditions of glycolytic flux elevated for a prolonged period of time in extended storage. The results indicated that anaerobic storage is compatible with the existing additive solution for up to 9 weeks⁵¹.

Study III

Neither additive solutions currently in use, nor the experimental additives that demonstrate extended storage times^{43-46,142}, specifically aim to reduce oxidative damage. We hypothesized that anaerobic storage conditions will be synergistic with advanced additives to further prolong the shelf life of units by reducing the rate of development of storage lesions due to oxidative damage in addition to the direct effect of anaerobic conditions on metabolic status of RBCs (**Figure 2**). We tested this hypothesis in Study III for an advanced experimental additive solution with high pH (8.4) which showed success in a 9-week storage under the conventional aerobic conditions⁴⁶.

To avoid the large subject-to-subject variability inherent in studies with a small number of subjects, this study was also designed as a dual-arm trial in which RBCs from the same subject were collected on two separate occasions, stored under aerobic and anaerobic conditions in a random order, and tested to isolate the effect from anaerobic storage conditions on storage duration.

Unexpectedly, our results showed that no further gain was achieved when anaerobic storage was combined with the alkaline additive solution. We also found that subjects whose RBCs stored better in one arm had comparable results in the other arm of this study. Two plausible explanations for the observed lack of synergy are that: (i) the cytosol of RBCs became *too* basic for optimal function because of over-alkalinization produced by the gas exchange (since both O₂ as well as CO₂ are removed in this process) and due to the activity of carbonic anhydrase; and (ii) factors other than oxidative damage (e.g., metabolic deficiency) limited post-transfusion survival of RBCs⁵¹.

Study IV

Based on *in vitro* experiments completed after Study III, we conducted another dual-arm study, this time using an *acidic* experimental additive (OFAS3). In the Control arm, RBCs were stored with OFAS3 additive in aerobic conditions. In the Test arm, RBCs were stored anaerobically with OFAS3, and PIPA supplementation was added (without warming the units) at weeks 7 and 11 of storage. We measured 24-hr *in vivo* recovery for both Test and Control arms after 10 weeks of storage. Units in the Test arm were re-examined after 12 weeks and, depending on the observed recovery, again at the 14th or 16th week of storage (**Table I**)[§]. For the Test arm (anaerobic storage), an acceptable recovery rate was measured at week 12 for all units[&], and two units with good recovery at week 14 also showed adequate recovery at week 16.

This study demonstrated that when anaerobic storage was combined with PIPA supplement additions, RBCs stored for 12 weeks showed acceptable survival *in vivo* and maintained high 2,3-DPG levels such that full oxygen delivery capacity would be available immediately after transfusion (and not only 8-24 hrs later when transfused RBCs stored conventionally recover their function *in vivo*)⁵⁰.

Study V

In this dual-arm, 8-subject study we stored leukoreduced RBCs anaerobically in OFAS3 additive solution (Test arm) and aerobically in AS-3 additive solution (Control arm). We measured the 24-hr recovery at 6 weeks (Test and Control) and at 9 weeks (Test only); we also determined the rate of long-term survival of transfused RBCs after each of these three infusions.

The results of this study demonstrated that 6-week Control and 9-week Test units were equivalent in terms of 24-hr recovery, morphology scores, and ATP levels. Two subjects with relatively high hemolysis after 6 weeks (Control) showed higher than 1% hemolysis after 9 weeks (Test). The long-term survival rate was indistinguishable between all three infusions suggesting that for those RBCs that survive the initial stress related to infusion and/or scrutiny by reticuloendothelial system of the recipient, a normal life-span is expected regardless of the storage methods used⁴⁹.

Summary of *in vivo* studies

The following conclusions can be drawn from the results of these five clinical studies:

- Anaerobic conditions could extend the storage time by more than 50% while maintaining acceptable viability of stored RBCs *in vivo*.
- Anaerobic storage conditions in combination with an acidic additive solution had a synergistic beneficial effect on stored RBCs; a combination with an *alkaline* additive conferred no additional benefit.
- ATP and 2,3-DPG could be maintained at high levels throughout the extended duration of anaerobic storage by adding PIPA supplement two times during storage without warming the units.
- Maintaining high ATP levels throughout storage did not guarantee high 24-hr recovery post-transfusion.
- The level of PS exposure at the time of re-infusion did not correlate with the rate of RBC survival *in vivo*.
- The significant reduction in oxidative damage combined with metabolite precursor supplements could not extend storage beyond 12 weeks for most units. Therefore, other factors – such as accumulation of toxic products or cold denaturation of RBC proteins – may be damaging RBCs in storage and reducing their viability *in vivo*.
- No difference was observed in the long-term survival rates between RBCs stored in aerobic and anaerobic conditions once the cells survived the first 24 hr *in vivo*.

