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Oxygen delivery during extreme anemia with ultra-pure earthworm hemoglobin

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Abstract

Aim—*Lumbricus terrestris* (earthworm) erythrocrurin (LtEc) is a naturally occurring extracellular hemoglobin (Hb) with high molecular weight (3.6 MDa), low autoxidation rate, and limited nitric oxide (NO) dioxygenation activity. These properties make LtEc an potential candidate for use as red blood cell (RBC) substitute, i.e. Hb-based oxygen carrier (HBOC). Previous studies have shown that small amounts of LtEc can be safely transfused into mice, rats, and hamsters without eliciting major side-effects. Therefore, this study was designed to understand oxygen (O₂) transports to tissues and systemic/microvascular hemodynamics by LtEc during anemic conditions.

Main methods—Hamsters fitted with dorsal window chambers were hemodiluted to 18% hematocrit (Hct) using 6 g/dL dextran 70 kDa (**Dex70**). Hemodilution was then continued to 11% Hct using 10 g/dL **LtEc**, 6 g/dL **Dex70** or 10 g/dL human serum albumin (**HSA**). Blood pressure, heart rate, blood gas parameters, and microvascular hemodynamics, microvascular blood flow, functional capillary density (FCD), intravascular pO₂ and perivascular pO₂ were studied.

Key findings—**LtEc** maintained blood pressure without inducing vasoconstriction, while increasing microvascular perfusion and FCD relative to **Dex70** and **HSA**. **LtEc** increased blood O₂ carrying capacity, maintained systemic and microvascular parameters without decreasing arteriolar diameter or increasing vascular resistance with during extreme anemia. **LtEc** transfusion effects in blood flow and O₂ carrying capacity increased O₂ delivery compared to conventional plasma expanders.

Significance—**LtEc** or synthetic molecules that replicate the characteristics of LtEc, could be an effective O₂ carrier with potential to be used in transfusion medicine to prevent tissue anoxia resulting from severe anemia.

Keywords

Erythrocrurin; hemodynamics; hemodilution; anemia; tissue oxygenation; functional capillary density; albumin

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INTRODUCTION

The clinical need for an artificial oxygen (O₂) carrying solution is highlighted by the short shelf-life, frequent shortages, and storage-induced damage associated with the use of allogeneic red blood cells (RBCs) in transfusion medicine (Spinella, et al. 2011). Numerous attempts have been made to develop a synthetic and safe hemoglobin (Hb)-based O₂ carrier (HBOC), but these attempts failed during clinical trials (Spahn 2000). The first attempts at transfusing cell-free human Hb (HbA) failed when the HbA tetramer was shown to quickly dissociate in the blood stream, extravasate through the blood vessel wall, and cause kidney failure (Brandt, et al. 1951, Miller and McDonald 1951, Savitsky, et al. 1978). The next generation of HBOCs prevented tetramer dissociation by intramolecularly crosslinking the HbA tetramer (HemAssist, Baxter, Irvine, CA) (Schubert, et al. 2002) or polymerizing Hb tetramers with glutaraldehyde; PolyHeme, Northfield, Evanston, IL) (Gould, et al. 2002) and Hemopure OPK Biotech, Boston, MS (Winslow 2000). Unfortunately, these HBOCs were shown to scavenge nitric oxide (NO) and cause hypertension/vasoconstriction by interfering with endothelial smooth muscle NO signaling (Doherty, et al. 1998, Rohlf, et al. 1998).

The latest generation of HBOCs proposes to resolve the aforementioned problems by increasing the molecular weight (i.e. size) of polymerized Hbs (Matheson, et al. 2002), or encapsulating Hb tetramers within large diameter vesicles (Arifin and Palmer 2005). Based on the effects of Hb molecular size that we have previously observed, HBOCs with relatively large molecular weights (i.e. large molecular radii) do not leak into the perivascular space and have increased vascular retention times (Cabral, et al. 2009, Cabral, et al. 2010). Inspired by these results, we have investigated the use of the naturally occurring large extracellular erythrocrurin (i.e. mega Hb) from the common earthworm, *Lumbricus terrestris* (LtEc), which also has a high molecular weight (3.6 MDa) (Royer, et al. 2006, Strand, et al. 2004). Since earthworms lack RBCs, LtEc has naturally adapted to prevent the problems associated with acellular HBOCs (Hirsch, et al. 1997). LtEc is a complex structure, it has 144 globin subunits and 36 linker proteins that are extremely stable (Sharma, et al. 1996). LtEc also has a lower rate of oxidation than mammalian Hbs, with a positive redox potential ($E_0 = 112$ mV compared to $E_0 = -50$ mV for HbA) (Harrington, et al. 2007). In addition, LtEc and other erythrocrurins have an extremely low rate of NO scavenging (Elmer, et al. 2012). Hirsch *et al.* have shown that LtEc may be safe when injected into mice and rats without any noticeable side effects (Hirsch, et al. 1997).

