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REVIEW ARTICLE

Pre-clinical studies using OxyVita hemoglobin, a zero-linked polymeric hemoglobin: a review

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Abstract Hemoglobin-based oxygen carriers (HBOC) are being developed to provide the oxygen necessary in clinical situations when whole blood is not available. The safety and effectiveness of each HBOC must be determined before clinical approval. In the past several years animal studies have been conducted with zero-linked polymers to evaluate their effectiveness at delivering oxygen in vivo. Studies have addressed issues associated with interstitial extravasation, cerebral ischemia and blood flow, resuscitation, and coagulation interactions. Several of the investigations reviewed are based on early preparations of zero-linked polymerized bovine hemoglobins (ZL-HbBv), which contained a wide range of high-molecular-weight polymers. Recent studies using the Oxyvita product OxyVita Hb, which contains a more homogenous population (97%) of large-molecular-weight species ($\sim$17 MDa), are also included in this review.

Keywords Blood substitutes · Hemoglobin-based oxygen carrier · Zero-linked polymeric hemoglobin · Acellular hemoglobin

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Introduction

The evolution of HBOC (hemoglobin-based oxygen carrier) development over the past several decades has resulted in vital design considerations incorporated into new molecules with the intention of addressing crucial factors responsible for less than optimum results during early clinical applications [1–3]. One of these unique molecular design approaches emerged from the laboratory of Professor Enrico Bucci and his team at the University of Maryland using novel zero-linked polymerization technology [4].

Specific modification of this zero-linked approach by Oxyvita scientists resulted in the incorporation of several vital physical, chemical, and physiological properties into a "super-polymeric" hemoglobin molecule, with a mean molecular weight of 17 MDa (light scattering) and a methemoglobin content of less than 3%. Functionally, it has a low P_{50} (6 mmHg) and a Hill coefficient of $n = 1.0$ (non-cooperativity). A more extensive description of the physical and chemical properties of OxyVita Hb has recently been published [5].

Our focus in this review is to present an overview of early pre-clinical studies involving use of the zero-linked HBOCs (referred to as ZL-HbBv) that have emerged during this period of development. These studies were conducted by independent investigators at different institutions using early preparations of zero-linked polymeric hemoglobins. More recently, several studies have been conducted on OxyVita Hb, modified for production by Oxyvita. This review will address several important physiological issues associated with serious concerns in this field of endeavor: (a) the problem involving hemoglobin intravascular extravasation linked with NO binding; (b) observations of MAP (mean arterial pressure) elevation on

infusion of early HBOCs; and (c) the determination of adequate oxygen delivery by HBOCs.

Early generations and developments: retention versus intravascular extravasation

HBOCs are acellular oxygen-delivery proteins that carry oxygen and may also function as a plasma expander. Very early use of normal stroma-free tetrameric hemoglobin (human) resulted in rapid circulatory elimination, the appearance of hemoglobin in the urine, and coupled elevation of mean arterial pressure (MAP) within different animal models studied [6]. This effect was largely because of the dissociation of these tetramers into smaller molecular species (dimers). In response to these observations, HBOC development over the years has resulted in incorporation of important design considerations into new molecules with the intention of addressing the many factors responsible for these negative clinical results. In general, intramolecularly cross-linked HBOCs used reagents that formed molecular bridges within the central cavity, which are oriented along the dyad axis of the tetramer and are filled with charged residues and water molecules. The first generation using this new design approach [7] began with the introduction of an intramolecular cross-linking approach (HbXL99$\alpha\alpha$, HemAssist, M.wt. 64 kDa).

