Microcirculation and NO-CO Studies of a Natural Extracellular Hemoglobin Developed for an Oxygen Therapeutic Carrier

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Abstract: Extracellular soluble hemoglobins (Hbs) have long been studied for their possible use as safe and effective alternatives to blood transfusion. While remarkable progress has been made in the use of cell-free Hb as artificial oxygen carrier, significant problems remain, including susceptibility to oxidative inactivation and propensity to induce vasoconstriction. Hemarina-M101 is a natural giant extracellular hemoglobin (3600 kDa) derived from marine invertebrate (polychaete annelid). Hemarina-M101 is a biopolymer composed of 156 globins and 44 non-globin linker chains and formulated in a product called HEMOXYCarrier®. Prior work has shown Hemarina-M101 to possess unique anti-oxidant activity and a high oxygen affinity. Topload experiment with this product into rats did not reveal any effect on heart rate (HR) and mean arterial pressure (MAP). A pilot study with the hamster dorsal skinfold window chamber model showed absence of microvascular vasoconstriction and no significant impact on mean arterial blood pressure. In vitro nitric oxide (NO) and carbon monoxide (CO) reaction kinetics measurements show that Hemarina-M101 has different binding rates as compared to human Hb. These results revealed for the first time that the presence of this marine hemoglobin appears to have no vasoactivity at the microvascular level in comparison to others hemoglobin based oxygen carriers (HBOCs) developed so far and merits further investigation.

Keywords: Annelid, blood substitute, extracellular hemoglobin, hemodynamic activity.

INTRODUCTION

Blood substitute development has mainly centered on fluids that transport oxygen as this is a principal function of the circulation. This focus on oxygen carrying capacity has led to the development of two main groups of blood substitutes: perfluorocarbon emulsions and hemoglobin (Hb)-based oxygen carriers (HBOCs) [1]. HBOCs derived from human, bovine or recombinant Hb have been found to present different forms of toxicity [2]. This toxicity appears to be circumvented by polyethylene glycol conjugation and phospholipid encapsulation [3-5]. However, these approaches introduce significant quantities of additional material in the circulation whose effect and clearance are not well established. Molecular Hb in solution is an elegant method for implementing an oxygen carrier because it can reach and deliver oxygen to tissue regions inaccessible to red blood cells. However the natural tendency of molecular Hb solutions is to cause vasoconstriction and thus attainment of the goal to transport oxygen to tissue is hindered by most molecular Hb solutions. Vasoconstriction while not necessarily a negative effect per se, molecular Hb solutions are associated with significant incidence of adverse effects [4, 6].

A fundamental observation that defines how the circulation reacts to molecular Hb in blood was made by Sakai et al. [7] who showed that vasoactivity is an inverse function of molecular dimension. The validity of this tenent is supported by results obtained with the zero-link polymer bovine Hb and ultra-high molecular weight Hb polymers [8, 9]. These mechanistic aspects have been explored by studies with macromolecular plasma expanders that do not carry oxygen [10].

A molecular Hb that fulfills the dimensional paradigm of Sakai et al. [7] is the oxygen transporter of the marine invertebrate Arenicola marina [11, 12]. This is a natural extracellular respiratory pigment of high molecular weight (~3600 kDa) that does not require chemical modification. This giant extracellular O₂ carrier consists of globin and non-globin linker chain complexes that have a large Hb oxygen binding capacity, carrying up to 156 O₂ molecules when saturated [11, 13]. This material named Hemarina-M101, is purified from extracellular hemoglobin of Arenicola marina and used to produce HEMOXYCarrier® (Hemarina S.A., France), a product being developed as an oxygen carrying therapeutic. HEMOXYcarrier® could be used in a large range of concentrations in terms of biorheological considerations since its viscosity is similar to that of plasma. Furthermore, recent works showed that a different formulation of Hemarina-M101 (HEMO2life®, Hemarina S.A., France) considerably improves kidney preservation waiting to be transplanted [14].
An important physiological aspect of red blood cell (RBC) function is the management of endogenous gaseous messenger molecule transport such as nitric oxide (NO) and carbon monoxide (CO). These molecules are potent vasodilators and are effective at very small concentrations. NO bioavailability in the vessel wall is determined by very complex enzymatic mechanism [15] and also the balance between NO production by shear stress on the endothelium and its scavenging by hemoglobin [16]. CO bioavailability appears to be of significance in the hepatic microcirculation [17].

