12 Hemoglobin-Based Oxygen Carriers: Brief History, Pharmacology and Design Strategies, Review of the Major Products in Clinical Trials, On-Going Studies, and Coagulation Concerns

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Abbreviations

°C	degrees centigrade
2-3 DPG	2,3 Diphosphoglycerate
CABG	coronary artery bypass graft
cADP	collagen and adenosine diphosphate, a reagent for PFA-100 measurement of platelet adhesion
cEPI	collagen and epinephrine, a reagent for PFA-100 measurement of platelet adhesion
CO	carbon monoxide
Cu	copper
DBBF	bis(dibromosalicyl) fumarate
dL	deciliter
EU	European Union
FDA	United States Food and Drug Administration
g	gram
g/dL	gram per deciliter
Hb	hemoglobin
Hb_4	Four strands of hemoglobin to form tetramer
HBOC	Hemoglobin-based oxygen carrier
HIV	Human immunodeficiency virus
IND	Investigational New Drug Application
kDa	kilodalton
L	liter
Lys	lysine
M	molar concentration
M101	HEMO2-life
MA	Massachusetts
MAL-PEG	maleimido polyethylene glycol
mg/dL	milligrams per deciliter
NO	nitric oxide
O_2	oxygen
PEG	Polyethylene glycol
PFA-100	Platelet Function Analyzer-100
PLP	pyridoxal-5′-phosphate
pRBC	packed red blood cells
r	recombinant
RBC	red blood cell
SFH	Stroma Free Hemoglobin
SOD	superoxide dismutase
TACO	Transfusion associated circulatory overload
US	United States
Zn	zinc
α	alpha
β	beta

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Introduction

Transfusion of erythrocytes (RBC) to treat acute or chronic anemia has significant drawbacks, given the risks of transfusion, volunteer donor requirements, limited supply with increasing demand, especially during a pandemic such as COVID-19, and erythrocytes are often unavailable in emergency situations or where blood is not an option. Significant work has been done for almost 100 years to attempt to replicate the functions of RBCs with oxygen carriers/oxygen therapeutics based on hemoglobin (Hemoglobin-based Oxygen Carriers, HBOC). This chapter will outline the background of HBOCs, discuss how HBOCs can be designed and how developed HBOCs are different from each other based on the changed pharmacology and physiology, highlight all major products to undergo human trials including one extensively studied product approved for human use in two countries (Hemopure), introduce newer products still under preclinical development, and finally present translational and clinical trials studying whether or not certain HBOCs may cause coagulation issues.

HBOCs have been extensively studied since 1934, when Amberson first used bovine hemoglobin and administered in canines, then in 1949 [1], he purified human hemoglobin and infused it into anemic patients. The U.S. Army developed a tetrameric cross-linked Hb that then was produced as the Baxter Corporation product, diaspirin cross-linked, although this product failed clinical trials because of increased morbidity and mortality [2], in the mid-1980s, a number of companies developed second generation HBOCs, including Biopure Corporation (Cambridge, MA) with Oxyglobin and Hemopure. Hemopure was approved by the Medicines Control Council in South Africa for treatment of anemia in 2001 and later in Russia and other countries, and Oxyglobin, FDA and EU approved for canine anemia in 1997, and 1998 respectively. To date, there have been a large number of studies evaluating these products in animal models 3–5 and clinical trials documenting success in phase I and II trials [3–6]. Hemopure was athe first to complete a Phase 3 trial (the other being PolyHeme,discussed below), while other products have undergone Phase 3 trials, but due to adverse events, were either terminated prematurely or suspended by regulatory agencies due to safety concerns.

Transfusion Medicine

Dating from 1795, blood transfusions rank with the most frequently utilized hospital procedures. In spite of the frequency of use, significant risk is linked: iron overload and Transfusion Related Acute Lung Injury (TRALI) [7]. Risk reduction related to improved blood banking techniques have mitigated infectious etiologies such as Chagas disease, HIV, Hepatitis C, malaria, *etc.*, issue with compatibility, and Transfusion Associated Circulatory Overload (TACO) [8].

Orthopedic surgical procedures, such as total joint arthroplasty and spinal instrumentation, have high perioperative transfusion rates [9], for the elderly, the perioperative blood loss and reduced tolerance for anemia increase the transfusion probability [10]. Blood use for patients older than 65 accounts for greater than 50% of perioperative erythrocyte transfusions and is predicted to double over the next 30 years [11]. Based on older data, a predicted shortfall of 4,000,000 units of blood in the US, more surgical procedures in an aging population, increasing blood use, and decreased donor blood collection will impact erythrocyte use across the country and the world [12].

Despite implementation strategies to conserve red cell use and the increasing safety of blood, the supplies of erythrocytes and components are unable to keep pace with the increasing demand [13].

Blood transfusion risks in addition to the diminishing supply of donated erythrocytes and blood products have mandated the requirement to develop artificial oxygen carriers (AOCs) and other substitutes for blood, including coagulation factors and platelets. If artificial oxygen carriers were able to be produced efficiently, and cost effectively, and be proven safe and efficacious, with long shelf life and stability, then these products would revolutionize medical care, both in the pre-hospital setting and beyond.

History of Hemoglobin-Based Oxygen Carriers

Amberson, in the 1930's was the first to document creation of HBOCs, and he tested his bovine hemoglobin in canine models and then human hemoglobin in parturients who had suffered hemorrhage and were unable to be transfused with erythrocytes [1]. Unfortunately, while all animals and humans improved transiently with the hemoglobin infusion, all died related to complications of renal failure due to blockage of renal tubules with dimers of hemoglobin, as is seen with all forms of hemolysis.

Early work involved lysing the red blood cell (RBC) coat to produce hemoglobin free of RBC membrane and structural stroma (SFH). These infusions of SFH led to jaundice, chemical pancreatitis and esophagitis, and ultimately renal failure. Toxicities of early generation HBOCs include renal failure, nitric oxide (NO) scavenging with resultant vasoconstriction, and hyper-oxidation engendering methemoglobinemia, while newer generation HBOCs aim to ameliorate these symptoms [14]. These toxicities appear due to the dissociation of the Hb tetramer into dimers, although changes in

Table 12.1 Synopsis of artificial oxygen carriers that have been tested in clinical investigations

Product	Company	Source	Modification	State of development
PEG-Hb	Enzon, Piscataway, NJ	Bovine	Polyethylene glycol-conjugated (PEGylated)	Phase Ib, tumor radiosensitization, discontinued in 1996
HemAssist (DCLHb, diaspirin-crosslinked Hb)	Baxter, Deerfield, IL	Human	Intramolecular diaspirin α-α cross-linked tetramer	Phase III cardiac surgery, acute normovolemic hemodilution, trauma/stroke discontinued in 1999
Optro	Somatogen, Boulder, CO	Recombinant	Intramolecular cross-linked β-chain mutation (108 Lys)	Phase II discontinued in 1999
PHP/Hemoximer	Curacyte/Apex Bioscience, Triangle Park, NC	Human	Surface-modified polyoxyethylene-pyroxilated polymer	Phase III, distributed shock, discontinued in 2011
Oxygent	Alliance Pharmaceutical Corp, San Diego, CA	Chemical	Perfluorochemical emulsion	Phase III, discontinued in 2001
HemoLink (hemoglobin raffimer)	Hemosol, Toronto, Canada	Human	Intra- and intermolecular cross-linking with O-raffinose	Phase II/III, surgery, acute normovolemic hemodilution/cardiac surgery discontinued in 2004
PolyHeme (polymerized human Hb)	Northfield, Evanston, IL	Human	Glutaraldehyde polymerization	Phase III, trauma, surgery, discontinued in 2009
Hemospan (MP4)	Sangart Inc, San Diego, CA	Human	Maleimide-polyethylene glycol-modified Hb	Phase II published, phase III completed, discontinued in 2015
Hemopure (hemoglobin glutamer-250 [bovine])	Acquired by Hemoglobin Oxygen Therapeutics in 2014, Souderton, PA	Bovine	Glutaraldehyde polymerization	Phase III, perioperative transfusion, acute normovolemic hemodilution cardiac surgery. Hb glutamer-250 (bovine) approved for perioperative treatment of anemia in adult elective surgical patients in South Africa and Russia. Available for expanded access in the United States
Sanguinate	Prolong Pharmaceuticals, South Plainfield, NJ	Bovine	Polyethylene glycol-conjugated (PEGylated) carboxyhemoglobin	Phase II trials complete Phase III trial not complete
$HemO_2Life$	Hemarina, Morlaix, Brittany, France	Marine Invertebrate	Hexagonal-bilayer-linked globin molecules	Phase I in progress
OxyVita Hb	OXYVITA Inc, Windsor, NY	Bovine	Hb stabilized with sebacoyl diaspirin	Preclinical trials in progress

Adapted and modified from Jahr et al. [14]

the chemical molecular configuration of Hb, and issues with the microcirculation have also been suspect. Modifications such as cross-linkage, polymerization, and polyethylene glycol (PEGylation) conjugation, are all techniques that have been utilized in an attempt to diminish these toxicities and are reviewed in the section on "How HBOCs Are Made" [14]. See Table 12.1: Synopsis of HBOCs that have been Tested in Clinical Investigations [14].

