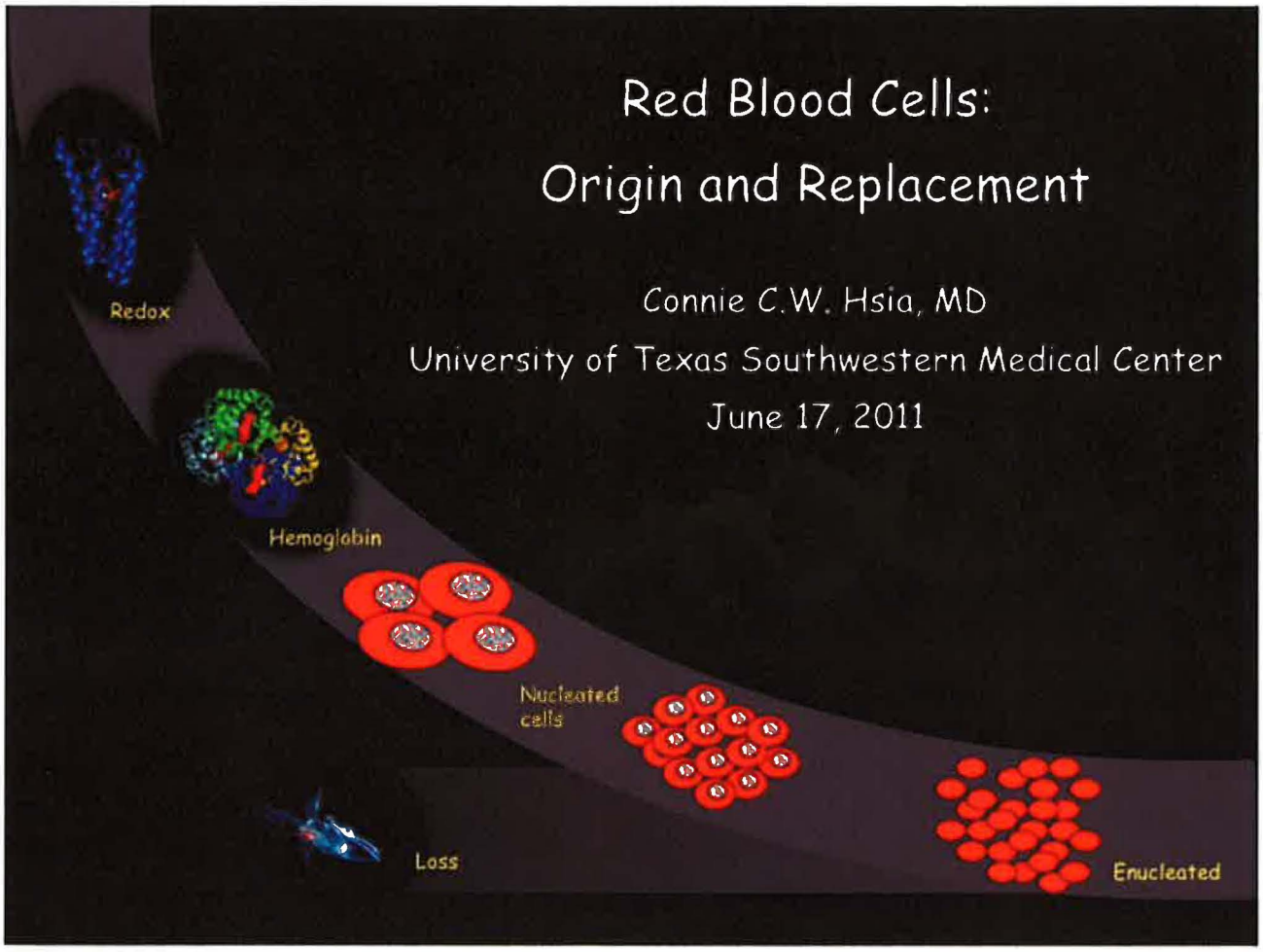
Department of Internal Medicine

Grand Rounds



This is to acknowledge that Connie Hsia, M.D. has no financial interests or other relationships with commercial concerns related directly or indirectly to this program. Dr. Hsia will not be discussing off-label uses in her presentation.

Connie C.W. Hsia, M.D. is Professor of Internal Medicine in the Division of Pulmonary and Critical Care. Her research interests concern the mechanisms of dysfunction and adaptation in pulmonary gas exchange, oxygen transport and compensatory lung growth. Her clinical interests center on patients with sarcoidosis.

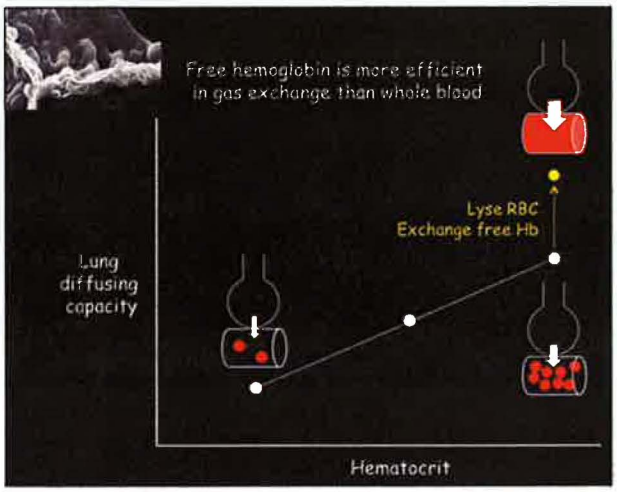
Learning Objectives

At the end of the presentation, the participants should be able to describe:

1. The nature of hemoglobin toxicity
2. How hemoglobin came to be sequestered in red blood cells
3. The regulatory functions of red blood cells
4. New advances in red cell substitute and replacement therapeutics.

