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Blood Substitutes: The Future Is Now

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I thank you for the tremendous honor of presenting this prestigious lecture. As eloquently stated by those preceding me, there is no greater professional satisfaction than to be recognized by your peers. Clearly no one stands here without the enduring support and counsel of those more capable and wise. My inventory of those to whom I am forever indebted is extensive. At the top of the list are Dr John H Davis, Dr Ben Eiseman, Dr Alden H Harken, and especially my incredibly patient and insightful spouse, Dr Sarah Van Duzer. I would like to take a moment, on behalf of the extended trauma community, to express gratitude to and remember Dr C James Carrico not only for his enormous contributions to trauma care but also for the ideals he exemplified as a loving husband, caring father, altruistic academic leader, and unselfish colleague. Today I am going to resist the overwhelming desire to lament the escalating crisis in US health care, but rather share with you what I believe is one of the most exciting research developments in my lifetime for care of the injured patient.

Trauma surgeons, perhaps more than any other health care providers, recognize the tremendous potential clinical benefit of a blood substitute (Table 1). Whether locally—the high school massacre in Denver,[1] nationally—the tragic 9/11 events in New York City and the Pentagon,[2] or internationally—the ongoing war against terrorism,[3] there is a sense of urgency to develop this life-sustaining resource. I believe the day this clinical benefit will finally be realized has arrived and, consequently, have chosen to review at this forum the scientific background, current status, and future application of blood substitutes in trauma care. This will be a biased perspective, because it is based principally on my opportunity to work closely with Steven A Gould, MD and Northfield Laboratories, Inc (Evanston, IL), in the insinuation of their human polymerized hemoglobin (Hb) solution, PolyHeme, into the care of the injured patient over the past decade.[4-6] The current generation of blood substitutes undergoing US Food and Drug Administration (FDA) phase III clinical testing are red blood cell (RBC) substitutes and fundamentally provide the respiratory function of hemoglobin. Agents have been developed to replace platelets[7,8] and plasma coagulation factors;[9,10] their combination with an RBC substitute will be a welcome refinement for the treatment of advanced hemorrhagic shock in the future. The most promising RBC substitutes at this time consist of extracted Hb from lysed RBCs, often referred to as hemoglobin-based oxygen carriers (HBOCs).

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Hemoglobin physiology (Fig. 1)

Hemoglobin is recognized as essential for the transport of oxygen (O_2).[11] Adult human Hb consists of two α and two β polypeptide chains, each bound to a heme group capable of binding one molecule of O_2 (1 g of Hb binds 1.39 mL of O_2). The molecular weight of the Hb tetramer is 64,500. The globin subunits of deoxyhemoglobin are held by electrostatic forces in a tense conformation with a relatively low affinity for O_2. When O_2 binds to a heme group, mechanochemical stresses weaken the electrostatic forces, resulting in a relaxed conformation; this exposes remaining binding sites and increases O_2 affinity 500-fold. The Hill coefficient reflects the cooperative effect of multiple O_2 binding sites on Hb, responsible for the sigmoid shape of the oxyhemoglobin dissociation curve. The Hill coefficient of the adult RBC is 2.7 (range 2.4 to 2.9). Factors that modify O_2-binding affinity include RBC 2,3-diphosphoglycerate (2,3-DPG) content, the concentration of carbon dioxide and hydrogen ion in blood, and body temperature. Binding of 2,3-DPG between the β chains of Hb stabilizes the tense conformation and reduces affinity for O_2. Conversely, loss of 2,3-DPG increases O_2 affinity; ie, it shifts the oxyhemoglobin dissociation curve to the left as defined by a reduced P_{50}. P_{50} is the O_2 tension when the Hb binding sites are 50% saturated; the normal P_{50} in adults at sea level is 26.3 mmHg. The addition of hydrogen ion or carbon dioxide to blood also reduces the

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Abbreviations and Acronyms

DCLHb = diaspirin cross-linked hemoglobin
2,3-DPG = 2,3-diphosphoglycerate
Hb = hemoglobin
HBOC = hemoglobin-based oxygen carrier
IL = interleukin
ISS = Injury Severity Score
MOF = multiple organ failure
NO = nitric oxide
P_{50} = O_2 tension when hemoglobin binding sites are 50% saturated
PMN = neutrophil

O_2-binding affinity of Hb, known as the Bohr effect; oxygenation of Hb reduces its affinity for carbon dioxide—the Haldane effect. Only 10% of carbon dioxide is exported from tissue as carbaminohemoglobin; 80% is transported as bicarbonate, and 10% in physical solution. Finally, decreased core temperature increases the affinity of Hb for O_2; ie, it reduces the P_{50}.

Hemoglobin interaction with nitric oxide (Fig. 2)

The interaction of Hb with nitric oxide (NO) is presently undergoing intense investigation, and is conspicuously relevant to the efficacy and safety of Hb solutions used in trauma care.[12] Hb is known to bind NO through high-affinity ferrous (Fe^{++}) sites on heme. When NO binds to the heme iron, it can engage in redox reactions with the metal ion, leading to the production of methemoglobin (Fe^{+++}), with nitrate, and the formation of additional reactive O_2 species. Recently, a different interaction between NO and Hb has been described by a second binding site at the β 93 cysteine residue on the

Table 1. Potential Clinical Benefits of Hemoglobin-Based Oxygen Carriers

Availability
Abundant supply
Universally compatible
Prolonged shelf-life
Storage at room temperature
Safety
No disease transmissions
No antigenic reactions
No immunologic effects
Efficacy
Enhanced oxygen delivery
Improved rheologic properties

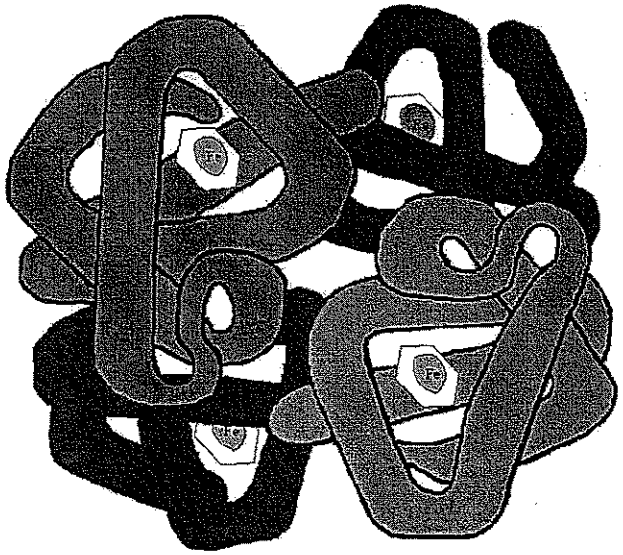


Figure 1. Adult human hemoglobin consists of two α and two β polypeptide chains, each bound to a heme group capable of binding one molecule of oxygen.

globin chain. Stamler and colleagues[13-15] propose this *S*-nitrosylation reaction as a key physiologic process designed to transport this vasoactive agent cooperatively with O_2. At high O_2 saturation, Hb assumes the relaxed conformation in which thiol affinity for NO increases, so both O_2 and NO are loaded onto Hb. At low O_2 levels, NO is unloaded along with O_2 from the allosterically modified tense Hb form, which exposes the β93 cysteine site. Gladwin and associates[16] argue that heme nitrosylation (Hb $Fe^{++}NO$) is the predominant NO transport mechanism for Hb in the human circulation,

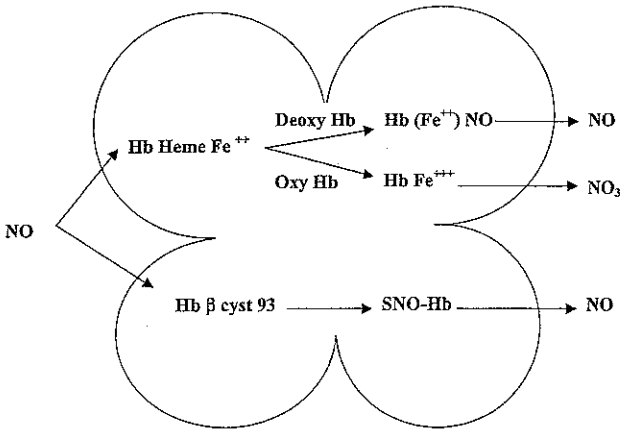


Figure 2. Adult human hemoglobin can transport nitric oxide by heme nitrosylation ($HbFe^{++}$ NO) or binding to the β 93 cysteine residue on the globulin chain (Hb β cyst 93). $HbFe^{+++}$, methemoglobin; NO, nitric oxide; NO_3, nitrate; SNO-Hb, *S*-nitrosylation.

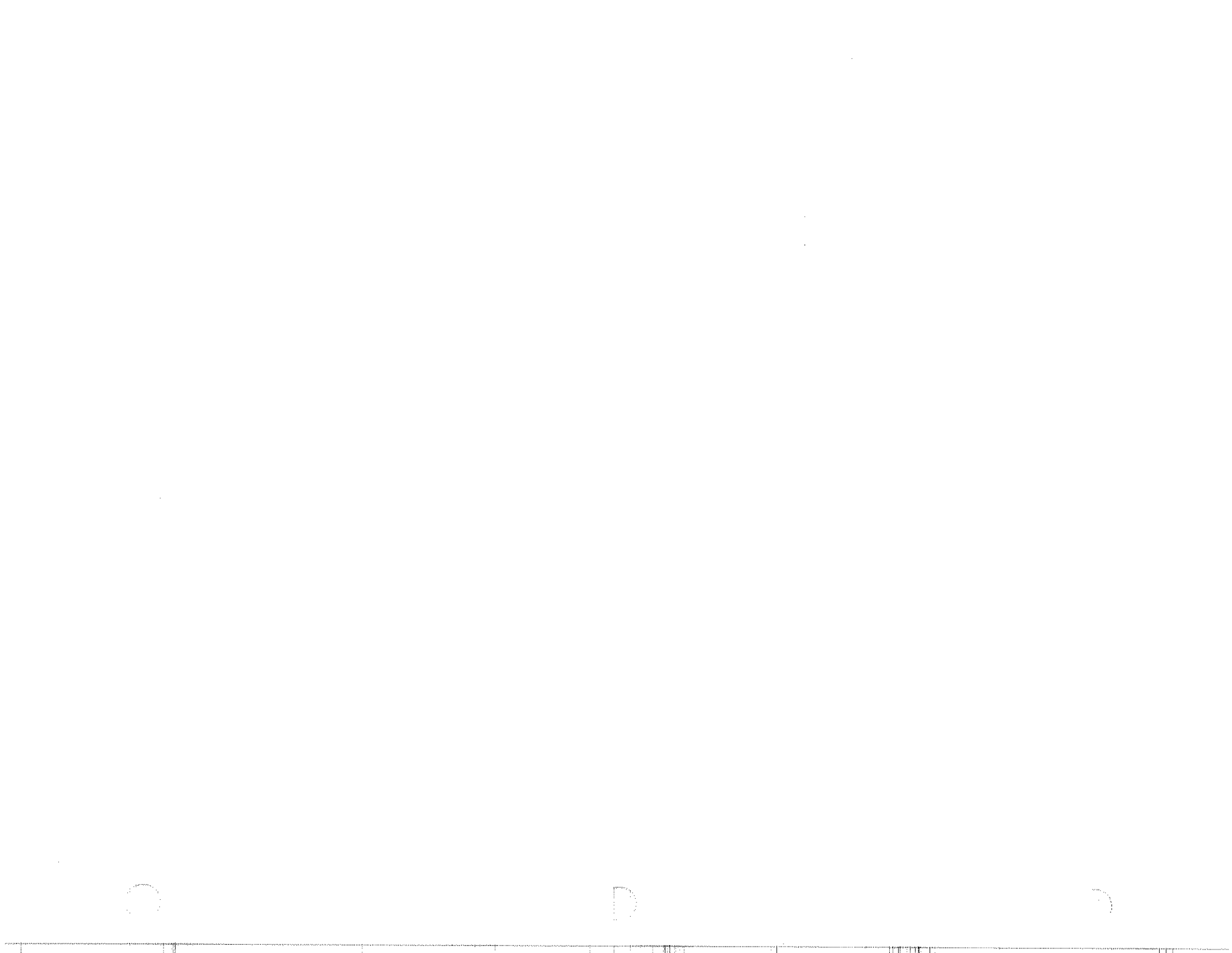


Table 2. Evolution of Hemoglobin-Based Oxygen Carriers

I. Hemolysates
Bovine hemolysates into dogs/cats: preservation of neurologic function, maintenance of oxygen consumption 1933 Amberson et al, Science[18]
Human hemolysates into patients: transport O_2, observed "pressor effect" 1949 Amberson et al, J Appl Physiol[20]
II. Modified hemolysates
Human filtered hemolysates into humans: renal dysfunction 1951 Miller et al, J Clin Invest[22]
III. Tetrameric hemoglobin
Human Hb tetramer into dogs: no renal dysfunction 1967 Rabiner et al, J Exp Med[25]
Human Hb tetramer into humans: renal toxicity, hypertension, abdominal pain 1978 Savitsky et al, Clin Phamacol Ther[28]
IV. Modified tetrameric hemoglobin
Human modified Hb into animals: improved survival as low-volume resuscitation agent, but concern for systemic and pulmonary vasoconstriction 1993 Hess et al, J Appl Physiol[93]
Human modified Hb into trauma patients: increased mortality 1999 Sloan et al, JAMA[91]
V. Polymerized hemoglobin
Human glutaraldehyde polymerized hemoglobin 1984 Gould et al, Surgery[41]
Bovine glutaraldehyde polymerized hemoglobin 1989 Vlahakes et al, Eur J Cardiovasc Surg[36]
Human *o*-raffinose polymerized hemoglobin 2000 Carmichael et al, Crit Care Med[42]

and additionally this complex facilitates O_2 delivery by an enhanced Bohr effect. Whether carried on heme Fe^{++} or β chains, it is now clear that human Hb can traffic NO, not just consume it.

