Hemoglobin-based Oxygen Carriers: First, Second or Third Generation? Human or Bovine? Where are we Now?

Lena M. Napolitano, MD, FACS, FCCP, FCCM,*

HEMOGLOBIN-BASED OXYGEN CARRIERS

The need for an alternative to allogeneic red blood cells (RBCs) for transfusion has been recognized for more than a century.¹–³ Concerns about the infectious and immunosuppressive risks of allogeneic blood products persist, and the increased disproportion between blood donation and consumption has reinforced the search for alternative erythrocyte transfusion strategies in recent years. The most serious motivation for the development of a blood substitute is the worldwide shortage of safe and viable allogeneic donor blood. A report on blood donations found that during 2001, 12.7% of hospitals reported cancellations of surgeries due to donor blood shortages and 18.9% reported shortages of blood for nonsurgical purposes.⁴ In addition, the stress on the donated blood supply is projected to increase in the coming years.⁵ Interestingly, even though blood transfusion has remained the standard of care, the efficacy and safety of allogeneic RBC therapy has never been rigorously tested via the clinical trial process.⁶–⁸ Thus, comparing the safety and efficacy of a blood substitute, hemoglobin-based oxygen carriers (HBOCs), to the standard of care may prove to be difficult. HBOCs are oxygen carriers that use purified human, animal, or recombinant hemoglobin (Hb) in a cell-free Hb preparation. They are infusible oxygen-carrying fluids that...
have long shelf lives, have no need for refrigeration or cross-matching, could be in abundant supply, and are ideal for treating hemorrhagic shock in remote settings where blood is not available. Despite significant effort in the development of HBOCs, currently no such product is approved for use in North America or Europe, although several are in the clinical trial stage. One product is approved for use outside of the United States. Hemopure (polymerized bovine Hb, Biopure Corporation, Cambridge, Massachusetts) is approved for use in South Africa for adult surgery patients to treat acute anemia and reduce allogeneic blood use.9

Hb is a logical choice for an RBC substitute because of its high capacity to carry oxygen and its oncotic properties.10 It also lacks the numerous and complex antigens of the RBC membrane, hence it is universally compatible. It is a robust molecule that withstands rigorous purification and viral inactivation processes, and it is stable under ordinary storage conditions.

The structure of Hb (Fig. 1) was determined in 1959 by Max Perutz, for which he was awarded a Nobel Prize. Human Hb is a 64-kDa tetrameric protein composed of two \( \alpha \) subunits and two \( \beta \)-globin subunits that fold into a compact quaternary structure (\( \alpha_2\beta_2 \)). Each \( \alpha \) and \( \beta \)-globin subunit contain an iron-heme group that binds to an oxygen molecule, allowing for transport. A fully saturated Hb molecule carries a maximum of four oxygen molecules. Environmental conditions such as \( \text{paO}_2 \), pH, temperature, and \( \text{paCO}_2 \) cause Hb to undergo conformational change from a high oxygen-affinity state to a lower oxygen-affinity state.

HBOCs are made by the lysis of RBCs releasing Hb molecules for purification and thorough sterilization and viral inactivation methods that are not possible with whole blood\(^{11} \) or by recombinant technology. The three potential sources for Hb to make HBOCs are outdated human RBCs, bovine RBCs, and recombinant Hb (Fig. 2). Only 5% to 10% of donated allogeneic blood becomes outdated, and therefore, the quantity of Hb available from this source may not be sufficient for mass production of an HBOC.\(^{12} \) Bovine Hb as a source for HBOCs has no quantity constraints.

Four main problems had to be overcome before Hb could be considered a serious candidate for a blood substitute. First, Hb in dilute solution is rapidly cleared by the

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**Fig. 1.** Structure of Hb. (From www.chem.prudue.edu/courses/chm333/hemoglobin.jpg)
Second, dissociable tetramers and dimers with resultant free Hb bind with nitric oxide, inducing vasoconstriction and renal dysfunction (Fig. 3). Third, dilute Hb has a high affinity for oxygen. Fourth, even exceedingly small amounts of stromal (cell membrane) contaminants in Hb solutions appear to be toxic.\(^\text{13}\)

Hemoglobin tetramer rapidly dissociates into dimers and monomers, gets filtered by the kidney, and causes potential damage to renal tubular cells. This issue was
resolved by polymerization of the free Hb molecules (Fig. 4). This advance also led to reduced vasoreactivity via reduced nitric oxide binding. Ongoing advances have significantly improved HBOCs and resolved many of the prior problems that had been noted with the early HBOC solutions.

With the absence of problems such as nephrotoxicity, increased colloid osmotic pressure, and sudden renal clearance, modern HBOCs have shown their effectiveness and tolerability in numerous animal studies and several clinical studies. HBOCs can be infused without prior cross-matching and are now available as stable formulations with a long shelf life. HBOCs may find application in differential indications, including as potent oxygen-delivering agents in addition to the globally recognized goal of being used as RBC substitutes in cases of significant bleeding.

Thus, eight different companies embarked on the development of an HBOC in the 1980s and 1990s. A number of HBOC products have been developed and have undergone preclinical and clinical testing (Table 1) as oxygen carriers and blood substitutes, although some have been discontinued related to safety issues. To date, only one product (Oxyglobin, polymerized bovine Hb, Biopure Corporation) is licensed for veterinary use and only one product (Hemopure) is approved for limited use in humans in South Africa when blood is not available.

In March 2006, a workshop sponsored by the National Heart, Lung, and Blood Institute was convened to identify the role of basic science in clarifying the issues that are impeding progress in the development of HBOC solutions. These discussions resulted in a consensus that, although HBOCs have shown clinical promise, various side effects have inhibited further development and regulatory approval, with cardiovascular events being of particular concern. Specific recommendations from this group included better understanding of the impact of HBOC infusion on human physiology, the need for development of rapid and noninvasive methods for the measurement of tissue oxygenation in human patients to better inform transfusion decisions, further investigation of the routes and consequences of Hb metabolism, optimization of clinical protocols for HBOC use, and assessment of the impact of HBOC formulation excipients.

The following section will review the history of HBOC development and specifically review the results of clinical trials of the more recent HBOCs.