We have also previously shown that LtEc can be easily highly purified via tangential flow filtration (TFF) and, when injected into hamsters in an experimental model that magnifies vasoactivity, LtEc did not produce any adverse reactions (Elmer, et al. 2012). In this study, 6g/dL dextran 70 kDa was used to induce an extreme anemic state in order to magnify the effects of LtEc in O₂ transport when most of the hamster native RBCs had been removed from the circulation (11% hematocrit, Hct). Our main goals were to (1) assess the contribution of LtEc to tissue O₂ delivery, (2) determine if LtEc induces hypertension and/or vasoconstriction, and (3) establish the circulatory half-life and pharmacokinetics of LtEc. Overall, LtEc improved systemic and microvascular parameters compared to conventional plasma expanders during extreme hemodilution, going beyond increasing O₂ carrying capacity to increase blood flow, while avoiding vasoconstriction and oxidation.

METHODS

Earthworm Preparation

For each round of purification, 1,000 Canadian nightcrawlers (*Lumbricus terrestris*) were purchased from Wholesale Bait Company (Hamilton, OH). Worms were rinsed in tap water to remove dirt, and then 1 L batches of worms were extensively washed with 20-30 L of tap

water to remove as much mucus as possible. A blender was used to homogenize the worms (puree mode for ~10 seconds) and the homogenate was immediately centrifuged at 3,716 g for 40 min at 4°C. Solid debris was discarded and the cloudy red supernatant was centrifuged at 18,000 g for 20 min at 10-15°C. The clear red supernatant (~2.0 L per 1,000 worms) was then put through filter paper to remove any remaining large particles.

Purification of LtEc

The earthworm homogenate was passed through two 0.22 µm TFF cartridges in parallel (1050 cm² surface area, Spectrum Labs, Rancho Dominguez, CA) at 450 mL/min until the majority of the sample volume was transmitted through the filter. The filter pores clogged several times during the filtration process (indicated by a clear filtrate) and were subsequently cleaned with deionized water. The red retentate of the 0.22 µm filter was stored at -80°C for future analysis. The 0.22 µm filtrate (1.8-2.0 L) was then concentrated on two 500 kDa TFF cartridges at 450 mL/min down to a final volume of approximately 200 mL. The 500 kDa retentate was then diafiltered by diluting it to 2 L with buffer and then concentrating it down to 200 mL ten times. The retentate was diluted with 20 mM Tris buffer (pH 7.0) during the first eight rounds of diafiltration and modified lactated Ringer's buffer (115 mM NaCl, 4 mM KCl, 1.4 mM CaCl₂, 13 mM NaOH, 12.25 mM N-acetyl cysteine, 0.3% sodium lactate, pH 7.0) during the last two rounds of diafiltration. During the final round of diafiltration, the retentate was concentrated to 20-50 mL and sterilized by passing it through a 0.45 µm syringe filter. The purified LtEc was then stored at -80°C until needed. After each round of purification, all filters were rinsed and soaked in 0.2 M NaOH for 1 hour, then rinsed with distilled water and stored at 4°C.

Estimation of Yield and Methemoglobin Level

The Hb concentration and the percent of oxidized Hb (i.e. methemoglobin [metHb]) was measured using the cyanomethemoglobin method (Crosby, et al. 1954). Before quantification, each sample was centrifuged at 10,000 g for 5 minutes to remove any debris. The concentration of each sample was then multiplied by its volume to obtain the mass of Hb in each filtrate or retentate sample.

Oxygen equilibrium curve

Oxygen equilibrium curves for the LtHb and the Hamster blood were obtained by deoxygenation of O₂-equilibrated samples in a Hemox buffer at 37°C, using a Hemox Analyzer (TCS Scientific Corporation, New Hope, PA). The Hemox buffer pH was adjusted to match the arterial blood pH of the animals using Tris and BisTris buffers. Tris and BisTris buffers were prepared by titrating the reagents with HCl before adjusting the pH of the solutions to keep Cl⁻ ions concentration equal to the buffer at the pH values.

Window Chamber Animal Preparation

Animal handling and care followed the NIH Guide for the Care and Use of Laboratory Animals and the experimental protocol was approved by the local animal care committee. Studies were performed in 55 - 65 g male Golden Syrian Hamsters (Charles River Laboratories, Boston, MA) fitted with a dorsal skinfold window chamber. The hamster window chamber model is widely used for microvascular studies without anesthesia. The complete surgical technique has been described previously (Endrich, et al. 1980). Arterial and venous catheters filled with heparinized saline solution (30 IU/mL) were implanted into the carotid and jugular vessels. Catheters were tunneled under the skin, exteriorized at the dorsal side of the neck, and securely attached to the window frame.

Inclusion Criteria

Animals were considered suitable for experiments if systemic parameters were as follows: heart rate (HR) > 340 beats/min, mean arterial blood pressure (MAP) > 80 mm Hg, systemic Hct > 45%, and arterial O₂ partial pressure (p_AO₂) > 50 mm Hg. Additionally, animals with signs of low perfusion, inflammation, edema, or bleeding in their microvasculature were excluded from the study.