How HBOCs Are Made

Stroma Free Hemoglobin

One of the early methods of isolating hemoglobin involves the creation of stroma-free hemoglobin (SFH). This is essentially purified hemoglobin isolated from human red blood cells (RBCs) free of the erythrocyte membrane and structural fragments (stroma). There are multiple ways to achieve this, one of which involves crystallization of hemoglobin. With crystallization, RBCs are lysed with water and toluene, centrifuged, and washed with a phosphate buffer. Hemoglobin crystals are then formed without the red cell membrane [15]. SFH can also be isolated with a commercially available blood cell separator. Using this motor-operated separator, donor blood is centrifuged to separate the plasma and isolate just the RBCs. The RBCs are then lysed by hypotonic shock and the stroma is separated, leaving just the Hb lysate [16]. Lastly, selective DEAE-cellulose absorption can be used to create SFH. Outdated RBCs are washed with saline, centrifuged at 5000 rpm at 4 °C, hemolyzed with distilled water for 30 minutes, then centrifuged at 5000 rpm at 4 °C for 30 minutes again. The lysate mixture is mixed with DEAE-52, then separated by vacuum filtration and dialyzed with a standard kidney dialysis buffer. The SFH is then isolated by pressure filtration [17].

α-α Cross Linked Hb

Normal hemoglobin in animals and humans is a tetramer, Hb4, comprised of two identical α chains and two identical β chains which are non-covalently bonded. It is possible to cross link the two individual alpha units of Hb in order to prolong its half-life in the body. The advantage of cross-linking the two alpha chains is because this alpha-alpha cross-link prevents dissociation of oxyhemoglobin into $\alpha\beta$ dimers which are readily excreted by the kidneys [18]. Snyder et al. found that the half-life of HbXL99 (α-α cross-linked Hb) in rats was increased to 3.3 hours compared to 90 minutes for α Hb alone. In order to cross link the two alpha chains, a commonly used cross-linking agent is bis (dibromosalicyl) fumarate (DBBF). To start, stroma-free hemoglobin is used and must initially be deoxygenated. Deoxygenation is achieved by reacting the Hb with a reducing agent such as sodium dithionite or ferrous citrate for 1–3 hours in room temperature. A cross linking agent, preferably DBBF is then added. DBBF has a high specificity for the Lys 99 residues on each of the Hb α chains. The newly α-α cross linked Hb can be separated from any unreacted Hb using chromatography. This α-α cross linked Hb is now resistant to alpha-beta dimerization and renal elimination [19].

Glutaraldehyde Polymerized Hbs

Hemoglobin can be polymerized with glutaraldehyde to create a large HBOC with less risk of extravasation and subsequent nitric oxide scavenging. Two notable HBOCs that are created using this method are Hemopure (HBOC-201), and Polyheme. Hemopure is a purified bovine Hb polymerized with glutaraldehyde for stability and formulated in a lactated Ringer's solution. PolyHeme is synthesized utilizing outdated human erythrocytes, modified with pyridoxal-5′-phosphate (PLP), and is then polymerized with glutaraldehyde [14]. As a whole, glutaraldehyde solutions are made up of polymers that react with hemoglobin at multiple cross-linking sites and create a heterogenous mixture of Hb. The process of glutaraldehyde polymerization involves a redox potential decrease, and an iron autoxidation rate increase [20]. To create soluble polymers, a solution of 0.05 M phosphate buffer (pH 6.8) is prepared along with 100 mg/mL of human oxyhemoglobin and 3.3 mg/mL of glutaraldehyde at 20 °C. After 20 minutes, glycine is added, and the solution is dialyzed with a phosphate buffer. The process by which insoluble polymers are created is as follows: a 0.05 M phosphate buffer (pH 6.8) is combined with 100 mg/mL of Hb and 3.3 mg/mL of glutaraldehyde. The solution is then frozen at – 30 °C for 2 hours and warmed slowly at 4 °C. The insoluble foam is then rinsed with a glycine solution and phosphate buffer.

Recombinant HBOC

Acellular recombinant hemoglobin can be used an oxygen carrier. Similar to HBOCs constructed from human or animal Hb, recombinant HBOCs (rHBOCs) potentially can be infused in place of erythrocytes. Recombinant Hb has the advantage that it can be mass produced utilizing molecular biology in unlimited amounts and does not require human Hb. Recombinant Hb can be produced in E. Coli by fusing alpha or beta globin cDNAs to the coding region of a bacteriophage. The fusion protein can then be recovered and refolded to produce tetrameric Hb [21]. A similar, newer, system involves incorporating the alpha or beta globin genes as a transcript in the tac promoter. Then, functional Hb tetramers are produced after exogenous heme is incorporated into the E. Coli cytoplasm [22].

Zero-Linked HBOCs

Another type of polymerized hemoglobin is zero-linked Hb. This is created by a procedure where hemoglobin's surface is activated with carbodiimide at the carboxyl groups. This causes the carboxyl groups to create covalent bonds to amino groups of an adjacent hemoglobin molecules. The name zero-link is derived from the fact that no chemicals are left behind in the linked polymers, unlike with glutaraldehyde or other polymerization techniques [23].

Pegylated HBOCs

PEGylated hemoglobin refers to the addition of polyethylene glycol to hemoglobin. Two important products that use pegylated Hb are PHP (Hemoximer), Hemospan and Sanguinate. The overall procedure involves deriving hemoglobin from bovine or outdated human sources and PEGylating it. Then, either maleimide is added to produce the Hemospan (MP4) product, or the Hb is PEGylated and carbon-monoxide hybridized to create Sanguinate [7]. Hemoglobin can be pegylated in two ways: or under anaerobic conditions to produce PEG-Hb(deoxy), or under aerobic conditions to produce PEG-Hb(oxy). To produce PEG-Hb (deoxy), a buffered solution of 50 mM sodium phosphate, and 100 mM KCl, 0.5 mM EDTA is placed in a 1 L bottle of pH 7.0 at 20 °C. Inositol hexaphosphate is added, Hb is reacted with iminothiolane, and then maleimido-polyethylene glycol (MAL-PEG) is added to the mixture. The solution is

dialyzed with a phosphate buffer saline to eliminate any unreacted MAL-PEG and passed through a filter to remove endotoxins. To produce PEG-Hb (oxy), PEG conjugation is done aerobically in a 1 L bottle at 5 °C. 3 mM Hb is treated with IMT in a phosphate buffer saline at pH of 7.4 for 4 hours, and then MALPEG is added. After an incubation period of 2 hours, a cysteine solution is added to terminate the reaction. The sample is passed through a syringe filter to remove endotoxins [24].

Invertebrate Hb

Hemarina's multiple products, including HEMO2-life, is derived from a large extracellular hemoglobin molecule that is naturally found in the invertebrate Arenicola marina, commonly known as the lugworm. Each Hb molecule from this species can transport 156 oxygen molecules, compared to just 4 oxygen molecules carried by human Hb [25]. In addition, HEMO2-life can function in the temperature range of 4–37 °C, and its size is 250 times smaller than a human RBC [26]. These properties give HEMO2-life remarkable utility for oxygen delivery in humans. A team of researchers led by Lupon et al., in France investigated optimal ways of packaging and delivering HEMO2-life. Using their technique, it is packaged in a 20 mL solution containing 1 g of active extracellular HEMO2-life, 203.3 mg of Magnesium chloride, 105.2 mg of sodium chloride, 100.3 mg of sodium gluconate, 73.5 mg of sodium acetate, 7.5 mg of potassium chloride, 7.3 mg of calcium chloride, 35.2 mg of ascorbic acid, and 20 mg of water for injection. The investigators found the half-life to be 48–72 hours and that the washout period maximum is 4 days. The team led by Alix et al., studied the addition of HEMO2-life (M101) to preserve liver grafts. The investigators discovered that when added to a cold storage solution liver graft, M101 effectively oxygenates the grafts during preservation, preventing post-transplant injury. The team also reported that M101 has intrinsic Cu/Zn superoxide dismutase (SOD) activity, likely contributing to its utility for preventing post-transplant injury.