FIRST-GENERATION HBOCs

The first-generation HBOCs were based on observations that cross-linking with, for example, glutaraldehyde, overcame Hb subunit dissociation and renal toxicity. Experience with these solutions showed that they can be vasoactive—sometimes increasing

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**Fig. 4.** PolyHeme-human Hb substitute.
blood pressure, sometimes decreasing tissue perfusion, and sometimes producing both actions. Clinical trials were disappointing because of unexpected toxicity.

A first-generation HBOC, diaspirin cross-linked Hb (DCLHb; HemAssist), was under development by Baxter Hemoglobin Therapeutics (Baxter International, Inc., Deerfield, Illinois) during the 1990s. This product had circumvented the safety concerns related to dimerization of the Hb tetramer by cross-linking the alpha chains chemically. Animal studies were promising.\(^1^7\) The clinical studies performed with human cross-linked Hb (DCLHb) were stopped because of an increased rate of mortality in two clinical trials for patients who received DCLHb after stroke and multiple injury shock.\(^1^8,1^9\) Additional studies in cases of cardiac and noncardiac surgery documented additional safety concerns, with early study termination related to serious adverse events.\(^2^0-2^2\)

### SECOND-GENERATION HBOCs

The second-generation HBOCs are based on a better understanding of the mechanisms of this vasoconstriction and specific modifications to reduce nitric oxide binding and resultant vasoconstriction. Four products have undergone recent clinical investigation (Table 2).

Three HBOC products (Hemopure, PolyHeme, MP4 (formerly Hemospan)) are currently in phase III clinical trials. The largest number of patients enrolled in HBOC clinical trials are in those investigating Hemopure and PolyHeme, and a comparison of the specific characteristics of these two HBOCs is delineated in Table 3.
Hemopure (HBOC-201) is a polymerized form of bovine Hb with a P-50 of 30 mm Hg, which is closer to that of human Hb than stroma-free Hb. It has an intravascular half-life of from 8 hours to 23 hours and a shelf life of 36 months at room temperature. One unit of Hemopure contains 30 g of ultrapurified, chemically cross-linked Hb in 250 mL of a balanced salt solution.

When infused, these linked Hb molecules circulate in the plasma, are smaller and have a lower viscosity, and more readily release oxygen to tissues than those of allogeneic RBCs (Fig. 5). Hemopure is compatible with all blood types and is purified through patented techniques that have been validated to remove infectious agents, including bacteria, viruses, prions, and other potential contaminants. A similar bovine Hb substitute is used in veterinary medicine as Oxyglobin.

**Hemopure: Clinical Trials**

Phase II and III studies with HBOC-201 have documented that infusion of HBOC-201 can avoid or reduce allogeneic blood transfusion needs for patients in specific perioperative settings.

**Table 2**

<table>
<thead>
<tr>
<th>HBOC</th>
<th>Product</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemopure</td>
<td>Bovine Hb that is glutaraldehyde cross-linked to produce a polyHb</td>
<td>Biopure Corporation, Cambridge, Massachusetts</td>
</tr>
<tr>
<td>PolyHeme</td>
<td>Human Hb from donated human blood that is pyrioxylated to decrease the oxygen binding affinity and glutaraldehyde cross-linked to produce a polyHb</td>
<td>Northfield Laboratories Inc., Northfield, Illinois</td>
</tr>
<tr>
<td>HemoLink</td>
<td>Human Hb from donated human blood and O-raffinose cross-linked to produce a polyHb</td>
<td>Hemosol Corporation, Mississauga, Canada</td>
</tr>
<tr>
<td>MP4</td>
<td>PEG-conjugated human Hb</td>
<td>Sangart Inc., San Diego, California</td>
</tr>
</tbody>
</table>

**Abbreviations:** MP4, Maleimide PEG-Hb; PEG, Polyethylene glycol.

**Hemopure**

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**Table 3**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hemopure</th>
<th>PolyHeme</th>
<th>RBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g%)</td>
<td>13</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Unit equivalent (g)</td>
<td>30</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Molecular weight (&gt; 64 k Da)</td>
<td>≥ 95%</td>
<td>≥ 99%</td>
<td>≥ 100%</td>
</tr>
<tr>
<td>P50 (mm Hg)</td>
<td>38</td>
<td>29</td>
<td>26</td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>1.4</td>
<td>1.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Oncotic pressure (mm Hg)</td>
<td>25</td>
<td>23</td>
<td>25</td>
</tr>
<tr>
<td>Viscosity (cp)</td>
<td>1.3 cp</td>
<td>2.1 cp</td>
<td>(Whole blood = 5–10 cp)</td>
</tr>
<tr>
<td>Methemoglobin (%)</td>
<td>&lt;10</td>
<td>&lt;8</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Half-life</td>
<td>19 hours</td>
<td>24 hours</td>
<td>31 days</td>
</tr>
<tr>
<td>Shelf life at 4 C</td>
<td>≥ 3 years</td>
<td>≥ 1.5 years</td>
<td>42 days</td>
</tr>
<tr>
<td>Shelf life at 21 C</td>
<td>≥ 2 years</td>
<td>≥ 6 weeks</td>
<td>≥ 6 hours</td>
</tr>
</tbody>
</table>
One clinical study evaluated HBOC-201 as a substitute for allogeneic RBC transfusion in patients undergoing elective infrarenal aortic operations (Table 4). In a single-blind, multicenter trial, 72 patients were prospectively randomized two-to-one to receive an HBOC-201 (n = 48) or allogeneic RBC (n = 24) at the time of the first transfusion decision, either during or after elective infrarenal aortic reconstruction. Patients randomized to the HBOC-201 group received 60 g of HBOC-201 for the initial transfusion and had the option to receive three more doses (30 g each) within 96 hours. In this group, any further blood requirement was met with allogeneic RBCs. Patients randomized to the allogeneic RBC group received only standard RBC transfusions. The efficacy analysis was a means of assessing the ability of the HBOC to eliminate the requirement for any allogeneic RBC transfusions from the time of randomization through 28 days later. The two treatment groups were comparable for all baseline characteristics. Although all patients in the allogeneic RBC group required at least one allogeneic RBC transfusion, 13 of 48 patients (27%; 95% confidence interval (CI), 15% to 42%) in the HBOC group did not require any allogeneic RBC transfusions. The only significant changes documented were a 15% increase in mean arterial pressure and a threefold peak increase in serum urea nitrogen concentration after HBOC transfusion. The complications were similar in both groups, with no allergic reactions. There were two perioperative deaths (8%) in the allogeneic RBC group and three perioperative deaths (6%) in the HBOC group (P = 1.0). This study concluded that the HBOC significantly eliminated the need for allogeneic RBC transfusion in 27% of patients undergoing infrarenal aortic reconstruction, but did not reduce the median