History of hemoglobin-based oxygen carriers (Table 2)

Sellards and Minot[17] infused lysed RBCs into humans in 1916, but their intent was to evaluate the tolerance to hemolysis. Recognizing that extracellular Hb exists in certain invertebrates, Amberson and associates,[18] at the University of Tennessee in Memphis, are credited with the first in vivo studies to establish the ability of Hb solutions to transport O_2 in mammals. In 1933, using a bovine hemolysate, they reported successful exchange transfusions in cats and dogs with intact neurologic function. Perhaps most noteworthy was the preserved ability of cats to land on their feet when dropped upside down. They further observed, "Such animals finally die, after five or six hours, not because the hemoglobin is unable to carry on its respiratory function, but because it leaves the blood stream." A year later they confirmed maintenance of normal O_2 consumption in a series of experiments using the same animal models.[19] In 1949 this group[20] reported their collected clinical experience with lysed human RBCs infused into 14 patients, concluding that "dissolved Hb transports O_2 much as it does when confined to the RBC." The most dramatic response was the life-restoring infusion of 2,300 mL (250 g Hb) into a 22-year-old with massive postpartum hemorrhage; unfortunately she died on day 9 with renal failure. Of interest, Amberson and colleagues also observed a "chemical pressor principle." Shortly after, several groups[21,22] provided conclusive evidence that human hemolysates produced renal dysfunction and speculated that the RBC membrane (stromal lipid) was the toxic factor. Moss and coworkers[23] also provided experimental evidence that stromal contamination provoked intravascular coagulation. These observations led to the development of stroma-free Hb, pioneered by Rabiner and colleagues[24-26] at Michael Reese Hospital in Chicago. De Venuto and associates[27] at the Letterman Army Institute of Research produced a stroma-free Hb solution based on crystallization as a purification method. Stroma-free Hb appeared promising in extensive preclinical work, but in 1978 the meticulous clinical trial by Savitsky and associates,[28] infusing only 250 mL (16 g Hb) of stroma-free (99%) Hb into eight healthy volunteers, documented transient renal dysfunction, hypertension, and abdominal pain. With the virtual elimination of the RBC membrane, these adverse effects were attributed to instability of the Hb tetramer; ie, spontaneous dissociation into dimers and monomers. Additional limitations of stroma-free Hb included reduced O_2 affinity secondary to the loss of 2,3-DPG (P_{50} = 12 mmHg); relatively short intravascular retention (<6 hours), and relatively low colloid osmotic activity (COP = 20 mmHg at 7 g%).

Recognizing the life-sustaining potential of Hb solutions but unacceptable toxicity and physiologic short-

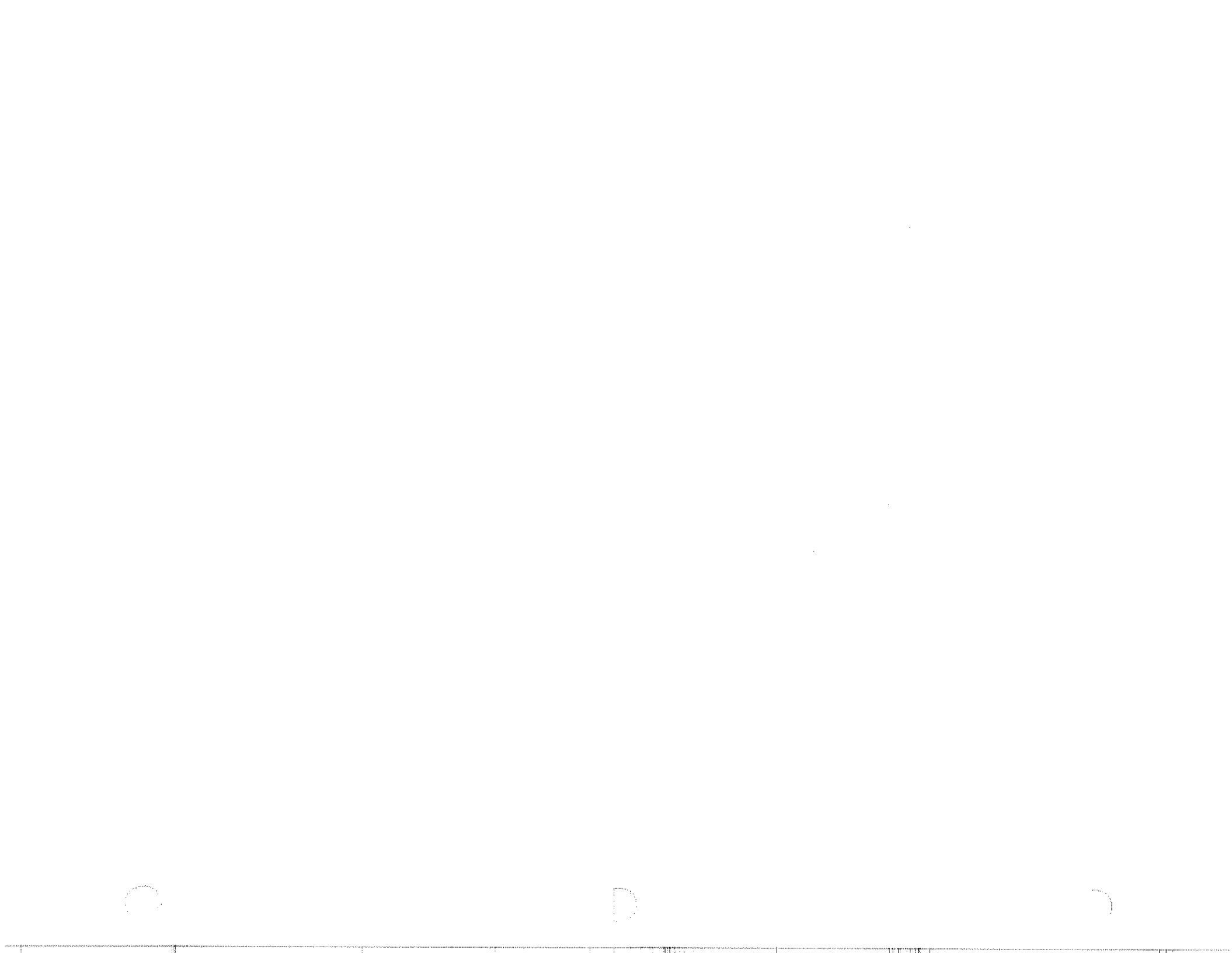


Table 3. Potential Role of Hemoglobin-Based Oxygen Carriers in Trauma Care

Application	Location
I. Perioperative applications	
Conserve stored RBCs	ED, angiography, OR, ICU
Reduce allogeneic RBC transfusions	ED, angiography, OR, ICU
II. Acute hemorrhagic shock	
When stored RBCs unavailable	Field, ED, OR, ICU, remote hospital, disaster, military
More efficient resuscitation	Field, ED, OR, ICU, remote hospital, disaster, military
III. Regional perfusion	
Enhance O_2 delivery	
Ischemic reperfused tissue	OR, ICU
Inflamed tissue	OR, ICU
Ex vivo organ perfusion	OR, ICU

ED, emergency department; OR, operating room.

comings of unmodified Hb tetramer, private industry, in the 1980s, embarked on a vigorous search for a viable option through several diverse conceptual modifications. To address O_2 affinity, there were at least four approaches: 1) compensate for the loss of 2,3-DPG by stabilization of deoxyhemoglobin—a common agent used was pyridoxal 5[1] phosphate, which binds to the β chain-valine 1;[29,30] 2) internal cross-linking of the Hb tetramer under deoxy conditions, such as diaspirin cross-linking of the α chains-lysine 99;[31,32] 3) recombinant DNA technology and site-directed mutagenesis—for example, the N108K mutation in the β chain was introduced into the human Hb gene constructed in *E coli*;[33,34] and 4) nonhuman Hb with a lower O_2 affinity (eg, bovine).[35,36] Prolonging intravascular retention was achieved by internal cross-linking of the Hb tetramer biochemically or with DNA technology;[29-32] surface conjugation of the Hb tetramer to polymers, such as polyethylene glycol or polyoxyethylene;[37,38] encapsulation of the Hb tetramer within a liposomal envelope;[39,40] and polymerizing Hb tetramers. Glutaraldehyde,[41] interacting with any of the 44 lysines in the Hb tetramer, and ring-opened raffinose[42] resulted in intratetrameric and intertetrameric cross-linking. Polymerizing tetrameric Hb also reduced colloid osmotic activity, permitting higher circulating concentrations of the Hb solution. Tetrameric Hb at a concentration of 7 g% exerts an oncotic pressure equivalent to normal plasma; polymerized Hb can be loaded at 12 g% without excessive colloid osmotic pressure.[43]

FDA guidelines to evaluate hemoglobin-based oxygen carriers (Table 3)

Approval for the clinical use of HBOCs is being pursued internationally, but for practical reasons I will confine my discussion to the regulatory efforts within the US. FDA approval of a new product generally proceeds through phase I, II, and III studies designed to establish safety and efficacy. FDA regulation defines efficacy as follows: "Effectiveness means a reasonable expectation that. . .pharmacologic or other effects of the biologic product. . .will serve a chemically significant function in the diagnosis, cure, mitigation, treatment or prevention of disease in man."[44] The Center for Biologics Evaluation and Research (CBER) is the review body for the FDA in the arena of biologies and has published a comprehensive listing of "points to consider in the safety evaluation of HBOCs."[45] These points encompass characterization of the product, animal safety testing, and human studies. These address the theoretic concerns of Hb solutions raised in previous reviews,[46-48] including pulmonary and systemic hypertension, organ dysfunction, oxidative tissue injury, synergy with bacterial pathogens, and immunomodulation.

In 1994 CBER convened a workshop with the National Heart, Lung and Blood Institute and the Department of the Army to develop "points to consider in the efficacy evaluation of HBOCs."[49] Clinical trial endpoints were divided into two categories. Direct measures of clinical benefit included improved patient survival and reduced complications; an example of a surrogate endpoint was a laboratory measurement expected to correlate meaningfully with clinical benefit. Documenting a direct clinical endpoint for HBOCs was viewed as challenging because this endpoint had never been established for RBCs. Specific recommendations for clinical studies were in three areas: perioperative applications, acute hemorrhagic shock, and regional perfusion. Field trials for severe trauma, where RBCs are not available, were labeled as difficult because of safety and ethical issues. Decreased perioperative allogeneic RBC transfusion was regarded as a clinical benefit, but the potential risks of HBOCs would have to be defined and evaluated as well. Examples for regional perfusion studies included enhanced tumor radiosensitivity and an adjunct during coronary angioplasty (the FDA had approved Fluosol DA in 1989 as an O_2-carrying drug for this setting).