This was followed by several different approaches involving intermolecular cross-linking polymerization: glutaraldehyde cross-linked bovine hemoglobins, M.wt. >150 kDa (Hemopure, human application; Oxyglobin, veterinary application), raffinose cross-linked human hemoglobin (Hemolink), M.wt. >100 kDa, and pyridoxylated glutaraldhyde cross-linked human hemoglobin (PolyHeme M.wt. >120 kDa). In addition, several Optro recombinant human hemoglobins (M.wt. 64 kDa) were also created. All of these approaches were an attempt to eliminate the formation of dimers from stroma-free hemoglobin (small molecular radii), which resulted in rapid elimination from the circulatory system and stress on the glomerulus and kidneys. Larger-molecular-weight cross-linked human and bovine hemoglobins were designed with the intention of reducing access to the vascular space and increasing retention time compared with the intra-cross-linked hemoglobin tetramers. The objective of this approach was to limit hemoglobin extravasation associated with NO binding and a concomitant increase in vasoconstriction and MAP [8, 9]. In the late nineties, Hemospan (selected pegylation, M.wt. ~90 kDa) was introduced to eliminate extravasation mitigating vasoconstriction and maintain mean arterial pressure [10]. These different chemical modifications resulted in several HBOCs with no observed dimer formation and increased molecular sizes which alleviated earlier clinical problems associated with rapid elimination from the circulation. Although these modifications did result in reduced extravasation with maintenance of normal mean arterial pressure, these same HBOCs have, unfortunately, continued to cause oxidative and cellular damage during phase I, II, and III studies [11, 12]. Table 1 summarizes these early acellular HBOCs.

A new generation HBOC based upon zero-linked polymerization technology (liquid and powder preparations)

The development of zero-linked polymeric hemoglobin technology uses an activation process to establish covalent pseudopeptide bonds between hemoglobin tetramers. This resulted in the production of very large polymeric species with a wide range of molecular sizes (ZLHb-Bv average size, 25 MDa). These preparations initially contained a heterogeneous distribution of high-molecular-weight species. When infused in a rat model this preparation led to elimination of hemoglobin from the urine but some hemoglobin did appear in the hilar lymph of the rat kidneys [13]. Residual polymers below 300 kDa and any unreacted hemoglobin were removed from these zero-linked polymeric hemoglobin preparations by additional filtration. Upon use of these larger polymeric hemoglobin molecules, no hemoglobin was evident in the hilar lymph of rats and no increase in MAP was observed in either anesthetized or awake cats [13].

Table 1 Early acellular HBOCs evaluated in clinical trials

Acellular HBOC product	Composition (M.wt. kDa)
HemAssist (Baxter)	Diaspirin cross-linked human Hb (64 kDa)
Hemopure (Biopure)	Glutaraldehyde cross-linked bovine Hb (polymeric, >150 kDa)
Hemolink (Hemosol)	Raffinose cross-linked human Hb (polymeric, >120 kDa)
PolyHeme (Northfield)	Pyridoxylated glutaraldehyde cross-linked human Hb (polymeric, >120 kDa)
Optro (Baxter/Somatogen)	Recombinant human Hb, rHb1.1 (64 kDa)
Hemospan (Sangart) (early product)	Pegylated human Hb (~90 kDa)

Table 2 Physicochemical properties of OxyVita hemoglobin

	OxyVita Hb solution 6 g%	Small volume resuscitation fluid 9 g%	OxyVita Hb powder in water 6 g%
Size exclusion LC-polymers (%)	100	99.20	100
Size exclusion LC-tetramers	0%	0.85	0%
Spectral ratio A_{576}/A_{540} after pasteurization	0.963	0.963	0.964
Autoxidation-MetHb (%)	3.70	3.00	7.92
Oxygen affinity, p50 mmHg[a]	5.95	5.26	6.59
Hill Coefficient (*n* value)	1.1	1.2	1.2
Dynamic light scattering[b] (radius, nm)	36.0	36.5	36.4
Dynamic light scattering[b] (M.wt., kDa)	17,337	17,400	17,154
pH	7.46	7.5	7.58

[a] Hemox Analyzer, pH 7.50

[b] Dyna-Pro, protein solutions (ref. [5])

Beginning in 2003, Oxyvita further modified this zero-linked polymeric hemoglobin for large-scale production in order to carry out extensive pre-clinical studies to determine the product's safety and efficacy as an oxygen-delivery system by means of a series of animal studies conducted by independent investigators. The production modifications have resulted in a more homogenous-molecular-weight product (referred to as OxyVita Hb) with an average molecular weight of 17 MDa. At 6 g% hemoglobin concentration, the relative viscosity of this preparation is 1.2 times higher than that of plasma and its colloidal osmotic pressure, 3 mmHg (in lactated Ringer's solution, pH 7.4, 23°C), is approximately 1/10 that of plasma.