Systemic Parameters

MAP and HR were monitored continuously (MP150, Biopac System Inc., Santa Barbara, CA). Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes. Hb content was determined spectrophotometrically (B-Hemoglobin, Hemocue, Stockholm, Sweden). Arterial blood was collected in heparinized glass capillaries (50 µL) and immediately analyzed for pO₂, pCO₂, base excess (BE), and pH (Rapidlab 248, Bayer, Norwood, MA). Arterial Hb saturations were measured using a IL482 CO-Oximeter (Instrumentation Laboratory, Lexington, MA).

Experimental Setup

The unanesthetized animal was placed in a restraining tube with a longitudinal slit from which the window chamber protruded, then fixed to the microscopic stage for transillumination with the intravital microscope (BX51WI, Olympus, New Hyde Park, NY). Animals were given 20 minutes to adjust to the tube environment and images were obtained using a CCD camera (4815, COHU, San Diego, CA). Measurements were carried out using a 40× (LUMPFL-WIR, numerical aperture 0.8, Olympus) water immersion objective.

Microhemodynamics

Arteriolar and venular blood flow velocities were measured using the photodiode cross-correlation method (Photo-Diode/Velocity, Vista Electronics, San Diego, CA) (Intaglietta, et al. 1975). The measured centerline velocity (V) was corrected according to blood vessel size to obtain the mean RBC velocity (Lipowsky and Zweifach 1978). A video image-shearing method was used to measure blood vessel diameter (D) (Intaglietta and Tompkins 1973). Blood flow (Q) was calculated from the measured values as $Q = \pi \times V (D/2)^2$. Blood vessel wall shear stress (WSS) was defined by $WSS = WSR \times \eta$, where WSR is the blood vessel wall shear rate given by $8VD^{-1}$, and η is the microvascular blood viscosity or plasma viscosity. Microvascular blood flow was determined by the perfusion pressure, vascular resistance, and blood viscosity. In a rigid tube, changes in apparent fluid viscosity would lead to parallel changes in resistance and reciprocal changes in flow. Microvascular resistance (MR) was calculated as the ratio of blood pressure and microvascular volumetric blood flow ($MR = MAP / \text{average microvascular } Q$). Microvascular hindrance (MH) was calculated as the ratio of MR and blood viscosity ($MH = MR / \eta$).

Functional Capillary Density (FCD)

Functional capillaries, defined as capillary segments that have RBC transit of at least one RBC in a 60 second period in 10 successive microscopic fields, were assessed in a region of 0.46 mm². FCD (cm⁻¹) is calculated as the total length of RBC perfused capillaries divided by the area (0.46 mm²).

Microvascular pO₂ and microvascular O₂ saturations

pO₂ measurements were made using phosphorescence quenching microscopy (PQM) (Torres Filho and Intaglietta 1993). PQM is based on the O₂ dependent quenching of phosphorescence emitted by albumin-bound metalloporphyrin complex after pulsed light excitation. PQM is independent of the dye concentration. Perivascular pO₂ was measured in

tissue regions between functional capillaries. PQM allows for precise localization of the pO₂ measurements without subjecting the tissue to injury. Intravascular Hb O₂ saturations were calculated using the O₂ equilibrium curves measured and the intravascular pO₂s measured with the PQM. The O₂ equilibrium curves were measured at arterial blood pH as described above.

Isovolemic Hemodilution

Acute anemia was induced by two isovolemic hemodilution steps. This protocol was described in detail in a previous publication (Cabrales, et al. 2004, Cabrales, et al. 2010). Briefly, the volume of each exchange-hemodilution step was calculated as a percentage of the animal's blood volume (BV), estimated as 7% of body weight (BW). The acute anemic state was induced by lowering the Hct to 18% in two progressive isovolemic hemodilution steps using 6 g/dL dextran 70 kDa (Pharmacia, Uppsala, Sweden). The first exchange was 40% of the BV and second exchange was 35% of the BV, respectively. After hemodilution to 18% Hct, the animals were randomly divided into three experimental groups: **Dex70** (further hemodiluted with 6 g/dL dextran 70 kDa to 11% Hct), **HSA** (further hemodiluted with 10% HSA to 11% Hct), or **LtEc** (further hemodiluted with 10% LtEc to 11% Hct). The properties of the test solutions are shown in Table 1. The viscosity of LtEc was higher than undiluted blood (4.2 cP) and the dextran 70 kDa and HSA solutions. In contrast, dextran 70 kDa and HSA solutions have higher COP compared to LtEc, which is also lower than the COP of plasma serum (18 mm Hg). The O₂ affinity and cooperativity of LtEc (P₅₀ = 28 mm Hg and n = 3.7) are similar to hamster blood (P₅₀ = 32 mm Hg and n = 2.9).