Pharmacology and Physiology of HBOCs

Table 12.2 shows the pharmacology and physiology of HBOCs.

Development of HBOCs

As mentioned previously, the first generation of blood substitutes were the SFH products. SFHs were manufactured utilizing ultrafiltration or crystallization. The SFH prepared by ultrafiltration method was reported to lack the vasoconstriction and ex vivo perfused myocardial depressant factors [7]. However, SFH was later found to elicit many adverse effects to progress forward, but the untoward outcomes were valuable in determining the cause of the adverse effects.

Using the feedback from the SFH experiments, it was hypothesized that crosslinking may help reduce the adverse effects seen in SFH. Researchers had been working towards crosslinking hemoglobin since the mid-1960s and that effort was accelerated during the 1980s. At that time, the HIV epidemic was beginning, and the military was concerned the epidemic would deplete the blood supply for transfusions. Blood availability for transfusions were essential to treating wounded soldiers, and so a viable blood substitute became an urgent unmet need.

Development of αα-Hb and HemAssist

The group of scientists from the Army Institute of Research at Letterman (LAIR) were successful in creating a crosslinked Hb that could reduce the renal toxicity issues seen in SFH [28]. To be able to mass produce, LAIR contracted out to Baxter Healthcare. Together, they successfully crosslinked the α chains of hemoglobin utilizing bis(dibromosalicyl) fumarate (DBBF) [28]. The army called the product DBBF-Hb and ultimately $\alpha\alpha$-Hb, while Baxter (Deerfield, IL) referred to the product as diaspirin-crosslinked Hb (DCLHb), with the trade name of HemAssist. The relationship between LAIR and Baxter however was not collaborative as Baxter's focus was on increasing production while LAIR was focused on resolving vasoconstriction effects and other biological problems. Following a study on a pig model simulating a battlefield injury, LAIR stopped attempting to produce a blood substitute. LAIR declared that based on the pig model, $\alpha\alpha$-Hb was extremely vasoactive and therefore too toxic to be considered as a blood substitute option [28].

This decision was validated again by further studies showing systemic and pulmonary hypertension as well as doubling of the vascular resistance which was equivalent to that seen with inhibition of nitric oxide synthesis [29]. Baxter however continued with production of HemAssist and went on to be tested in human clinical trials because in their application to the Food and Drug Administration (FDA), Baxter explicitly stated that their product, HemAssist, was critically different from DBBF-Hb by LAIR [28]. Ultimately, HemAssist failed as well for being too vasoactive, just like DBBF-Hb.

In the critical study of HemAssist published by Saxena et al., two major adverse events were identified. One patient experienced transient renal and pancreatic insufficiency while the other suffered from both a fatal brain edema as well as a pulmonary edema [30]. Similar results were seen in

Table 12.2 Pharmacology and physiology of HBOCs

Product	HemAssist	HemoLink	Hemoximer	PolyHeme	Oxyglobin	Hemopure	Hemospan	OxyVita	Sanguinate	Hemarina
Production method	Alpha-alpha crosslinked Hb	Polymerized human Hb, cross-linked with o-raffinose	Pyridoxalated Hb polyoxyethylene conjugate	Glutaraldehyde polymerized human Hb	Acellular polymerized Hb	Purified cell-free glutaraldehyde cross linked and polymerized	PEGylated Hb plus maleimide	Zero-linked polymerization	PEGylated carboxy-Hb	Derived from large extracellular Hb molecule
Source	Outdated human Hb	Outdated human Hb	Outdated human Hb	Outdated human Hb	Bovine	Bovine	Outdated human Hb	Bovine	Bovine	Arenicola marina
Hb concentration (g/dL)	10	10	8	10	13	13	4.2	6	4–5	4
P50 (mmHg)	32	39 ± 12	23.4	20–22	34	40	6	6.4	7–16	7.05 ± 0.93
Hill coefficient	2.9	1.0 ± 0.2		1.7	1.3	1	1.2	1.2		
Viscosity (cp)	1.2	1.1	3	1.9–2.2	2.8	1.3	2.5	2.8		1.23
COP (mmHg)	42	25.4	49	20–25	42	25	55	2.2		1
pH	7.4	7.5 ± 0.5	7.4		7.8	7.6–7.9	7.5 ± 0.2	7.5	8	6.5–7.5
Solution		LR			LR	LR	LR	LR	Phosphate buffered saline	Injectable buffer
Half-life in humans (hr)	6–12	15–18		24	18–43	19	20–36	72	7.9–13.8	48–72
Stability/ storage	<5 °C for 1+ yr	Oxy-ligand form: −80 °C De-oxy-ligand form: 4 °C		4–8 °C: 1+ yr	Room temp: 3 yr	Room temp: 3 yr		Lyophilized: 5 yr. Liquid: 1 yr. at room temp	48 °C: 4 yr., Room temp: 2 yr	-20 °C ± 5 °C/−80 °C ± 10 °C, for 5 years
Molecular weight (kDa)	64	>100	109	150	200	250	90	33,000	109–120	3600

Adapted and modified from Reference [27] and from personal communication February 2021 with:
HemAssist: Dr. Tim Estep, Hemolink: Dr. Davy C.H. Cheng, Hemoximer: Dr. Joe DeAngelo, PolyHeme: Dr. Eugene Moore & Dr. Alexis Craloey, Hemopure: Dr. Greg Dube', Hemospan: Dr. Peter Keipert, Sanguinate: Dr. Abe Abuchowski, OxyVita: Dr. Hanna Wollocko, Hemarina: Drs. Franck Zal and Eric Delpy

the study conducted by Sloan et al. in which 46% of the patients given HemAssist died 28 days after administration compared to the 17% who received only saline [31]. This failure by Baxter placed further emphasis on preclinical testing prior to administration to human subjects.

Development of Hemolink

Prior to the cessation of investigations of HemAssist, a second generation of HBOCs were developed, the earliest being Hemolink, made by Hemosol Inc. (Mississauga, ON, CA). Hemolink, from outdated human blood hemoglobin, is a raffinose crosslinked HBOC [27]. An indicated use was for cardiac surgery and achieved human clinical trial status. In a phase II clinical trial using the intraoperative autologous donation (IAD) concept during cardiac surgery, patients given Hemolink required less allogeneic erythrocytes, even past the operative period [32]. In another phase II dose escalation trial conducted by Hill et al., Hemolink was successful at reducing allogenic red cell transfusion for subjects having coronary artery bypass graft (CABG) surgery [33].

Hemolink clinical trials were stopped voluntarily during a Phase IIb trial due to concern over increased morbidity and mortality [27, 34]. There was a disproportional incidence of adverse cardiac events in the group administered Hemolink, although the higher occurrence of adverse events may have been due to the increased number of diabetics within the Hemolink treated group [34]. Hemolink was created with the rational that HBOC s would restore the oxygen carrying capacity of the blood lost and ultimately facilitate an increase in blood pressure [35]. However, although vasoconstriction does increase central blood pressure, it may, by the same pathway, decrease downstream capillary pressure, and might be responsible for causing myocardial ischemia.

Development of Polyheme

A reoccurring issue with HBOCs was the vasoconstrictive properties they exhibited due to the vasoactive tetramers. Researchers hypothesized the vasoactive tetramer properties were due to trans-endothelial extravasation of the small molecular weight tetramer, which lead to abluminal binding of nitric oxide (NO) and induced unrestricted vasoconstriction [36].

To alleviate extravasation, Northfield Laboratories produced a nearly tetramer-free and considerably larger HBOC from glutaraldehyde polymerized human Hb. PolyHeme's polymerization technique improves intravascular persistence and is then purified until nearly all unpolymerized tetramers are removed. This reduces interactions with nitric oxide and therefore potentially reducing vasoconstriction [36].