Stored RBCs: The control group (Table 4)

An obstacle to designing clinical trials with HBOCs compared with stored RBCs is the lack of agreement on a transfusion trigger; ie, the maintenance of sufficient circulating O_2-carrying capacity to ensure adequate O_2 delivery to tissue. Isovolemic anemia in healthy volunteers is tolerated at 5 g%,[50] the American Society of Anesthesiologists' recommendation is more than 6 g%,[51] the National Institutes of Health consensus is between 7 and 10 g%,[52] and in certain high-risk patients the suggested threshold is more than 10 g%.[53] The other side of the risk-to-benefit equation remains equally perplexing. Although the risk of transmitted disease appears to be declining[54] (excepting the recent West Nile virus), the list of immunologic consequences appears to be expanding. The concept of transfusion-related immunodysfunction stems from the 1973 report by Opelz and associates,[55] who identified a relationship between pretransplant transfusion and renal allograft survival. Subsequently, perioperative transfusion was associated with tumor recurrence[56] and postinjury and postoperative infections.[57-61] Transfusion-related, cell-mediated immunosuppression is multifactorial and includes depletion of cytotoxic T lymphocytes, reduced T-lymphocyte blastogenesis, altered cytokine response of recipient T cells (favoring release of interleukin $[IL]_4$, IL_5, and IL_{10} over IL_2 and γ-interferon), reduction in CD4+ cells, increased suppressor T-lymphocyte activity, impaired natural killer cell activity, and depressed macrophage function.[62,63]

We have been interested in the potentially adverse proinflammatory effects of stored RBCs and, specifically, the capacity to provoke neutrophil (polymorphonuclear [PMN]) cytotoxicity. The PMN is a key cellular mediator in the pathogenesis of postinjury multiple organ failure (MOF). Consequently, PMN functional responses are evaluated as a clinical surrogate for the two-event model of MOF; ie, inflammatory priming and subsequent activation.[64-65] In our ongoing epidemiologic studies, we have shown that more than six units of RBC transfusion within the first 12 hours postinjury is an independent risk factor for MOF.[66] Previous studies in our center have shown that after severe injury, patients at high risk for MOF have circulating PMNs that are primed for cytotoxicity within the first 6 hours postinjury, as marked by the increased surface expression of CD11b/CD18,[67] p38 MAPK activation,[68] release of cytotoxic products in response to fMLP,[69] and delayed apoptosis.[70] The precise mechanism(s) linking RBC transfusion and PMN priming remains to be established, but it is generally believed that passenger leukocytes accompanying RBCs in storage are important in the generation of proinflammatory agents.[71-73] Plasma from stored RBCs primes PMNs in vitro, and this effect increases progressively from 14 to 42 days of storage.[74,75] Shanwell and associates[76] have incriminated cytokines (TNF-α, IL_1, IL_6, IL_8) generated during storage, and we have focused on proinflammatory lipids presumably generated from the RBC membrane.[77-79] Metabolites of the arachidonic acid cascade have been strongly implicated in the pathogenesis of transfusion-related acute lung injury.[80-82] Although prestorage leukoreduction of RBCs decreases the generation of cytokines,[76] this process does not eliminate PMN priming.[83] Another emerging concern regarding stored RBCs is their altered rheologic state. Notable RBC shape changes occur by the second week of storage and progress during longer preservation.[84,85] The effects of reduced RBC deformability on microcirculation have been documented experimentally, and include impaired tissue access of the stiff RBC and

Table 4. The Risks and Benefits of Stored Red Blood Cells

Elusive transfusion trigger
Healthy volunteers (>5 g%)
American Society of Anesthesiologists (>6 g%)
NIH (7–10 g%)
Cardiopulmonary disease (>10 g%)
Disease transmission
Human immunodeficiency virus
Human T-cell lymphotrophic virus
Hepatitis B, C viruses
West Nile virus
Yersinia, malaria, babesia, chagas
? Variant Creutzfeldt - Jacob disease
RBC membrane compatibility
Hemolytic reactions
Allergic response
Fever, rash, bronchospasm, anaphylaxis
Immunodysfunction
Decreased allograft rejection
Increased tumor recurrence
Increased postoperative infection
Transfusion-related acute lung injury
Increased postinjury multiple organ failure
Compromised O_2 delivery
Reduced RBC deformability
Decreased 2,3-diphosphoglycerate

RBC entrapment resulting in microvascular obstruction.[86,87] These experimental findings might explain the observation that blood transfusion failed to improve O_2 consumption in critically ill and injured patients[88,89] and in one study appeared to induce splanchnic ischemia.[90] Collectively, these in vitro, in vivo, and clinical studies invoke the untimely transfusion of stored RBCs in the pathogenesis of dysfunctional immunoinflammation that ultimately culminates in postinjury MOF.

Clinical evaluation of modified tetrameric hemoglobin

Of the modified Hb tetrameric solutions that looked promising in the late 1980s,[31-40] only one formulation was authorized by the FDA for a phase III study in trauma, and this product failed notoriously.[91] Regarded by some as a major setback for the clinical implementation of HBOCs, it is important to emphasize that this US multicenter trial of diaspirin cross-linked Hb (DCLHb) for the treatment of severe traumatic hemorrhagic shock was based on the explicit proposal that "DCLHb was tested not as a substitute for blood but rather as an adjunct to the currently used therapies for enhancing oxygen delivery: fluids, blood, and operative intervention." Although an unexpected outcome raises the issue of comparable study groups,[92] the difference in the primary study endpoint was alarming: the 28-day mortality for the DCLHb group was 46% (24 of 52), compared with 17% for the the control (normal saline) group (8 of 46). Much expert thought and preparation went into the study design of this human trial, but the scientific rationale of using a vasoconstricting agent for the initial resuscitation of acute hemorrhagic shock was questionable. The study objective was to determine if the infusion of up to 1,000 mL DCLHb (100 g Hb) during the first 2 hours of hospitalization could reduce 28-day mortality in injured patients with evidence of persistent hypoperfusion secondary to acute blood loss. The control was an equivalent volume of normal saline. The authors justified this study design because in preclinical trials "DCLHb has been shown to be effective in enhancing perfusion in small volumes, suggesting a pharmacologic effect that is independent of hemoglobin." But the pharmacologic effect was not always reported as beneficial.

In 1993, Hess and coauthors,[93] at the Letterman Army Institute of Research, reported that in a swine model of hemorrhagic shock DCLHb infusion doubled systemic and pulmonary vascular resistance, and these responses were associated with a fall in cardiac output. In fact, these changes were equivalent to resuscitation with unmodified tetrameric Hb (stroma-free Hb). The authors concluded, "The decrease in cardiac output associated with the vasoconstriction in the Hb-treated animals was equal to the increase in oxygen-carrying capacity—crystalloid or colloid solutions provided equally rapid correction of the elevated whole blood lactate." In a followup study,[94] the infusion of low-dose (4 mL/kg = 14 g Hb) DCLHb into swine subjected to hemorrhagic shock prompted the authors to further warn "pulmonary hypertension and low peripheral perfusion may offset benefits for trauma patients." In an animal model of pulmonary contusion, Cohn and colleagues[95] observed that DCLHb resuscitation led to "pulmonary hypertension, greater pulmonary contusion size and stiffer lungs." Finally, in a trial of critically ill patients, DCLHb was reported to have a "marked vasopressor action, allowing norepinephrine requirements to be reduced," but at 7.5 hours after DCLHb administration there was increased pulmonary vascular resistance with decreased cardiac index and O_2 delivery[96]—a familiar theme with this product. Although the authors of the DCLHb trial cited several animal models that appeared to support their study hypothesis, none of these models replicated their study design—a lesson for future conduct of clinical trials with HBOCs. Of note, the mechanisms responsible for the vasoconstriction resulting from DCLHb administration were elucidated before the trauma clinical trial. The increased vascular resistance was shown to be predominantly mediated by the scavenging of NO with an additional component of enhanced endothelin release.[97-99] Production of DCLHb has been terminated, but the relevance of these basic mechanisms to trauma care with HBOCs is clear.

Clinical evaluation of polymerized hemoglobin: Safety

At this moment, the most successful HBOCs clinically are polymerized Hb solutions (Table 5). Perhaps a coincidence but, as discussed earlier, polymerization addresses several of the problems inherent in tetrameric Hb: enhanced intravascular retention and reduced colloid osmotic activity. Polymerization also appears to attenuate vasoconstriction associated with the infusion of Hb solutions. In fact, PolyHeme, which contains less than 1% tetrameric Hb, is devoid of vasoconstriction in

Table 5. Current Hemoglobin-Based Oxygen Carriers Undergoing FDA-Approved Phase III Clinical Trials

Product	Manufacturer	Hemoglobin source	Polymerization	Status in USA
Hemolink	Hemosol Inc, Mississauga, ON	Human	*o*-Raffinose	—
Hemopure	Biopure Corp, Cambridge, MA.	Bovine	Glutaraldehyde	BLA filed at FDA
PolyHeme	Northfield Lab, Evanston, IL	Human	Glutaraldehyde	BLA filed at FDA

BLA, Biological License Application.

our clinical experience with this product over the past decade.[4-6] A proposed explanation is that tetrameric Hb (65 KDa) extravasates through the endothelium to bind abluminal NO, leading to unopposed vasoconstriction; but polymerized Hb (>130 KDa) remains in the vasculature to bind only luminal NO.[43] Of interest, Hb of the common earthworm, *Lumbricus terrestric,* is a polymer with a molecular weight of 400 KDa that circulates extracellularly.[100] Mice and rats undergoing exchange transfusion with this naturally occurring polymeric Hb showed no changes in behavior, and nuclear magnetic resonance spectroscopy of the heart confirmed normal O_2-carrying capacity.[101] Polymerized HBOCs have undergone extensive preclinical and clinical testing; PolyHeme is the only product to be evaluated in severely injured patients in the US to date. This is likely because of the anticipated challenges in designing clinical trials in trauma identified by the FDA.[49] Hemopure (Biopure Corp, Cambridge, MA), a polymer of bovine Hb, has been used successfully to reduce allogeneic RBC transfusion in elective cardiac,[102] aortic,[103] and hepatic[104] surgery. One study with abdominal aortic reconstruction raised concern about increased systemic vascular resistance,[105] an effect identified in normal volunteers.[106] Hemopure has also been evaluated for efficacy with in vitro[107] and in vivo[108,109] regional reperfusion models; these studies suggest an advantage over stored RBCs. Animal studies designed to replicate prehospital hypotensive resuscitation for hemorrhagic shock have been encouraging,[110,111] although the issue of compromised tissue perfusion because of vasoconstriction has surfaced.[112-114] Hemopure has been approved for replacement of acute blood loss in South Africa, but there are no published results to date.

Clinical testing of Hemolink (Hemosol, Inc, Mississauga, Ontario, Canada), o-raffinose polymerized Hb, has targeted conservation of allogeneic RBC transfusion through enhanced intraoperative autologous donation during coronary artery bypass grafting.[115,116] The pulmonary and systemic vasoconstrictions associated with Hemolink infusion[117] is attenuated by general anesthesia.[118] Assessment of Hemolink for hemorrhagic shock[119] and regional reperfusion has been limited but promising.[120] The mechanism(s) responsible for vasoconstuction after infusion of Hemopure and Hemolink has not been established. NO binding appears to be involved but alternative mechanisms have been proposed, including increased endothelin, release-enhanced adrenergic receptor sensitivity, and reduced arterial wall shear stress.[121] The relative proclivity for increased pulmonary and systemic vascular resistances with the various polymerized Hb solutions might be explained by the amount of residual tetrameric Hb after polymerization and purification (Table 6). Additional issues reported with the clinical use of polymerized Hb solutions include interference of laboratory tests that are based on colorimetric changes from dissolved plasma Hb, inaccuracy of O_2 saturation monitoring because of methemoglobin, mild elevations of serum amylase (but without evidence of pancreatitis), and skin rashes. None of these have been considered clinically important adverse events.

