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Invited Review

Hemoglobin-based Blood Substitutes and the Hazards of Blood Radicals*

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Cell-free hemoglobins, chemically altered or genetically expressed in microbial host systems, have been developed as oxygen-carrying therapeutics. Site-directed modifications are introduced and serve to stabilize the protein molecules in a tetrameric and/or a polymeric functional form. Animal studies, as well as recent clinical studies, have suggested these proteins probably deliver oxygen to tissues. However, concerns still persist regarding the interference of hemoglobin and its oxidation products with the vascular redox balance, potentially impeding its clinical usefulness. This article reviews our current understanding of heme-mediated toxicities and some of the emerging protective strategies used to overcome hemoglobin side reactions.

Keywords: Hemoglobin, blood substitutes, free radicals

INTRODUCTION

The development of blood substitutes for a variety of clinical applications has progressed rapidly in recent years. The potential benefits of a blood substitute include universal compatibility, immediate availability, freedom from disease transmission, and long term storage.[1] The proposed indications for an oxygen-carrying blood substitute are primarily emergency resuscitation of trauma patients and preoperative hemodilution during surgical procedures. Other likely applications of clinical benefit to patients include the use of these products in treatment of ischemic diseases, tumor sensitization, and the treatment of sickle cell anemia.[1] Several oxygen-carrying products have advanced to Phase II/III clinical trails in the United States. The starting material is stroma-free hemoglobin (SFH) or chromatographically purified hemoglobin A_0, obtained from sources including outdated human or bovine blood. In some cases the protein is fully expressed in a bacterial host system. Various

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TABLE I Common chemical and non-chemical approaches used in developing hemoglobin-based blood substitutes

Classes of modifications	Sites of modifications	Developers
Intramolecular cross-linkage		
2 nor-2-formylpyridoxal 5-phosphate	Val(1)β_1-Lys(82)β_2	NFPLP (Dutch Red Cross)[a]
bis(3,4-dibromosaliacyl)fumarate	Lys(99)α_1-Lys(99)α_2	DCLHb (Baxter);[a] DBBF (US Army)[a]
Intramolecular and intermolecular cross-linkage		
Glutaraldehyde	Surface/multisites	Hemopure (Biopure)
Polyaldehyde (o-raffinose)	DPG pocket and surface/multisites	Hemolink (Hemosol)
Polyethylene glycol (PEG)	Surface/multisites	PEG-Hb (Enzon)
Pyridoxal phosphate and polyoxyethylene (POE)	Val(1)β_1-Lys(82)β_2 and surface/multisites	PHP (Apex)
Pyridoxal phosphate and glutaraldehyde	Val(1)β_1-Lys(82)β_2 and surface/multisites	Polyheme (Northfield)
Site-directed mutagenesis		
Glycine bridge	Cross-bridging of 2-α chains in Presbyterian mutant Hb	Optro (Somatogen)[b]
Encapsulation		
Phospholipids	Envelops SFH or DBBF	US Navy

[a]Programs have terminated.
[b]Baxter acquired Somatogen and is currently developing second generation recombinant hemoglobins.

chemical and/or genetic alterations have been employed by industry to produce a stable and functional blood substitute including intra-tetrameric cross-linked hemoglobin; polymers of hemoglobin tetramers, (intra- and inter-tetrameric cross-linked), hemoglobin tetramers conjugated to non-protein macromolecules or genetically stabilized tetramers. Hemoglobin molecules have, in some cases been encapsulated within phospholipid liposomes (Table I) (for review see [1]). A major problem with hemoglobin-based products has been the adverse reactions encountered in some ongoing clinical trials. The basis of toxicity is poorly understood as most research carried out by industry is proprietary, and only a minimal free exchange of information among investigators occurs. A recent well publicized clinical trial in the United States for traumatic hemorrhagic shock was terminated early by Baxter Healthcare Corporation because of a significantly increased mortality in the patient group treated with diaspirin cross-linked hemoglobin (DCLHb) relative to the control group.[2] This has intensified the search for safe and effective blood substitutes and provided new impetus for researchers to gain a better understanding of molecular basis of hemoglobin toxicity.