PolyHeme was hypothesized to have indications as a resuscitation fluid, especially helpful in avoiding the onset of anemia and delaying the inevitable mortality until surgical intervention and red cell transfusion would be available [27]. PolyHeme was designed as an asset for shock and organ ischemia, especially in emergencies.

Early clinical trials suggested PolyHeme was successful in providing oxygen-carrying capacity at life-threatening Hb levels and maintaining oxygen transport during intense blood loss. With these results, the FDA approved a Phase III clinical trial. This pivotal multicenter trial published by Moore et al. was significant due to its clinical results but also for the ethical issues that arose. The protocol was centered on two hypotheses regarding survival benefits: early replacement of oxygen-carrying capacity in a setting in which blood is not available, and PolyHeme administration in place of blood transfusion during the first 12 hours after injury to decrease the immunoinflammatory response and ensuing organ dysfunction [36].

The study enrolled over 700 patients and was divided into two phases, the "pre-hospital" and "in-hospital". During the pre-hospital phase, incapacitated trauma patients were either given the standard of care (saline) or given PolyHeme. In the in-hospital phase, the subjects administered saline in the pre-hospital phase then were administered erythrocytes (allogeneic), whereas subjects who were given PolyHeme in the pre-hospital phase continued with PolyHeme administration, instead of the standard of care administration of allogeneic blood. While the patients given PolyHeme required and were administered fewer units of erythrocytes, more PolyHeme cohort subjects were also designated as having "probable myocardial infarction" [36].

The study was discontinued in Phase III following negative results [27]. The ethical issues stemmed from the portion of the trial occurring post hospital admission. Since 1996, the FDA had published regulations that waive the requirements for informed consent in specific emergency research protocols. Researchers in this clinical trial did not therefore need to obtain personal consent once the subjects arrived at the hospital prior to administration of PolyHeme. Once at the hospital, blood transfusion is the standard of care for trauma patients; however, these subjects were not able to consent to waive the erythrocytes (standard of care) and instead administered an experimental protocol.

Development of Hemoximer

See Table 12.3 for development of Hemoximer (PHP).

Table 12.3 Development of Hemoximer (PHP)

Compelling evidence suggests that NO is the causative agent responsible for vasodilation and hypotension in distributive shock. In the successfully conducted Phase II clinical development program PHP has previously been demonstrated to reverse vasodilation and resolve hypotension associated with this type of shock
The PHOENIX trial was a European, placebo-controlled, Phase III study treating patients in catecholamine-resistant, distributive shock with the Nitric Oxide (NO) scavenger PHP/Hemoximer.
This phase III, multi-center, randomized, placebo-controlled study compares the effectiveness of continuous infusion of PHP/Hemoximer plus conventional vasopressor therapy against placebo (normal saline) plus conventional vasopressor therapy in patients with catecholamine-resistant distributive shock. In addition, the safety and tolerability of this new treatment modality will be evaluated.
For inclusion into the trial the patients had to be adequately resuscitated with fluids and must require a norepinephrine dose of ≥0.3 mcg/kg/min to maintain a mean arterial blood pressure of ≥65 mmHg. Furthermore, patients had to fulfill at least two criteria indicative of a systemic inflammatory response ("SIRS" criteria). PHP/Hemoximer as active compound or placebo was administered by continuous intravenous infusion at 0.25 mL/kg/hr. for a maximum of 150 hours.
Efficacy was to be demonstrated by PHP significantly reducing 28-day all-cause mortality. Secondary endpoints include: Survival time, survivor days in the intensive care unit ("ICU") and time on mechanical ventilation and on vasopressors.
The study was launched by Curacyte AG in Austria, Belgium, Germany, Spain, The Netherlands and in the United Kingdom in 2009. The outcome of the third interim analysis on safety and mortality data of 300 patients, or 66% of the study population in the trial is reported. Statistical results were reviewed in an unblinded fashion by an independent Data Monitoring Board (DMB). The DMB, consisting of intensive care physicians, one bioethical expert and one statistician from the US and Europe came to the unanimous conclusion, that the study should be terminated.
The trial was designed to statistically prove survival benefit of patients treated with PHP after 28 days compared to placebo (+ standard care), and at day 60 and day 90 as secondary endpoints. After treatment of two thirds of the patient population the numerical number of deaths in the PHP/Hemoximer cohort exceeded that of the placebo group. Given the current status of the study there is no more chance that the study could demonstrate a statistically significant efficacy on 28-day all-cause mortality in favor of PHP/Hemoximer. Therefore, the continuation of the PHOENIX study with enrolment of new patients under the premise to detect an unlikely difference would be unethical. Based on the DMB's recommendation Curacyte AG suspended the PHOENIX trial.

Reprinted from: vm-lifescience.com/curacyte-phase-iii-distributive-shock-trial-phoenix-trial-terminated-for-futility/ Accessed 2.16.2021

Development of Hemopure and Summary/ Discussion of Pivotal HEM-0115 Trial

The final second generation HBOC product to be discussed is Biopure's Hemopure (HBOC-201), one of the most extensively studied HBOCs (now produced by Hemoglobin Oxygen Therapeutics, Souderton, PA, USA). Hemopure's hemoglobin is extracted from bovine RBC, purified and then polymerized with glutaraldehyde, after extensive cleaning processes, validated by the US FDA to ensure removal of bacteria, fungi, parasites, viruses and prions. Hemopure draws its benefit from maintenance of oxygen delivery with anemia or low blood flow situations to ischemic tissues. Unlike its other fellow second generation HBOCs, Hemopure has gained regulatory indication for human use in South Africa (2001), and in Russia (2006) to treat acute surgical anemia.

Hemopure is a glutaraldehyde cross-linked, polymerized bovine hemoglobin that is cell free and purified positioned in a changed lactated Ringer's solution with 13.6 g/dL Hb (30–35 g Hb/250 mL unit), a pH of 7.6–7.9, and a P50 of 40 mmHg. Hemopure may be kept at 20 degrees centigrade for up to 3 years, is not in need of cross-matching, has an oxygen release that is independent of 2,3-diphosphoglycerate, and has a half-life of 19 hours in the circulation [37].

Drawing from four different studies in which RBC transfusion was used as the control, Hemopure administration led to lower allogeneic RBC units transfused [27]. In a randomized and double-blind trial conducted by Levy et al., HBOC-201 was evaluated for its treatment of moderate anemia following cardiac surgery due to blood and hemodilution. In the study, 98 patients scheduled to cardiac surgery were randomly divided into the control group, set to receive RBC transfusion, and the other to receive HBOC-201, and then monitored during the course up to their third postoperative transfusion. Of the 50 patients given Hemopure, 17 of them did not require any RBC transfusion following the operation [5]. An important point is that although this study concluded that Hemopure administration decreased the requirement for transfusion of erythrocytes postoperatively in one third of its participants, the study was not designed to compare the mortality and morbidity and so no conclusions were able to be reached with regard to its efficacy relative to blood.

A more detailed description of the HEM-0115 study follows [37].

The research study evaluated Hemopure as an alternative blood transfusion and its safety and efficacy in a Phase III, single blind, multicenter trial in elective orthopedic surgery. The whole trial study population included male and female (nonpregnant/lactating women) subjects, 18 years or older. The investigators randomized six hundred eighty-eight patients to treatment with Hemopure or erythrocytes. Paired/matched group analysis was performed comparing groups directly, determined by a predetermined prospective dichotomy (success of treatment compared to failure in the Hemopure arm and similarly predetermined dichotomy in the erythrocyte arm, determined by the requirement for additional oxygen carrying capacity [moderate compared to high]). The hypothesis

was to prove the ability of Hemopure to safely reduce or eliminate perioperative transfusion.

In the majority of subjects, Hemopure administration demonstrated the eliminate of need for erythrocytes. Comparing the Hemopure and erythrocyte arm in a safety analysis demonstrated disequilibrium and was linked to subject's age, volume overload, and under treatment, and occurred in those subjects in whom Hemopure alone was insufficient. The investigators concluded that in subjects under 80 years of age with a clinical requirement deemed moderate, may be infused with up to 10 units of Hemopure to avoid erythrocytes safely.