Our experience is limited to PolyHeme, and investigation with this polymerized Hb has focused on its potential role in trauma care. Gould and colleagues[122] established the safety of infusing 1 unit (50 g) of PolyHeme in healthy volunteers. Under FDA guidance, we initiated clinical trials in trauma to confirm safety with escalating doses of PolyHeme and to explore study endpoints to establish efficacy. In the first clinical trial,[4] 39 patients received 1 (n = 14), 2 (n = 2), 3 (n = 15), or 6 (n = 8) units of PolyHeme instead of stored RBCs as part of their initial resuscitation after acute blood loss. Infusion rates ranged from 1 unit in 175 minutes to 6 units (300 g) in 20 minutes. Although the RBC Hb fell to 2.9 ± 0.2 g%, total Hb was maintained at 7.5 ± 0.2 g% with PolyHeme. With respect to safety, the patient's temperature, mean arterial pressure, heart rate, and creatinine clearance did not change during the 72-hour study period. Liver function tests and amylase varied substantially because of patient injuries. Excluding patients with abnormal preinfusion values, there were no notable changes in these serum enzymes. Transitioning

Table 6. Characteristics of Polymerized Hemoglobin Solutions Versus Stored Red Blood Cells

Characteristic	Hemopure	Hemolink	PolyHeme	RBCs
Hemoglobin (g%)	13 g%	10 g%	10 g%	13 g%
Unit equivalent (g)	30 g	25 g	50 g	50 g
Molecular weight (> 64 KDa)	≥95%	≥65%	≥99%	100%
P_{50} (mmHg)	38	34	29	26
Hill coefficient	1.4	1.0	1.7	2.7
Oncotic pressure (mmHg)	25	24	23	25
Viscosity	1.3 cp	1.1 cp	2.1 cp	(whole blood = 5–10 cp)
Methemoglobin (%)	<10	<7	<8	<1
Half-life	19 h	18 h	24 h	31 d
Shelf-life @ 4° C	≥3 y	≥1 y	≥1.5 y	42 d
Shelf-life @ 21° C	≥2 y	–	≥6 wk	<6 h

cp, centipoise; P_{50}, O_2 tension when hemoglobin-binding sites are 50% saturated.

from safety to efficacy, the O_2-carrying capacity from infused PolyHeme versus the patients' RBCs was determined by simultaneous assessment of the O_2 content of these compartments in venous and arterial blood. O_2 utilization was 27% ± 3% for RBCs and 37% ± 2% for PolyHeme (Fig. 3), a finding consistent with previous work in healthy volunteers attributed to the mildly elevated P_{50}. Relevant to potential endpoints for efficacy, 23 patients (59%) avoided allogeneic RBC transfusion during the first 24 hours.

Cognizant of the vasoconstriction associated with the DCLHb clinical trial, we designed a study to specifically evaluate the vascular response to PolyHeme infusion in acutely injured patients.[123] Patients requiring urgent transfusion were randomized to either PolyHeme (up to 6 units) or stored RBCs during their initial resuscitation. Systemic arterial pressure, pulmonary arterial pressure, cardiac index, and pulmonary capillary wedge pressure were measured every 4 hours postinfusion. There were no major differences between the groups for these indices or the calculated systemic or pulmonary vascular resistance (Table 7).

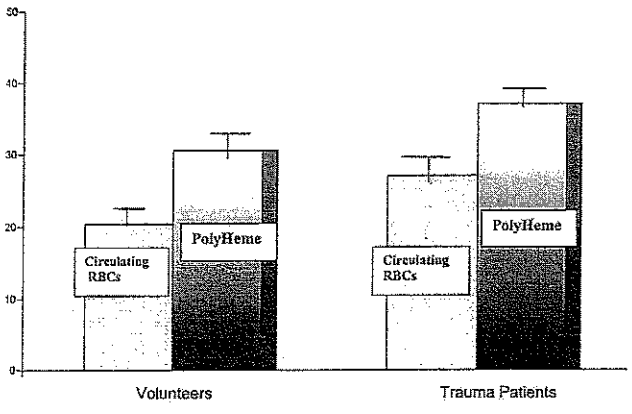


Figure 3. Oxygen utilization was compared between circulating RBC Hb and PolyHeme in healthy volunteers and acutely injured patients. The increased oxygen utilization for PolyHeme might be in part from the mildly increased P_{50} (29 mm). P_{50}, O_2 tension when hemoglobin-binding sites are 50% saturated.

Clinical Evaluation of Polymerized Hemoglobin: Efficacy

Perioperative applications: Reduce allogeneic RBC transfusions

Prompted by the FDA guidelines to demonstrate efficacy (Table 3), all HBOC companies have pursued what appeared to be the simplest clinically; ie, to reduce the need for allogeneic RBC transfusions. Northfield Laboratories has been the only one to focus on trauma so far.

In collaboration with David B Hoyt, MD, and the University of California at San Diego, we conducted a randomized trial in patients requiring urgent transfusion.[5] The 44 trauma patients (Injury Severity Score [ISS] = 21 ± 1.3) were allocated to receive stored RBCs or up to 6 units of PolyHeme as their initial blood replacement. The RBC Hb was equivalent preinfusion (10.4 ± 0.4 g% versus 9.4 ± 0.3 g%); at end infusion, the RBC Hb of the PolyHeme patients fell to 5.8 ± 0.5 g% versus 10.6 ± 0.3 g% in the control. The PolyHeme group received 4.4 ± 0.3 units, resulting in a plasma Hb of 3.9 ± 0.2 g%. The total number of allogeneic RBC transfusions for the control versus PolyHeme was 10.4 ± 0.9 units versus 6.8 ± 0.9 units ($p < 0.05$), respectively, through day 1, and 11.3 ± 0.9 units versus

Table 7. Pulmonary Vascular Resistance after PolyHeme Infusion for Acute Blood Loss

Group	+4 h	+8 h	+12 h	+16 h
Pulmonary Vascular Resistance Index				
PolyHeme	288.0 ± 34.4	311.2 ± 35.2	278.4 ± 42.1	293.0 ± 61.7
RBC	232.7 ± 59.7	281.8 ± 44.0	312.6 ± 62.6	251.4 ± 35.6

7.8 ± 0.9 units ($p = 0.06$), respectively, through day 3 (Fig. 4). After the initial phase, infusion of 4.6 units of stored RBCs in the control group was equivalent to the 5.2 units in the PolyHeme group. Both volumes presumably represent the infused RBCs or PolyHeme lost during the acute hemorrhage. Subsequent replacement volumes were the same, ultimately sparing the PolyHeme group approximately four units of allogeneic RBC transfusion.

As mentioned previously, other polymerized HBOCs have been used in elective surgery to achieve a similar study endpoint.[102-104,115,116] The primary conceptual difference is that these HBOCs were used to augment pre- or intraoperative autologous donation in the controlled setting of elective surgery. Similar to our trauma study, these randomized trials with other polymerized HBOCs demonstrated a compelling reduction in allogeneic RBC transfusion. But at this time, because of the logistic problems in conducting this trauma study, including the inability to obtain waiver of informed consent, we pursued an alternative study design. Because of our long-term interest in the pathogenesis of postinjury MOF, our revised study hypothesis became PolyHeme, in lieu of stored RBCs during initial resuscitation, which would attenuate the adverse immunoinflammatory effects of allogeneic RBC transfusion.

In preparation for these clinical trials, we conducted in vitro studies to test our hypothesis that PolyHeme—free of inflammatory cytokines and lipids—would eliminate the PMN priming previously documented with stored RBCs.[77-83] Human PMNs were isolated from healthy volunteers and the plasma fraction was separated from packed RBCs at 42 days of storage in our blood bank (the last day stored RBCs can be transfused clinically, but often the first RBCs infused into trauma patients).[124,125] The isolated PMNs were incubated with either RBC plasma or PolyHeme at concentrations calculated to be equivalent to 8 units of transfusion. The plasma fraction from three or more units of stored RBCs primed the human PMNs for enhanced superoxide production and elastase release (Fig. 5). Because activated vascular endothelium interacts with primed PMNs early in the pathogenesis of postinjury MOF, we further compared the effects of PolyHeme versus stored RBCs on endothelium in vitro.[126] Human microvascular endothelial cells were incubated with the plasma fraction of RBCs at 42 days storage in concentrations simulating transfusion of 2, 4, or 8 units, or with PolyHeme at similar transfusion volumes. Intercellular adhesion molecule 1 ($ICAM_1$) the counterligand for the CD11b/CD18 adhesion molecule on the primed PMN, was measured as a marker of endothelial activation. $ICAM_1$ surface expression was increased twofold after endothelial exposure to stored RBC plasma but was not altered by PolyHeme (Fig. 6). With this supportive in vitro work, we proceeded to test our hypothesis in trauma patients.

In our clinical trial, injured patients requiring urgent transfusion were administered either PolyHeme (up to 20 units = 1,000 g) or stored RBCs for their initial resuscitation.[127] PMN priming was determined by the surface expression of CD11b/CD18 and superoxide production. The study groups (stored RBC [n = 10] versus PolyHeme [n = 9]) were comparable with respect to injury severity (ISS = 27.9 ± 4.5 versus 21.9 ± 2.7), physiologic compromise (emergency department pH =

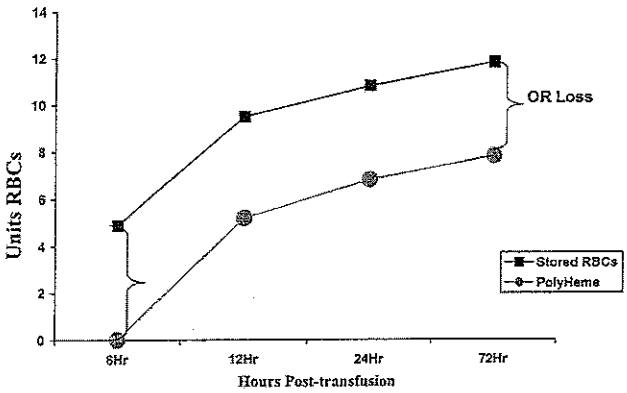
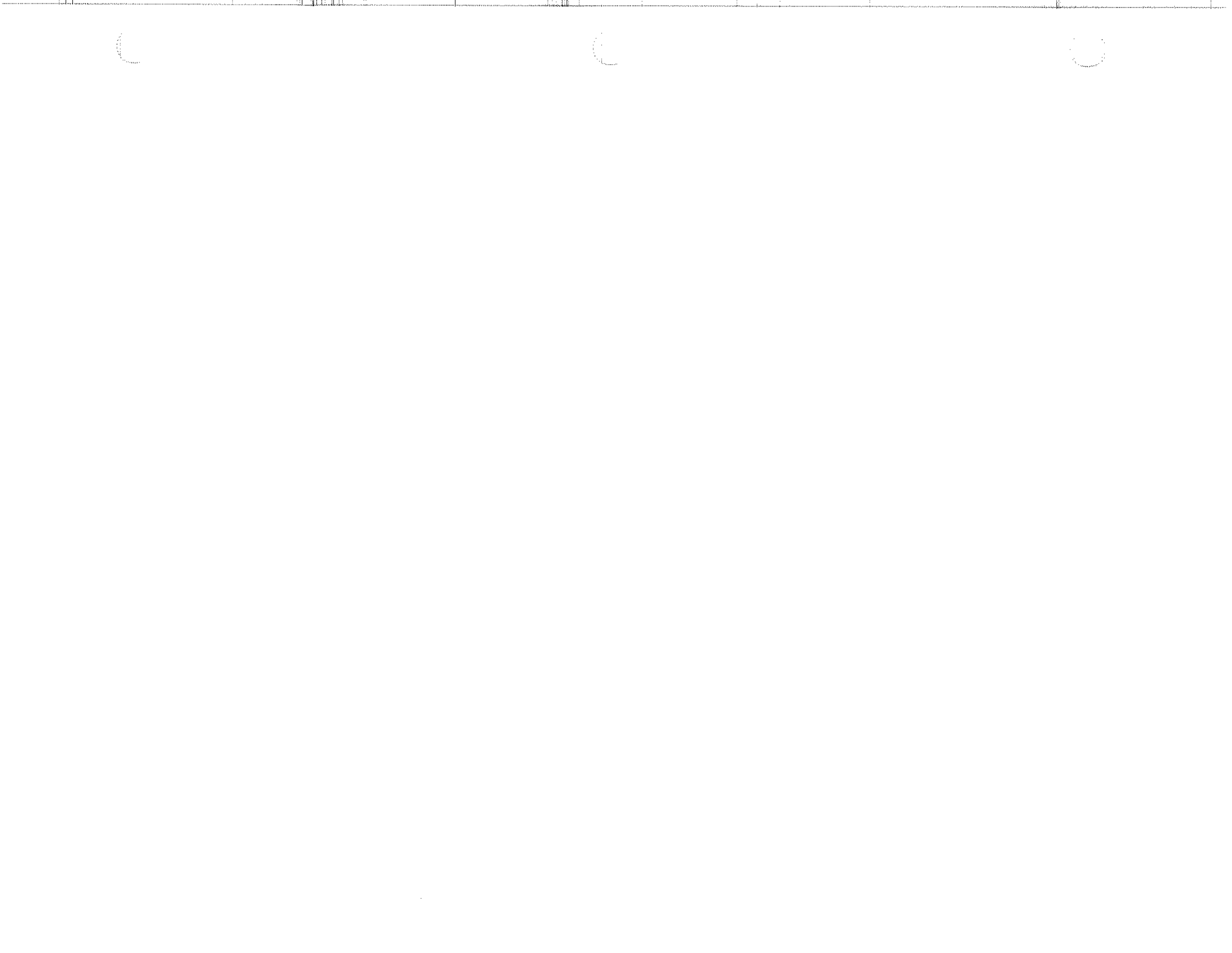


Figure 4. Acutely injured patients were randomized to receive stored RBCs or up to 6 units of PolyHeme as their initial blood replacement. The net difference in allogeneic RBC transfusion persisted for 72 hours, and was due to the initial operating room loss.