The present study reports a pilot microvascular investigation of the effect of a topload intravenous infusion of HEMOXYCarrier® on a) microhemodynamics in the hamster dorsal skinfold window chamber model, and b) systemic parameters in the rat. The in vitro biochemical activity in terms of NO and CO reaction kinetics was also explored.

**MATERIAL AND METHODS**

**HEMOXY Carrier® Production and Sample Preparation**

HEMOXYCarrier® (Hemarina SA, France) was manufactured using GMP standards governing medicinal products [18]. HEMOXYcarrier® is formulated in an injectable saline solution and its properties are presented in Table 1.

**Table 1. Functional Properties of HEMOXYCarrier®**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P50 (mmHg)</td>
<td>7.05 ± 0.93 (n=9)</td>
</tr>
<tr>
<td>n50</td>
<td>2.54 ± 0.23 (n=9)</td>
</tr>
<tr>
<td>Bohr coefficient</td>
<td>-0.5</td>
</tr>
<tr>
<td>ΔH (KJ mol−1)</td>
<td>-19</td>
</tr>
<tr>
<td>COP (mmHg)</td>
<td>1.0</td>
</tr>
<tr>
<td>Viscosity (cP)</td>
<td>1.23</td>
</tr>
<tr>
<td>SOD activity (U/mg Hb)</td>
<td>3.53 ± 0.02 (n=3)</td>
</tr>
<tr>
<td>CN inhibition</td>
<td>100%</td>
</tr>
<tr>
<td>Fe (atom/molecule)</td>
<td>156 (1)</td>
</tr>
<tr>
<td>Cu (atom/molecule)</td>
<td>3.58 ± 1.17 (n=5)</td>
</tr>
<tr>
<td>Zn (atom/molecule)</td>
<td>5.13 ±0.75 (n=5)</td>
</tr>
</tbody>
</table>

a) The oxygen equilibrium binding data were collected in dissolved lyophilized plasma (Sigma) in 10 mM HEPES, pH 7.35 at 37°C for a final heme concentration of 40 mg/mL. The Bohr coefficient was measured over the pH range 7.2-7.6, and the temperature effect from 33°C to 41°C, which corresponds to the range of values encountered in human, including pathological cases.

b) The Bohr coefficient and temperature sensitivity of hemoglobin are calculated as previously described [24].

c) COP and viscosity were determined on a M101 sample of 58 mg/mL at room temperature on an Onkometer BMT 293 (BMT MESSTECHNIK GMB, Germany) according to the company protocol and on a Brookfield viscometer (Brookfield Engineering Laboratories Vertriebs, Germany) according to the manufactures protocol.

d) The SOD activity was determined using the Flohé and Otting method [25] and value come from [12].

e) HEMOXYCarrier® was incubated for 10 min with 50 mM KCN prior to the addition of xantine oxidase.

f) Fe was determined by mass spectrometry [11].

g) Cu and Zn contents were determined by inductively coupled plasma-MS [26].

**Hamster and In Vitro Gas Studies**

HEMOXYCarrier® (68 mg/mL) was defrosted slowly by exposing the vial to room temperature. The sample was then diluted with 0.9% normal saline solution to produce 40 mg/mL solution for the study (4%). This diluted solution was then aliquoted and returned to the -80°C freezer. In preparation for infusion into the animal, samples were removed from the -80°C freezer and warmed to room temperature. HEMOXYCarrier® concentration was reduced in order to compare the results to a previous study with several molecular Hbs also tested at the same concentration [19]. The dose for these animals was approximately 280 mg/kg.

**Rat Studies**

For this study, HEMOXYCarrier® was administered by i.v. infusion at the dose level of 600 mg/kg, a dose that has shown a tissular oxygenation potential on rodent model (unpublished data). Concentration adjustment was performed using an injectable saline solution developed by Hemarina as previously mentioned. Prior to infusion, HEMOXYCarrier® was removed from the -80°C freezer and warmed to room temperature.