[§] Two were dropped from the study after 10 weeks because of lower than 75% recovery for the Test arm.

[&] Out of 6 subjects with 75% recovery or more at 10 weeks of storage, 4 were tested at week 12 and week 14; 2 subjects with highest values at week 10 were tested at week 14 and week 16 (both subjects scored higher than 75% at week 14).

Effects of CO₂ depletion during gas exchange

The process of gas exchange used in these studies was effective in removing O₂ as well as dissolved CO₂. Depletion of CO₂ may have caused alkalosis in RBC cytosol, which may have contributed to the observed increase in glycolytic flux yielding high levels of ATP^{51,143}. To rule out this possibility, we used CO (instead of Ar) for gas exchange in order to force Hb into the R-state (COHb), while still alkalinizing RBC by CO₂ removal. In these experiments, gas exchange with CO depleted O₂ and CO₂ (as with Ar in all other anaerobic experiments), but the ATP levels were *suppressed* (instead of enhanced) relative to the aerobic controls⁵¹.

This observation can be explained by CO binding to Hb: because the affinity of Hb to CO is 200 times higher than for O₂, Hb was locked into R-state and thus released 2,3-DPG into cytosol. This process also freed the binding domain of Band 3 for PFK and other glycolytic enzymes to bind (**Figure 2C**). Since RBCs contain neither mitochondria nor metabolic enzymes to be inhibited by CO, the reduced ATP levels can only be attributed to a reduced glycolytic flux¹⁴⁴. On the other hand, the Hb-CO complex is extremely stable and once formed it prevents any auto-oxidation from taking place and causing oxidative damage. These results showed that even if cytosolic alkalization caused by CO₂ removal played a role in elevating the level of ATP, the contribution was insignificant in comparison to the effect on glycolytic flux from the conformational transition of Hb.

Practical implementation of anaerobic storage

In all clinical studies described above, the initial depletion of O₂ was carried out manually using an experimental protocol of gas equilibration in a large transfer bag. Deoxygenated RBCs were then stored in an anaerobic canister to prevent re-oxygenation. We are now designing and fabricating a prototype blood collection / storage system to replace this manual experimental protocol. Our goal is to make this new anaerobic storage system: (i) self-contained; (ii) disposable; (iii) inexpensive; (iv) easy to gain approval under current blood banking regulations; (v) to require no or minimal additional infrastructure; and (vi) to require minimal departure from the current operational modality of blood banks.

The prototype system consists of a device for the initial O₂ depletion and a multi-layered anaerobic storage bag impermeable to O₂. Initially, the system will be made compatible with packed RBC units prepared by the currently available blood collection sets. Anaerobic RBCs will be prepared by sterilely connecting a unit of packed leukoreduced RBCs to the O₂-depletion device, passing the RBCs through the device to deplete O₂, and collecting the deoxygenated RBCs into the anaerobic storage bag. Future versions will include a complete blood collection set incorporating the anaerobic system with additive solution, blood component bags and a leukoreduction filter. The O₂-depletion device will resemble a dialysis cartridge commonly seen in renal dialysis instruments. In place of the flowing dialysate, O₂ sorbent granules will be packed around the hollow fibers to scavenge O₂ from the suspension of RBCs passing inside the fibers. Deoxygenated RBCs will be placed in a conventional PVC blood storage bag – this bag will be either laminated on both sides to make it impermeable to O₂, or will be contained within an O₂-barrier bag containing a sachet with an O₂ sorbent to compensate for possible leakage of O₂ and to further deplete O₂ during storage.

Utility of anaerobic storage

The use of RBC units with a higher proportion of viable cells at the time of transfusion would benefit chronically transfused patients directly: transfusing fewer non-viable RBCs reduces iron overload and may increase the clinical efficacy of transfusion.