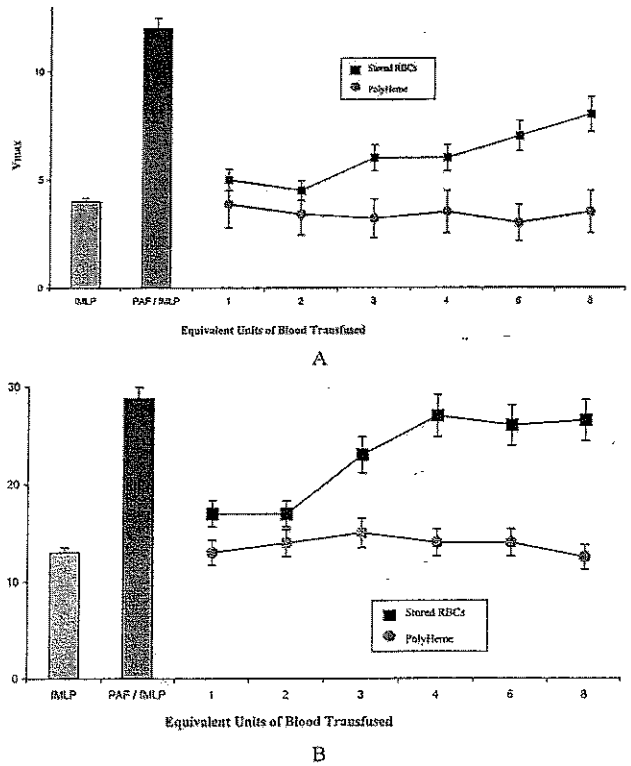


Figure 5. Isolated human neutrophils (PMNs) were incubated with either the plasma fraction from stored RBCs or PolyHeme at concentrations equivalent to one through eight units of acute transfusion. (A) PMN superoxide production; (B) PMN elastase release. fMLP, formyl-methionyl-leucyl-phenylalanine; PAF, platelet activiting factor.

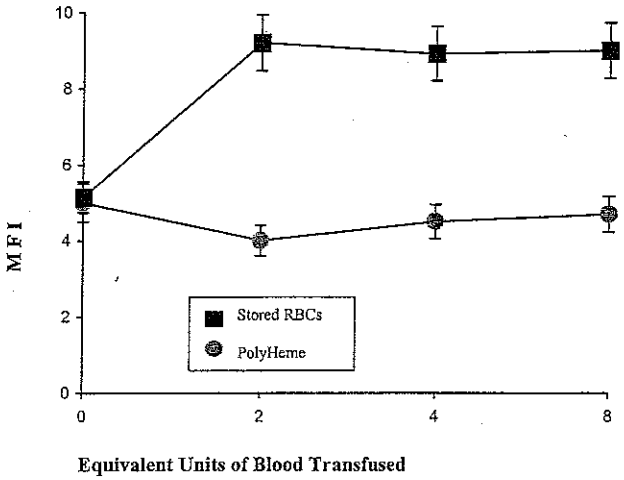


Figure 6. Human microvascular endothelial cells (HMVEC) were incubated with either the plasm a fraction from stored RBCs or PolyHeme at concentrations of 2, 4, and 8 units of acute transfusion. MFI, mean fluorescence intensity.

7.22 ± 0.04 versus 7.19 ± 0.08), and Hb transfusion in the first 24 hours (units = 14.1 ± 2.0 versus 14.5 ± 1). Circulating PMNs from patients resuscitated with stored RBCs manifested evidence of priming through increased CD11b/CD18 expression and enhanced superoxide production (Fig. 7). All patients in the PolyHeme group survived; three (30%) in the stored RBC group died of MOF.

To further investigate the impact of early resuscitation with PolyHeme in lieu of stored RBCs, we extended our clinical trial to evaluate the systemic levels of proinflammatory cytokines (IL_6, IL_8), counterregulatory cytokines (IL_{10}, IL_{11}), and markers of endothelial activation (sICAM, sE-selectin).[128] The study groups (stored RBC [n = 7] versus PolyHeme [n = 18]) were comparable with respect to injury severity. Patients resuscitated with stored RBCs had higher levels of the proinflammatory cytokines IL_6 and IL_8, and higher levels of the counterregulatory cytokine IL_{10} (Fig. 8), with a trend toward higher sICAM, and sE-selectin levels. We have not enrolled a sufficient number of injured patients to definitively address the ultimate study objective—reduction of postinjury MOF. But the incidence of MOF in the acutely injured patients given PolyHeme during their initial resuscitation for whom we had complete data (n = 20) was 15%, contrasted with a predicted incidence of 37% ($p < 0.05$) based on our MOF prediction model (age × 33.25, ISS × 27.25, units × 18.05, base deficit × 8.94, lactate × 4.30).[65] In sum, these clinical trials in trauma patients suggest that PolyHeme, used in the early resuscitation of patients with hemorrhagic shock, will attenuate the immunodysfunction associated with stored RBC transfusion and reduce the risk of postinjury MOF.

Acute hemorrhagic shock: When stored RBCs are unavailable

The most compelling indication for an HBOC is the scenario in which stored RBCs are unavailable. This potential benefit for military use has largely driven the development of HBOCs, but there are also a number of key applications in civilian trauma care. Most conspicuous is the role in prehospital care, particularly for extended transport times. But there are also remote hospitals throughout the country in which stored blood is simply not available or is rapidly depleted when multiple casualties are encountered. There have been a number of well-designed animal models that strongly suggest prehospital low-volume resuscitation with HBOCs can save

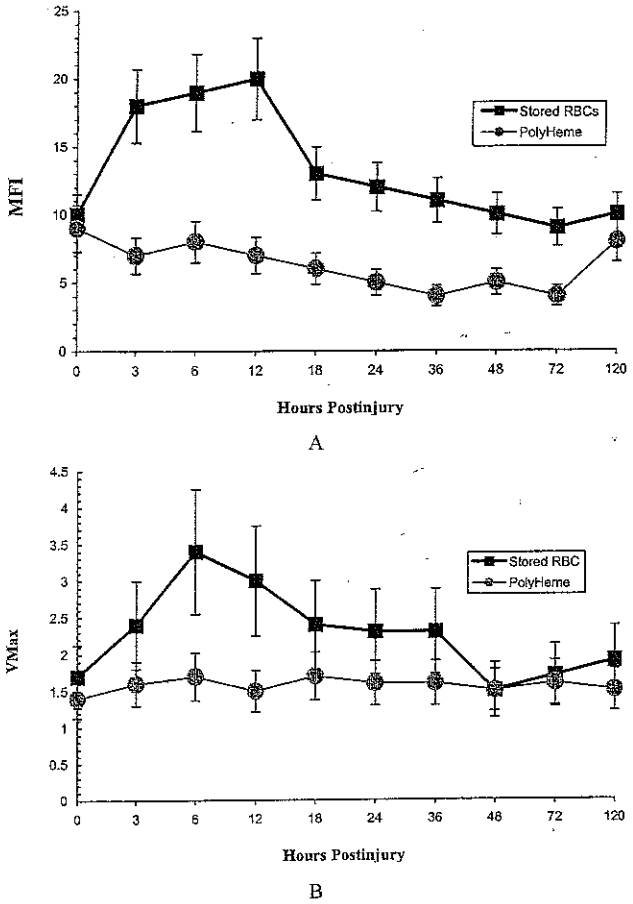


Figure 7. Circulating neutrophils (PMNs) from injured patients who underwent initial resuscitation with either stored RBCs or PolyHeme. (A) PMN CD11/CD18 receptor expression; (B) PMN superoxide production. (From: Johnson JL, Moore EE, Offner PJ, et al. Resuscitation with a blood substitute abrogates pathologic postinjury neutrophil cytotoxic function. J Trauma 2001;50:449–456, with permission.)

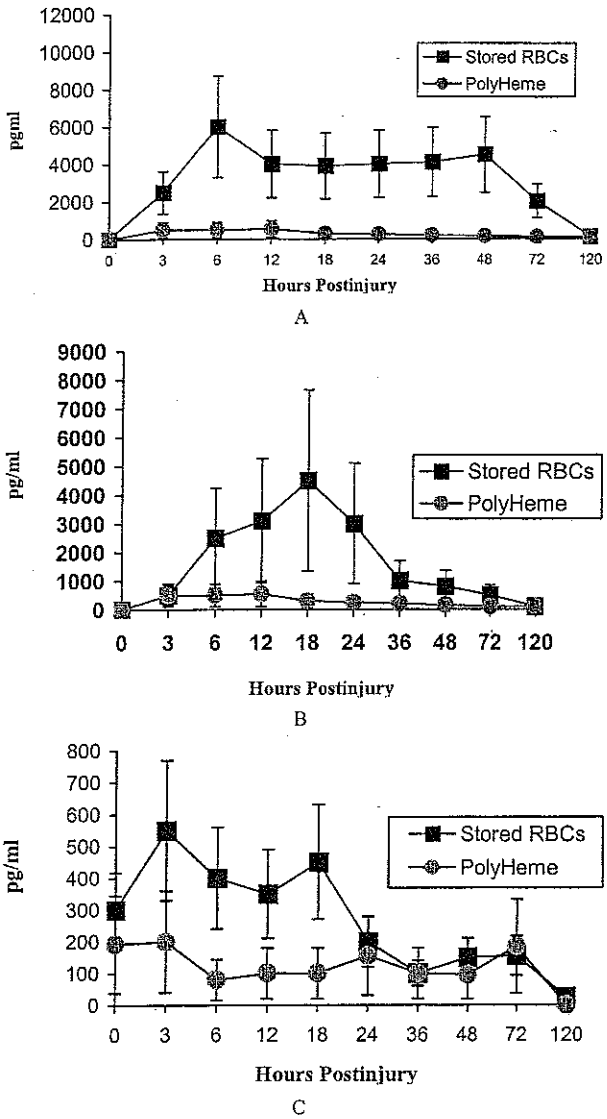


Figure 8. Systemic interleukin IL_6, IL_8, and IL_{10} from injured patients who underwent initial resuscitation with either stored RBCs or PolyHeme. (A) IL_6, (B) IL_8, and (C) interleukin 10 IL_{10}. (From: Johnson JL, Moore EE, Gonzalez RJ, et al. Alteration of the postinjury hyperinflammatory response via resuscitation with a red cell substitute. J Trauma [in press]; with permission.)

lives. Despite the appeal, the scientific design and ethical conduct of clinical trials to establish efficacy of HBOCs when RBCs are unavailable remain a challenge. To best approximate this scenario, we compared the 30-day mortality in 171 trauma patients given up to 20 units (1,000 g) of PolyHeme, compared with a historic control of 300 surgical patients who refused stored RBCs on religious grounds.[6] The trauma patients received rapid infusion of 1 to 2 units (n = 45), 3 to 4 units (n = 4.5), 5 to 9 units (n = 47), or 10 to 20 units (n = 34) of PolyHeme; 40 patients had a nadir RBC Hb $\leq$ 3 g% (mean = 1.5 $\pm$ 0.7 g%). Total Hb was adequately maintained (mean = 6.8 $\pm$ 1.2 g%) by plasma Hb added by PolyHeme. The 30-day mortality was 25.0% (10 of 40 patients), compared with 64.5% (20 of 31 patients) in the control patients at these RBC Hb levels (Fig. 9).