Observation: Free circulating hemoglobin is more efficient in gas exchange than whole blood

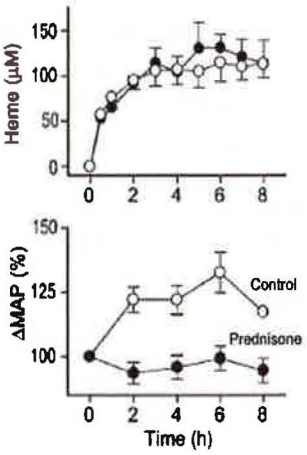
The red blood cells (RBCs) are a respiratory organ. They carry hemoglobin (Hb) for oxygen binding in the lung and release in the periphery. At a given work intensity, increasing Hb concentration increases oxygen transport. Hemolysis or isovolemic exchange transfusion of free Hb solution to replace whole blood further augments oxygen uptake and release at a given Hb concentration (1). This is because the RBC membrane and plasma add resistance to gas diffusion during the short transit time through capillaries. Therefore, Hb solution is more efficient than whole blood for capillary gas exchange.



Q: If so, why is hemoglobin sequestered within red blood cells at the expense of gas exchange efficiency?

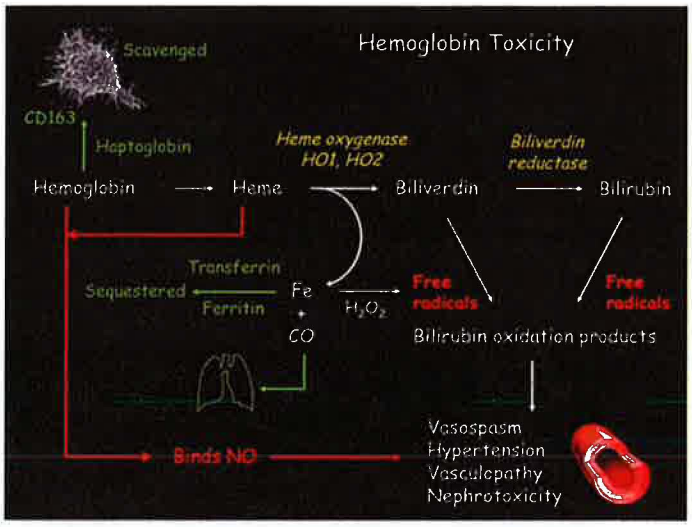
A: Free hemoglobin is toxic

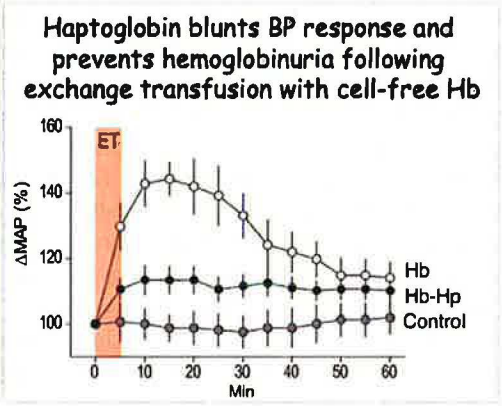
1. Hemoglobin scavenges NO to induce vasospasm. Hb itself and the oxidation of Hb also contribute to vasospasm and vascular hypertension. Hb and its breakdown products release free radicals and pro-inflammatory mediators (2, 3). Infusion of free Hb solution increases mean arterial blood pressure (ΔMAP); the effect is prevented by prednisone pre-treatment (4) *(Figure at right)*.



2. Circulating heme can release "free" redox-active iron, which catalytically amplifies the production of reactive oxygen species (ROS). These oxidants oxidize lipids, proteins and DNA; they activate cell signaling pathways and pro-inflammatory transcription factors, alter protein expression, perturb membrane channels and induce apoptosis and cell death. Heme-derived oxidants recruit leukocytes, platelets, and RBCs to the vessel wall, oxidize low-density lipoproteins and consume NO (3).

3. Hemoglobin breakdown products cause vasculopathy (2). Heme is metabolized to biliverdin by heme oxygenase (HO)-1 and -2. Constitutive HO-2 is expressed in neurons and vascular cells; inducible HO-1 is expressed in marcrophages and other cells. Biliverdin is metabolized to bilirubin via biliverdin reductase. Free radicals act on bilirubin, biliverdin, and possibly heme to produce bilirubin oxidation products (BOXes). Extracellular free radicals also act on heme to produce BOXes. Iron released by heme degradation binds ferritin intracellularly or transferrin extracellularly. Any free iron can interact with H_2O_2 to produce hydroxyl free radicals and oxidize bilirubin, biliverdin or heme. Inhibiting HO or biliverdin reductase decreases production of bilirubin and BOXes. The BOXes diffuse into vascular smooth muscle cells to produce chronic vasospasm and vasculopathy.



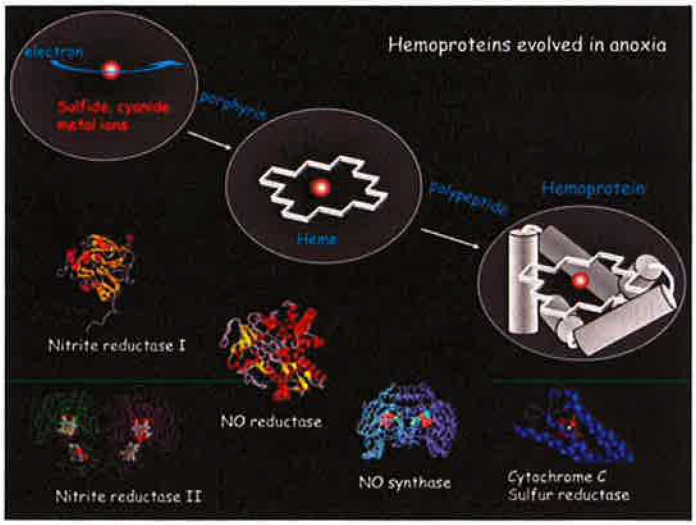


To minimize toxicity, free Hb is efficiently and irreversibly conjugated to the abundant plasma protein, haptoglobin (Hp) to form a stable high-molecular-weight complex that is cleared by the macrophage scavenger receptor, CD163. Binding to Hp prevents extravascular Hb oxidative processes and oxidative tissue damage. The Hb-Hp complex is one of the strongest protein-protein interactions, indicating that the system evolved under high evolutionary pressure (4, 5).