As a result of unique modifications of the zero-linked polymerization process used for production of OxyVita Hb in the liquid phase, it is now possible to lyophilize this polymeric hemoglobin into an amorphous powder preparation. When this powder preparation is re-constituted back into an intravenous (IV) fluid, it has the same physicochemical properties (Table 2) as the original liquid preparation [5]. This unique powder product is suitable for long-term storage, up to 5 years under a wide range of climatic conditions. Several different formulations of this powder form of OxyVita Hb are available, enabling delivery in a variety of infusion media, including Ringer's solution, Ringer's lactate, saline solutions, and water.

Examples of pre-clinical studies using ZLHb-Bv and OxyVita Hb

During the past several years a series of in-vivo animal studies conducted with the zero-linked polymers have addressed issues associated with interstitial extravasation [13], cerebral ischemia and blood flow [14, 15], resuscitation [16], and coagulation behavior [17, 18]. Some examples of these studies that are reviewed herein are largely based on ZlHb-Bv preparations, which contained a wider range of high-molecular-weight polymers. Several investigations [16–18] using the Oxyvita product (OxyVita Hb) are also included in this review. Many other studies conducted during the past decade have demonstrated the viability of this zero-linked polymerization technology, but space does not permit inclusion at this time.

Extravasation, vasoconstriction, and pressor effect

Most of the early HBOC studies showed that infusion of acellular stroma-free tetrameric (M.wt. 64 kDa) hemoglobin as an oxygen-delivery system was associated with rapid disappearance from the circulation and elimination in the urine. Dissociation of the hemoglobin tetramers into dimers was shown to be a major factor in this behavior. Introduction of cross-linked HBOCs [7] significantly reduced their appearance in the urine by preventing dissociation of hemoglobin into the smaller-molecular-weight dimers (32 kDa). Although Matheson et al. [13] demonstrated that several $\beta\beta$ cross-linked hemoglobins did lead to elimination of hemoglobin in the urine and greater circulatory retention time, significant extravasation still occurred, as was evident from the hemoglobin's presence in the hilar lymph of rat kidneys. Unfortunately, this approach still did not eliminate increases in mean arterial pressure (MAP) in the animal models employed. Further complications were evident in that the vasoconstriction associated with this MAP increase was not uniformly observed in the various internal organs [13].

This association of vasoconstriction and increased MAP has been linked to the high affinity of these acellular HBOCs for nitric oxide (NO). HBOCs have different NO binding because of their distance or potential interaction with the vascular tissue wall which is a major site of NO generation [19]. The observation that vasoconstriction is not evenly distributed within the internal organs is clearly consistent with the presence of membrane pores of different sizes, permitting extravasation of HBOCs of different molecular size. This would explain the absence of cross-linked Hb in the urine but its continued appearance in the

hilar lymph of the rat kidneys and the associated increase in MAP described previously.