**Animal Models and Preparation**

### I. Microcirculation Study with the Hamster Dorsal Skinfold Window Model

Studies were performed in golden Syrian male hamsters (Charles River Laboratories, Boston, MA), weight range of 50-70g. Animals were handled according to the Guide for the Care and Use of Laboratory Animals (US National Research Council, 1996) and experiments were approved by the University of California, San Diego Institutional Animal Care and Use Committee. The hamster dorsal skinfold window chamber model has been widely used for microvascular studies and allows for the study of intact tissue in an awake animal, thus reducing complications due tissue exposure and anesthesia. Chamber implantation and vascular catheterization were performed under general anesthesia (pentobarbital 50 mg/kg i.p.) as previously described [20, 21]. Mean arterial blood pressure (MAP) and heart rate (HR) were continuously monitored using a data acquisition system (MP 150; Biopac Systems, Inc., Santa Barbara, CA). Blood was sampled from the arterial catheter into heparized microcapillary tubes and centrifuged to determine systemic hematocrit (Hct). Arterial blood chemistry (pH, pO2, pCO2) was measured using a blood gas analyzer (RAPIDLab 248, Siemens, Deerfield IL). Animals were included into the study if their MAP > 80 mmHg, and systemic Hct > 45% which are within the normal physiological range [22]. After baseline systemic and microvascular measurements were made, animals were given an i.v. infusion of the study material (100-150 μL/min), volume equal to 10% of its blood volume which was estimated as 7% of the body weight. MAP and HR were assessed at 10, 30 and 60 min after completion of the infusion. Hematocrit and arterial blood gases were reassessed at the end of the experiment (60 min after load).

The unanesthetized animal was placed into a restraining tube, which was then affixed to the stage of an intravital microscope (BX51WI, 40 x objective, NA 0.7 SW; Olympus, Central Valley, PA). The tissue image was projected onto a CCD camera (4815-2000; COHU, San Diego, CA) connected to a timer and viewed on a monitor. Animals had at least 30 minutes to adjust to the tube environment prior to
measuring baseline parameters (MAP, HR, blood gases and Hct). Arterioles and venules, chosen by their visual acuity (4-6 each type), were characterized by their blood flow velocity and diameter. Vessels were chosen from baseline observations and the same vessels were followed throughout the experiment. Microvessel diameters were measured with an image-shearing system (Digital Video Image Shearing Monitor 908, Vista Electronics, San Diego, CA), while arteriolar and venular blood flow velocities were measured with photodiodes using the cross-correlation technique (Velocity Tracker Mod-102 B, Vista Electronics., San Diego, CA). Blood flow rates \( Q \) were calculated as: \( Q = \pi RBC \text{velocity}/R_r \cdot (\text{diameter}/2)^2 \) where \( R_r = 1.6 \) is the ratio of the centerline velocity to the bulk velocity.

II. In Vivo Cardiovascular Effects in the Anaesthetized Rat

These experiments were carried out on male Wistar rats (Elevage R. Janvier, France) weighing 250-300g (7 weeks old). Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.). A catheter was placed into the left carotid artery to measure arterial blood pressure and heart rate. A Millar-type pressure probe was placed into the left ventricular cavity via the right carotid artery to measure left ventricular pressure (LVP). Lastly, a catheter was placed into the pudendal vein for infusion of the test sample. The animal’s body temperature was maintained at 37°C with a water circulating warming blanket.

After stabilization of hemodynamic parameters, baseline measurements were performed and HEMOXYCarrier® (600 mg/kg) or saline solution 0.9% (used as a control) was infused intravenously (5 min, 1.2 mL/kg/min). Experiments were carried out in 18 rats. Six animals received HEMOXYCarrier® (600 mg/kg) and twelve animals received saline solution as control. Hemodynamic parameters were monitored for a period of 45 min post infusion. Heart rate was derived from the phasic arterial blood pressure signal. Left ventricular \( \text{dP/dt}_{\text{max}} \), an index of myocardial contractility, was calculated as the maximal rate of rise of left ventricular pressure signal.

III. In Vitro Measurement of NO and CO Binding

The time course of the ligand binding was analyzed during rapid mixing of the deoxygenated HbV (human Hb vesicles) and Hb solutions and a NO- or CO-containing solution using a stopped-flow rapid scan spectrophotometer (RSP-1000; Unisoku Co. Ltd., Osaka, Japan). Briefly, 3 mL of a deoxygenated PBS solution containing HbV [23], human HbA, Oxyglobin (Biopure) and HEMOXYCarrier® at [heme] = 3 \( \mu \)M was rapidly mixed with NO or CO containing PBS solution. The change of absorption at 430 nm was monitored. The methods are described in detail in a previous report [23].

Data Analysis

Statistics were performed using Prism version 4.0 for Windows (GraphPad, San Diego, CA). Data are presented as mean ± sem.