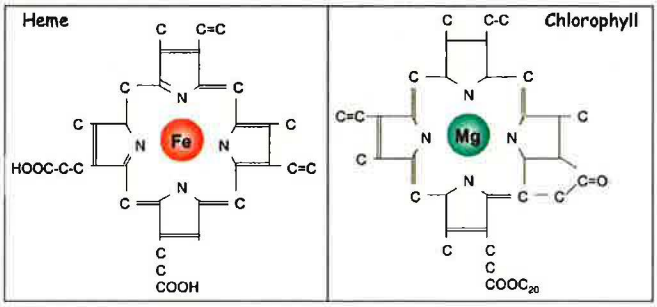
Q: How could hemoglobin, so essential to respiration, be so toxic?

A: Hemoglobin did not evolve originally to carry oxygen but was coerced into that role.

Anaerobic origin of hemoglobin



The earliest prokaryote, termed the Last Universal Common Ancestor (LUCA), is thought to have evolved ~3.8 billion years ago (BYA) in a nearly anoxic atmosphere (6, 7). These earliest organisms were probably similar to the extant methanococci. They derived energy from inorganic material, ingesting hydrogen sulfide, hydrogen cyanide and metal ions (Fe, Mg, Cu) capable of transferring electrons. In time a molecular cage, the porphyrin ring, known as the "*ring of life*", evolved to trap redox ions. Eventually these rings became associated with polypeptides to form metalloproteins capable of modifying the specific functions of the rings by altering the 3D conformation of the protein complexes and making the metal ion binding sites more or less exposed. Porphyrin rings form the backbone of both heme and chlorophyll, two of the most important molecules in biology (8). The

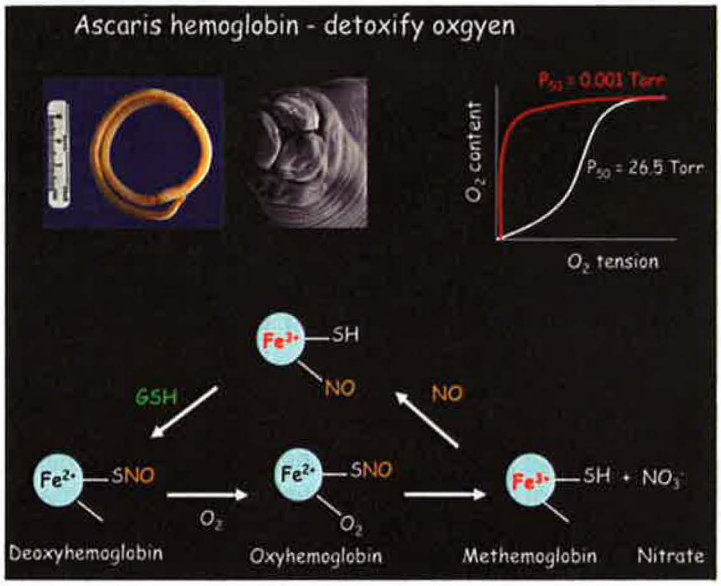


earliest hemoproteins were cytochromes that served widespread non-oxygen related functions, e.g., as nitrite reductase, NO reductase, NO synthase, and sulfur reductase. Remnants of ancient hemoglobins that produce energy from nitrogen- or sulfur-based respiraton persist to this day, e.g., in the deep sea tubeworm and other extremophiles living near the hydrothermal vents (9), and anaerobic parasites such as *Ascaris Lumbricoides* (below).

The ancestral cyanobacteria (blue-green algae) migrated from the deep ocean to the surface exposed to sunlight, eventually acquiring chlorophyll to begin photosynthesis (10). As a result, atmospheric oxygen concentration gradually rose over ~2 billion years, reaching the present day level ~500 million years ago (MYA). Selection pressure progressively mounted for the anaerobes to detoxify and eliminate oxygen, hide under deep sea or soil, or become extinct.

Co-opting hemoglobin for detoxification of oxygen

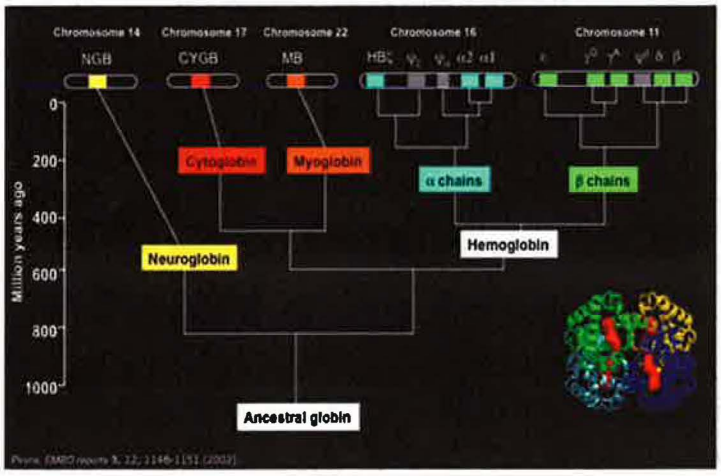
The roundworm *Ascaris lumbricoides* infects ~1 billion people. Their eggs, once ingested, hatch into a larval worm that penetrates the duodenal wall to enter the blood where it is carried to the liver, heart and pulmonary circulation. The larva breaks free into the alveoli where it grows, molts, and is eventually coughed up, then swallowed back into the gastrointestinal tract. In the second transit the parasites mature into adult worms in the anoxic small intestine (PO_2~10 Torr), reaching up to 30 cm in length, produce more eggs and perpetuate the cycle. *Ascaris* eggs can persist in soil for years, being resistant to chemicals, desiccation and cold temperatures.