TABLE II Summary of pre-clinical and clinical experiences with hemoglobin-based blood substitutes

Pre-clinical
Nitric oxide inactivation leading to vasoconstriction and hypertension
Macrophages activation leading to cytokine release, vasculitis and thrombosis
Platelet and red cell aggregation
Rapid oxidation to non-oxygen carrying methemoglobin
Cellular damage markers of free radical injury
Enhancement of endotoxin effects
Clinical
Vasoconstriction and hypertension
Gastrointestinal distress
Excessive mortality in patients with acute ischemic stroke and in patients with traumatic hemorrhagic shock

Pre-Clinical Experience with Hemoglobin Solutions

Pre-clinical experience with these modified hemoglobins, based largely on studies carried out in a variety of animal model systems, is now available in the literature (Table II). A side effect of

most hemoglobin-based blood substitutes is constriction of the peripheral vessels, which increases blood pressure by up to 35–40%. Vasoconstriction is a phenomenon that is still largely unexplained, however, one widely accepted mechanism is the removal of nitric oxide (NO), a natural "vasodilator", by cell-free hemoglobin.[3] Extracellular hemoglobin modified chemically and stabilized in the tetrameric or polymeric forms, unlike the red cell, can reach NO production sites, in the microvasculature where this reaction is believed to initially occur. Inflammatory responses to the infusion of a variety of hemoglobin solutions have been reported. The observation of transient hemorrhagic lesions with the infusion of glutaraldehyde-polymerized bovine hemoglobin (polyHb) that was characterized by "small vessel vasculitis" led to the recognition that endothelial cell injury plays a central role in the mechanism of toxicity of hemoglobin solutions.[4] Recent studies on the hemostatic and hematological effects of cell-free hemoglobin showed that pyridoxylated hemoglobin polyoxyethylene (PHP) produced thrombocytopemia and bleeding by promoting aggregation of platelets and caused loss of sequestration of red cells due to continued bleeding and clumping of cells which greatly compromised the survival of dogs with severe hemorragic shock.[5] The rapid oxidation (autoxidation) of hemoglobin to non-functional methemoglobin is an important concern in the use of hemoglobin as an oxygen-carrying product. The uncontrolled spontaneous oxidation of ferrous iron compromises the efficacy and in some instances the safety of the infused hemoglobin-blood substitute. A case in point is the published report on the limitation of efficacy due to autoxidation of bovine glutaraldehyde-polymerized hemoglobin in an ovine model of exchange transfusion.[6] The circulating levels of methemoglobin were increased from 3% to 40% in the first 24-hour of infusion with hemoglobin. The question of how much methemoglobin is too much was recently addressed in a rat model of 30% exchange transfusion of conjugated hemoglobin with polyethylene glycol (PEG–Hb).[7] It was estimated that metHb levels greater that 10% can significantly decrease the ability of this hemoglobin to oxygenate tissues. No direct, but considerable circumstantial evidence suggests that the production and interaction of potentially harmful free radicals can result from the infusion of cell-free hemoglobin preparations. Pancreatic and liver enzymes in a number of animal models of exchange transfusion were reported to be elevated, suggesting possibly a free-radical mediated injury. Increased levels of amylase and lipase after hemoglobin infusion in animal models have been frequently observed.[8] Plasma creatine phosphokinase (CK) and lactate dehydrogenase (LDH) activities, indicators of tissue injury, were reported to be unusually high in a severe hemorrhage swine model resuscitated with diaspirin cross-linked hemoglobin (DBBF).[9] Interestingly, the pattern and magnitude of lactate, CK and LDH elevations seen in these studies were similar to those reported in humans in a recent clinical trial infused with DCLHb, the commercial analog of DBBF.[10] Direct implication of hemoglobin mediated free radical injury was recently reported in neuronal cell cultures. Hemoglobin killed neurons while leaving glial cells intact; however addition of antioxidants and iron chelators were protective against hemoglobin mediated cytotoxicity.[11]

Endotoxin, a bacterial product, and hemoglobin have been shown to exert synergistic toxicity when hemoglobin is given in a clinically relevant dose as an oxygen-transporting resuscitation system.[12] This is quite worrisome since these products are given in some instances to trauma victims with contaminating wounds.[12] Biochemically, hemoglobin has been shown to act as a binding protein for bacterial endotoxin (lipopolysaccharide, LPS) and that both the structure and biological activity of LPS is altered in the presence of hemoglobin.[13] *In vitro* studies

showed that LPS induces oxidation of chemically modified hemoglobin (DBBF).[14]