Oxygen Delivery and Extraction. The microvascular methodology used in our studies allows a detailed analysis of O₂ supply to the tissues. Calculations were made using equations 1 and 2 (Cabrales, et al. 2010):

$$O_2 \text{ delivery} = \left[(RBC_{Hb} \times Y \times S_A) + (Plasma_{Hb} \times Y \times \check{S}_A) \right] \times Q \quad (1)$$

$$O_2 \text{ extraction} = \left[(RBC_{Hb} \times Y \times S_{A-V}) + (Plasma_{Hb} \times Y \times \check{S}_{A-V}) \right] \times Q \quad (2)$$

Where, RBC_{Hb} is the Hb encapsulated inside RBCs, total Hb - plasma Hb [g_{Hb}/dlblood], $Plasma_{Hb}$ is the extracellular Hb [g_{Hb}/dlblood], γ is the O₂ carrying capacity of saturated Hb [1.34 mlO₂/g_{Hb}], S_A is the arteriolar blood O₂ saturation and $\check{S}_A$ is the arteriolar extracellular Hb O₂ saturation, the subscript (A-V) indicates the arteriolar/venular differences, and Q is the microvascular flow rate. O₂ saturations were calculated using the measured pO₂ and oxygen equilibrium curve.

Pharmacokinetic Analysis of LtEc

LtEc pharmacokinetics was studied in hamsters fitted with the dorsal window chamber (described in the animal preparation section) after an exchange transfusion of 40% of the estimated BV. Pharmacokinetic parameters were determined for total LtEc in the plasma using a non-compartmental method (NCOMP Pharmacokinetics Analysis Software, Philadelphia, PA). At each time point (0.5, 1, 2, 4, 8, 12, 24, and 48 h) after the exchange transfusion, a 50 µL aliquot of blood was collected, and plasma was obtained by centrifugation. The plasma Hb and metHb concentration were measured via the cyanomethemoglobin method (Sakata, et al. 1982).

Statistical Analysis

Results are presented as the mean $\pm$ standard deviation and the values are presented relative to the baseline. Data between time points in the same group were analyzed using analysis of variance for repeated measures (ANOVA, Kruskal-Wallis test) and *post hoc* analyses with the Dunn's Multiple Comparison, when appropriate. Statistics were calculated using Prism 4.01 (GraphPad, San Diego, CA) and differences were considered statistically significant if $P < 0.05$.

RESULTS

Eighteen animals were included in the study. All animals tolerated the entire protocol without any visible signs of discomfort. Animals were assigned to the following experimental hemodilution groups: **Dex70** (n=6; 61.7 ± 6.2 g); **HSA** (n=6; 63.6 ± 4.6 g); and **LtEc** (n=6; 65.7 ± 4.7 g). Systemic data at baseline and during moderate hemodilution (18% Hct) were calculated by combining values from all of the experimental groups (n=18). All animals passed the Grubbs' test, meaning that all the measured values at baseline were within a similar population ($P < 0.05$). Similarities between groups at baseline and moderate hemodilution (18% Hct) were also statistically verified between groups ($P > 0.30$).

Biophysical Properties of Solutions and Blood

Dex70 and **HSA** have higher COP values (44 and 48 mm Hg) compared to **LtEc** (6 mm Hg), and the viscosity of **LtEc** is 3 and 5 times greater than **Dex70** and **HSA**. Blood rheological properties and COP after hemodilution are presented in Table 2. **LtEc** showed higher blood and plasma viscosity compared to **Dex70** and **HSA**. However, the plasma COPs for all the study groups were similar, and not different from baseline. Interestingly, no pathophysiological effects were observed in the hamsters transfused with **LtEc**. Even after 1 hour post infusion of **LtEc**, very few leukocytes and macrophages were observed in the microcirculation. In addition, the urine collected from each animal's bladder after the experiments showed no signs of **LtEc**.

Blood Gas Parameters

Blood gas parameters are also presented in Table 2. After extreme hemodilution with **Dex70**, **HSA**, and **LtEc**, the Hct decreased to 11% following the experimental protocol. Total Hb concentration also decreased in the **Dex70** and **HSA** groups ($\text{Hb} = 3.6\text{--}3.7$ g/dL), but the Hb concentration in the **LtEc** group (6.1 ± 0.5 g/dL) was slightly higher than at moderate hemodilution (5.8 ± 0.4 g/dL). Arterial pO_2 increased significantly relative to baseline and moderate hemodilution for all groups, but only the **LtEc** group had arterial pCO_2 levels similar to moderate hemodilution. Hemodilution with **LtEc** increased arterial pO_2 compared to baseline and moderate hemodilution, however lower than hemodilution with **Dex70**. Arterial blood pH was not significantly different after hemodilution with any of the test solutions. Blood BE was significantly decreased after hemodilution with **Dex70** and **HSA** compared to baseline, but no significant differences in blood BE were observed in the **LtEc** group.

MAP and HR

Changes in MAP and HR are presented in Figure 1. The MAP after hemodilution with **LtEc** was lower relative to baseline, but not significantly different from moderate hemodilution at 18% Hct. The additional O_2 carrying capacity provided by **LtEc** maintained MAP after hemodilution, while the MAP values of the **Dex70** and **HSA** groups significantly decreased relative to baseline and moderate hemodilution. No significant changes in HR were observed in any of the test groups.