The Hb of *A. lumbricoides* has an exceedingly high oxygen binding affinity (P_{50} ~0.001 Torr), >26,000 times that of human Hb (26.5 Torr). *Ascaris* Hb binds oxygen and does not release it. The worm uses endogenous NO to oxidize oxyhemoglobin (Fe^{+2}) to methemoglobin (Fe^{+3}) while converting oxygen to nitrate (NO_3^-), which is safely excreted. Methemoglobin is reduced back to deoxyhemoglobin in a glutathione (GSH)-mediated reaction. Thus, *Ascaris* Hb acts as deoxygenase to detoxify oxygen (11).

Co-opting hemoglobin again for oxygen transport

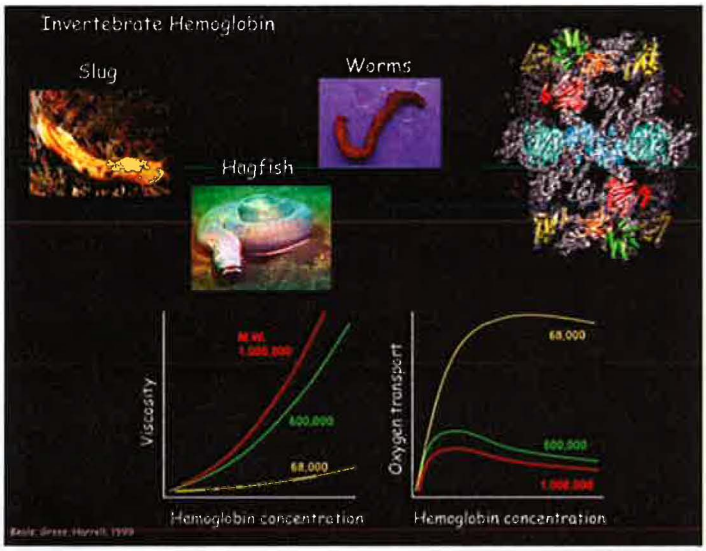
Eventually, the ability of oxygen to accept electrons was exploited by early organisms to produce ATP more efficiently than anaerobic metabolism, giving rise to a wide variety of intracellular and extracellular oxygen transport



pigments. From a common ancestral globin gene, Hb diverged from neuroglobin ~800 MYA and from cytoglobin and myoglobin about 400 MYA. The α- and β- globin chains progressively diverged until their genes are carried on separate chromosomes (12). All intracellular globins are oxygen-sensitive.

Origin of red blood cells

Most invertebrates possess cell-free circulating Hb or an equivalent respiratory pigment. The small Hb molecules are rapidly eliminated by excretory filters. To retain circulatory Hb, the invertebrate Hb forms giant molecules, some with over 100 subunits (13) *(Figure at right)*. Large Hb molecules are cumbersome, with a high blood viscosity and a low oxygen-carrying capacity at a given concentration, thereby restricting the organisms to low speeds of locomotion. Their primitive blood cavities do not require vasoregulation. As more active organisms evolved with higher oxygen requirements, selection pressures increased for smaller Hb molecules that have a lower viscosity, higher oxygen carrying capacity and longer circulatory retention. The primitive Hb-producing lining cell of the hemolymph cavity (precursor of the endothelial cell) that periodically sloughed off into the circulating blood fulfilled these requirements and eventually became early dedicated RBCs, eventually to be mass produced in pre-splenic tissue, spleen, liver, kidney and finally bone marrow.

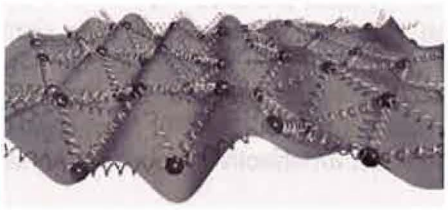


The shrinking red blood cell

All vertebrates possess RBCs. Average RBC size decreases ~50 fold with increasing metabolic demand, from amphibians to reptiles, birds and mammals. The salamander has the largest (~100μm) and the deer mice the smallest (~2μm) RBCs. Fish, amphibians and reptiles have large spherical or oval nucleated RBCs. Birds have smaller nucleated RBCs. Mammals have the smallest RBCs devoid of nuclei or organelles. In 2003 a skeleton of a 68 million year old *Tyrannosaurus Rex* was discovered in Montana. Its bone fragments contained preserved soft tissue as well as blood vessels containing nucleated RBCs (14).

RBC diameter is ~30% larger than capillary diameter and must deform during capillary transit (15). As RBC size decreases across species there is a corresponding exponential increase in capillary-length-to-tissue-volume ratio and a decrease in average capillary-to-tissue diffusion distance. A smaller vascular diameter increases flow resistance, so a higher blood pressure is needed to maintain tissue perfusion. The need for high vascular pressure in turn drives the evolution of heart chambers. At the same time the pulmonary blood-gas diffusion interface becomes larger and thinner, which requires a low pressure to avoid fluid build-up or mechanical damage. These divergent selection pressures are thought to drive the separation of systemic and pulmonary circulations, which began with the amphibians. Thus, RBCs evolved in close interaction with the cardiopulmonary and vascular systems.

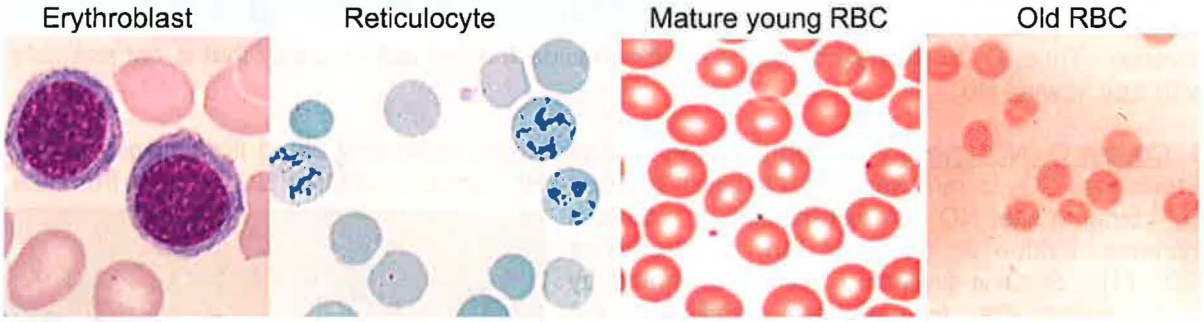
Flexible cytoskeleton

RBC cytoskeleton is made of spectrin molecules attached to the inside of the cell membrane in a mesh network. Mechanical energy (from squeezing or shearing) or chemical energy (ATP) break the bonds between spectrin molecules or between spectrin and actin embedded in the cell membrane, allowing holes to open in the cytoskeleton and the cell to become fluidic. *Left panel*: Electron micrograph of RBC cytoskeleton (16). *Right panel*: RBC cytoskeleton modeled as a network of springs (spectrin) and anchors (junction complex) (17). Any pathology that alters the cytoskeleton (e.g., malaria infection, sickle cell disease) reduces RBC deformability.