Clinical Experience with Hemoglobin Solutions

Hemoglobin-induced hypertension remains a prominent obstacle to clinical application of hemoglobin-based blood substitutes. Although the mechanism of this hypertensive effect is not yet fully understood, most investigations have focused on the interaction between hemoglobin and NO.[3] NO is believed to be the endothelial relaxing factor (EDRF), induces blood vessel dilation and reduces blood pressure by relaxing vascular smooth muscle. Removal of NO by hemoglobin, as it extravasates the endothelium lining of blood vessels, results in an unopposed vasoconstriction of blood vessels and hypertension. In human trials with current generation blood substitutes, gastrointestinal discomfort has been universally reported. The symptoms include nausea, vomiting, diarrhea, dyspagia or generalized abdominal pain. These symptoms are believed to be related to NO scavenging by hemoglobin, causing localized spasm throughout the gastrointestinal tract, particularly lower esophageal sphincter muscles.[15] The hemodynamic effects and toxicity of small doses of DCLHb in critically ill patients were initially reported with positive outcome.[16] The vasopressor action of this hemoglobin has been shown to normalize hemodynamic imbalances in these patients.[16] However, in a recent controlled safety study of DCLHb in acute ischemic stroke patients, there were more adverse events and deaths in the DCLHb-treated patients than in the control group.[10] Mortality was also higher in DCLHb-treated patients with severe hemorrhagic shock than control group treated with saline. Of the total number of patients (112), 98 (88%) were infused with DCLHb or saline solution. After 28 days, 24 (46%) of the 52 patients infused with DCLHb died, compared to 8 (17%) of the 46 patients infused with saline solution.[2]

Molecular Basis of Hemoglobin Toxicity

Hemoglobin is a Redox Active Molecule

Hemoglobin within its natural red blood cell environment is found in high concentrations and in a medium rich in organic phosphates, 2,3-diphosphoglycerate (2,3-DPG) which stabilizes the tetrameric form, the only functional form of hemoglobin. The red cells provide a highly specialized enzymatic machinery that catalyze the breakdown of superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) produced by the spontaneous oxidation of the ferrous iron. Reductase systems, which catalyze the reduction of the ferric iron back to ferrous state, that reversibly bind oxygen, are also present. In spite of these protective mechanisms 1–3% of the hemoglobin in the red cell is oxidized at any given time.[17] Chemical and genetic modifications of cell-free hemoglobin are undertaken in order to prevent tetramer dissociation so as to prolong its intravascular retention and to lower the oxygen affinity, thus ensuring tissue oxygenation. Cross-linking either by the use of bifunctional reagents or genetic stabilization of the hemoglobin tetramer, polymerization or decoration of the surface of the hemoglobin molecule has for the most part successfully resolved the problems of circulatory retention and oxygen delivery. However, the redox reactivity of cell-free hemoglobin and its impact on the physiological processes has not been fully appreciated (Figure 1).[18] Hemoglobin outside the red cell undergoes constant and uncontrolled redox transition into non-functional form, the ferric (metHb) as it reversibly binds to oxygen (autoxidation) (Equations 1 and 2)

$$Hb^{2+}O_2 \Leftrightarrow Hb^{2+} + O_2 \quad (1)$$

$$Hb^{2+} \Rightarrow Hb^{3+} + O_2^{\bullet -} \quad (2)$$

Another redox transition of hemoglobin that may occur in the presence of hydrogen or lipid peroxide is the conversion of the ferrous or the ferric form of hemoglobin into a higher oxidation

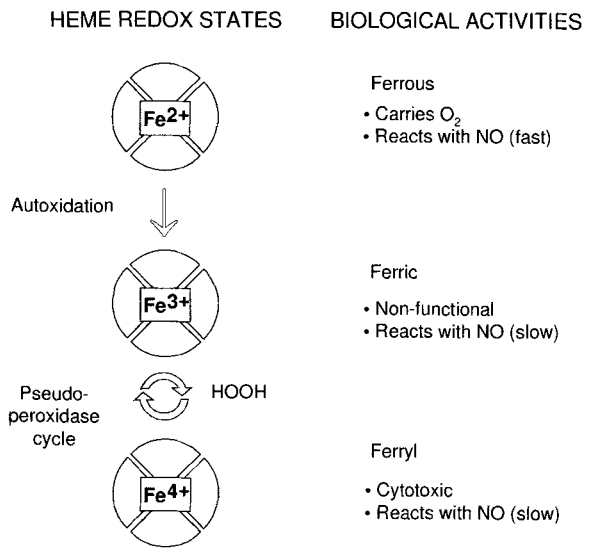

FIGURE 1 Redox transition of hemoglobin. Hemoglobin exists predominantly in the ferrous form, which reversibly binds oxygen. Ferrous hemoglobin undergoes spontaneous oxidation to the ferric form, which dose not carry oxygen (autoxidation). Ferri-hemoglobin (met) is also formed by a NO mediated oxidation of the heme iron. In the presence of H_2O_2, both the ferrous and the ferric forms of hemoglobin undergo rapid transformation to higher oxidation states, the ferryl, an extremely reactive form of hemoglobin.