Microvascular Hemodynamic Measurements

Changes in microvessel diameter and blood flow are shown in Figure 2, absolute values are listed in the figure legend. For the **Dex70** and **HSA** groups, arteriolar diameters significantly decreased relative to moderate hemodilution. In contrast, the arteriolar diameters in animals transfused with **LtEc** were significantly higher than baseline and the **Dex70** and **HSA** groups. Venular diameters for **Dex70** and **HSA** were not significantly different from baseline, but the venular diameter of the **LtEc** group was significantly lower relative to baseline. The blood flow in the arterioles and venules were significantly lower for the **Dex70** and **HSA** groups relative to baseline. In addition, the venular blood flow of the **Dex70** and **HSA** groups was significantly lower than at moderate hemodilution, but venule blood flow was maintained after hemodilution with **LtEc**. On the other hand, arteriolar blood flow in the **LtEc** group was significantly higher than **Dex70**, **HSA**, and baseline, resulting from the significant increase in arteriolar diameter.

Changes in FCD are presented in Figure 3. The FCD was significantly reduced in all experimental groups compared to baseline and 18% Hct. However, the FCD of the **LtEc** group was significantly higher relative to the **Dex70** and **HSA** groups. Arteriolar and venular shear stress and microvascular resistance and hindrance are also presented in Table 3. There were no significant differences in the wall shear rate of the **LtEc**, **Dex70**, and **HSA** groups, but wall shear stress was significantly higher for **LtEc** compared to **Dex70** and **HSA**. Assuming that microvascular blood pressure is proportional to MAP, the estimated microvascular resistance of the **LtEc** group (62% of baseline) was significantly lower than the **HSA** (68% of baseline) and **Dex70** (77% of baseline) groups. Vascular hindrance (i.e. the contribution of vascular geometry to blood flow) for **LtEc** (96% of baseline) was similar to baseline, but significantly higher in the **Dex70** (146% of baseline) and **HSA** (147% of baseline) groups. These results confirm that **LtEc** does not induce a vasoconstrictive response upon infusion. This also shows that extreme hemodilution with the plasma expanders used in this study increases peripheral vascular resistance in order to maintain O₂ supply to vital organs.

Microvascular Oxygen Tensions

Arteriolar, venular and perivascular pO₂s are presented in Figure 4. Arteriolar pO₂ was significantly higher in the **LtEc** group than the **Dex70** and **HSA** groups. The **LtEc** group also had significantly higher perivascular and venular pO₂ than the **Dex70** and **HSA** groups.

Microvascular Oxygen Delivery

Oxygen transport parameters were calculated using the *ex vivo* P₅₀ of LtEc. The O₂ delivery and extraction values are presented in Figure 5. **LtEc** provided superior O₂ delivery and maintained high O₂ extraction. Arterial (systemic) and microvascular O₂ delivery were significantly higher for **LtEc** compared to the **Dex70** and **HSA** groups. Arterial to arteriolar O₂ extraction was not different between the groups. However, the arteriolar to venular (microcirculation) O₂ extraction was significantly higher for **LtEc** compared to the **Dex70** and **HSA** groups. These results show that LtEc is able to maintain O₂ delivery after hemodilution compared to the plasma expanders used in this study.

Pharmacokinetics of LtHb

Four animals were used for the pharmacokinetic study (n=4; 65.7 ± 6.7 g) presented in Figure 6 and Table 3. The initial dose of LtEc was 189 ± 52 mg and the maximum plasma concentration (C_{max}) was 3.36 ± 0.61 g/dL. The volume of distribution and retention time of LtEc indicates a mean residence time of 23.2 ± 1.2 hours. MetHb levels remained lower than 20% during the entire observation period. The measurements of reduced (Fe²⁺) and oxidized

(Fe³⁺, metHb) LtEc show that LtEc is able to resist oxidation *in vivo*. Therefore, most of the transfused LtEc remained in the bloodstream in the O₂ transporting form (Fe²⁺) a full day after the initial transfusion.

DISCUSSION

The principal result of this study is that naturally occurring extracellular megaHb complex defines the properties to generate a new generation of safe and efficacious HBOC, which can be advantageous in extreme anemic conditions when blood is not available. In addition, if highly purified LtEc were to be used as HBOC, it may avoid many costly synthetic steps necessary in order to modify mammalian Hb into HBOC. Our results show that **LtEc** effectively maintains systemic hemodynamics, preserves blood gas parameters, and delivers O₂ during extreme anemia (11% Hct). **LtEc** improved O₂ delivery to the tissues and sustained two-fold higher perivascular pO₂ values and 50% higher oxygenation than the plasma expander groups (see Figures 4 and 5). These improvements in O₂ delivery are mostly due to the O₂ affinity and binding equilibrium of **LtEc**, which is similar to RBCs. Earthworm Hb has evolved during the course of natural selection to possess unique structural and functional properties that impart its efficacy as a naturally occurring extracellular O₂ carrier. LtEc possesses a unique structure in the form of a megaHb complex that meets the molecular size requirement that we have previously defined for synthetic polymerized bovine Hb to minimize side effects of safe HBOCs (Cabral, et al. 2009, Cabral, et al. 2010). However, LtEc includes additional structural and oxidative stability properties, that appear to be essential for the development of efficacious extracellular HBOCs.