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Hemopure (HBOC-201)</th>
<th>RBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage</td>
<td>Room temperature (20–30 °C)</td>
<td>Refrigerated</td>
</tr>
<tr>
<td>Shelf life</td>
<td>36 months</td>
<td>42 days</td>
</tr>
<tr>
<td>Preparations</td>
<td>Ready to use</td>
<td>Testing, typing, and cross-matching</td>
</tr>
<tr>
<td>Compatibility</td>
<td>Universal</td>
<td>Type specific</td>
</tr>
<tr>
<td>Effectiveness</td>
<td>Immediate oxygen delivery</td>
<td>Dependant on length of storage</td>
</tr>
<tr>
<td>Purity</td>
<td>Processed to remove infectious agents</td>
<td>Tested and screened for infectious agents</td>
</tr>
<tr>
<td>Raw material</td>
<td>Bovine Hb abundant, controlled source</td>
<td>Limited availability, not controlled</td>
</tr>
</tbody>
</table>
allogeneic RBC requirement. HBOC transfusion was well tolerated and did not influence morbidity or mortality rates.24

A multicenter, randomized, single-blind trial (n = 81) compared HBOC-201 (n = 55) with an equivalent volume of Ringer’s solution (n = 26) in surgical patients and evaluated the tolerability of a single intraoperative dose of HBOC-201. No deaths were reported; however, a delayed dose-dependent increase in plasma methemoglobin concentration was noted. Intraoperative administration was well tolerated, up to a maximum of 245 g.25

Another randomized, double-blind, RBC-controlled, multicenter efficacy clinical trial (US Phase II Post-Cardiopulmonary Bypass Surgery Trial–1997) of the bovine Hb solution HBOC-201 (Hemopure) was performed in patients undergoing cardiac surgery and requiring blood transfusions. The object of this trial was to avoid RBC transfusions in patients for 28 days after the procedure. There were 98 patients in the study, 50 who received Hemopure and 48 who received RBC transfusions. Up to 120 g (4 units of Hemopure) could be used up to 3 days after the surgery. The use of Hemopure eliminated the need for RBC transfusions in 34% of cases, and oxygen extraction was greater in the HBOC group. This study documented that Hemopure reduced the need for allogeneic RBC transfusions without significant clinical side effects, with the exception of nonsignificant vasoconstriction reflected by increased mean blood pressure in the treatment group.26

A phase III, noncardiac surgery trial was initiated in 1998 in Europe and South Africa, and the goal again was to avoid RBC transfusions for 28 days after the surgery. This study enrolled 160 patients, 83 who were treated with Hemopure and 77 who were treated with RBC transfusions. Up to 210 g of Hb (7 units of Hemopure) were allowed during a 6-day treatment time. This trial obtained its goal because 43% of the patients treated with Hemopure were able to avoid RBC transfusions.

Most recently, the report of the largest clinical trial (US Phase III Orthopedic Surgery Trial, initiated in 1998) was published.27 The ability of HBOC-201 to safely reduce or eliminate the need for perioperative transfusion was studied in orthopedic surgery patients. A randomized, single-blind, RBC-controlled, parallel-group multicenter study was conducted. Six hundred and eighty-eight patients were randomized to receive treatment with HBOC-201 (H, n = 350) or RBCs (R, n = 338) at the first transfusion decision. Primary endpoints were transfusion avoidance and blinded assessment of safety noninferiority. A total of 59.4% of patients in the H arm avoided the need for RBC transfusion. Adverse events (8.47 versus 5.88) and serious adverse events (0.35 versus 0.25) per patient were higher in the H versus R arms (P<.001 and P<.01). HBOC-201 eliminated the need for transfusion in the majority of subjects. The between-arms safety analysis (H versus R) was unfavorable and likely related to patient age, volume overload, and undertreatment, and was isolated to patients who could not be managed by using HBOC-201 alone. However, patients younger than 80 years old with moderate clinical needs may safely avoid transfusion when treated with up to 10 units of HBOC-201.

As a consequence, HBOC-2001 was approved for treatment of perioperative anemia in elective adult surgical patients in South Africa in 2001. Hemopure is approved in South Africa for the treatment of adult surgical patients who are acutely anemic, with the intention of eliminating or reducing the need for allogeneic RBC transfusions.

Per Biopure Corporation’s report, Hemopure has been administered to more than eight hundred human subjects in 22 completed clinical trials, including four advanced, RBC-controlled trials in patients undergoing cardiac, vascular, general noncardiac, and orthopedic surgery, respectively. These trials represent a logical progression in the study design that has expanded the dosing limits from 4 units (120 g Hb) of
Hemopure administered after surgery over a maximum period of 3 days to 10 units (300 g Hb) administered before, during, or after surgery over a 6-day period.\textsuperscript{28}

In the United States, phase III trials have been put on hold due to safety issues. In December 2006, the Blood Products Advisory Committee of the US Food and Drug Administration (FDA) voted against recommending that the US Navy proceed with late-phase clinical trials of Hemopure. The main reason for this was the adverse effect profile of the compound, because previous studies had shown that Hemopure could increase the risk of strokes and myocardial infarction. Hemopure’s manufacturer, Biopure Corporation, is currently addressing the FDA’s questions regarding the safety and efficacy of the product.