Leaner is meaner

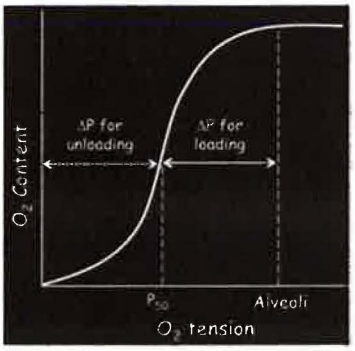
The rigid nucleus restricts deformation and occupies space that could be used to pack more Hb. Once the cell is committed to O_2 transport, the nucleus and organelles are extruded from the erythroblast via formation of a contractile actin ring on the plasma membrane (18). Reticulocytes contain remnant ribosomal material, which is soon lost. Enucleation improves O_2 carrying capacity of the red cell. Mature RBCs package their waste products into membrane vesicles, which are spit out. Aging RBCs lose membrane and cytoplasm to become progressively smaller in size and membrane surface charge, leading their degradation in the reticuloendothelial system.



Not just a bag of hemoglobin

Far from being a passive oxygen carrier, RBCs serve multiple regulatory functions:

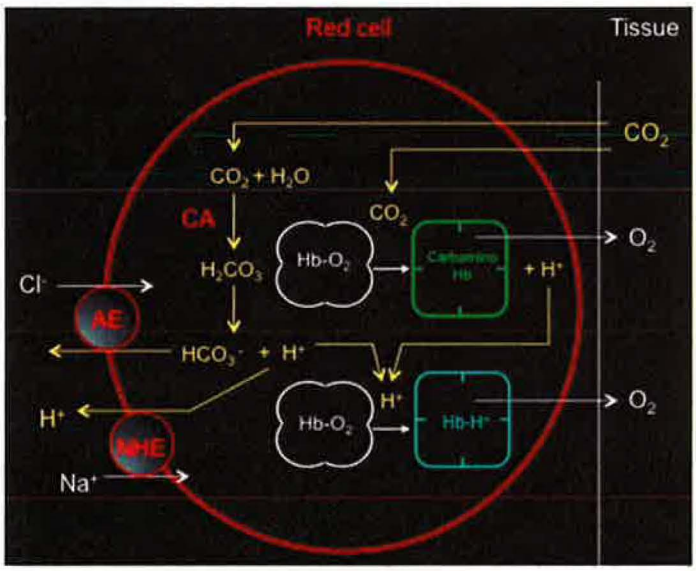
1. Retain Hb molecules within the circulation without toxicity. Free Hb has a half-life of several hours. Human RBCs circulate on average for 120 days.



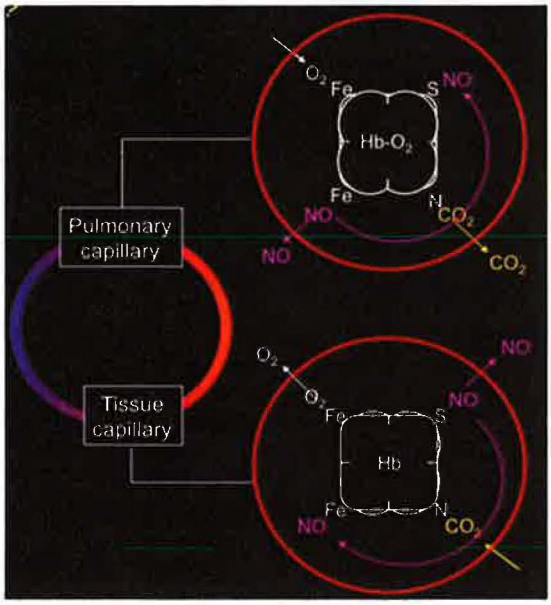
2. Act as a metabolic sensor (19). Oxygen transport by Hb is regulated by its binding affinity (P_{50} of oxyhemoglobin dissociation curve). At any given mean alveolar oxygen tension (PA_{O2}) the pressure gradient (ΔP) from alveoli to blood drives oxygen loading onto Hb. The ΔP from blood to tissue mitochondria drives oxygen unloading from Hb. Shifting the P_{50} alters the balance between loading and unloading of oxygen.

RBC metabolism of depends on glycolysis, producing 2,3-bisphosphoglycerate (BPG) as an intermediate product. 2,3-BPG binds deoxy-Hb in a pocket between $\alpha 1$-$\beta 2$ chains to stabilize the deoxy- (Tense) molecular conformation, thereby increasing P_{50} and improving oxygen unloading in the periphery. In this way the oxygen-carrying function of RBC is intrinsically coupled to its metabolism. An RBC under stress (e.g., during tissue hypoxia or acidosis) produces more 2,3-BPG from glycolysis, thereby releasing more oxygen to the tissue, making the RBC an effective metabolic sensor of cellular oxygenation state.

3. Couple O_2-CO_2 transport to maintain acid-base balance. Tissue CO_2 diffuses into RBC and is converted by carbonic anhydrase (CA) to bicarbonate (HCO_3^-) and proton (H^+). Proton binds to a histidine residue on globin to stabilize the deoxy-Hb conformation- thereby releasing O_2 to the tissue. CO_2 also binds directly to oxy-Hb to form carbamino-Hb and cause O_2 release. Bicarbonate is shuttled out of the cell via the membrane transporter anion exchanger (AE)-1. Any excess H+ ions are shuttled out of the cell via another membrane transporter, sodium-proton exchanger (NHE)-1. In pulmonary capillaries these reactions run in reverse. Thus, O_2 to CO_2 exchange is coupled inside the red cell in a way that is not possible with free flowing Hb.