This landmark, pivotal study aimed to demonstrate efficacy, safety and adverse event profile of HBOC-201 use. Treatment with Hemopure exceeded the pre-determined primary endpoint of 35% avoidance of transfusion with an overall 59% avoidance observed at the 6-week follow-up assessment.

One of the most interesting evaluations for the management of low hematocrits is whether the transfusion criteria is relevant to current transfusion practice. The study demonstrates that this clinical decision must be tempered by the patient's clinical status with respect to cardiac disease, pharmacologic agents, and a host of other factors [38, 39].

The concept of a universal transfusion trigger has fallen out of favor with the recognition that the decision to transfuse is actually quite complex, based upon individual patient factors beyond total Hb concentrations as reflected in the most current United Kingdom (2007) and United States (2006) guidelines [40, 41]. The term transfusion "threshold" is now preferred.

The results of matching group and root cause analyses suggest that the future use of Hemopure in patients over age 80 and/or when the need for additional oxygen carrying capacity exceeds what can be met with Hemopure alone should be limited to when blood is not available. Vigilance to patient vascular volume status is required when infusing Hemopure; avoidance of fluid overload and, if present, aggressive management is required. Patients with preexisting cardiac disease are less tolerant of lower total Hb concentrations and may be at greater risk of morbid events and increased mortality.

The most robust observations have come from large retrospective studies which suggest that for older patients and patients with cardiac disease relatively small deviations from normal Hb and Hct levels may be associated with an increased risk of morbidity and mortality [42, 43].

In the Hemopure arm, more than 59% of subjects did not require erythrocytes. Consequences deemed adverse and those deemed serious (serious adverse events [SAEs]) per subject were greater in the Hemopure versus erythrocyte groups. Volume overload and undertreatment may explain this difference.

Transfusion was eliminated in the Hemopure arm in most subjects. The safety analysis of the two different study groups (H vs. R) did not reach favorable status probably due to subject's age, hydration status and possible volume overload, and potential under administration of Hemopure, and was isolated to subjects who were unable to be cared for only with Hemopure. Nonetheless, octogenarians with modest acute anemia safely may avoid transfusions when managed with up to 10 units of Hemopure.

Adverse Events of Particular Interest

The vasoactivity of HBOCs as a drug class has been of great general interest, with some HBOCs showing greater vasoactivity than others [44]. Blood pressure elevations were transient with mean SBP [Systolic blood pressure] 10–15 mm Hg above that observed with PRBCs.

No significant changes were noted in the cardiac biomarker CK-MB between treatment arms, differences in troponin elevations were observed; with 5 of the 18 HBOC-201 patients with elevations of troponin showed serial elevations.

The analyses described in the 'Study Limitations and Critique' section suggest that when the Schedules for Maximum Surgical Blood Orders predicts a "need" of three units of erythrocytes or less, in the patients under 80 years old who are undergoing elective orthopedic surgical procedures, safe, and effective avoidance of red cell transfusion is possible with the use of up to 10 units of Hemopure. Whenever erythrocytes are not an option, treatment with Hemopure may be appropriate and might be optimal.

In conclusion, the HEM-0115 Phase III study demonstrated Hemopure's reduction or elimination of erythrocyte requirements in patients undergoing orthopedic surgery. Six hundred, eighty-eight subjects were randomized to be administered Hemopure or erythrocytes. For up to 6 days, Hemopure subjects were infused with a maximum of 325 g/2500 mL of Hemopure. Erythrocytes were then transfused as needed. Hemopure's reduction of the requirement for more erythrocytes occurred in more than 59% of the subjects. The Hemopure cohort reported an increased rate of adverse effects that consider was significantly greater [45].

Recent Clinical Trials on Hemopure

In a study examining the preoperative therapeutic effects of Hemopure, LaMuraglia et al. concluded preoperative

Hemopure administration to anemic patients undergoing aortic surgery decreased the need for erythrocytes in almost one third of the patients [46]. Based on the two studies, Hemopure administration before or after helped alleviate the need for RBC transfusion. The FDA has yet to approve a trial testing Hemopure's efficacy on trauma patients due to apprehensions regarding trial design and study justification [45]. As discussed earlier, there were significant ethical concerns raised over the trial design of Polyheme's use in trauma settings and further trauma trials will need thoroughly designed protocols to receive approval.

However, recently, the US Department of Defense approved and funded a trauma trial to be conducted in South Africa (https://www.dvidshub.net/news/printable/311421 accessed 1/31/2021) with Hemopure. See Table 12.4 describing the plans for the study.

Table 12.4 Plans for the Hemopure Trauma Trial in South Africa

Experts from the US Army Institute of Surgical Research and Emergency Medicine and South Africa's Stellenbosch University (SU) have embarked on a large, multi-institutional clinical trial to evaluate the use of synthetic blood-products for the resuscitation of trauma victims before they arrive at a hospital.
The Institute of Surgical Research is coordinating through the US Africa Command Science department and under an agreement implemented by the US Embassy Pretoria, Office of Defense Coordination on Research, Development, Testing and Evaluation (RDT&E) in 2016.
The San Antonio based center, which focuses on combat casualty care, will be partner with Stellenbosch University's division of emergency medicine Faculty of Medicine and Health Sciences.
Other universities involved in the clinical trial include the University of Cape Town, KwaZulu-Natal, Witwatersrand and Pretoria. These entities collectively represent the body of academic emergency medicine in South Africa.
All parties met in Cape Town for the first major planning session 27–30 November at Stellenbosch University's Tygerburg multi-center, prospective, randomized clinical study of bioplasma freeze dried plasma and Hemopure for use in treatment of trauma patients with significant Haemorrhage.
The study will evaluate the use of the hemoglobin-based oxygen carrier Hemopure (HbO2 therapeutics LLC) together with bioplasma FDP (National Biologics Institute), freeze-dried plasma, to resuscitate trauma victims prior to arrival at a hospital emergency room.
Trauma is the leading cause of death and disability among young adults, which is often due to severe blood loss. Bleeding following trauma causes 1.5 million deaths a year worldwide.
South Africa has a high burden of trauma, especially amongst young adults.
Improvements in survival and better clinical outcomes from trauma result from early diagnosis, rapid bleeding control, and the early deployment of ambulances to rapidly transport patients to advanced medical care.

Reprinted from https://www.dvidshub.net/news/printable/311421 accessed 1/31/2021

Development of Third Generation HBOCs

After the setbacks experienced by the researchers of earlier generation HBOCs, manufacturers set out to develop products that would eliminate the toxicities, biophysical and chemical adverse events caused by the crosslinked/polymerized HBOCs. In lieu of creating products to replace donor blood for transfusions, researchers shifted focus to development of artificial oxygen carriers designed to be administered when erythrocytes are unavailable or in cases where a blood transfusion is impossible because of health or religious reasons (e.g., Jehovah's Witness). Researchers concluded that a blood substitute as an artificial oxygen carrier may be designed to prevent or treat ischemia-related morbidity and consequently decrease the mortality from anemia, hypoperfusion, or ischemia [7].

A product focused on alleviating ischemia related morbidities would establish enough effectiveness to gain regulatory approval, rather than a product designed to replace donor blood. Hemospan (MP4) was produced by Sangart in San Diego, CA, Hemospan was manufactured from outdated human blood and modified with maleimide-polyethylene glycol. Related to proposed non-hypertensive action, Hemospan was regarded as a plasma expander, in sharp distinction from previous vasoconstrictive HBOCs. MP4 initially had favorable results in its Phase I study, where healthy subjects did not become hypertension or experience gastrointestinal adverse effects [47].

While the MP4 itself was showing promise, further studies on it within the United States were halted. In 2008, Natanson et al. published a meta-analysis concerning the risk of myocardial infarction (MI) and death in patients participating in HBOC trials [48]. The meta-analysis itself was inconclusive and exhibited major statistical analysis incongruencies. Despite the study's methodological inconsistencies, the FDA implemented an immediate halt to all HBOC studies [49]. Fortunately, the regulatory agencies in the European Union and many other countries allowed HBOC clinical trials to continue once they had completed their own safety review.

Following trials testing Hemospan in trauma settings across various countries worldwide, the FDA requested more information to approve a Phase 2c. Although the FDA eventually allowed the trial, Sangart could not obtain enough funding to continue with the trial [49].