Hemopure is currently in phase III clinical trials in South Africa and Europe (Table 5). In the United States, Hemopure is currently under review by the FDA, and animal studies are being conducted. In March 2003, the US Naval Medical Research Center signed a collaborative research-and-development agreement with Biopure Corporation to help fund and conduct a trial on the effects of Hemopure in out-of-hospital resuscitation of patients with severe hemorrhagic shock. This trial was named Restore Effective Survival in Shock (RESUS), and more than $14 million in Congressional, Navy, Army, and Air Force funding has been given so far to support the trauma development program for Hemopure. Hemopure has also been approved for compassionate use.

PolyHeme

PolyHeme (Northfield Laboratories Inc., Evanston, Illinois) is a first-generation, pyridoxylated, polymerized Hb made from outdated human blood (Fig. 6). The development of PolyHeme originally began as a military project following the Vietnam War, and it has since shown great potential for both military and civilian use. It is one of the few HBOC products currently being evaluated in phase III clinical trials. It has a half-life of 24 hours, a shelf life longer than 12 months when refrigerated, and a p-50 from 28 mm to 30 mm Hg. The extraction and filtration of human Hb from RBCs is the first step in PolyHeme production. Then, using a multistep polymerization process, the purified Hb is associated into tetramers and, as the final step, is incorporated into an electrolyte solution.

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Hemopure clinical trials</th>
</tr>
</thead>
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<tr>
<td>Studies (#)</td>
<td>Source of Patients</td>
</tr>
<tr>
<td>4</td>
<td>Healthy volunteers</td>
</tr>
<tr>
<td>4</td>
<td>Nonsurgery</td>
</tr>
<tr>
<td>3</td>
<td>Surgery with ANH</td>
</tr>
<tr>
<td>6</td>
<td>General surgery</td>
</tr>
<tr>
<td>1</td>
<td>Military surgery trial</td>
</tr>
<tr>
<td>4</td>
<td>Major surgery trials</td>
</tr>
<tr>
<td>22</td>
<td>Other clinical trials</td>
</tr>
</tbody>
</table>

Data do not include more than 250 postclinical trial applications in South Africa.

Abbreviation: ANH, Acute normovolemic hemodilution.

\textsuperscript{a} Total does not include compassionate use patients treated under emergency investigational new drug applications. Adapted from www.biomed.brown.edu.
PolyHeme was developed as a temporary solution to blood loss. As a military project, the focus was to develop a blood substitute to keep trauma patients alive in remote areas where allogeneic blood is not available. It is recognized that PolyHeme has a short circulation half-life of only 24 hours. Conditions requiring blood for longer than the circulation time of PolyHeme would require repeated transfusions of PolyHeme or later replacement with donor blood. Another factor that can limit the effectiveness of PolyHeme is the fact that it is manufactured using human Hb. Although such Hb can be reclaimed from expired RBC products, it does not completely eliminate the need for donors because there must be a source of the outdated erythrocytes. The use of human Hb could limit the supply and manufacturing potential of PolyHeme.

**PolyHeme: Clinical Trials**

Multiple clinical trials have already been completed in the hospital setting, which have increasingly tested the safety and effectiveness of PolyHeme. Testing has included transfusion of PolyHeme during resuscitation as well as both intraoperatively and postoperatively, and has shown the effectiveness of PolyHeme at different rates, from 1 unit to 20 units. The most rapid transfusion occurred during a severe hemorrhage, in which 20 units were transfused in 20 minutes during a phase II clinical trial.

In an initial Phase II clinical trial (n = 39) up to 6 units of PolyHeme were administered in patients after acute trauma and surgery. No safety issues related to PolyHeme were reported. The plasma Hb mean was 4.8 ± 0.8 g/dL (reflecting PolyHeme Hb concentration) and RBC Hb fell to 2.9 ± 1.2 g/dL (reflecting the patients’ endogenous RBC Hb), but total Hb was maintained at 7.5 ± 1.2 g/dL with infusion of 6 units (300 g) of PolyHeme.

In a phase II randomized trial in 44 patients with acute trauma, PolyHeme reduced the required number of allogeneic RBC transfusions. The patients (33 men, 11 women), aged from 19 to 75 years with an average Injury Severity Score of 21 ± 10, were randomized to receive RBCs (n = 23) or up to 6 units (300 g) of PolyHeme (n = 21) as their initial blood replacement after trauma and during emergent operations. There were no serious or unexpected adverse events related to PolyHeme. The PolyHeme infusion of 4.4 ± 2.0 units (mean ± standard deviation) resulted in a plasma Hb of 3.9 ± 1.3 g/dL, which accounted for 40% of the total circulating
There was no difference in total Hb between the groups before infusion (10.4 ± 2.3 g/dL control group versus 9.4 ± 1.9 g/dL experimental group). At end infusion, the experimental RBC Hb group fell to 5.8 ± 2.8 g/dL versus 10.6 ± 1.8 g/dL (P < .05) in the control group, although the total Hb was not different between the groups or different from that at the time of preinfusion. The total number of allogeneic RBC transfusions for the control and experimental groups was 10.4 ± 4.2 units versus 6.8 ± 3.9 units, respectively, (P < .05) through day 1, and 11.3 ± 4.1 units versus 7.8 ± 4.2 units, respectively, (P = .06) through day 3. This study documented that PolyHeme was safe in cases of acute blood loss, maintained total Hb in lieu of RBCs despite the marked fall in RBC Hb, and reduced the use of allogeneic blood. PolyHeme appeared to be a clinically useful blood substitute.