Our current results show that when extreme anemic states are induced by hemodilution with **LtEc**, hemodynamics and oxygenation are superior compared to plasma expanders (HSA & dextran 70 kDa). The systemic hemodynamics benefits of **LtEc** were paralleled with positive microvascular changes, where blood flow and FCD were significantly greater for **LtEc** compared to plasma expanders. Hemodilution decreased the Hct and dramatically reduced blood viscosity; however, when **LtEc** was used to further reduce the Hct, it increased plasma and blood viscosity relative to **HSA** or **Dex70**. The increase in blood viscosity with **LtEc** did not increase blood pressure, and on the contrary **LtEc** decreased microvascular resistance. In addition, the hemodynamic changes and the pharmacokinetic results indicate that **LtEc** can be used as a potential HBOC, since it improves blood flow, O₂ delivery and has a longer circulation time than commercially developed HBOCs (Elmer, et al. 2010, Vandegriff, et al. 2006).

The infusion of current commercially developed HBOCs results in vasoconstriction, which is evident as a rapid rise in peripheral vascular resistance and blood pressure, commonly associated with bradycardia, decreasing cardiac output and perfusion to vital organs (Rohlf, et al. 1998). Paradoxically, microvascular resistance was lower for **LtEc** relative to **HSA** and **Dex70**, even though **LtEc** has a significantly higher viscosity than **HSA** and **Dex70**. Consequently, the group hemodiluted with **LtEc** had higher plasma viscosities than both of the plasma expander groups. It has been shown that higher high plasma viscosity increases shear stress on the endothelial vessel walls, thereby inducing mechanotransduction responses that activate endothelial NO production, causing relaxation of the smooth muscle and increasing blood vessel diameter (Tsai, et al. 2005). This phenomenon has been previously observed with high viscosity plasma expanders (Tsai, et al. 2005), and may be one of the mechanisms that preserves vascular tone after hemodilution with **LtEc**. Hemodilution with low viscosity plasma expanders decreased vascular resistance, but, on the other hand, it increased vascular hindrance, which accounts for the geometric contribution of the vasculature to the resistance, suggesting generalized vasoconstriction,

whereas the hemodilution with **LtEc** reduced vascular resistance and preserved vascular hindrance. **LtEc** acted as a viscogenic O₂ carrier, maintaining both shear stress and oxygenation, without vasoconstriction, leading to improved transmission of central pressure to the microcirculation to preserve FCD. In a similar extreme hemodilution study, a commercially available polymerized bovine Hb (Oxyglobin, OPK Biotech Inc., Cambridge, MA) developed for veterinarian use reduced the FCD to 40% of baseline, and increased vascular resistance (Tsai 2001). Consequently, hemodilution with Oxyglobin reduced O₂ delivery to the surrounding tissues (Tsai 2001). This result was found to be due to vasoconstriction, which lowers microvascular blood flow, capillary pressure and FCD (Cabrales, et al. 2004). These adverse effects are not only due to the extracellular nature of the HBOC, since non-O₂ carrier plasma expanders, such as HSA and Dex70, decreased FCD, blood flow, and capillary pressure (Cabrales, et al. 2004). In contrast, hemodilution with **LtEc** maintained microvascular hemodynamics and FCD, which preserved oxygenation and wash out of metabolic by products.