4. Couple O_2-NO transport to regulate regional vasomotor tone and blood flow. In pulmonary capillaries, CO_2 is released and O_2 binds to deoxy-Hb, which facilitates NO release from the Fe^{+2} binding site. NO can exit the cell or bind to a cysteine residue on globin to form S-nitrosothiol (20, 21). SNO is protected from scavenging by Fe^{+2} binding site. In peripheral capillaries, the opposite interactions occur. As CO_2 from tissue loads onto Hb, O_2 unloads and facilitates the release of NO from the SNO binding site. The released NO can be scavenged by Fe^{+2} or can exit the red cell to induce local vasodilation. Via these allosteric interactions, regional perfusion is coupled to regional O_2 delivery in a way that would not be possible had blood contained only free Hb.



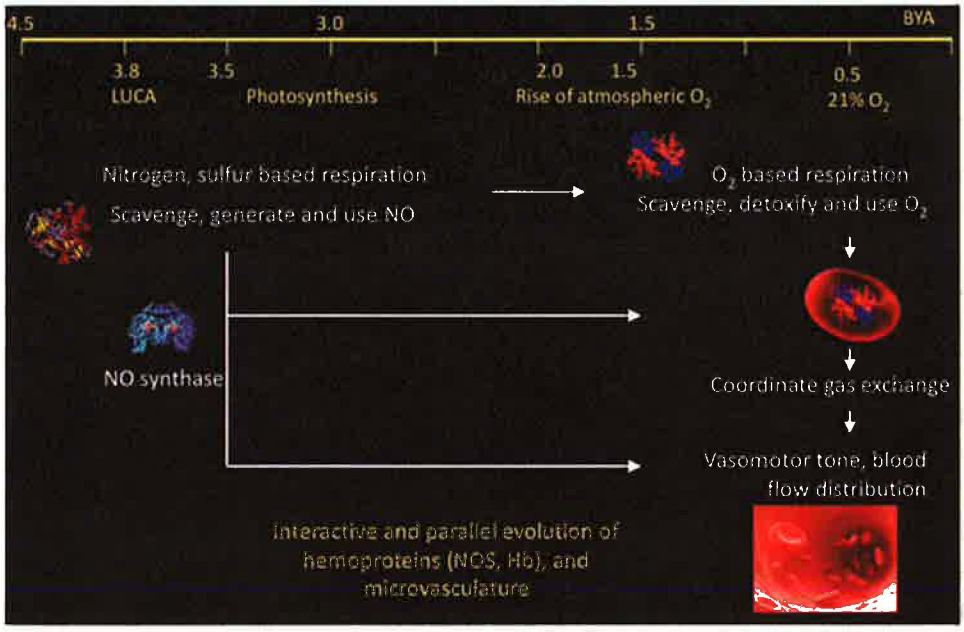
Under shear stress, RBCs can synthesize NO using L-arginine as substrate, just like endothelial cells (22, 23). Glycolytic ATP from RBCs is also exported to stimulate production of NO and other vasodilators by endothelium, thereby actively regulate local blood flow to match O_2 demand (24).

5. Modulate circadian rhythm. RBCs exhibit distinct 24-hour cyclic oscillations in their content of certain antioxidant proteins (e.g., peroxiredoxins) and could modulate circadian fluctuations in various metabolic pathways (25).

6. Immunosurveillance. During infection by hemolytic bacteria, free Hb that is released can be directly activated by microbial proteases, leading to production of reactive oxygen species that in turn kill the pathogen. This is a fundamental and universal antimicrobial strategy (26).

Origin of hemoglobin toxicity

Hemoproteins evolved only once, then repeatedly adapted their structure and function in response to changing environmental as well as organismal needs, a process known as "*exaptation*". Early hemoproteins participated in anaerobic respiration that involved scavenge, detoxification and eventually cell signaling based of reactive nitrogen species including NO. Some of these hemoproteins came to scavenge, detoxify and eventually deliver oxygen, but retained their ancestral NO-related functions. At the same time NO evolved into a key mediator of vasodilation, setting up a fundamental conflict and the selection pressure for Hb to be sequestered in dedicated cells for protection and for fine regulation of gas exchange as well as vasomotor tone. Along each step, Hb/RBCs evolved in close interaction with the pulmonary and microvascular systems to sustain progressively higher levels of oxygen demands. The interactive + parallel evolution of oxygen and NO homeostasis is a classical biological trade-off responsible for both the optimization of gas exchange and the potent toxicity of cell-free Hb.

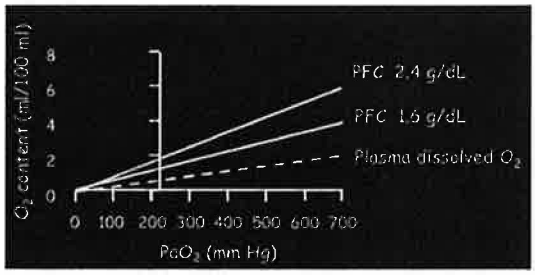


Implications for blood substitutes and replacement

Substitute oxygen carriers
Perfluorocarbon based
Hemoglobin based

Red blood cell replacement
Synthetic RBCs
RBCs derived from stems cells

Perfluorocarbon (PFC)-based blood substitutes



PFCs are chemically inert, thermally stable, colorless, with a high density and molecular weight, and low viscosity, and surface tension. They vaporize at body temperature. Because of a low solubility they are produced as emulsions, nanoparticles (200nm) that can be given intravenously or instilled into the lungs. PFCs dissolve 50 times more oxygen than plasma (but carry much less oxygen than hemoglobin); therefore large quantities must be administered frequently.