A nonrandomized, prospective trial enrolled 171 trauma or surgical patients who received rapid infusion of from 1 unit to 20 units (1,000 g, 10 L) of PolyHeme in lieu of RBCs as an initial oxygen-carrying replacement in trauma and urgent surgery.31 The protocol simulated the unavailability of RBCs, and the progressive fall in RBC Hb in bleeding patients was quantified. The 30-day mortality of this group was compared with that of a historical control group of 300 surgical patients who refused RBCs on religious grounds. A total of 171 patients received rapid infusion of from 1 unit to 2 units (n = 45), 3 units to 4 units (n = 45), 5 units to 9 units (n = 47), and 10 units to 20 units (n = 34) of PolyHeme. Forty patients had a nadir RBC Hb ≤ 3 g/dL (mean, 1.5 ± 0.7 g/dL), but total Hb was adequately maintained (mean, 6.8 ± 1.2 g/dL) because of plasma Hb added by PolyHeme. The 30-day mortality was 25.0% (10/40 patients) in the PolyHeme group compared with 64.5% (20/31 patients) in the historical control group at these low RBC Hb levels. Additionally, 75% of patients with RBC Hb levels less than 1 gm % survived traumatic injury after receiving PolyHeme as compared with 16% of patients in the historical control group at the same RBC Hb level. The authors concluded that PolyHeme increases survival at life-threatening RBC Hb by maintaining total Hb (plasma and RBC Hb) in the absence of RBC transfusion. PolyHeme should be useful in the early treatment of urgent blood loss and resolve the dilemma of the unavailability of RBCs.

The USA Multicenter PolyHeme Trauma Trial was recently completed, the first trial in the United States of an HBOC in the prehospital setting using waiver of informed consent. This was a 720-patient, phase III trial in trauma patients in which subjects were randomized to receive either PolyHeme or the standard of care at the time of injury.32 On reaching the hospital, patients in the control arm received allogeneic blood transfusion as indicated, whereas patients in the PolyHeme arm received PolyHeme for 12 hours and then received allogeneic blood as indicated. Some have commented that it was unethical to continue the study protocol for 12 hours of the in-hospital phase of the study, that is, in the absence of a requirement to administer allogeneic blood to trauma victims in the PolyHeme group upon hospital arrival.33 Preliminary results have indicated that there was no statistically significant difference in mortality (on day 1 and day 30) between the PolyHeme and control cohorts. For detailed analyses of the results of this trial, see the article by Dr. Ernest E. Moore and colleagues elsewhere in this issue.

Hemolink

Hemolink (Hb-raffimer, Hemosol Corporation, Mississauga, Canada) is a polymerized Hb product manufactured from donated human blood and O-raffinose cross-linked to produce a polyHb. A Phase I study was performed in healthy volunteers (n = 42), of
whom 33 received Hemolink, and it was well tolerated, with no evidence of organ dysfunction.\textsuperscript{34}

**Hemolink: Clinical Trials**

The first phase II, randomized, controlled, single-blind, dose-escalation, multicenter trial was performed in 60 adult patients undergoing elective coronary artery bypass graft (CABG) surgery at Duke University.\textsuperscript{35} After induction of anesthesia, autologous whole blood was collected to achieve a Hb of 7 g/dL on cardiopulmonary bypass. Patients were randomized to receive either Hemolink (treatment) or 6% hetastarch (control) in sequential, escalating dose blocks of 250 mL, 500 mL, and 750 mL. After the return of the autologous blood, allogeneic RBCs were transfused according to pre-determined Hb triggers. Serious adverse events were distributed evenly between the two groups of patients. Elevated blood pressure was more frequent in the treatment group than in the control group (16/28 mmHg versus 9/32 mmHg, $P = .036$). In the group of 40 patients in the 750-mL dose block, eight of the 18 treatment patients and four of the 22 control patients avoided needing allogeneic RBC transfusion ($P = .093$). The median volume of allogeneic RBCs transfused was lower in the treated subjects than in the control subjects ($P = .042$). Hemolink was well tolerated and could be effective in reducing transfusion for patients undergoing CABG surgery. Although perioperative hypertension was more frequent in the treated patients, blood pressure management prevented serious adverse sequelae.

A second phase II, dose-response study in patients receiving elective CABG surgery ($n = 60$) was performed in a single-blind, multicenter, placebo-controlled, open-label trial at London Health Sciences Center in Canada.\textsuperscript{36} This study aimed to determine the dose-response of Hemolink administered in conjunction with intraoperative autologous donation in patients undergoing CABG. A secondary objective was to evaluate the effectiveness of Hemolink in reducing the incidence of the need for allogeneic RBC transfusions. Patients were randomized to receive a single dose of Hemolink or a control (10% pentastarch). Patients were sequentially enrolled in a dose block of 250 mL, 500 mL, 750 mL, and 1000 mL. Sixty patients received Hemolink ($n = 30$) or the control ($n = 30$). Hemolink was well tolerated. Most (98%) adverse events were mild or moderate in severity. There was an expected dose-dependent increase in the incidence of blood pressure increases and jaundice in Hemolink-treated patients. In a dose-pooled analysis of Hemolink versus the control, increased blood pressure (43% versus 17%), nausea (37% versus 33%), and atrial fibrillation (37% versus 17%) were the most frequently reported adverse events. All serious adverse events were considered unrelated or unlikely to be related to the study drug. No Hemolink-treated patient required an intraoperative allogeneic RBC transfusion, compared with 5 (17%) pentastarch-treated patients ($P = .052$). This advantage of Hemolink was maintained at 24 hours after surgery (7% versus 37%; $P = .010$) and up to 5 days after surgery (10% versus 47%; $P = .0034$). Hemolink was effective in facilitating decreased exposure or avoidance of allogeneic RBC transfusions when used in conjunction with intraoperative autologous donation.

A phase III, multicenter clinical trial was undertaken next. The purpose of this study was to determine if intraoperative autologous donation alone or in conjunction with Hemolink confers a reduction in RBC or blood component transfusion compared with results in standard clinical practice. The trial was a multicenter, randomized, double-blind study to determine the efficacy and safety of Hemolink versus 10% pentastarch when used to facilitate intraoperative autologous donation in 299 patients undergoing primary CABG. The patients received Hemolink or pentastarch as an adjunct to intraoperative autologous donation immediately before cardiopulmonary
bypass. Results were compared with transfusion requirements for 150 matched patients in the reference group. The frequency of allogeneic RBC transfusion in the Hemolink, pentastarch, and reference groups was 56%, 76%, and 95%, respectively. The number of allogeneic RBC units used was 49 in the Hemolink group, 104 in the pentastarch group, and 480 in the reference group \( (P < .001) \). The total number of non-RBC units administered was 150 in the Hemolink group, 238 in the pentastarch group, and 270 in the reference group. In this study, patients treated with Hemolink in conjunction with intraoperative autologous donation received fewer transfusions overall and a lower volume of allogeneic RBCs and non-RBC allogeneic blood products than did those in the two comparison groups. This potentially confers a real benefit on the overall blood supply by decreasing use and increasing availability.