Another HBOC product focused on alleviating hypoxia and relieving ischemic tissue is Sanguinate, produced by Prolong Pharmaceuticals. Sanguinate is PEGylated bovine carboxyhemoglobin, which releases carbon monoxide (CO) and transfers O_2. However, this cannot be done simultane-

ously as they both bind the same binding site. It is suspected that they initially make carbonmonoxy-Sanguinate, and once infused, it is purported to release CO during circulation and subsequently it oxygenated (oxy- Sangunate) in the lung and delivers oxygen to the tissues. But the manufacturer never explicitly demonstrated data that explained how Sanguinate actually works.

This dual action was hypothesized to help inhibit vasoconstriction, diminish extravasation, decrease free oxygen radial formation, enhance blood rheology, and oxygenate tissues; however, these claims were not independently validated [50]. The dual action component, the purported safety profile and mechanism of action, sets it apart from other HBOCs, yet the manufacturer also attempted to develop and test CO-MP4.

Misra et al. led a Phase I clinical study, a single-center, single-blind, placebo-controlled, single dose study of the safety of Sanguinate in a group of healthy patients. The study indicated a good safety profile when administered at 80, 120, or 160 mg/kg.

Once its safety was established in healthy patients, Sanguinate was administered under an expanded access emergency Investigational New Drug (IND). The patient had sickle trait as well as beta thalassemia trait, and due to being a Jehovah's Witness, refused blood transfusions. The patient had a brain oximetry reading between the 40 and 50 mmHg range and was no longer responsive. Following administration to the patient brain oximetry values rose to normal range, patient regained mental status, and became responsive. The patient's improvement indicated Sanguinate had transported oxygen to the ischemic cerebral tissue [50].

In a randomized, open label, Phase Ib open label safety study of Sanguinate in sickle cell subjects, researchers found Sanguinate to be safe, with no serious treatment-related adverse events [51]. This study supports the concept that Sanguinate may be beneficial for the management of comorbidities associated with sickle cell anemia.

Another area in which Sanguinate might be beneficial is in the treatment of subarachnoid hemorrhage (SAH). Dhar et al. conducted a study evaluating administration of Sanguinate to patients with SAH at risk for delayed cerebral ischemia (DCI). The study was small, with only 12 patients. However, Sanguinate administration improved cerebral blood flow (CBF) and flow metabolism balance in the weak brain regions [52].

A newer HBOC product, Oxyvita is showing promise by eliminating the issues presented by earlier HBOC generations, however has not yet been tested clinically. Oxyvita is a zero-linked hemoglobin polymer, created by using cross-linked bovine tetramers instead of any exogenous binding agents previously designed in the manufacture of HBOCs, such as raffinose or glutaraldehyde. Without those exogenous binding agents remaining in the product and the supra-high molecular weight (20–30 million Dalton), Oxyvita has a much lower tendency to extravasate into the subendothelial space where NO scavenging may readily occur. Therefore, Oxyvita is purported to avoid NO scavenging, which was a common problem in previous HBOCs. Oxyvita can be stored safely at room temperature or stored in lyophilized form for up to 5 years. In its liquid form, it can be stored for 12 months at room temperature [27]. Its storing capabilities are a major advantage. In studies, Oxyvita has eliminated extravasation associated with HBOCs and shown to deliver oxygen to ischemic tissues more effectively than red blood cells [53]. Future studies are needed but so far, the product appears promising.

OxyVita is an HBOC that is developed to be different from others in that it ostensibly avoids vasoconstriction, hypothesized to be related to nitric oxide induction locally due to HBOC extravasating from the circulatory system (including lymph). OxyVita was not found in lymphatic fluid after an infusion in a feline model, nor did it demonstrate any vasoconstriction. Possibly, decreased extravasation thereby minimized the effects of nitric oxide being scavenged, therefore not causing the expected vasocontrictive issues.

The final HBOC product to be discussed is HemO2Life. HemO2Life is derived from extracellular hemoglobin (M101) from *Arenicola marina*, an invertebrate marine worm that experiences periods of hypoxia and hypothermia during high tide [14]. The heavy (3600 kDa) biopolymer exhibits a large O_2 binding capacity and releases O_2 without the assistance of allosteric effectors, via a simple gradient [54]. It has been marketed as an organ preservative to be added to solutions used for donated organs during transport.

It is being studied for organ preservation. A common complication in organ transplantation is the high chance of ischemia-reperfusion injuries [55]. There are procedures aimed at reducing enzymatic activity, and thus reducing the risk of ischemia, but the need for an efficient oxygen delivery system remains. Researchers wanted to examine the beneficial effects of $HemO_2$Life for kidney preservation prior to transplantation. Bovine kidneys were subjected to 60-minutes warm ischemia prior to preservation to increase post-transplant complications, in order to mimic kidney conditions witnessed in extended donor and deceased after circulatory death kidneys. The addition of $HemO_2$Life to the preservation solution decreased short-term function loss and preserved tissue integrity, overall improving organ preservation [55]. Following the success in bovine studies, $HemO_2$Life was tested for the first time in humans.

A multicenter safety study was conducted to study the addition of $HemO_2$Life to the preservative process of 60 kidneys harvested from deceased donors. The study conducted by Le Meur et al. indicated the transplanted kidneys from the $HemO_2$Life added solution presented less delayed graft function than the contralateral group of kidneys which were kept

in the preservation solution not containing $HemO_2Life$, in addition to presenting better renal function as measured by creatinine levels [56]. Preliminary data from completed trials looks favorable, with no adverse events, graft loss related to product, no deaths, and no allergic or immunological effects from $HemO_2Life$.

Coagulation and HBOCs

Introduction

Coagulation is a complex process that works by creating a chemical reaction, eliminating negative charges that cause particles to repel each other. The action of these bubbles forces clots or flocs of particles to the water surface where they can be skimmed off. Understanding the importance of coagulation, different scientists have developed different methods and coagulants in order to have the right procoagulants for the necessary indication [57]. However, whenever biologic therapeutics, such as HBOCs are developed, it is critical to determine if they are procoagulant or anticoagulant, as their effect to improve oxygen carrying capacity may be diminished or negated by clot formation or anticoagulant effects.

One initial concern with HBOC was possible activation of platelets as Hb can scavenge endothelial NO which has an anti-PLT aggregation effect. Therefore, with the development of Oxyvita, its possible coagulation side-effects, a new generation HBOC or Oxyglobin (HBOC-200, the veterinary congener to Hemopure) were studied [58]. This research aimed to describe and analyze the effects of Oxyvita or Oxyglobin and compared these effects with other hemodiluants and investigated if molecular weight and molecule specificity affects the results. Comparisons were made in low, medium, high and very high concentrations, using the measurements made with thrombelastography (TEG) [58].

TEG analysis demonstrated that OxyVita and Oxyglobin have the greatest deviation on clot strength. The size of this activity is equivalent to 6% hetastarch at low and medium dilutions, yet was significantly larger that that of 0.9% normal saline solution. At the highest dilutions, both OxyVita and Oxyglobin clot strength was decreased significantly in comparison to 6% hetastarch, 0.9% normal saline, and whole blood [58].

Oxidized Oxyglobin cause elevated methemoglobin levels may implicate that additional coagulation issues which are resulting most likely, by platelet function and coagulation proteins reaction to the effects of oxygen free radicals. These may interact with coagulation factors cause modification of the redox-sensitive areas on the platelet surface. It is entirely possible that methemoglobinemia, related to HBOC infusion, may cause levels to be greater than 10%, considered significant, that there may be coagulopathies as a result [59].

OxyVita is now also produced in a powder form, basically lyophilized from the liquid form [60]. This has been tempered with the need for proper buffering, ensuring appropriate electrolytes are maintained in the delivered product, and that the osmolarity is maintained in the final infusion solution. Reconstitution time may also be a factor in military and civilian trauma use [60].

Ex vivo comparisons of coagulation interference of two different molecular weight HBOCs (OxyVita, a new-generation zero-linked polymerized bovine hemoglobin-based oxygen carrier, 17–33 megadalton and Oxyglobin, 200 kilodalton) and hetastarch 6% (670 kilodalton) with normal saline, using thrombelastography, showed that the two HBOCs decreased clot strength (MA and G) at low and medium dilutions [61].