In the hamster studies, N and n denote the number of animals and vessels studied, respectively. Differences within groups were first tested with one-way analysis of variance (ANOVA) for repeated measures and for multiple comparisons between groups. Bonferroni post hoc test was used if significance was obtained. In haemodynamic studies performed on anaesthetized rat, homogeneity of baseline values (T0) between the two groups was tested for each parameter using a two-tailed Student's t test for independent samples. For each parameter, changes from baseline value (delta %) at each measurement time were compared between the two groups using a two-way ANOVA (group, time) with repeated measurements over time. If group x time interaction was significant, comparison between the two groups was performed at each time by a student t test. Changes were considered statistically significant if P < 0.05.

RESULTS AND DISCUSSION

I. Hamster Studies

The study was performed in 5 animals of 60 ± 5 g. All animals tolerated and completed the protocol without any adverse events. Table 2 presents the changes in hematocrit and arterial blood gas parameters at baseline and 60 min after topload infusion of HEMOXYCarrier®. Blood gases parameters were not significantly affected by the administration of HEMOXYCarrier® while Hct was slightly reduced (P < 0.05). This slight change in Hct, while being statistically significant, is not considered physiologically significant. These data are consistent with previous experiments done on mice and rats with this product [12]. Figs. (1A) and (1B) show the effect of HEMOXYCarrier® on MAP and HR. HEMOXYCarrier® induced slight and transient changes in MAP and HR after completion of topload compared to baseline. No significant changes were observed 10 minutes after the infusion until the end of the observation period after 1 hr. Arteriolar (n=31, n: number of vessels) and venular (n=25) diameters, microvascular flow velocity and flow were not significantly modified by HEMOXYCarrier® (Figs. 2A and 2B). We speculate that HEMOXYCarrier® in circulation may not react with NO as found with others cell free Hbs or that other properties such as its ability to transport CO may lead to the arteriolar vaso-inactivity. Thus, it is important to extend these microvascular studies of HEMOXYCarrier® to measure perivascular NO to validate this hypothesis.

Table 2. Hematocrit and Arterial Blood Gases before and after i.v. Topload Infusion of HEMOXYCarrier®

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>60 min</th>
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<tbody>
<tr>
<td>Hct</td>
<td>49.8 ± 1.6</td>
<td>48.3 ± 0.9*</td>
</tr>
<tr>
<td>Ph</td>
<td>7.37 ± 0.02</td>
<td>7.37 ± 0.05</td>
</tr>
<tr>
<td>PO2</td>
<td>55.9 ± 4.1</td>
<td>57.5 ± 2.4</td>
</tr>
<tr>
<td>PCO2</td>
<td>56.3 ± 1.2</td>
<td>55.7 ± 6.5</td>
</tr>
<tr>
<td>BE</td>
<td>7.0 ± 1.3</td>
<td>5.8 ± 1.4</td>
</tr>
</tbody>
</table>

Figs. (3) and (4) present a summary of our previous findings concerning MAP and microvascular response for several molecular Hbs (sourced from human and bovine) when infused with the same protocol as the current study [19]. The previous study used (1) \( \alpha \alpha \) cross-linked Hb (\( \alpha \alpha \)Hb, HemAssist®, Baxter); (2) polymerized bovine Hb (PBH4, Oxyglo-
bin®, Biopure Corp.), (3) polyethylene glycol decorated Hb (PEG-Hb, Hemospan®, Sangart) or (4) 0.9% NaCl (saline). Saline infusion was the no-treatment group/volume control used to assess the effect of the experimental protocol. It was a more extensive study where changes in microvessel diameter, blood flow, and perivascular NO concentration along with systemic hemodynamic parameters were followed. The results obtained in the current study with HEMOXYCarrier® were introduced into these figures for comparison.

The smaller molecular Hbs PBH4 and ααHb cause arteriolar constriction and reduced microvascular perfusion, while both HEMOXYCarrier® and PEG-Hb (higher molecular size of human and bovine Hbs, 95 kDa) maintained arteriolar diameter. However, PEG-Hb induced blood volume expansion in comparison with HEMOXYCarrier® that maintained microvascular flow.

All these solutions resulted in an increased mean arterial pressure due to vasoconstriction for PBH4 and ααHb and to blood volume expansion and maintenance of cardiac output for PEG-Hb. This previous work with included perivascular NO measurement using microelectrodes concluded that perivascular NO was reduced to the same level for all Hb solutions because NO binding affinities are similar. However, effects on vascular resistance are related to the type of molecular modification, molecular volume and oxygen affinity.