In light of the limited resources available to Hemosol Corporation, as well as the time and expense likely required to address certain adverse results noted in the course of the clinical trials of Hemolink, the company elected to discontinue further development of Hemolink in June 2004.

**Maleimide PEG-Hb**

Maleimide PEG-Hb (MP4) (Sangart Inc., San Diego, California) is a polyethylene glycol–conjugated human Hb currently undergoing clinical trials in Europe as a colloid oxygen therapeutic and not as a blood substitute.\(^3\)\(^7\) To further increase the circulation time, Hb can be linked to a macromolecule to increase its size. Human or bovine Hb that is conjugated with polyethylene glycol (PEG) is protected from renal excretion. The PEG-Hb has a larger molecular size and has a viscosity lower than whole blood but higher than colloids in clinical use.

MP4 was developed by introducing additional surface thiols with iminothiolane onto the Hb. This process usually adds about six additional thiols, and it is then linked to on average eight PEG-5000. MP4 then requires no more purification steps. MP4 has a lower Hb concentration, higher viscosity (but lower than blood), higher oxygen-affinity, and higher colloidal oncotic pressure than most other HBOCs in development (Table 6). MP4 did demonstrate an improvement in microcirculatory blood flow and tissue oxygenation in animal studies. In animal models, MP4 has been shown to be effective in reversing lactic acidosis in studies of hemorrhagic shock.\(^3\)\(^8\) Adverse effects associated with the vasoactive properties of first-generation blood substitutes are not seen with MP4. At relatively low concentrations, MP4 is capable of transporting oxygen.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PolyHeme (Northfield Laboratories Inc.)</th>
<th>Hemopure (Biopure Corporation)</th>
<th>MP4 (Sangart Inc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>500</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Hb concentration (g/dl)</td>
<td>10</td>
<td>13</td>
<td>4.3</td>
</tr>
<tr>
<td>Hb mass (g)</td>
<td>50</td>
<td>~ 30</td>
<td>~ 10</td>
</tr>
<tr>
<td>( P_{50} ) (mm Hg)</td>
<td>26–32</td>
<td>38</td>
<td>5</td>
</tr>
<tr>
<td>Methemoglobin (%)</td>
<td>&lt;8.0</td>
<td>&lt;15.0</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>Tetramer (%)</td>
<td>( \leq 1.0 )</td>
<td>( \leq 3.0 )</td>
<td>( &lt;1.0 )</td>
</tr>
<tr>
<td>Shelf life (years)</td>
<td>&gt;1</td>
<td>3</td>
<td>&gt;1</td>
</tr>
</tbody>
</table>
large amounts of oxygen. Pharmacokinetic analysis of plasma Hb yielded an estimated half-life of 43 hours in subjects in the phase I trial who received 100 mg/kg.

**MP4: Clinical Trials**

Sangart Inc. announced positive results from a phase I study of MP4 in 2005. A Phase Ib/II clinical trial evaluated increasing doses of MP4 in orthopedic surgery patients (n = 30), with doses of from 200 mL to 1000 mL of either MP4 or Ringer’s acetate before induction of spinal anesthesia was subsequently completed. This study demonstrated that MP4 was well tolerated at these doses, with no new safety concerns raised.

A Phase II trial, conducted in Sweden, involved 90 patients undergoing hip arthroplasty in a multicenter, double-blind, clinical trial. Patients were randomized to receive either MP4 or Ringer’s acetate (control) before induction of spinal anesthesia. MP4 was found to be well tolerated in the study group, with no serious adverse effects attributed to the product during the trial period. The percentage of hypotensive episodes in the MP4 group was about 45%, compared with 87% among those in the control group. Incidence of intraoperative vasopressor use was about 15% in the MP4 group, compared with 32% among those in the control group. A special feature of this study was Holter monitoring, starting 1 hour before induction of anesthesia and continued for 24 hours. Blinded analysis of these data did not find any significant imbalances or safety concerns.

A single-center, double-blind, phase II study of MP4 was completed in November 2007 at the Johns Hopkins Medical Center in Baltimore. In this study, patients undergoing elective open prostatectomy were randomly assigned to receive either crystalloid (Ringer’s solution) or MP4 after an estimated surgical blood loss of 250 mL. A special feature of this study was the assessment of pulmonary artery hemodynamics using transesophageal echo (ClinicalTrials.gov Identifier NCT00425334).

A randomized, single-blind, controlled, phase II pilot study of the use of MP4 compared with colloid (Voluven, Fresenius Kabi, BAD Homburg, Germany [6% hydroxyethyl starch 130/0.4 in 0.9% sodium chloride], a commercial starch-based plasma expander) to evaluate vascular resistance and blood flow in the forearm and to assess local skin blood flow and tissue oxygenation in an ischemic region of the foot in patients with chronic critical limb ischemia recently completed at the Karolinska University Hospital in Stockholm, Sweden. The main study objective was to investigate the effect of MP4 on vascular resistance and demonstrate the absence of vasodilation in patients with chronic critical limb ischemia. The secondary study objective was to evaluate the effects of MP4 on local skin blood flow and tissue oxygenation in an ischemic region of the foot in these patients.

MP4 is undergoing further evaluation in two pivotal, multicenter, international, double-blind, controlled, phase III studies in Europe. One study (a prevention trial) completed enrollment of 376 patients at 18 centers in six countries in May 2008 and evaluated the ability of MP4 to prevent acute hypotension in orthopedic surgery patients undergoing first-time hip replacement procedures under spinal anesthesia. The primary objective was to demonstrate that MP4 is a superior colloid compared with Voluven for preventing hypotensive episodes during the operative and early postoperative period. The secondary objective was to show that MP4 can also reduce the incidence of operative and postoperative morbidity. (Additional details can be found at http://clinicaltrials.gov/NCT00420277).