The number of RBC units discarded due to outdating in the developed countries is low. In the US, outdate rates are usually <2%, and most of this is due to A⁺ and AB blood types. Furthermore, in 2007, the average age of transfused units was 19.5 days in the US nationally¹⁴⁵, 21±11 days for ICU patients⁷ and 20±11 days for trauma patients, only 18% of whom received RBC units stored for longer than 40 days¹⁴⁶. Thus, there is little urgency to extend the shelf-life of RBC units in general transfusion therapy (with a possible exception of the military setting¹⁴⁷). There is little doubt, however, that RBCs are damaged during refrigerated storage and that this damage increases progressively with the duration

of storage. There is a growing (yet controversial) concern regarding the negative consequences of transfusing “old” RBCs¹⁶. Strategies to reduce the rate of development of storage lesions are particularly timely and should warrant rigorous pursuit. Extending shelf-life of stored RBCs is one way of reaching this goal. The use of RBCs with a shelf life extended by 50% under current normal practices would effectively amount to the use of relatively ‘fresher’ blood even though the “nominal age” would be the same.

Currently, we expect anaerobic storage to benefit cardiac surgery and critical care patients as numerous recent reports have suggested an association between the age of transfused units and poor clinical outcomes in these groups of patients (for a recent comprehensive review see Lelubre et al¹⁶). More general conclusions must await results of two ongoing large clinical trials of the efficacy of RBC transfusion^{**} aimed to determine a correlation between the age of RBC units and patient outcomes. The potential utility of anaerobically stored RBCs would not be limited to specific groups of patients and could be made universally available if an inexpensive anaerobic storage system could be developed and incorporated into the current blood banking operation without causing a major disruption to the normal procedures.

Conclusions

In the past, oxidative damage to RBCs has been studied extensively in the context of patients suffering from hereditary hemoglobinopathies. The mechanisms and the impact of oxidative reactions on the physiological function of RBCs are now well understood. Because of its abundance and high reactivity, hemoglobin plays a central role in promoting the oxidation in RBC with oxygen (present at a relatively high concentration in the cytosol of the cell) acting as a fuel in this process. Despite multiple reports implicating or hypothesizing oxidative damage mediated by denatured Hb as the major driving force in the development of storage lesions^{109,148}, until recently, little effort has been focused on reducing oxidative damage directly. Moreover, a mere handful of reports exists unequivocally demonstrating evidence of specific oxidative damage in the early stages of RBC storage.

An attractive way to reduce oxidative damage during refrigerated storage is to eliminate for the entire duration of storage a critical substrate of oxidative reactions – oxygen. In a series of pilot clinical studies, we have tested this hypothesis by stripping oxygen away from hemoglobin at the onset of storage and maintaining the anaerobic conditions throughout storage. Our 24-hr recovery data suggest that anaerobic conditions combined with existing additive solutions can reduce the rate of development of storage lesions and extend the refrigerated storage of RBCs by 50% or more. On the other hand, anaerobic storage reduces the rate of storage lesion development during storage and therefore has the potential for improving the functional quality of RBCs stored for any given duration. In addition to these 24-hr *in vivo* recovery data, a recent comprehensive proteomic study of the early development of oxidative damage to membrane proteins and the cytoskeleton of RBCs showed a significant reduction of oxidative damage in anaerobic conditions¹¹⁴.

At this time, there are no definitive data available to correlate the extent and/or type of oxidative damage and the viability and efficacy of stored RBCs *in vivo*. It appears prudent, however, to look for strategies for combating the development of oxidative damage during storage. In pursuing this goal, storing RBC in anaerobic conditions offers an attractive alternative to the additions of anti-oxidant chemicals to the additive storage solution primarily because anaerobic storage completely avoids the safety concerns associated with infusing these chemicals into the recipient during transfusion. A larger clinical study that will examine the correlations between the extent and nature of oxidative damage and the 24-hr *in vivo* recovery of stored RBCs is currently underway. If a causal relationship can be established, RBCs preserved in a user-friendly self-contained anaerobic blood collection / storage will provide more viable, “fresher” RBC to all patients receiving transfusions.

^{**} Red Cell Storage Duration and Outcomes in Cardiac Surgery, n=2800; and Red Cell Storage Duration Study (RECESS), n=1612 (<http://clinicaltrials.gov>).

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