Since Oxyvita utilizes a modification of the zero-link polymerization, which uses chemical activators to add the inter-dimerically bovine hemoglobin that is cross-linked into "super-polymeric" macromolecules [62]. This unique design may also be of value with regard to coagulation concerns as demonstrated in the study previously discussed.

The new OxyVita that is lyophilized has properties similarly to the original liquid form after its reconstitution in an aqueous format. The time to reconstitute, based on solubility, with a time range of 10–30 s in water and has been demonstrated to function similarly to the liquid format [63].

Small volume resuscitation is now the recommended strategy for hemorrhaging patients; since most fluids currently advocated are larger volume with lack of oxygen carrying opportunities, HBOCs may be the idea solution to aid with oxygen deliver to deprived tissues [64].

This thinking then may be applied to the use of Hemopure in trauma victims: since it may not replace erythrocytes, its value to allow severely compromised victims to be rescued in the place of injury or in more advanced care settings, when either hematinic effects may allow for regeneration of erythrocytes, or transfusion is an option [65].

As a new generation HBOC, zero-linked hemoglobin polymer may begin to address the issues presented by the first two earlier generations of HBOCs, and may hold promise as a universally applicable HBOC [27].

The requirement for an alternative to erythrocytes to transport oxygen and moderate transfusions has driven the search for artificial oxygen carriers, mostly in the HBOC realm, as products derived from human or bovine Hb, or chemical modification or genetic engineering [66].

Finally, a citation of the Oxyvita study commented that the study was successful in mimicking resuscitation of traumatic hemorrhagic shock with the resultant hemodilution of crystalloids or colloid solutions [67]. Effects on coagulation when whole blood is diluted to 1:11, 1:5, 1:2, or 1:1 with

normal saline, 6% hetastarch(670 KDa), Hb-200, or OxyVita were analyzed accurately, and the results were considered valid and reproducible [67].

Single Site Clinical Study of Hemopure on Platelet Function

A single-site analysis from the previous HEM-0115 trial assessed platelet function in patients, before and after transfusion of erythrocytes or infusion of Hemopure [61]. The analysis using a PFA-100 (Platelet Function Analyzer-100, Siemens Healthcare Diagnostics Inc., Tarrytown, NY). "The PFA-100 System measures the complex process of primary haemostasis and aids in the rapid detection of platelet dysfunction. It is the first commercially available *in vitro* testing system to incorporate high-shear flow in which the process of platelet adhesion and aggregation following a vascular injury is simulated *in-vitro*" [68]. From the databank and unpublished, it was determined that those using Hemopure did not require more non-red blood cell products, such as fresh frozen plasma, cryoprecipitate and platelets, than the erythrocyte group (published as AABB Abstract: Williams J et al. Transfusion 2002). There was also a significant difference found in the "after transfusion" time period between the Hemopure and erythrocyte group, where the Hemopure group had increased cEPI and cADP (measurements of platelet adhesion that are aspirin-independent and aspirin-dependent). An increase in these measurements can correlate with an increased risk for bleeding. This increase, however, reversed about one Hemopure half-life on "Day 1 After transfusion." The increased cEPI may be explained by the lower level of Hb concentration found in the study in patients given Hemopure *versus* erythrocytes. Possibly, the increase in cEPI and cADP was due to haemodilution of the Hemopure blood product, and not the Hemopure itself. No change that was statistically significant in cEPI or cADP measurements from "before transfusion" and baseline was noted [61].

In this clinical trial, Hemopure's effects on subjects that undergo orthopaedic surgical interventions were evaluated. The research compared both packed red blood cells *versus* Hemopure at multiple periods [61], true baseline (before surgical incision and induction of anaesthesia), and at the following time periods: prior to the decision to transfuse, following an episode of transfusion, on the first, second, and third through ninth days and at follow-up of 21 days following a transfusion episode. In this subset study, 27 subjects were evaluated, 12 in the Hemopure group and 15 in PRBC.

Prior to transfusion and at baseline comparisons did not reveal a significant changes statistically in any of cEPI-collagen/epinephrine or cADP-collagen/ADP evaluations. cEPI results of the Hemopure cohort elevated (not statistically significant) compared with the true baseline, after transfusion, before transfusion and a day after transfusion. cADP results in the Hemopure cohort were larger post-transfusion in comparison to the actual baseline and before transfusion. At all intervals, no differences that were statistically valid were noted in the PRBC group in cEPI and cADP results.

The trial revealed that Hemopure infusion appears to create some platelet aggregation issue. Despite statistically significant differences after Hemopure infusion, the mean values of cEPI and cADP were greater than the documented upper normal limits, the values were not above the non-closure time, indicating that clotting could still occur.

In an animal study for the effects of Hemopure on platelet function, it was demonstrated that animals that were resuscitated with Hemopure or hetastarch 6% both, initially, had significant increases in the platelet function analyser closure time (PFA-CT). This change returned to normal in the hetastarch group by 24 hours but reached a maximum in the HBOC-201 group at 24 hours and normalised in 72 hours after transfusion [69]. This increase in both Hemopure and hetastarch is likely due to the glycoproteins that are on the surface of both these molecules that have transient effects on platelet function, yet do not destroy function such that the platelets are functionless, as with chronic aspirin therapy.

Ex vivo comparisons of coagulation interference of two different molecular weight HBOCs and hetastarch 6% with normal saline, using thrombelastography, showed that the two HBOCs decreased clot strength (MA and G) when evaluated at dilutions considered to be low and medium. The effect's noted size was similar to 6% hetastarch but was larger significantly than a normal saline solution would be expected to produce [58]. This *ex-vivo* study sheds important light on the platelet function study in that many subjects in the HEM-0115 trial were treated with 6% hetastarch as a volume expander and it has definite effects on platelet function as demonstrated in this TEG study.

The analysis using a PFA-100 determined that those using Hemopure did not require more blood products than the erythrocyte group. There was also a significant difference found in the "after transfusion" time period between the Hemopure and erythrocyte groups, where the HBOC-201 group had increased cEPI and cADP. An increase in these measurements can correlate with an increased risk for bleeding [70]. This increase, however, reversed about one Hemopure half-life on "Day 1 After transfusion."

The increased cEPI may be explained by the lower level of Hb concentration found in the study in patients given Hemopure *versus* erythrocytes. It is also possible that the increase in cEPI and cADP was due to haemodilution of the Hemopure blood product, and not the Hemopure itself. There was no statistically significant change in cEPI or cADP measurements from "before transfusion" and baseline.

How do cEPI and cADP values change in different diseases and conditions according to this work? In PFA-100 measurements, the cEPI cartridge is more sensitive overall

(86%) than the cADP cartridge (81%) and is especially sensitive to drug-related platelet dysfunction [70]. The clinical predictive value of PFA-100 is debatable, because severe bleeding caused by thrombocytopenia is rare and occurs only in patients with a concomitant coagulopathy or anatomical defects in the vascular system [71].

The effects on platelet function that are made by anaesthetic inhalational agents such as halothane, sevoflurane, and intravenous anesthetic agents such as propofol cause a reversible inhibition of platelet function, which is dose dependent in doses utilized in clinical practice. There is a residual suppressive effect 1 hour postoperatively with sevoflurane and propofol [72]. Although the research study has shown that by comparing the baseline values with before transfusion time period, the anaesthetic drugs or surgery has no significant effect on PFA-CT. Certain food molecules and foods are known to inhibit platelet function as well such as fatty acids and cocoa [72].

Cell-free Hb is prone to auto-oxidation to methaemoglobin; Hemopure infusion in surgical patients demonstrated that the percent of plasma methaemoglobin increased in a dose-dependent manner with a delayed onset and reached maximal value 3 days after transfusion [73]. The mean value of methaemoglobin concentration was 3.66% and, in patients who received high doses of Hemopure (2.5 g/kg), the mean was 7.1%. In the process of oxidation, reactive oxygen species are generated. Platelets contain several glycoprotein receptors with thiol groups and vicinal thiols, making them redox-sensitive structures. These glycoprotein receptors include adhesion receptor glycoprotein IIb/IIIa and the P2Y12ABT ADP receptor, which is instrumental in the function of platelet's activation and aggregation. Disulphide isomerase such as protein disulphide isomerase, which has a role in platelet aggregation, is another redox-sensitive structure. A redox homeostasis exists in blood as a result of a trans-plasma membrane redox system of platelets, which can be impaired by free radicals [73].