Compared to previous work by Hirsch *et al.* (Hirsch, et al. 1997), this study is the first to use LtEc at a concentration capable of significantly increasing O₂ transport, as verified by our results. In situations where bulk O₂ transport capacity is required, LtEc can serve a potentially safe and efficacious HBOC. The acid-base balance at 11% Hct after extreme hemodilution with **LtEc** was not different from baseline (48% Hct), while the plasma expanders used in this study showed negative acid-base balances. Additionally, the arterial PO₂ of animals hemodiluted with plasma expanders was drastically increased, suggesting significant adjustments in the respiratory system, in an attempt to improve oxygenation, which only increases the dissolved O₂ within the plasma having minimal effects in the O₂ transported by the blood. O₂ transport calculations show that **LtEc** increased the arterial O₂ transport by 60% compared to conventional plasma expanders, and similarly at the microcirculatory level, **LtEc** increased O₂ transport by at least 50% compared to **HSA** and **Dex70**. The O₂ transport similarities between LtEc and RBCs suggest that each species contribution to O₂ transport is proportional to their concentration. Therefore, the physiological O₂ affinity of LtEc preserves LtEc's O₂ transport benefits along the entire cardiovascular system. In this study, the perivascular pO₂ was low due to the limited O₂ carrying capacity of the hemodiluted blood. Although, LtEc showed higher perivascular pO₂ compared to plasma expanders resulting from its higher O₂ carrying capacity. The O₂ transport benefits of LtEc may be magnified by the hamster respiratory gas exchange characteristics and their Hb O₂ affinity with a P50 of 32 mm Hg. Moreover, hamsters are fossorial animals, which explains their low arterial PO₂ at baseline, since their respiratory exchange is adapted to their burrow environment with a greater buffering capacity from higher HCO₃ and decreased sensitivity to blood pH (Tomasco, et al. 2010). LtEc meets the requirements of a HBOC and/or O₂ carrier plasma expander as demonstrated by its lack of acute toxicity, long retention time and reduced oxidation. The pharmacokinetic data show that LtEc has a longer circulation time than most other HBOCs (13-17 hrs) (Elmer, et al. 2010, Vandegriff, et al. 2006). The *in vivo* oxidation rate of LtEc appears to be quite low, since no significant increases in metLtEc (Fe³⁺ form) were observed over a period of 48 hours. In contrast, HBOCs in clinical trials have report levels of Fe³⁺ higher than 40% in less than 24 hours after infusion (Siegel, et al. 1997). By resisting oxidation, LtEc is able to maintain O₂ delivery for long periods of time and reduce oxidative stress induced by the presence of Fe³⁺ in the bloodstream. Additionally, the results obtained with LtEc led to the hypothesis that its molecular size and stability (structural and oxidative) are important characteristics that allow it to function as a non-toxic HBOC in vertebrates. The importance our results during hemodilution with LtEc are many fold, since vasoconstriction is perceived to be the critical barrier hampering the commercial development of HBOCs. Regardless of the exact mechanism for vasoconstriction, ultrapure naturally extracellular LtEc did not produce vasoconstriction. Finally, it is important to mention that none of the animals showed

significant changes in behavior following transfusion of LtEc. LtEc appears to be safe in hamsters, but future work will be aimed to establish any potential side-effects and immunogenicity.

Conclusion

This study shows that LtEc is an effective HBOC which does not elicit any significant vasoconstriction or hypertension, and it is able to effectively and safely deliver O₂ during extreme anemia. The large molecular size, O₂ binding characteristics, high viscosity, stability, and high circulation half-life, contribute to LtEc superior O₂ delivery. Thus seem to be essential properties required for the next generation HBOC. Additionally, highly purified LtEc might have the potential to be used as a HBOC, since it does not require costly synthetic modification steps and appears to avoid the vasoconstriction and hypertension regarded as major limitations of current HBOCs. While these results are promising, much more work will have to be done before synthetic molecules inspired in LtEc or purified LtEc can enter clinical trials. Transfusion of higher LtEc concentrations must be explored to determine immunogenicity and clearance mechanism of LtEc. All extracellular Hbs are known to deplete NO, thus detailed *in vitro* and *in vivo* studies must be done to determine the exact nature of the interactions between LtEc and NO. Nonetheless, in light of our current results, LtEc characteristics should be further studied to be able to incorporate these properties in the new generation of O₂ carriers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1. Mean arterial pressure (MAP) and heart rate (HR) after extreme hemodilution with *LtEc*, *HSA* and *Dex70*. The broken line represents the baseline level. †, $P < 0.05$ relative to baseline; ‡, $P < 0.05$ compared to *Dex70*; §, $P < 0.05$ compared to *HSA*.

FIGURE 2.

Relative changes to baseline in arteriolar and venular hemodynamics after extreme hemodilution with *LtEc*, *HSA* and *Dex70*. The broken line represents the baseline level. †, $P < 0.05$ relative to baseline; ‡, $P < 0.05$ compared to *Dex70*; §, $P < 0.05$ compared to *HSA*. Diameters (μm , mean $\pm$ SD) at baseline in each animal group were as follows: *LtEc* (arterioles (A): 62.1 ± 7.2 , $n = 48$; venules (V): 62.6 ± 5.8 , $n = 46$); *HSA* (A: 61.9 ± 9.1 , $n = 42$; V: 63.6 ± 7.2 , $n = 46$); and *Dex70* (A: 62.7 ± 8.6 , $n = 48$; V: 64.8 ± 7.4 , $n = 46$). n = number of small blood vessels studied. RBC velocities (mm/s, mean $\pm$ SD) at baseline in each animal group were as follows (data was not presented in the figure, but used in the calculation of blood flow): *LtEc* (A: 4.3 ± 0.9 ; V: 1.9 ± 0.7); *HSA* (A: 4.3 ± 1.0 ; V: 2.0 ± 0.8); *Dex70* (A: 4.4 ± 0.8 ; V: 2.1 ± 0.7). Blood flow (nL/s, mean $\pm$ SD) at baseline in each animal group were as follows: *LtEc* (A: 12.7 ± 5.3 ; V: 5.4 ± 2.1); *HSA* (A: 12.4 ± 4.2 ; V: 5.7 ± 2.1); *Dex70* (A: 12.0 ± 4.7 ; V: 6.2 ± 2.4).