With the availability of ZLHb-Bv, an extensive investigation by Matheson et al. [13] on the vascular response to infusion of this high-molecular weight-polymer with an average M.wt. of 25 MDa resulted in elimination of this hemoglobin's extravasation through vessel walls coupled with a pronounced reduction in vasoconstriction, effectively maintaining normal MAP. This study was carried out using anesthetized and fully conscious cats. The large radius of the hemoglobin polymer resulted in a greater retention time in the circulatory system with a half-life 8–12 times longer than for the unmodified hemoglobins. In particular, this behavior is consistent with our improved understanding of the complex nature of the mechanism of intracellular extravasation through endothelial capillary walls associated with a wide range of cellular pore sizes [20]. Small pores (radius = 30–35 Å) allow dimeric hemoglobins (radius = 24 Å) to pass through, whereas larger tetrameric hemoglobins (radius = 32 Å) are more hindered from movement across the membrane. Large pores (radius = 200–250 Å) allow lower-molecular-weight polymerized molecular hemoglobin species to extravasate, leading to NO binding, vasoconstriction, and an increase in MAP. Another factor helping to eliminate extravasation is a permeability selection; this is associated with an array of negative charges on the epithelial wall which reduce the interaction with ZLHb-Bv, which has a large negative surface charge [4]. OxyVita Hb retains the same large negative surface charge.

Cerebral microcirculation and blood flow

The complex nature of the cerebral blood flow has been well demonstrated by many investigations over the past decade [21, 22]. This complexity is even more apparent when attempts are made to understand and evaluate the effectiveness of HBOCs at delivering oxygen and reducing infract volume during cerebral ischemia. In an extensive study using the rat model, wherein the transfusion of large polymers of hemoglobin with a wide range on molecular sizes (ZLHb-Bv preparation) was carried out, it was determined that reduction (by 39%) of the infarct volume was dependent on the concentration (6 g%) of this high-affinity hemoglobin (P_{50} = 4 mmHg) and on the range of intermediate size hemoglobin polymers (500–14,000 kDa) transfused [15]. Little or no reduction in the infract volume was observed for: (1) transfusion in 5% albumin solution; (2) a lower concentration of hemoglobin polymers of similar size; (3) hemoglobin polymers without removal of polymers <500 or >14,000 kDa; or (4) cross-linked hemoglobin tetramers with normal oxygen affinity.

The effective polymeric hemoglobin transfusion solution did not improve the distribution of cerebral blood flow during an ischemic event, nor did it affect blood flow to the brain or other major organs in mice without ischemia. This latter finding also demonstrates that these hemoglobin polymers do not initiate significant vasoconstriction in the brain or in peripheral vascular beds [15]. Thus, in evaluating the potential of HBOCs for overcoming cerebral ischemia, a range of critical factors, for example hemoglobin molecular size, concentration, and oxygen affinity, must be taken into consideration. Other HBOCs currently under development may have the potential to address and augment our understanding of cerebral ischemia transfusion processes and protect the brain from ischemic strokes [23].

Trauma and resuscitation response

Uncontrolled hemorrhage is a leading cause of preventable morbidity during trauma. Crucial aspects of this kind of death are circulatory volume loss, tissue oxygen deprivation, and uncontrolled hemorrhage. In many instances, logistic conditions associated with these kinds of injuries may preclude the resuscitation efforts necessary for timely delivery to a hospital trauma center. In military situations, the standard management of penetrating trauma has been to use an aggressive resuscitation fluid until control of the hemorrhage has been achieved. The assumption has been that early and rapid fluid resuscitation will restore blood pressure, reduce severe shock, and prevent multiple organ failure. This approach has recently been challenged on the basis of recent data indicating that aggressive attempts to normalize blood pressure with large fluid boluses result in increased bleeding, hemodynamic decompensation, and mortality. A newer procedure for permissive hypotensive resuscitation employing smaller volumes of hypertonic fluids is now favored.

Several immediate advantages of this approach include: (a) restoration of tissue perfusion accompanied by a modest increase in blood pressure with concomitant lowering of clotting factor dilution and rebleeding; and (b) alleviation or reversal of lung damage.

This latter approach using a small volume of resuscitation fluid (SVRF) is more compatible with battlefield conditions. Within this approach, restoration of adequate blood pressure must be accompanied by low risk of rebleeding, and enable adequate oxygen delivery to ensure organ perfusion, reducing the danger of multiple organ failure when full resuscitation is achieved. This SVRF is augmented with a hemoglobin-based oxygen carrier (HBOC) for adequate oxygen delivery.