I want to share a personal experience with PolyHeme in our institution that convinced me the time has arrived for the FDA approval of an HBOC for trauma care. An 18-year-old man arrived by ground ambulance at our emergency department in extremis after a gunshot wound to the abdomen with a high-velocity elk-hunting rifle (30.06, hollow soft point 220 gr, muzzle energy 2,840 ft/lb). Because of immediate availability, 10 units

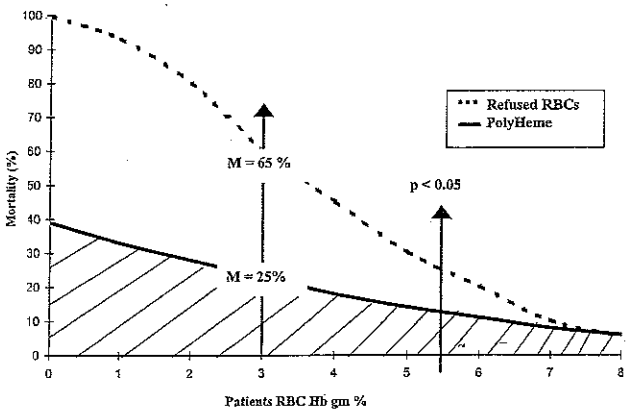


Figure 9. The 30-day mortality (M) is compared in patients who refused stored RBC transfusion versus injured patients who were initially resuscitated with PolyHeme. Mortality was significantly less in the PolyHeme group when RBCHb ≤ 5.3 g%. (From: Gould SA, Moore EE, Hoyt DB, et al. The life-sustaining capacity of human polymerized hemoglobin when red cells might be available. J Am Coll Surg [in press]; with permission.)

of PolyHeme were administered during the first 14 minutes of in-hospital resuscitation, representing greater than 91% of total circulating Hb at end infusion (RBCHb = 0.7 g%). The missile entered the left mid-abdomen and exited directly posteriorly. At laparotomy, we encountered an avulsed shattered left kidney with secondary aortic and vena caval perforations, a partially transected superior mesenteric vein, and destructive injuries to his distal duodenum, proximal jejunum, mid-ileum, and descending and sigmoid colon. In addition, he had massive soft tissue loss in the retroperitoneum, including the psoas and paraspinous muscles, and suffered a concussive spinal cord lesion with resultant paraplegia. The patient received an additional 40 units of packed RBCs during initial laparotomy but ultimately this gentleman survived to discharge without organ failure. We believe the immediate infusion of this HBOC was pivotal in maintaining sufficient O_2 delivery during the critical period of massive blood loss to save this man's life.

Hemoglobin-based oxygen carriers in trauma care: the future is now

War on terrorism

I encourage the ongoing collaboration of the US military, FDA, and private industry to deploy HBOCs for use in the current Middle East conflict. (Northfield Laboratories and the US Army are working together on a treatment investigational new drug application for combat casualties.) The US Army recognizes an evolving paradigm shift to a "dispersed battlefield," where prolonged evacuation will become the rule rather than exception. The reduction in overall mortality from combat wounds in our lifetime has been largely from improved care after evacuation from the battlefield. Bellamy[129] has projected that the percentage of wounded soldiers who die in the field (killed in action) will increase from 20% with evacuation times less than 2 hours, to 26% with 6 hours, to 32% with 24 hours. The majority of soldiers killed in action are anticipated to die from acute blood loss and the sequelae of hemorrhagic shock when evacuation is delayed. Kaytchuch and Sullivan[130] estimate that more than one-third of combat casualties are potentially salvageable with earlier intervention at the point of wounding or far-forward in the continuum of care. One-fifth of modern battlefield deaths attributable to acute blood loss are from compressible wounds; ie, those accessible for direct pressure. The US Army has already devised methods to improve survival in these soldiers, including more efficient multitiered combat medical assistance, hemostatic pressure bandages, and more effective tourniquets.

Addressing the noncompressible life-threatening wounds (chest, abdomen, and pelvis) remains a challenge. Enhanced hemostasis through intraosseous or intravenous infusion of procoagulants (factor VII a, factor Xa, phospholipids, desmopressin, etc) and antifibrinolytics (aprotinin, ϵ-aminocaproic acid, tranexamic acid, etc) is an attractive concept, but restoring O_2-carrying capacity is equally compelling. Perhaps a combination of these modalities will be more effective.[131]

Homeland security

Preparing for disaster is now virtually a daily exercise in trauma centers throughout the country.[132] Although systematic planning for chemical, biologic, and nuclear weapons is clearly essential,[133] it is important to remember that the incomprehensible devastation and widespread chaos generated by powerful explosions have made this the most common means of inflicting terror worldwide.[134] The events of 9/11 illustrated the fragility and inflexibility of the current blood reserve in the US. Extensive blood donation represented impressive public support for victims of the World Trade Center and Pentagon calamities, but relatively little blood was used because of the extreme lethality of the massive detonations and building collapses. In addition, the end result was

the loss of massive quantities of human blood that became outdated. At the World Trade Center, where more than 3,000 were killed, the dead-to-wounded ratio was an astounding 5 to 1. The blast outside the Murray Federal Building in Oklahoma City, by contrast, resulted in 167 deaths with a dead-to-wounded ratio of nearly 1 to 5.[135] This relative proportion of seriously injured patients is more typical of natural disasters, such as earthquakes.[136] So, the acute demand for blood will vary considerably depending on the inciting mechanism, population density, physical setting, and access to trauma care. The universal compatibility and shelf-life of HBOCs of more than 1 year make them particularly attractive in disaster preparation, along with the stockpile of antibiotics, antidotes, and immunizations.

Civilian trauma care

The existing safety profile and emerging data on efficacy for HBOCs (Table 1) warrant continued active clinical investigation in the three areas outlined by the FDA (Table 3). The pressing question is: Is it time for the FDA to approve one or more of these HBOCs for clinical use? I submit that the current generation of HBOCs can save lives and should be licensed, in limited quantities, for scenarios where stored blood is not available within the hospital.

Compassionate use when blood could not be matched[137] or for critically injured patients who refused blood on a religious basis[138] is very compelling. We recently transfused 18 units of PolyHeme, over 2 weeks, into a 39-year-old Jehovah's Witness and mother of four young children. This woman was transferred to our institution with a Hb = 2 g%, heart rate > 135/minute, and acute ST segment depression on her electrocardiogram after a class III abruptio placentae with fetal demise, disseminated intravascular cogulation, ARDS, and ruptured uterus. She survived urgent abdominal hysterectomy and had an otherwise uneventful recovery (manuscript in progress).

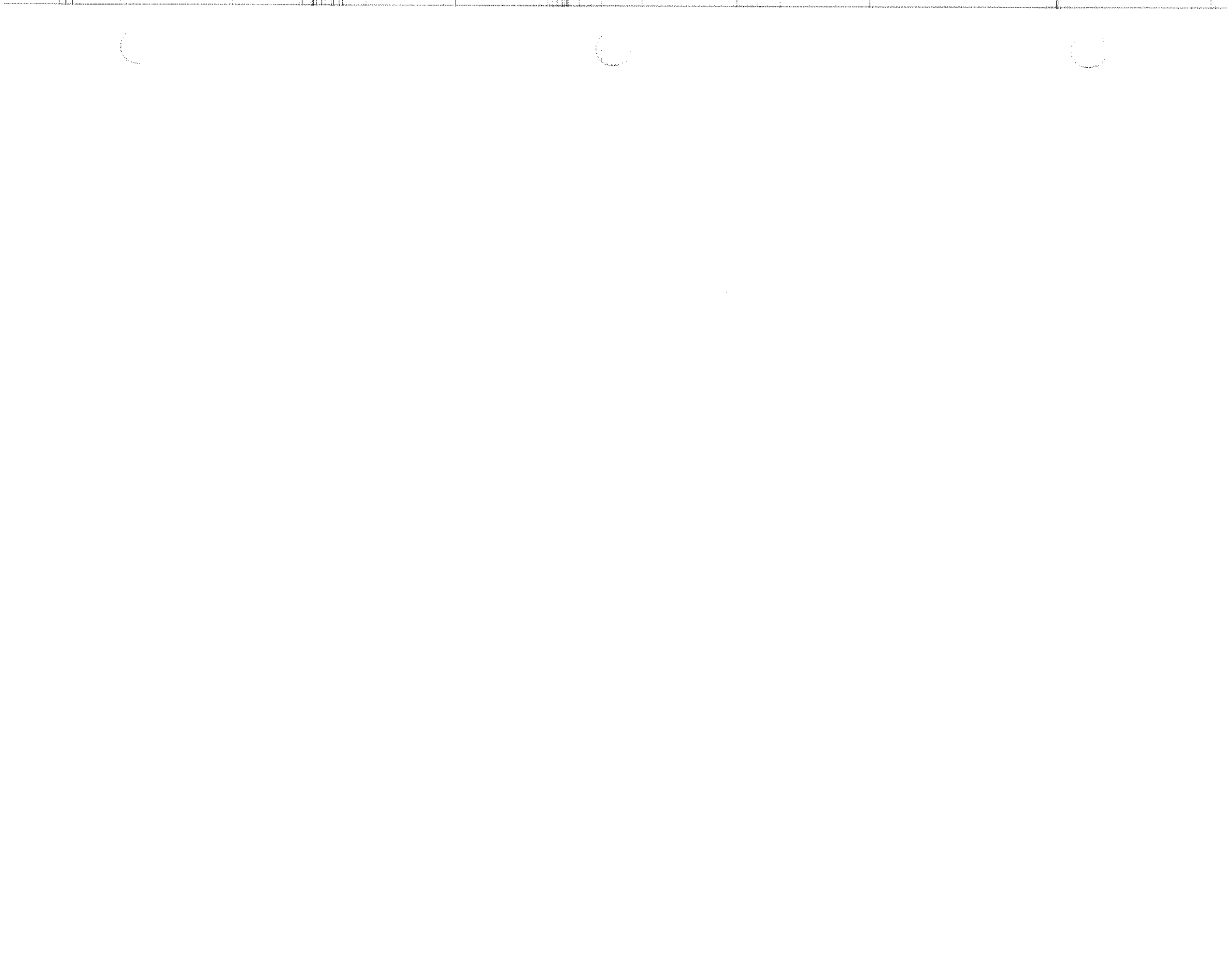
Trauma surgeons will attest that delays in blood availability for acute life-threatening hemorrhagic shock occur in urban trauma centers today, and there are certainly remote hospitals where multiple casualties can rapidly deplete the blood reserve. There is always concern about off-label use and abuse, but this is a challenge with any product newly licensed by the FDA. Extension of clinical investigation to prehospital care is well founded by appropriately designed animal models. The Israel Defense Forces Medical Corps has been administering type O packed RBCs for more than a decade.[139] But a major impediment to establishing the scientific basis for the appropriate use of HBOCs in the field is waiver of informed consent.[140,141] Unfortunately, acutely injured patients who are most likely to benefit from prehospital infusion of HBOCs are incapable of providing informed consent and proxy consent is logistically impractical. Undoubtedly, the trauma experience with DCL Hb will encumber the waiver of consent issue. Another confounder is the study control; ie, when is it ethical to withhold treatment that is proved beneficial to the patient?[142] Finally, the potential efficacy of HBOCs expands well beyond the temporary replacement for stored RBCs. Hemoglobin solutions might ultimately prove superior in delivering O_2 to ischemic or injured tissue.[143] An undisputable byproduct of the intense competition to license HBOCs for clinical use is the enhanced knowledge of the fundamental physiology of hemoglobin. Although the current generation of HBOCs can save lives today, the next generation might be biochemically tailored for specific clinical indications.[144]

In sum, the development of HBOCs spans 70 years but the progress in the last decade has been meteoric. Indeed, with FDA approval, this might represent the next chapter in transfusion medicine as blood banking worldwide has evolved from whole blood to component therapy and now the integration of HBOCs. As academic trauma surgeons, my colleagues and I have had the unique opportunity to participate in the translation of basic science to saving lives; that is the ultimate reward of academic medicine.

Acknowledgment: I am indebted to: Dr Steven A Gould and Northfield Laboratories for the unique opportunity to study PolyHeme; Biopure Corporation and Hemosol, Inc, for providing comprehensive reference lists for their respective products; trauma colleagues at Denver Health Medical Center (Jeffrey L Johnson, MD, Walter L Biffl, MD, Jon M Burch, MD, David J Ciesla, MD, C Clay Cothren, MD, Reginald J Franciose, MD, John B Moore, MD, David A Partrick, MD, James B Haenel, RRT, and Jeffrey S Long, RRT) for their active participation in the bench and clinical investigation of PolyHeme; research colleagues at University of Colorado Health Science Center (Christopher C Silliman, MD, PhD, Alden H Harken, MD, and Anirban Banerjee, PhD) for their invaluable collaboration; and Victoria Martin for illustration and manuscript preparation.