state, the ferryl form.[18] Chemical and genetic manipulations were also found to accelerate both autoxidation of hemoglobin and in some instances promote the formation and stability of hemoglobin in its ferryl form. With a redox potential value close to that of hydroxyl radical, ferryl hemoglobin may thus represent a significant toxic pathway. If these redox reactions of hemoglobin were to occur uncontrollably in human physiology, it might explain some of the unique toxicological effects associated with the use of hemoglobin-based products. Recently, we described a mechanism to account for this unique redox chemistry of hemoglobin, based largely on studies carried out on both DBBF and recombinant myoglobins.[19,20] The model consists of a simple reaction scheme, reflecting H_2O_2 reactions with ferrous ($Hb^{2+}O_2$) to form the ferryl (Hb^{4+}) intermediate. This is followed by an autoreduction of the Fe^{4+} iron back to Fe^{3+}. In the presence of additional H_2O_2, Hb^{3+} is converted back to Hb^{4+} completing the catalytic cycle (Equation 3)

$$Hb^{2+}O_2 \xrightarrow[k_1]{HOOH} Hb^{4+} \underset{k_3}{\overset{k_2}{\rightleftharpoons}} Hb^{3+} \quad (3)$$
(HOOH at k_3)

We have shown that, besides modifying ligand interactions, clamping of α subunits with DBBF can also affect the tendency of this hemoglobin to undergo oxidative modification and to produce the Hb^{4+} species in solution. The reaction of DBBF with H_2O_2 produces a persistent Hb^{4+} in solution suggesting that it possess less effective enzymatic activity (pseudoperoxidase).[19] A new radical that was detected by EPR ($g = 2.006$) in the reaction of ferric DBBF with H_2O_2, not found in native methemoglobin A_0 was recently reported.[21] The differences in the extent and nature of the radicals formed in the cross-linked Hb may have important implications in the amount of oxidative damage it can induce. *In vitro* evidence on the detection of the ferryl form of DBBF has recently been documented in a number of experimental settings; monolayer of endothelial cells, endothelial cells subjected to ischemia/reperfusion and in cells that lack the antioxidant mechanism i.e., glutathione.[22–24] Documented cellular toxicity that has been attributed to the ferryl heme includes increases in lipid peroxidation, LDH release, and DNA fragmentation. There are markers of cell injury and death by apoptosis and necrosis. Although no *in vivo* evidence suggests that the cytoxicity of cell-free hemoglobin is directly associated with the ferryl iron, a causative role for the redox cycling of myoglobin in rhabdomyolysis-induced renal failure has recently been reported.[25]

Hemoglobin is a Scavenger of Vascular NO

Nitric oxide has emerged as an important chemical messenger in the control of many

physiological processes *in vivo*, including neurotransmission, inflammation, platelet aggregation, and regulation of gastrointestinal vascular smooth muscle.[26] The biological actions of NO are brought about by its binding and activating smooth muscle guanylyl cyclase, which trigger a biochemical cascade resulting in a variety of tissue-specific responses. A large number of *in vitro* and *in vivo* studies concluded that soluble (i.e. cell-free) forms of hemoglobin causes vasoconstriction by scavenging endogenously produced NO and that hypertension seen in patients infused with hemoglobin preparations is due to the depletion of NO in the wall of the vasculature.[3] NO reacts rapidly with an extremely high-affinity to the "deoxy" form of hemoglobin (Equation 4).[27] In the case of oxyhemoglobin, NO does not bind to the heme but reacts directly with the bound oxygen of the HbO_2 molecule to form methemoglobin and nitrate (Equation 5).[28] As a consequence the bound oxygen oxidizes both the heme iron and NO.