An additional study (a treatment trial) completed enrollment of 474 patients at 21 centers in five countries in March 2008 and evaluated the ability of MP4 to treat acute hypotension in orthopedic surgery patients undergoing first-time hip replacement
procedures under spinal anesthesia. The primary objective was to demonstrate that MP4 is a superior colloid compared with Voluven for treating hypotensive episodes during the operative period. The secondary objective was to show that MP4 can also reduce the incidence of operative and postoperative morbidity. (Additional details can be found at http://clinicaltrials.gov/ct/show/NCT00420277?order=1).

RECOMBINANT HUMAN HEMOGLOBIN

Recombinant human Hb (rHb) is manufactured using *Escherichia coli* with recombinant technology. This technology can be used to induce a variety of cell types to synthesize functional Hb. In addition, modifications of the Hb molecular structure can alter the properties of the molecule, allowing researchers to create Hbs with improved functionality or enhanced safety when used as Hb therapeutics. One very positive feature of rHb is that it can be manufactured resulting in an unlimited supply.

Two first-generation HBOCs were under development by Baxter Hemoglobin Therapeutics and Somatogen (Boulder, Colorado) during the 1990s, DCI Hb (HemAssist) and a modified rHb (rHb1.1, Optro), respectively. Each of these products had circumvented the safety concerns arising from dimerization of the Hb tetramer by cross-linking the alpha chains (either chemically in the case of DCI Hb or through recombinant engineering with rHb1.1).42

rHb1.1 was a first-generation HBOC with a nitric oxide scavenging rate similar to that of native human Hb. rHb 2.0 was a second-generation HBOC, created via genetic manipulation of the distal heme pocket of both the alpha and beta subunits of Hb, leading to steric hindrance for nitric oxide entry, with a nitric oxide scavenging rate from 20- to 30-fold lower than rHb1.1 but with maintenance of effective oxygen binding and release.43 Preclinical animal studies were promising. rHb2.0 was associated with decreased pulmonary hypertension, diminished capacity to scavenge nitric oxide, and lack of modulation of pulmonary vascular permeability. These findings therefore lend promise for the use of HBOCs with low nitric oxide reactivity as oxygen therapeutics.

rHb2.0 has been investigated in a swine model of uncontrolled hemorrhage. rHb 2.0 performed as well as heterologous blood for resuscitation in hemorrhage, did not cause sustained pulmonary hypertension, maintained adequate cardiac output and oxygen delivery, and was superior to lactated Ringer’s solution and the first-generation HBOC DCI Hb in survival rates.44 Additional preclinical studies documented positive results.45–48 Although rHb 2.0 appeared promising, no clinical trials were performed and Baxter Corporation suspended funding of this initiative.

NEXT-GENERATION HBOCs

A number of new, advanced HBOCs are undergoing development.49 A complete discussion of these HBOCs is beyond the scope of this review. A few interesting compounds deserve mention.

Pyridoxylated Hb polyoxyethylene conjugate (PHP, Apex Bioscience, Chapel Hill, North Carolina) is a conjugated Hb that is currently undergoing a phase III trial in patients with shock associated with systemic inflammatory response syndrome. The study has been designed to evaluate the safety and efficacy of continuous intravenous infusion of PHP plus conventional vasopressor treatment versus continuous intravenous infusion of Plasma-lyte A (Baxter Healthcare Corp., Deerfield, Illinois) plus conventional vasopressors as a treatment for restoring hemodynamic stability in patients with systemic inflammatory response syndrome with shock. The trial has
an estimated enrollment of 1000 patients, and the study start date was March 2001 (ClinicalTrials.gov Identifier NCT00021502).

For HBOCs cross-linked with enzymes, there has been an effort to synthesize compounds that not only perform the function of carrying oxygen, as do the molecules mentioned previously, but also harbor some of the enzyme activity that normal RBCs possess. PolyHb has been cross-linked with catalase and superoxide dismutase to form a compound that, in animal models, can not only carry oxygen but also remove oxygen radicals that are responsible for ischemia reperfusion injuries (Figs. 7 and 8). PolyHb has also been cross-linked with tyrosinase to form a soluble complex that can carry oxygen and decrease the systemic levels of tyrosine. This agent can help increase the efficacy of chemotherapy and radiation therapy in tumor tissue, and in a melanoma model, it has been shown to delay tumor growth without having significant adverse effects.

Cellular HBOCs

Cellular HBOCs consist of Hb molecules encapsulated inside oxygen carriers of different natures, aimed at mimicking features of RBCs. The advantages of cellular HBOCs consist of protecting the surrounding tissues and blood components from direct contact with potentially toxic tetrameric Hb, avoiding the Hb colloidal osmotic effect, prolonging Hb circulation half-life, and not requiring the direct modification of the Hb molecule. Additionally, with the application of nanotechnology, it is possible to achieve a submicron-sized oxygen carrier and thus ensure oxygen availability to all body compartments. Two types of cellular HBOCs have been studied: liposome systems and polymeric micro/nanoparticle systems.

Efforts have been made to encapsulate Hb within a lipid membrane to create a compound capable of carrying oxygen while not being associated with significant

Fig. 7. In conditions of tissue ischemia, RBCs may not be able to get to the location of the ischemia due to their size. PolyHb can get there but will provide oxygen for only a short period. PolyHb cross-linked with superoxide dismutase (SOD) and catalase (CAT) can supply oxygen and remove oxygen radicals, thus treating ischemic-reperfusion injury. (From Chang TMS. Therapeutic applications of polymeric artificial cells. Nature Reviews 2005;4:221–35; with permission.)
vasoconstriction. These liposomes appear to be retained in plasma for a significant period. However, they are difficult to produce and can activate the reticuloendothelial system, the complement pathway, and platelets. At present, the only institutions working actively on this product are in Japan.

The ultimate RBC substitute would contain not only Hb but also other contents of the RBC encapsulated in an artificial membrane. However, production of such a product would be extremely challenging. Efforts have been made to use polyactide, a biodegradable polymer that is converted to lactic acid in the body, to create artificial RBCs. These cells contain Hb along with the RBC enzyme complement, including superoxide dismutase, methemoglobin reductase, and catalase. (For more on this topic, see the article by Chang elsewhere in this issue.)