REFERENCES

1. High school massacre—Columbine bloodbath leaves up to 25 dead. The Denver Post. April 21, 1999.
2. Day of infamy—twin terrors. Time. September 11, 2001.
3. The valley of death—Inside the war's bloodiest battle. Time. March 18, 2002.
4. Gould SA, Moore EE, Moore FA, et al. Clinical utility of human polymerized hemoglobin as a blood substitute after acute trauma and urgent surgery. J Trauma 1997;43:325–332.
5. Gould SA, Moore EE, Hoyt DB, et al. The first randomized trial of human polymerized hemoglobin as a blood substitute in acute trauma and emergent surgery. J Am Coll Surg 1998; 187:113–122.
6. Gould SA, Moore EE, Hoyt DB, et al. The life-sustaining capacity of human polymerized hemoglobin when red cells may be available. J Am Coll Surg (in press).
7. Alving B. Potential for synthetic phospholipids as partial platelet substitutes. Transfusion 1988;38:1004–1010.
8. Lee DH, Blajcham MH. Novel treatment modalities. New platelet preparations and substitutes. Br J Haematol 2001;114: 496–505.
9. Martinowitz U, Kenet G, Segal E, et al. Recombinant activated factor VII for adjunctive hemorrhage control in trauma. J Trauma 2001;51:431–439.
10. Schraber MA, Holcomb JV, Hedner U, et al. The effect of recombinant factor VII on coagulopathic pigs with grade V liver injuries. J Trauma 2002;53:252–259.
11. Hsia CCW. Respiratory function of hemoglobin. N Engl J Med 1998;338:239–247.
12. Szabo C, Billiar TR. Novel roles of nitric oxide in hemorrhagic shock. Shock 1999;12:1–9.
13. Gow AJ, Luchsinger BP, Pawloski JR, et al. The oxyhemoglobin reaction of nitric oxide. Proc Natl Acad Sci 1999;96:9027–9032.
14. Gow AJ, Stamler JS. Reactions between nitric oxide and hemoglobin under physiological conditions. Nature 1998;391: 169–173.
15. Stamler JS, Jia L, Eu JP, et al. Blood flow regulation by S-nitrosohemoglobin in the physiologic oxygen gradient. Science 1997;276:2034–2020.
16. Gladwin MT, Ognibene FP, Pannell LK, et al. Relative role of heme nitrosylation and B-cysteine 93 nitrosation in the transport and metabolism of nitric oxide by hemoglobin in the human circulation. Proc Natl Acad Sci 2000;97:9943–9948.
17. Sellards AW, Minot GR. Injection of hemoglobin in man and its relation to blood destruction, with special reference to anemias. J Med Res 1916;34:469–494.
18. Amberson WR, Mulder AG, Steggerda FR, et al. Mammalian life without red blood corpuscles. Science 1933;78:106–107.
19. Mulder AG, Amberson WR, Steggeida FR, et al. Oxygen consumption with hemoglobin-Ringer. J Cell Comp Physiol 1934;5:383–397.
20. Amberson WR, Jennings JJ, Rhode CM. Clinical experience with hemoglobin-saline solutions. J Appl Physiol 1949;1:469–489.
21. Brandt JL, Frank NR, Lichtman HC. The effects of hemoglobin solutions on renal functions in man. Blood 1951;6:1152–1158.
22. Miller JH, McDonald RK. The effect of hemoglobin on renal function in the human. J Clin Invest 1951;30:1033–1040.
23. Moss GS, De Woskin R, Cochin A. Stroma-free hemoglobin—Preparation and observations on in vitro changes in coagulation. Surgery 1973;74:198–203.
24. Peskin GW, O'Brien K, Rabiner SF. Stroma-free hemoglobin solution—the "ideal" blood substitute. Surgery 1969;66:185–193.
25. Rabiner SF, Helbert JR, Lopas H, et al. Evaluation of a stroma-free hemoglobin solution for use as a plasma expander. J Exp Med 1967;126:1127–1142.
26. Rabiner SF, O'Brien K, Peskin GW, Friedman LH. Further studies with stroma-free hemoglobin solution. Ann Surg 1970; 171:615–622.
27. De Venuto F, Moores WY, Zegna AI, et al. Total and partial blood exchange in the rat with hemoglobin prepared by crystallization. Transfusion 1977;17:555–556.
28. Savitsky JP, Doczi J, Black J, Arnold JD. A clinical safety trial of stroma-free hemoglobin. Clin Pharmacol Ther 1978;23: 73–80.
29. Greenberg AG, Hayashi R, Siefert I, et al. Intravascular persistence and oxygen delivery of pyridoxylated stroma-free hemoglobin during gradations of hypotension. Surgery 1979;86: 13–16.
30. Sehgal LR, Rosen AL, Noud G, et al. Large volume preparation of pyridoxylated hemoglobin with high P_{50}. J Surg Res 1981; 30:14–20.
31. Chatterjee R, Wetty EV, Walder RY, et al. Isolation and characterization of a new hemoglobin derivative cross-linked between and ∂ chains. J Biol Chem 1986;261:9929–9937.
32. Przybelski RJ, Malcolm DS, Burris DG, et al. Cross-linked hemoglobin solution as a resuscitation fluid after hemorrhage in the rat. J Lab Clin Med 1991;117:143–151.
33. Looker DD, Abbott-Brown D, Cozart P, et al. A human recombinant hemoglobin designed for use as a blood substitute. Nature 1992;356:258–260.
34. Siegel JH, Fabian M, Smith JA, et al. Use of recombinant hemoglobin solution in reversing lethal hemorrhagic hypovolumic oxygen debt shock. J Trauma 1997;42:199–212.
35. Bunn HF. Differences in the interaction of 2-3 diphosphoglycerate with certain mammalian hemoglobins. Science 1971; 172:1049–1050.
36. Vlahakes GJ, Lee R, Jacobs EE, et al. Efficacy of a new blood substitute based on ultrapure polymerized bovine hemoglobin. Eur J Cardiothoracic Surg 1989;3:353–354.
37. Ajisaka K, Iwashita Y. Modification of human hemoglobin with polyethylene glycol—a new candidate for blood substitute. Biochem Biophys Res Commun 1983;97:1076–1081.
38. Matsushita M, Yabuki A, Melchesky PS, et al. In vivo evaluation of a pyridoxylated hemoglobin-polyoxyethylene conjugate. Biomater Artf Cells Artf Organs 1988;16:247–260.
39. Rabinovici R, Rudolph AS, Feuerstein G. Characterization of hemodynamic, hematologic, and biochemical responses to administration of liposome encapsulated hemoglobin in the conscious freely moving rat. Circ Shock 1989;29:115–132.
40. Rabinovici R, Rudolph AS, Vernick J, et al. Lyophilized liposome encapsulated hemoglobin—evaluation of hemodynamic, biochemical and hematologic responses. Crit Care Med 1994; 22:480–485.
41. Moss GS, Gould SA, Sehgal LR, et al. Hemoglobin solution—From tetramer to polymer. Surgery 1984;95:249–255.
42. Carmichael FJL, Ali ACY, Campbell JA, et al. A phase I study of oxidized raffinose cross-linked human hemoglobin. Crit Care Med 2000;28:2283–2292.



43. Gould SA, Moss GS. Clinical development of human polymerized hemoglobin as a blood substitute. World J Surg 1996;20: 1200–1207.
44. Department of Health and Human Services: 21CFR601: 25 (d) (2).
45. Center for Biologics Evaluation and Research. Points to consider in the safety evaluation of hemoglobin-based oxygen carriers. Transfusion 1991;31:369–371.
46. Alayash AI. Hemoglobin-based blood substitutes—oxygen carriers, pressor agents, or oxidants? Nature Biotech 1999;17: 545–549.
47. Creteur J, Sibbald W, Vincent JL. Hemoglobin solutions—Not just red blood cell substitutes. Crit Care Med 2000;28: 3025–3034.
48. McFaul SJ, Bowman PD, Villa VM. Hemoglobin stimulates the release of proinflammatory cytokines from leukocytes in whole blood. J Lab Clin Med 2000;135:263–269.
49. Center for Biologics Evaluation and Research. Points to consider on efficacy evaluation of hemoglobin and perfluorocarbon based oxygen carriers. Transfusion 1994;34:712–713.
50. Weiskopf RB, Viele MK, Feiner J, et al. Human cardiovascular and metabolic response to acute, severe isovolemic anemia. JAMA 1998;279:217–221.
51. Practice guidelines for blood component therapy—a report by the American Society of Anesthesiologists Task Force on Blood Component Therapy. Anesthesiology 1996;84:732–747.
52. National Institutes of Health Concensus Conference—perioperative red blood cell transfusion. JAMA 1988;260: 2700–2703.
53. Wu WC, Rathore SS, Wang Y, et al. Blood transfusion in elderly patients with acute myocardial infarction. N Engl J Med 2001;345:1230–1236.
54. Schrieber GB, Busch MP, Kleinman SH, et al. The risk of transfusion-transmitted viral infections. N Engl J Med 1996; 334:1685–1690.
55. Opelz G, Mickey MR, Sengr DPS, et al. Effect of blood transfusion on subsequent kidney transplants. Transplant Proc 1973;5:253–259.
56. Burrows L, Tarrter P. Effect of blood transfusions on colonic malignancy recurrence rate. Lancet 1982;18:662.
57. Hyman NH, Foster RS, DeMeules JE, et al. Blood transfusions and survival after lung cancer resection. Am J Surg 1985;149: 502–508.
58. Nowak Mm, Ponsky JL. Blood transfusion and disease-free survival in carcinoma of the breast. J Surg Oncol 1984;27: 124–127.
59. Agarwal N, Murphy JG, Cayten CG, et al. Blood transfusion increases the risk of infection after trauma. Arch Surg 1993; 128:171–177.
60. Graves TA, Cioffi WG, Mason AD, et al. Relationship of transfusion and infection in a burn population. J Trauma 1989;29: 948–952.
61. Offner PJ, Moore EE, Biffl WL, et al. Increased rate of infection associated with transfusion of old blood after severe injury. Arch Surg 2002;137:711–717.
62. Kao KJ. Mechanisms and new approaches for the allogeneic blood transfusion-induced immunomodulatory effects. Transfus Med Rev 2000;14:12–22.
63. Kirkley SA. Proposed mechanisms of transfusion-induced immunomodulation. Clin Diag Lab Immun 1999;6:652–657.
64. Botha AJ, Moore FA, Moore EE, et al. Postinjury neutrophil priming and activation states—therapeutic challenges. Shock 1995;3:157–166.
65. Sauaia A, Moore FA, Moore EE, et al. Early predictors of postinjury multiple organ failure. Arch Surg 1994;129:39–45.
66. Moore FA, Moore EE, Sauaia A. Blood transfusion—an independent risk factor for postinjury multiple organ failure. Arch Surg 1997;132:620–625.
67. Botha AJ, Moore FA, Moore EE, et al. Postinjury neutrophil priming and activation-An early vulnerable window. Surgery 1995;118:358–365.
68. Zallen G, Moore EE, Tamura DY, et al. Postinjury neutrophil priming in severely injured patients is mediated by p38 mitogen activated protein kinase activation. Surg Forum 1998; XLIX:100–101.
69. Zallen G, Moore EE, Johnson JL, et al. Circulating postinjury neutrophils are primed for the release of proinflammatory cytokines. J Trauma 1999;46:42–48.
70. Biffl WL, Moore EE, Zallen G, et al. Neutrophils are primed for cytotoxicity and resist apoptosis in injured patients at risk for multiple organ failure. Surgery 1999;126:198–202.
71. Bordin JO, Heddle NM, Blajchman MA. Biologic effects of leukocytes present in transfused cellular blood products. Blood 1994;84:1703–1721.
72. Dzik S, Aubuchon J, Jeffries L, et al. Leukocyte reduction of blood components—public policy and new technology. Transfus Med Rev 2000;14:34–52.
73. Vamvakas EC, Blajchman MA. Prestorage versus poststorage white cell reduction for the prevention of the deleterious immunomodulatory effects of allogeneic blood transfusions. Transfus Med Rev 2000;14:23–33.
74. Zallen G, Offner PJ, Moore EE, et al. Age of transfused blood is an independent risk factor for postinjury multiple organ failure. Am J Surg 1999;178:570–572.
75. Nielsen HJ, Reimert CM, Pedersen AM, et al. Time-dependent spontaneous release of white cell and platelet derived bioactive substances from stored human blood. Transfusion 1996;36:960–965.
76. Shanwell A, Kristiansson M, Remberger M, et al. Generation of cytokines in red cell concentrates during storage is prevented by prestorage white cell reduction. Transfusion 1997;36:678–684.
77. Aiboshi J, Moore EE, Ciesla DJ, et al. Blood transfusion and the two-insult model of postinjury multiple organ failure. Shock 2001;15:302–306.
78. Silliman CC, Johnson CA, Clay KL, et al. Compounds biologically similar to platelet activating factor are present in stored blood components. Lipids 1993;28:415–418.
79. Silliman CC, Clay KL, Thurman GW, et al. Partial characterization of lipids that develop during the routine storage of blood and prime the neutrophil NADPH oxidase. J Lab Clin Med 1994;124:684–694.
80. Kopko PM, Marhall CS, MacKenzie MR, et al. Transfusion-related acute lung injury—report of a clinical look-back investigation. JAMA 2002;287:1968–1971.
81. Silliman CC, Paterson AJ, Dickey WO, et al. The association of biologically active lipids with the development of transfusion-related acute lung injury. Transfusion 1997;37: 719–726.
82. Silliman CC, Voelkel NF, Allard JD, et al. Plasma and lipids from stored packed red blood cells cause acute lung injury in an animal model. J Clin Invest 1998;101:1458–1467.