A 1st generation PFC product was FDA approved in the early 1990's for IV use in high risk angioplasty (27) for the acute relief of tissue hypoxia, but it was a commercial failure. The quantities of drug required are prohibitive. No improvement was shown in oxygenation over a colloidal solution. FDA approval was later rescinded.

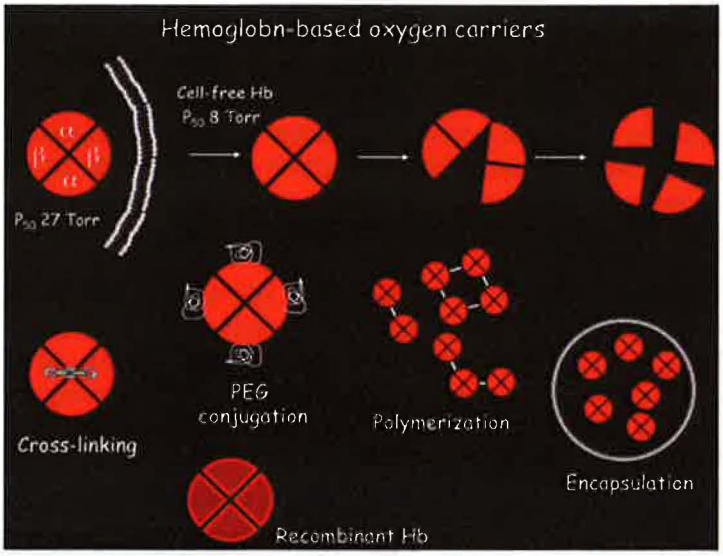
Reasons for class failure		
Short plasma half life (3-4 hr)	→	Requires frequent dosing, large quantity
Long reticuloendothelial retention (~1 wk)	→	Reduces platelet count Essentially single dose drug
Do not transport CO_2	→	Hypercapnia
Enhancement of O_2 delivery depends on high alveolar PO_2	→	ICU use Not for high altitude or severe lung disease
In vitro cytostatic effects	→	Proliferation, mitochondrial activity, surfactant synthesis
Environmental issue	→	Greenhouse gas, lifetime 50,000 years
Alternative uses	→	Contrast agent Chemotherapy

A 2nd generation product (perflubron emulsion), tested in a European Phase III trial, was found to reduce the need for blood transfusions during non-cardiac surgery (28). A subsequent U.S. Phase III trial in cardiac surgery patients was suspended in 2001 due to excessive stroke events in the control group. It was determined that the problem was related to the trial's design. Issues of protocol compliance also arose and the trial was terminated without reaching a conclusion.

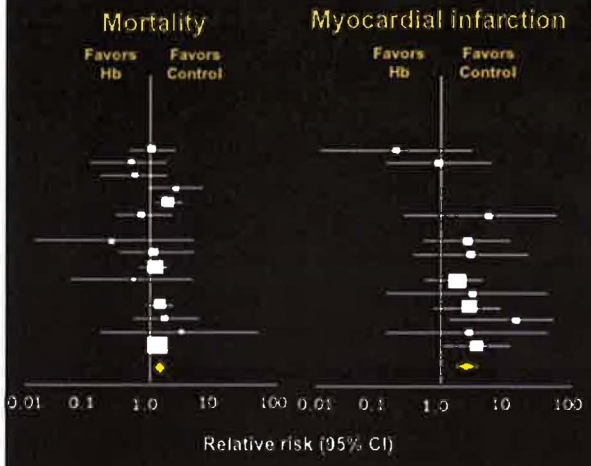
Hemoglobin-based oxygen carriers (HBOCs)

Early transfusion of native Hb preparations caused hypertension and renal failure because cell-free Hb dimerize from its tetrameric conformation; the dimers are filtered by the kidney. Subsequent efforts used chemical and recombinant techniques to intramolecularly cross-link, polymerize or conjugate Hb to increase its molecular size and retard renal clearance. Others tried to encapsulate Hb to mitigate vascular side effects (29, 30).

Numerous HBOC products went on to phase II/III trials. However, reports of serious cardiovascular adverse events accumulated (30, 31), see figure below. Presently, no clinical trials of HBOC products are being conducted in the U.S. Some products are being evaluated outside of the U.S. and marketed in South Africa.



16 phase II/III studies
1996 – 2007
3,711 patients



Adverse events		
Biopure (Hemopure®)	Bovine Hb, GA polymerized	Death, HTN, MI, CVA, CI, TIA, AKI
Northfield (Polyheme®)	Human Hb Pyridoxal-5'-P cross-linked and GA polymerized	Death, HTN, MI, CVA, CI, TIA, AKI
Hemosol (Hemolink®)	Human Hb, open chain raffinose cross-linked and polymerized	Death, HTN, MI, CVA, CI, TIA, AKI
Baxter (HemAssist®)	Human Hb, bis-3,5-dibromosalicicyl fumarate cross-linked	Death, HTN, MI, CVA, CI, TIA, AKI
Apex Bioscience (PHP®)	Human Hb, pyridoxal-5'-P cross-linked and polyoxy-ethylene conjugated	Death, HTN, MI, CVA, CI, TIA, AKI
Sangart (Hemospan®)	Human Hb, maleimide-polyethylene glycol conjugated	Death, HTN, MI, CVA, CI, TIA, AKI

Sources of adverse effects from HBOCs (30)

- Depletion of NO by binding to oxy- and deoxy-Hb
- Physical endothelial stress caused by colloidal osmotic pressure or viscosity of HBOC
- Induction of vasoactive factors (angiotension, endothelin, prostaglandins)
- Complex peroxidative reactions between ferrous (Fe^{2+}) and ferric (Fe^{3+}) Hb with hydrogen peroxide or NO that create reactive oxygen and nitrogen species such as superoxide ($O_2^{\cdot -}$), nitrate (NO_3^-) and peroxynitrite ($ONOO^-$)
- Focal to diffuse myocardial degeneration. Lesions are more likely to develop in the presence of existing cardiovascular disease.