In a recent Defense Advanced Research Projects Agency (DARPA) study [16], the objective was to determine a resuscitation strategy after 60% hemorrhage in conscious

male Long–Evans rats that improved survival for 3 h in the absence of conventional large-volume crystalloid support. This study focused on evaluation of the ability of zero-linked polymerized OxyVita Hb, in conjunction with a hypertonic saline solution and Hextend, to enhance survival compared with standard small-volume resuscitation using Hextend only. Direct outcomes included survival up to 3 h and duration of a MAP greater than 60 mmHg without additional fluid infusion.

The test fluids administered included OxyVita Hb in a pressure-titrated infusion, Hextend titration, OxyVita Hb infused in a bolus method, and a Hextend bolus infusion. During the course of these experiments, an array of cardiovascular data, arterial gases, acid–base data, metabolites, electrolytes, Hb levels, and oxygen saturation were determined at baseline, at each 20% hemorrhage increment, and over 1–3 h after the initial hemorrhage. The most pronounced finding from these studies was that small-volume resuscitation with OxyVita Hb significantly improved survival to 3 h and enhanced adequate MAP support for the duration, irrespective of the method of administration. These results clearly indicate that an OxyVita Hb-augmented hypertonic "cocktail" is an encouraging alternative to the standard method for improving survival and MAP support using a small-volume resuscitation approach.

Coagulation behavior

The use of HBOCs as a transfusion medium whether in the context of an exchange fluid or a replacement fluid must not interfere with the coagulation processes normally found in whole blood. Earlier studies reported that hetastarch solutions resulted in elevated coagulopathy, increasing with molecular weight via several different mechanisms [24, 25]. Given these findings, the risk of coagulopathy in the application and use of high-molecular-weight HBOCs has been investigated [17]. Using hemodilution during clinical resuscitation after hemorrhagic shock with different amounts of OxyVita Hb, 6% hetastarch, oxyglobin (HBOC-200) coagulopathy behavior in 1:11 to 1:1 dilutions in whole blood was evaluated by a thromboelastographic (TEG) technique in real time. This method enables direct analysis of clot strength and formation kinetics, and indirect determination of platelet and coagulation factor functionality and availability [26]. These ex-vivo TEG measurements used blood from healthy donors, which eliminated interfering factors such as anticoagulants and other intravenous fluids, enabling evaluation of the direct effect of the HBOC on the TEG determinations.

Findings from this study [17] indicated that both HBOCs had a similar effect on coagulation, and that this was more pronounced than that of 6% hetastarch at the highest levels of hemodilution. However, minimal coagulopathic effects are expected with the use of OxyVita Hb at the manufacturer's expected maximum dose of 10 g or 2–3 ml/kg. Hence some caution should be used when attempting to use OxyVita Hb as a large-volume oxygen plasma expander. Its application is not expected to lead to an increased risk of clinical bleeding when used in relatively small volumes (2–3 ml/kg).

An important related finding regarding the effect of an HBOC on coagulation is the presence of methemoglobin [18]. A dose–response study with HBOC-200 (oxyglobin) was carried out using the thromboelastogram technique. The results from this investigation revealed that high concentrations of methemoglobin in HBOC-200 led to increased coagulation impairment. This negative effect may be because of the effects on platelet function and on other coagulatory proteins of concomitant oxidative species associated with increased methemoglobin levels. Platelets contain functionally important glycoprotein receptors containing thiol groups providing increased sites of interaction for these redox-sensitive structures [27]. A significant characteristic of OxyVita Hb is the low methemoglobin levels associated with its preparation and its extraordinary resistance to molecular unfolding, which significantly reduces its conversion to methemoglobin in vitro and in vivo [28].

Hopefully this modest review of several investigations of pre-clinical studies using early ZLHb-Bv and later OxyVita Hb has provided some insight into the rationale for this unique molecular design approach and the resulting physicochemical and functional properties necessary for safe and effective HBOC that will become available for clinical applications in the near future.

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