83. Biffl WL, Moore EE, Offner PJ, et al. Plasma from aged stored red blood cells delays neutrophil apoptosis and primes for cytotoxicity—abrogation by poststorage washing but not prestorage leukoreduction. J Trauma 2001;50:426–432.
84. Berezina TL, Zaets SB, Morgan C, et al. Influence of storage on red blood cell rheologic properties. J Surg Res 2002;102:6–12.
85. Fitzgerald RD, Martin CM, Diltz GE, et al. Transfusing red blood cells stored in citrate phosphate dextrose adenine 1 for 28 days fails to improve tissue oxygenation in rats. Crit Care Med 1997;25:726–732.
86. Doyle MP, Walker BR. Stiffened erythrocytes augment the pulmonary hemodynamic response to hypoxia. J Appl Physiol 1990;69:1270–1275.
87. Parthasarathi K, Lipowsky HH. Capillary recruitment in response to tissue hypoxia and its dependence on red blood cell deformability. Am J Physiol 1999;277:H2145–H2157.
88. Marik PE, Sibold WJ. Effect of stored—blood transfusion on oxygen delivery in patients with sepsis. JAMA 1993;269: 3024–3029.
89. Shoh DM, Gottlieb ME, Rahm RL, et al. Failure of red blood cell transfusion to increase oxygen transport or mixed venous PO_2 in injured patients. J Trauma 1982;22:741–746.
90. Simchon S, Jan KM, Clien C. Influence of reduced red cell deformability on regional blood flow. Am J Physiol 1987;253: H898–H903.
91. Sloan EP, Koenigsberg M, Gens D, et al. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock—a randomized controlled efficacy trial. JAMA 1999;282:1857–1864.
92. Sloan EP, Koeigsberg M, Burnett PA, et al. Post hoc mortality analysis of the efficacy trial of diaspirin cross-linked hemoglobin in the treatment of severe traumatic hemorrhagic shock. J Trauma 2002;52:887–895.
93. Hess JR, Macdonald VW, Brinkley WW. Systemic and pulmonary hypertension after resuscitation with cell-free hemoglobin. J Appl Physiol 1993;74:1769–1778.
94. Poli de Figueiredo LF, Mathree M, Solanki D, et al. Pulmonary hypertension and systemic vasoconstiction may offset the benefits of acellular hemoglobin blood substitutes. J Trauma 1997; 42:847–856.
95. Cohn SM, Zieg PM, Rosenfield A, et al. Resuscitation of pulmonary contusions—effects of a red cell substitute. Crit Care Med 1997;25:484–491.
96. Reah G, Bodenham AB, Mallick A, et al. Initial evaluation of diaspirin cross-linked hemoglobin (DCLHb) as a vasopressor in critically ill patients. Crit Care Med 1997;25:1480–1488.
97. Gulati A, Sen AP, Sharma AC, et al. Role of ET and NO in resuscitative effect of diaspirin cross-linked hemoglobin after hemorrhage in rat. Am J Physiol 1997;273:H827–836.
98. Katsuyama SS, Cole DJ, Drummond JC, et al. Nitric oxide mediates the hypertensive response to a modified hemoglobin solution (DCLHb) in rats. Artif Cells Blood Substit Immobil Biotechnol 1994;22:1–7.
99. Schultz SC, Grody B, Cole F, et al. A role for endothelin and nitric oxide in the pressor response to diaspirin cross-linked hemoglobin. J Lab Clin Med 1993;122:301–308.
100. Fushitan K, Imai K, Riggs AF. Oxygen properties of hemoglobin from the earthworm *Lumbricus terrestric*. J Biol Chem 1986;261:8414–8423.
101. Hirsch RE, Jelicks LA, Wittenberg BA, et al. A first evaluation of the natural high molecular weight polymeric *Lumbricus terrestric* hemoglobin as an oxygen carrier. Artif Cells Blood Substit Immobil Biotechnol 1997;25:429–444.
102. Levy JH, Goodnough LT, Greilich P, et al. Polymerized bovine hemoglobin solution as a replacement for allogeneic red blood cell transfusion after cardiac surgery: results of a randomized, double-blind trial. J Thorac Cardiovasc Surg 2002;124:35–42.
103. LaMuraglia GM, O'Hara PJ, Baker WH, et al. The reduction of the allogenic transfusion requirement in aortic surgery with hemoglobin-based solution. J Vasc Surg 2000;31:299–308.
104. Standl T, Burmeister MA, Horn EP, et al. Bovine haemoglobin-based oxygen carrier for patients undergoing haemodilution before liver resection. Br J Anaesth 1998;80:189–194.
105. Kasper SM, Walter M, Grune F, et al. Effects of a hemoglobin-based oxygen carrier (HBOC-201) on hemodynamics and oxygen transport in patients undergoing preoperative hemodilution for elective abdominal aortic surgery. Anesth Analg 1996; 83:921–927.
106. Hughes GS, Antal EJ, Locker PK, et al. Physiology and pharmacokinetics of a novel hemoglobin-based oxygen carrier in humans. Crit Care Med 1996;24:756–764.
107. Page TC, Light WR, McKay CB, et al. Oxygen transport by erythrocyte/hemoglobin solution mixtures in an in vitro capillary as a model of hemoglobin-based oxygen carrier performance. Microvasc Res 1998;55:54–64.
108. Horn EP, Standl T, Wilhelm S, et al. Bovine hemoglobin increases skeletal muscle oxygenation during 95% artificial arterial stenosis. Surgery 1997;121:411–418.
109. Standl T, Horn P, Wilhelm S, et al. Bovine haemoglobin is more potent than autologous red blood cells in restoring muscular tissue oxygenation after profound isovolemic haemodilution in dogs. Can J Anaesth 1996;43:714–723.
110. Manning JE, Katz LM, Brownstein MR, et al. Bovine hemoglobin-based oxygen carrier (HBOC-201) for resuscitation of uncontrolled, exsanguinating liver injury in swine. Shock 2000;13:152–159.
111. McNeil JD, Smith DL, Jenkins DH, et al. Hypotensive resuscitation using an polymerized bovine-based oxygen carrying solution leads to reversal of anaerobic metabolism. J Trauma 2001;50:1063–1075.
112. Lee R, Neya K, Svizzero TA, Vlahakes GJ. Limitations of the efficacy of hemoglobin-based oxygen-carrying solutions. J Appl Physiol 1995;79:236–242.
113. Knudson MM, Lee S, Erickson V, et al. Tissue oxygen monitoring during hemorrhagic shock and resuscitation—a comparison of lactated Ringer's solution, hypertonic saline and HBOC-201. J Trauma (in press).
114. Koch T, Duncker HP, Heller A, et al. Effects of stroma-free hemoglobin solutions of pulmonary vascular resistance and mediator release in the isolated perfused rabbit lung. Shock 1994;1:146–152.
115. Carmichael FJ, Biro GP, Agensky L, et al. The safety and efficacy of the red substitute Hemolink in patients undergoing elective coronary artery bypass surgery. Blood 1999;94:1166.
116. Greenburg AG, Mazer CD. The use of an oxygen therapeutic as an adjunct to intraoperative autologous donation to reduce transfusion requirements in patients undergoing CABG surgery. Presented at the American College of Surgeons Clinical Congress, San Francisco, CA, Oct 2002.
117. Lieberthal W, Fuhro R, Freedman JE, et al. Raffinose cross-linking markedly reduces systemic and renal vasoconstrictor effects of unmodified human hemoglobin. J Pharmacol Exp Ther 1999;288:1278–1287.

118. Ning J, Wong LT, Christoff B, et al. Hemodynamic response following a 10% top load infusion of Hemolink in conscious, anesthetized and treated spontaneously hypertensive rats. Transfus Med 200;10:13–22.
119. Kerger H, Tsai AG, Saltzman DJ, et al. Fluid resuscitation with O_2 vs non O_2 carriers after 2 h of hemorrhagic shock in conscious hamsters. Am J Physiol 1997;272:H525–H537.
120. Whitley D, Patterson R, Greenburg AG, et al. Cell-free hemoglobin preserves renal function during normothermic ischemia. J Surg Res 1998;77:187–191.
121. Rohifs RJ, Bruner E, Chiu A, et al. Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide. J Biol Chem 1998;273:12128–12134.
122. Gould SA, Sehgal LR, Sehagl HL, et al. The development of hemoglobin solutions as red cell substitutes—hemoglobin solutions. Transfus Sci 1995;16:5–17.
123. Johnson JL, Moore EE, Offner PJ, et al. Resuscitation of the injured patient with polymerized stroma-free hemoglobin does not produce systemic or pulmonary hypertension. Am J Surg 1998;176:612–617.
124. Partrick DA, Moore EE, Barnett CC, et al. Human polymerized hemoglobin as a blood substitute avoids transfusion-induced neutrophil priming. Surg Forum 1996;XLVII:36–38.
125. Partrick DA, Moore EE, Barnett CC, et al. Human polymerized hemoglobin as a blood substitute avoids transfusion-induced PMN priming for superoxide and elastase release. Shock 1997;7:24.
126. Aiboshi J, Moore EE, Zallen G, et al. Use of a polymerized hemoglobin blood substitute instead of store blood prevents endothelial cell activation. Surg Forum 1999;L:202–203.
127. Johnson JL, Moore EE, Offner PJ, et al. Resuscitation with a blood substitute abrogates pathologic postinjury neutrophil cytotoxic function. J Trauma 2001;50:449–456.
128. Johnson JL, Moore EE, Gonzalez RJ, et al. Alteration of the postinjury hyperinflammatory response via resuscitation with a red cell substitute. J Trauma (in press).
129. Bellamy RF. The causes of death in conventional land warfare—implications for combat casualty care research. Mil Med 1984;149:55–62.
130. Zajtchuk R, Sullivan GR. Battlefield trauma care—focus on advanced technology. Mil Med 1995;160:1–7.
131. Fischer TH, Merricks EP, Nichols TC, et al. The coinfusion of rehydrated, lyophilized platelets with HBOC-201 for hemostasis in dilutional thrombocytopenia. Blood 2001;98:538a.
132. Eiseman B. Combat casualty management for tomorrow's battlefield—urban terrorism. J Trauma 2001;51:821–823.
133. Giovachino M, Carey N. Modeling the consequences of bioterrorism response. Mil Med 2001;166:925–930.
134. Frykberg ER. Medical management of disasters and mass casualties from terrorist bombings—how can we hope? J Trauma 2002;53:201–212.
135. Mallonee S, Shariat S, Stennies G, et al. Physical injuries and fatalities resulting from the Oklahoma City bombing. JAMA 1996;276:382–387.
136. Schultz CH, Koenig KL, Noji EK. A medical disaster response to reduce immediate mortality after a earthquake. N Engl J Med 1996;334:438–444.
137. Mullon J, Giacoppe G, Clogett C, et al. Transfusions of polymerized bovine hemoglobin in a patient with severe autoimmune hemolytic anemia. N Engl J Med 2000;342:1638–1643.
138. Cothren CC, Moore EE, Johnson JL, et al. Combined blood substitute and erythropoietin therapy in a severely injured Jehovah's Witness. N Engl J Med 2002;346:1097.
139. Barkana Y, Stein M, Maor R, et al. Prehospital blood transfusion in prolonged evacuation. J Trauma 1999;46:176–180.
140. Department of Health and Human Services: 45 CFR 46:116 d.
141. McRae AD, Weijer C. Lessons from everyday lives—a moral justification for acute care research. Crit Care Med 2002;30: 1146–1151.
142. Huston P, Peterson R. Withholding proven treatment in clinical research. N Engl J Med 2001;345:912–913.
143. Chang TM. Oxygen carriers. Curr Opin Invest Drugs 2002; 3:1187–1190.
144. Winslow RM. Blood substitutes—a moving target. Nat Med 1995;1:1212–1215.