Thus, in the current study, the slight rise in MAP could be explained by changes to blood volume expansion. However
HEMOXYCarrier® at 600 mg/kg (5-min i.v. infusion).

Compared to saline-treated animals, injection of HEMOXYCarrier® caused arteriolar constriction leading to reduced microvascular perfusion compared to PEG-Hb and HEMOXYCarrier®. Both HEMOXYCarrier® and PEG-Hb did not change arteriolar diameter compared to baseline and saline. However, increased arteriolar blood flow levels were achieved with PEG-Hb, while HEMOXYCarrier® had unchanged levels. ααHb and PBH4 induced reduced arteriolar blood flow levels. Blood flow data (Q) is presented as mean ± sem. Parameters are presented relative to baseline, thus no change from baseline would be denoted as 1, while 1.05 would mean a 5% increase from baseline. , p < 0.05 relative to saline;  ‡ , p < 0.05 relative to HEMOX;  § , p < 0.05 relative to PEG-Hb.

without significant changes to vessel diameter compared to baseline, one needs to consider expanding the current study with HEMOXYCarrier® to make perivascular NO and cardiac output measurements in order to better compare to the other molecular Hbs. It is important to note that these findings that indicate the absence of vasoconstriction in the dorsal window chamber which consists of a resting skeletal muscle and connective tissue may not be reflective of the absence of vasoconstriction in organs. We have shown in parallel experiments which measure organ blood flow distribution that results deduced from findings in the hamster chamber window model, which allow the observation and quantitative characterization of the microcirculation in the intact and unanesthetized state, are representative of even in some of the major organs that are not accessible by microvascular techniques [10].

II. In Vivo Cardiovascular Effects in the Anaesthetized Rat

All animals completed the experimental protocol and are included in these results.

Compared to saline-treated animals, injection of HEMOXYCarrier® at 600 mg/kg (5-min i.v. infusion) showed only minor effects on mean arterial pressure (differences not statistically significant compared to saline), heart rate and left ventricular dP/dt max (an index of myocardial contractility), as shown on Figs. (5, 6 and 7), respectively.

The maximal effect measured on left ventricular dP/dt max was a slight increase (9%, P<0.05) observed 15 min after the end of the infusion and was followed by a return to baseline value. Similarly, the effect of HEMOXYCarrier® on heart rate was minor as heart rate increased slightly within 30 min following the treatment (P<0.05) and tended to return to baseline value thereafter. Since heart rate and myocardial contractility are almost not affected by HEMOXYCarrier® and are the major components of cardiac output, this parameter may not be affected by HEMOXYCarrier®. The lack of effects of HEMOXYCarrier® on arterial blood pressure in the anaesthetized rats suggests that HEMOXYCarrier® may also not have a vasoconstrictor effect in the rat. These results are consistent with those obtained in the hamster dorsal skin-
HEMOXYCarrier® is derived from molecules designed by an organism presents biophysical transport properties appropriate for an oxygen carrier for transfusion medicine. HEMOXYCarrier® is derived from molecules designed by evolution to maintain life in hypoxic conditions for prolonged periods, hence is suitable for transporting and delivering oxygen. The extremely low P50 of this molecular Hb targets its oxygen delivery to highly hypoxic tissue and it is unknown how this may affect the function of metabolically active tissues. HEMOXYCarrier® is stable without needing chemical modification over a wide range of ionic compositions and osmolarities and has superoxide dismutase like antioxidant properties. The current findings taken as a whole do not provide a compelling explanation for HEMOXYCarrier®’s lack of local vasoactivity in the hamster dorsal window chamber model. However, these preliminary data on rat and hamster demonstrates that the product has different properties in comparison to previous HBOC formulations and thus warrants more in depth investigations and its continued pursuit as an oxygen transporter for transfusion medicine or other applications where oxygen delivery is needed.

CONFLICT OF INTEREST

F.Z. and M.R. are founders and hold stock in Hemarina which produces the substance being investigated. Materials for the studies were provided by Hemarina. Biotrial was contracted by Hemarina to perform the rat studies presented in this publication. E.D. and C.D.L.R. are employees of Biotrial. Hamster studies were performed at University of California, San Diego. All other authors declare that they do not have any conflicts of interest with this work.

ACKNOWLEDGEMENTS

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REFERENCES