This recent supportive citation of the earlier work helps understand how these platelet function changes might be explained.

Hemopure has a major impact as an HBOC in research on the human liver based on a study that used Hemopure in a novel indication, where oxygen supply is needed and prior to an HBOC availability, only erythrocytes could deliver such.

Investigators published the first use of Hemopure in a model of liver perfusion requiring machine perfusion (NMP-L). This study suggests that Hemopure compared to erythrocytes, may provide the following advantages: in terms of logistics, rheology, and immunology, in the evaluation of a perfusion model using normothermia [74].

In dealing with myocardial infarction (MI) and its incidence with HBOC studies based on investigator diagnosis, imbalances have been noted and possible toxicity mechanisms have been proposed to help understand these differences [75]. Experiments have been conducted that evaluated in vitro combining of HBOCs (made with human haemoglobin, unlike Hemopure, which uses bovine) and whole blood, the mixture may not cause aggregation of platelets or their activation, nonetheless, certain agonists' responses may be augmented [76]. However, HBOCs with a bovine haemoglobin have been suggested to cause clot formation impairment [75]. This important review by one of the investigators in a first generation HBOC, is important and supportive, in that if HBOCs cause coagulopathies, then it may diminish their ability to help resuscitate a trauma victim or other patient with massive blood loss and acute anaemia.

Coagulation is largely dependent on adequate platelet activity. High molecular weight polymers present in hetastarch solutions have been demonstrated to be related to coagulopathy. In this study, Hemopure was compared to erythrocytes. Mild platelet dysfunction was documented [77]. This review also reconfirms the statements from Reference [76].

Donated human blood is the source of all erythrocyte and plasma products, and so its viral safety is crucial Currently, lipid-enveloped viruses, such as human immunodeficiency virus, hepatitis C virus and hepatitis B virus risks from donated blood products is almost non-existent with appropriate testing in blood banks [78]. While the comments about the incidence of viral contaminants to the blood supply in the past have been widely dealt with, the newer viruses may always cause issues (COVID-19) and the supply of donors is shrinking as populations age and demand increases. Therefore, it may be short-sighted to dismiss the need for these types of products, notwithstanding the inherent risks of transfusion.

Summary of Hemopure

Hemopure represents the most widely studied commercially developed polymerized Hbs. The chemical cross-links within these HBOCs are random and occur both intermolecularly and intramolecularly. The HBOCs generated as a result are large molecules and were hypothesized to negate the previously mentioned side-effects associated with transfusion of acellular HBOCs, such as, vasoconstriction and hypertension engendered by the remants of cell-free Hb. Unfortunately, clinical studies conducted with both of these polymerized Hbs elicited substantial hypertension in vivo upon transfusion [79].

Since erythrocytes and all other blood products come from donated human blood, safety of viral contamination is paramount, not so much for the risk of transmission of lipid-enveloped viruses, such as HIV, Hepatitis C, and Hepatitis B, the transmission by plasma-derived solutions is likely minimal [78]. However, there are new viruses constantly being identi-

fied, such as COVID, which does not seem to be transmitted in donated blood, although the detection and removal processes of blood banks take years to initiate and implement.

Summary and Moving Forward

This chapter has reviewed HBOCs from early until the most recently completed trials, as well as discussed how various HBOCs are made and their pharmacology and physiology. Donated human blood and the derived products that have been available for around 50 years have allowed for replacement of erythrocytes, platelets and blood clotting factors and plasma/albumin. Based on the available published articles on products and in some cases recent website reviews, this chapter has updated the reader on the current usage of some products and attempted to focus on the risk/benefit of current products and attempted to introduce the reader to newer products that may someday improve outcomes from situations where blood is not available or an option [70, 80–83].

Donor erythrocyte transfusion may be a lifesaving treatment for severe anemic conditions. However, donor blood is a precious commodity that is in chronic shortage because there is no alternative. Aging populations and changes in the population dynamics worldwide will further worsen blood shortages. In the coming decades, it is predicted that the number of older people who will need blood transfusion will far exceed the younger people who can donate blood. Without alternatives, this imbalance may cause a blood shortage crisis that could result in countless preventable deaths. To alleviate the current shortages and prevent future blood supply crisis, we must invest now to reinvigorate efforts to develop of safe and effective blood substitute(s).

Since the early 1980's, HBOCs based on modified (cross-linked, polymerized or conjugated) human or bovine Hbs have been developed as leading candidates for donor RBC substitutes and some have recently been tested in clinical trials (HemAssist, Hemopure, PolyHeme, Hemolink, Hemospan, Hemoximer, Sanguinate). However, in phase 2–3 clinical trials, some adverse events/serious adverse events occurred in the HBOC treated subjects including pulmonary and systemic hypertension, cardiac dysfunction, coagulopathy and enzyme abnormalities [76]. This engendered the early cessation of studies or completed Phase 3 trial but failure to obtain regulatory clearance due to safety concerns.

Although Hemopure secured regulatory approval for human use in anemia in South Africa and Russia, none of the HBOC products tested have been approved for clinical use in the US and other advanced economies, except for Oxyglobin, the veterinary product approved by the United State Food and Drug Administration and its counterparts in the European Union and United Kingdom.

It is generally agreed that the pulmonary and systemic hypertensive responses observed following HBOC administration were caused by the iron-heme, a common prosthetic group in all the HBOCs, which avidly scavenges the vascular endothelium derived NO, a potent vasodilator, causing vasoconstriction leading to hypertension. NO has also been discovered to be produced endogenously in the cardiomyocytes to contribute significantly in the regulation of cardiac contractility and rhythm. Therefore, a possibility has been raised that HBOC interference with cardiac NO signaling might have caused some of the cardiac adverse events observed. In addition, HBOC redox-mediated radical generation and other unknown toxicity mechanism may also be involved. However, mechanisms for other adverse events/serious adverse events have not been elucidated.

Considering the issues encountered in HBOC clinical trials, a newer generation of HBOCs must be developed that significantly reduces NO reactivity and yet maintains optimal oxygen binding and delivery characteristics. In addition, it is highly desirable that newer HBOCs have favorable redox properties and stability for an extended storage preferably without refrigeration.

These are scientifically challenging goals but, with strong research support for better understanding of chemistry, physiology and pharmacology of HBOCs in clinically relevant models, they are achievable. Unfortunately, recent failures in obtaining regulatory approval and negative media coverage have caused a devastating impact on the HBOC field. However, there are a number of promising new products being developed and tested, many at the most basic level either in university or manufacturer settings.

Despite the critical need and urgency, current negative environment and lack of government support, make it extremely difficult to secure necessary funding for new HBOC development. Under the current circumstances and limited resources, a collaborative and coordinated approach among academia (basic research with funds from government and industry), industry (scaleup and manufacturing with angel/venture capital funding) and government (provide startup funding and regulatory support) would help create development of new or improved HBOCs for successful regulatory approval and eventual clinical use [84]. With an initial government funding, such a consortium could be implemented and start operationalized with an aim to help facilitate development of one or more safe and effective blood substitutes in a decade or two. Additionally, currently tested HBOCs, such as Hemopure, and newer products like Hemarina's HEMO2-life may be safe enough to provide a life-savings advantage, despite the side effect profile, in those subsets of patients who are less affected by them and benefit from the value of improved oxygen delivery in the face of severe anemia or when blood is not available or an option.

Key Points

- Blood is a donated product which is soon to be in short supply due to older population, fewer donors and more demand
- Hemoglobin-based oxygen carriers have been developed to ameliorate this shortfall and deliver oxygen to hypoxic tissues for any etiology: acute anemia or hemorrhage
- Hemoglobin-based oxygen carriers have intrinsic side effects, like all drugs, which must be understood and circumvented to achieve the desired benefits
- Newer generation HBOCs have been formulated to avoid the side effects of NO scavenging, hypertension, and chemical pancreatitis, or products to give as adjuncts
- Hemopure is approved for human use in South Africa and Russia and available for Expanded Use; Oxyglobin is approved for veterinary use in the US and EU
- Hemarina, made from sea worm's hemoglobin that can survive in air at low tide, may provide oxygenation from a natural and unaltered hemoglobin that is naturally able to provide more oxygen due to its biomimicry

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