Potential approaches towards safer HBOCs that are yet to be explored:

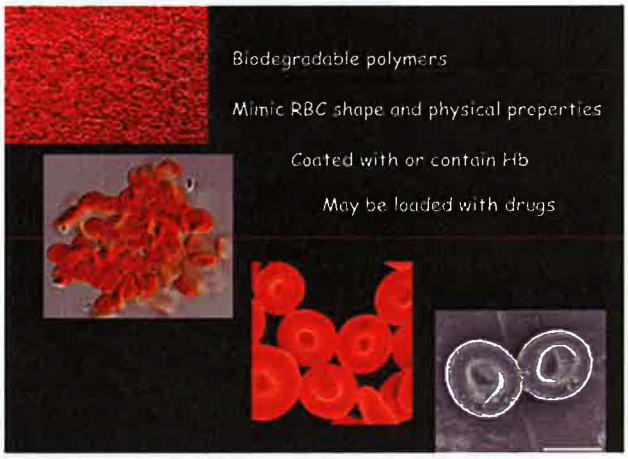
1. Minimize tissue pro-inflammatory responses to intravascular HBOCs, e.g., downregulate the Hb-CD163-hemoxygenase axis.
2. Hemoglobin retains its ancient function as a nitrite reductase, capable of converting nitrite (NO_2^-) into NO as it deoxygenates. Nitrite infusion may promote NO generation from Hb while maintaining oxygen delivery; this effect could be harnessed to treat hemolysis and to detoxify Hb-based blood substitutes.(32)

3. Conjugate HBOC to haptoglobin: The irreversible Hb:Hp complexes do not alter heme reactivity, do not extravasate and can theoretically protect organs such as the kidney from heme exposure. During oxidant exposure, Hb:Hp complex protects structural integrity of Hb by preventing amino acid oxidation, heme release, globin crosslinking **and** lipid peroxidation. The Hb:Hp complex could be a high-molecular weight, oxidatively protected, non-hypertensive and intravascularly retained Hb.
4. Conjugate HBOC to synthetic scavenger molecules – under development.

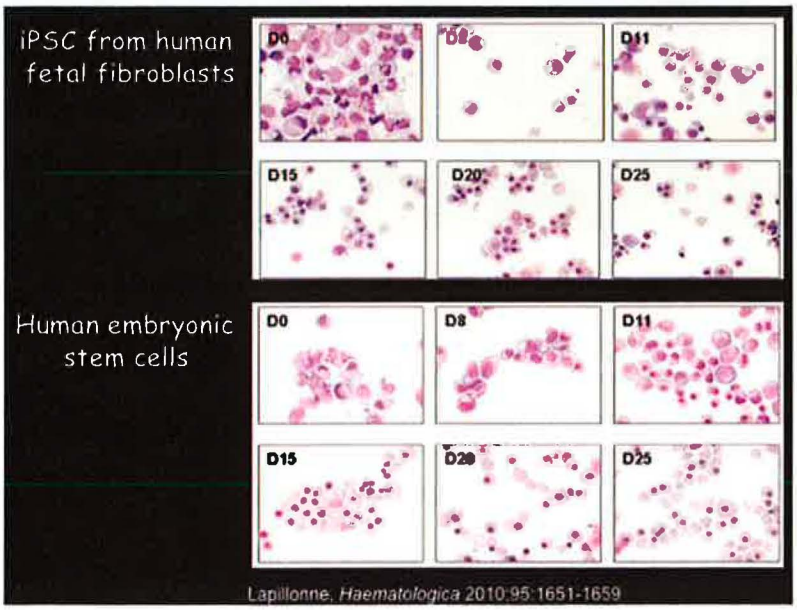
Blood replacement options

Synthetic red blood cells:

Not ready for prime time

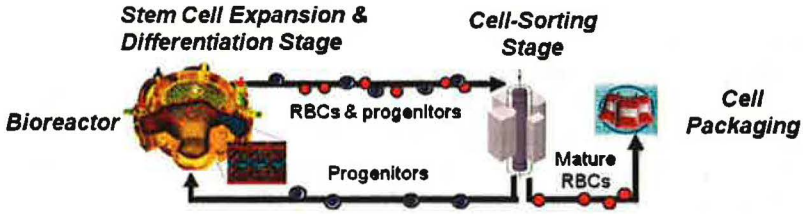


Pharming red blood cells from stem cells



Both human fetal embryonic stem cells (hESCs) and human inducible pleuripotent stem cells (hiPSCs) can be coaxed to differentiate into mature RBCs that exhibit a normal P_{50} and responds to 2,3-BPG (33, 34). The differentiation pathway has been simplified to a few intermediate cell types under controlled culture conditions.

From hESCs, investigators were able to grow blood types A, B, O, and both Rh D positive and Rh D negative, but none of the hESC lines approved in the USA contain the O-negative gene – the "universal" donor blood type (33). Using hiPSCs to grow Rh D negative blood can obviate this problem.



Closed loop operation. Expansion & differentiation in parallel in same bioreactor.

Development is underway in the U.S. and Europe for bioreactors that mimic bone marrow conditions suitable for large-scale production of universal donor type RBCs from hiPSCs. Ideally, the blood would be untainted and free of storage defects, providing an unlimited and safe blood supply. The Defense Advanced Research Program Agency (DARPA) has a Blood Pharming program to develop an automated, portable cell culture and packaging system for producing transfusable amounts of universal donor RBCs on the battlefield.

Theoretically, there should be no difference between native RBCs and those grown from hiPSCs. In practice, many challenges remain: The half-life, immunogenicity, and *in vivo* transport and regulatory functions of pharmed blood cells are unknown. These cells have yet to be tested *in vivo*. Any residual undifferentiated cells must be eliminated to avoid the risk of malignancy. The scale of production needed is immense. One unit of packed RBCs contains ~2 trillion cells. Production using existing technology is in billions of cells and at a prohibitive cost. Major bioengineering innovation as well as testing in animals will be needed before these products are ready for testing in humans. Nonetheless, red cell pharming is currently the most promising long-term approach for the repletion of a nearly optimal product of evolutionary compromise.

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