$$Hb^{2+} + NO \Rightarrow HbNO \quad (4)$$

$$Hb^{2+}O_2 + NO \Rightarrow Hb^{3+} + NO_3^- \quad (5)$$

NO can bind, at much slower rate with the met "ferric" state of hemoglobin to form ferric–NO complex, which can be reduced by additional NO. These processes could contribute to redox cycling of hemoglobin, thus NO acts as an oxidant or reductant in reactions with hemoproteins (Equations 6 and 7).[29]

$$Hb^{3+} + NO \Leftrightarrow Hb^{3+} - NO \quad (6)$$

$$Hb^{3+} - NO \Rightarrow Hb^{2+} - NO + NO^+ \quad (7)$$

Another, less appreciated role for NO in mammalian physiology, which may directly impact the redox chemistry of hemoglobin, is its antioxidant function.[30] The two major antioxidant mechanisms of NO are: (1) direct oxygen-radical scavenging capable of maintaining a balance between reactive oxygen and nitrogen intermediates at the tissue level (Equation 8) and (2) direct redox interaction with hemoproteins that suppresses the formation of potent cytotoxic oxidants, i.e. heme-associated ferryl radicals (Equation 9).[31]

$$NO + O_2^{\bullet -} \Rightarrow ONOO^- \quad (8)$$

$$Hb^{4+} + NO \Rightarrow Hb^{3+} + NO_2^- \quad (9)$$

The balance between NO and $O_2^{\bullet -}$ in the vasculature can, however, be disrupted under a variety of non-physiological processes (e.g., inflammation, ischemia, diabetes and sepsis)[32] and/or in the presence of cell-free hemoglobin in favor of more powerful oxidants, i.e. $ONOO^-$ (product of NO with $O_2^{\bullet -}$) and H_2O_2. Hemoglobin present in close proximity to $ONOO^-$ production sites in the vasculature can contribute to possible *in vivo* toxicity by a two step mechanism involving (1) direct oxidation of the heme iron and (2) nitration of tyrosine residues on the molecule, leading to subsequent instability and heme loss from the protein.[33]

Cell-free hemoglobin may present a low risk to healthy individuals with normal redox status, however, patients with a compromised vasculature and poor antioxidant status, i.e., diabetes, hypertension, myocardial infraction and acute ischemic stroke, may be at greater risk, as recent clinical trial failures with DCLHb have demonstrated.[2,10] Although the primary event responsible for the microvascular effects of hemoglobin solutions is believed to be the removal of NO by hemoglobin, subsequent oxidative reactions between hemoglobin and oxidants of the vascular system (i.e. H_2O_2 and $ONOO^-$) may potentially lead to a vascular inflammatory cascade of reactions progressing to multi-organ failure.

FUTURE DIRECTIONS

The major thrust in blood substitute research is directed towards the suppression or the elimination of hemoglobin side reactions, which are believed to be detrimental to both hemoglobin

and the surrounding tissue, i.e. vascular endothelium. One obvious strategy that is aimed at preventing the interference of hemoglobin with NO metabolism is to increase the molecular size of hemoglobin from a tetramer (64 kDa) to larger molecular size(s) that average between 300–400 kDa. In some instances the smaller molecular component of hemoglobin ($\leq$ 64 kDa), which is believed to extravasate readily the endothelial barrier are all but eliminated ($\leq$ 1–3%).[34,35] However, several recent reports based on animal studies and calculation of NO kinetics at the vascular level argue against extravation as a singular mechanism because of the extremely high affinity of hemoglobin for NO, which creates large diffusion gradients. Intravascular flow has recently been shown to have no effect on the reaction between NO and cell-free hemoglobin in the lumen, because the latter forms a homogenous solution and is not subject to the hemodynamic separation whereas in the case of intraerthyrocytic hemoglobin, an RBC-free zone in the microcirculation has been shown to effectively decrease erythrocyte consumption of NO.[36] Intuitively, it can be argued that cell-free hemoglobin, regardless of its molecular size can readily reach NO production sites and hemoglobin encapsulation within a red cell is the only viable strategy to prevent hypertension. Indeed attempts to polymerize hemoglobin molecule into oligomers attenuated hemodynamic responses but still triggered blood pressure increases.[37,38] Another approach that specifically targets the reaction sites of NO on the hemoglobin molecule appears to be a promising strategy in resolving the hemoglobin pressor effect. Genetic modification, intended to alter the size and polarity of naturally occurring amino acids in and around the distal heme pocket of both α- and β-chains of hemoglobin, can reduce the rate of NO scavenging by hemoglobin, and thus, reduced hemoglobin vasoactivity in animals.[39,40] To prevent free radical mediated toxicity of hemoglobin, trace amounts of superoxide dismutase (SOD) and catalase (CAT) have been cross-linked to hemoglobin. PolyHb–SOD–CAT has been shown to be effective in reducing ferryl hemoglobin formation and other free radicals in animal models.[41] Peroxide and superoxide-mediated neutrophil adherence to human endothelial cells was inhibited by hemoglobin coupled to stable nitroxyl radicals (nitroxides). Nitroxides possess the added advantage of SOD and CAT-like properties.[42]

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