Modifying the method of preparing microdimensional, polymeric, artificial cells can result in the creation of nanodimensional artificial cells. In the case of blood substitutes, nanodimensional liposomes of about 200 nm that contain Hb have been prepared. Biodegradable polymer membranes are being used to form nanodimensional, artificial RBCs as a third-generation blood substitute. These nanodimensional, artificial RBCs (80–150 nm in diameter) contain all the RBC enzymes. Recent studies show that using a polyethylene glycol–polylactide (PEG–PLA) copolymer membrane, it is possible to increase the circulation time of these nanodimensional, artificial RBCs to double that of polyHb.

**ADVERSE EFFECTS OF HBOCs**

Adverse effects associated with HBOCs include hypertension, abdominal pain, skin rash, diarrhea, jaundice, hemoglobinuria, oliguria, fever, stroke, and laboratory anomalies such as an elevation in lipase levels. Although most of these side effects were transient and clinically asymptomatic, many clinical trials involving these agents...
have been discontinued or held due to the associated adverse side effects. Although current formulations appear to cause fewer and less severe side effects compared with previous products, there remain concerns associated with HBOCs, including the following:

**Vasoactivity**—HBOC products are known to cause vasoconstrictive effects. As nitric oxide binds to Hb, nitric oxide becomes less available to cause vascular smooth muscle relaxation. Hence, vasoconstriction occurs.

**Hemostasis**—Studies have shown an increased hemostatic effect in HBOCs due to reversal of the inhibition effect of nitric oxide on platelet aggregation.

**Gastrointestinal side effects**—Studies have observed gastrointestinal side effects such as nausea, vomiting, diarrhea, and bloating. The binding of nitrous oxide to gastrointestinal intestine tissues is the proposed cause.

**Interference with laboratory assays**—High concentrations of Hb in plasma due to the infusion of HBOCs interfere with laboratory assays. Tests for liver enzymes, bilirubin, amylase, and other substances often yield inaccurate results because of the presence of HBOCs.

It has been difficult to discern whether the adverse events that have been observed following the infusion of HBOCs in patients subjected to elective orthopedic procedures, cardiopulmonary bypass surgery, and vascular surgical procedures are related solely to the HBOCs or to other treatments administered to these patients during their routine care. Along with all three of the HBOCs, the patients received Ringer’s d,l-lactate as the resuscitative fluid, Ringer’s d,l-lactate in the excipient medium for the HBOC, and liquid preserved red blood cells that had been stored at 4°C for longer than 2 weeks. The Ringer’s d,l-lactate solution has been shown to be toxic in both animals and patients. The current formulation of Ringer’s lactate contains only the l-isomer which has been shown in animals to be less toxic than the d-isomer of lactate.

In a recent publication morbidity and mortality have been reported associated with the length of storage of red blood cells at 4°C in patients subjected to reoperative cardiac surgery. Current clinical studies to assess the safety and therapeutic effectiveness of a HBOC must consider the effects of the composition of the resuscitation solution (Ringer’s l-lactate), the composition of the excipient medium (Ringer’s l-lactate or 0.9% NaCl) for the HBOC, and the length of storage of the liquid preserved red blood cells infused with the HBOC.52

A recent meta-analysis reviewed data on death and myocardial infarction as outcome variables in 16 trials in adult patients (n = 3711) involving five different HBOCs in varied patient populations.53 They reported a statistically significant increase in the risk of death (164 versus 123 deaths; relative risk [RR] 1.30, 95% CI, 1.05–1.61) and the risk of myocardial infarction (59 versus 16 myocardial infarctions; RR 2.71; 95% CI, 1.67–4.40). There are, however, many limitations to this analysis.54 For instance, multiple products (HemAssist, PolyHeme, Hemolink, Hemopure, MP4) were all included in this analysis, and there was a lack of consistent monitoring of cardiac events in these studies, a lack of consistent treatment in the perioperative period to prevent cardiac events in the surgical studies, no identification of specific cardiac risk in patients enrolled in these studies, and a lack of control for risk of myocardial events and mortality that may have been related to allogeneic transfusion.55,56

But these adverse effects of HBOCs may also occur with the transfusion of aged human RBCs. It has been documented that as human blood is stored, hemolysis occurs and increased concentrations of free Hb are present in these units of blood.57 Abnormal hemolysis in an individual RBC unit may be caused by several factors, including inappropriate handling during the processing of blood, inappropriate or extended duration of storage, bacterial hemolysins, antibodies that cause
complement lysis, defects in the RBC membrane, or an abnormality in the blood donor. The acceptable level of hemolysis has not been established in North America, but the value of 1% is currently used to assess biocompatibility of blood storage materials.58

Free plasma Hb, in addition to generating reactive oxygen species such as the hydroxyl and superoxide radicals, is also a potent scavenger of nitric oxide. Nitric oxide, which is normally produced by the endothelium, regulates basal vasodilator tone, inhibits platelet and hemostatic activation, and reduces superoxide levels through radical–radical scavenging. The vasodilator activity of nitric oxide is possible only because most Hb is normally compartmentalized within erythrocytes.

The clinical consequences of RBC storage for patients who are critically ill are particularly concerning.59 Duration of RBC storage has been associated with adverse outcomes. In patients undergoing cardiac surgery, transfusion of RBCs that had been stored for more than 2 weeks was associated with a significantly increased risk of postoperative complications as well as reduced short-term and long-term survival.60 Additional studies have documented significant risks associated with RBC transfusion61–63 and lack of efficacy.64,65

SUMMARY

There is still a significant unmet medical need for HBOCs in a variety of medical situations. There are now available additional viable approaches to modify the intrinsic biologic properties of Hb to produce improved HBOCs. The ultimate goal is availability of an HBOC for clinical use in appropriate clinical situations.

Polymerized Hb preparations have proved most successful in clinical trials due to their improved side effect profile. The goal is to evaluate blood substitutes with enhanced intravascular retention, reduced osmotic activity, and attenuated hemodynamic derangements such as vasoconstriction. Although not without substantial morbidity and mortality, the current safety of allogeneic blood transfusion demands that comparative studies show minimal adverse effects as well as efficacy and potential for novel applications.

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