FIGURE 3.

Functional capillary density (FCD) after extreme hemodilution with *LtEc*, *HSA* and *Dex70*. †, $P < 0.05$ relative to baseline; ‡, $P < 0.05$ compared to 18% Hct. FCD (capillaries per unit of area, cm^{-1}) at baseline for *LtEc* were 112 ± 11 , *Dex70* were 114 ± 12 and for *HSA* were 109 ± 10 , respectively.

FIGURE 4.

Intravascular and perivascular partial pressure of oxygen after moderate hemodilution and extreme hemodilution with *LtEc*, *HSA* and *Dex70*. Intravascular and perivascular PO_2 s for the tissues comprising the hamster window model are 51.8 mm Hg for arterioles, 32.7 mm Hg for venules and 21.7 mm Hg for perivascular areas.

FIGURE 5.

Arterial oxygen delivery and extraction after extreme hemodilution with *LtEc*, *HSA* and *Dex70*. Oxygen transport is not directly measurable; however, it can be calculated using the measured parameters. Hatched portion of bar indicated the amount of oxygen transported by the *LtEc*.

FIGURE 6.
LtEc pharmacokinetics after a 40% exchange transfusion.

TABLE 1

Physical properties of transfusion solutions.

	Solution properties			
	g/dL	Viscosity* CP	COP mmHg	P50 mmHg n
Plasma Expander				
Dextran 70 kDa (Dex70)	6.0	2.8	48	-- --
Human serum albumin (HSA)	10.0	1.6	44	-- --
Hb based O ₂ carriers				
LiEc	10.0	8.2	6	28.0 3.7

* Shear rate of 160 s^{-1} at 37°C

TABLE 2

Blood chemistry and rheological parameters.

	Baseline	Hemodilution	Extreme hemodilution		
			Dex70	HSA	LtEc
n	18	18	6	6	6
Hct, %	48.4 ± 0.9	18.5 ± 0.7 [†]	11.2 ± 0.3 ^{†‡}	11.3 ± 0.5 ^{†‡}	11.6 ± 0.7 ^{†‡}
Total Hb, g/dl	14.6 ± 0.4	5.8 ± 0.4 [†]	3.6 ± 0.3 ^{†‡}	3.7 ± 0.4 ^{†‡} $\longleftrightarrow$ $\xrightarrow{P=0.05}$	6.1 ± 0.5 ^{†‡§}
Plasma Hb, g/dl					2.7 ± 0.4
P_aO_2 , mmHg	58.8 ± 5.6	78.3 ± 6.1 [†]	116.5 ± 7.4 ^{†‡}	99.6 ± 4.7 ^{†‡}	97.2 ± 7.8 ^{†‡§}
P_aCO_2 , mmHg	53.3 ± 5.1	46.3 ± 5.8 [†]	36.2 ± 6.4 ^{†‡}	40.6 ± 5.0 [†]	45.1 ± 4.8 [†]
PH _a	7.356 ± 0.020	7.381 ± 0.024	7.387 ± 0.028	7.364 ± 0.027	7.372 ± 0.029
BE _a , mmol	2.6 ± 1.7	1.0 ± 1.1	-2.0 ± 1.2 ^{†‡}	-0.7 ± 0.9 ^{†‡}	0.8 ± 0.9 [§]
Blood Visc., cP	4.2 ± 0.2	2.3 ± 0.2 [†]	2.3 ± 0.2 [†] $\longleftrightarrow$ $\xrightarrow{P=0.05}$	2.0 ± 0.2 [†] $\longleftrightarrow$ $\xrightarrow{P=0.05}$	2.7 ± 0.2 ^{†§}
Plasma Visc., cP	1.2 ± 0.1	1.6 ± 0.1 [†]	1.6 ± 0.1 [†]	1.4 ± 0.2 [†] $\longleftrightarrow$ $\xrightarrow{P=0.05}$	2.0 ± 0.2 ^{†§}
COP, mm Hg	18 ± 2	16 ± 2	16 ± 2	18 ± 2	16 ± 2

Values are means ± SD. Baseline and hemodilution included all the animals. No significant differences were detected between baseline or hemodilution values between the experimental groups.

Hct, hematocrit; Hb, hemoglobin; P_aO_2 , arterial partial O₂ pressure; P_aCO_2 , arterial partial pressure of CO₂; BE_a, arterial base excess.[†]P<0.05 compared to baseline[‡]P<0.05 compared to MH[§]P<0.05 compared to Dex70.

TABLE 3

Pharmacokinetics of LtEc.

	LtHb
CL, mL h ⁻¹	3.36
MRT, h	23.2
V _{ss} , mL	78.1
Terminal K, h ⁻¹	0.045
Terminal T _{1/2} , h	14.5
C _{max} , Mg h ⁻¹	3.40

CL, plasma clearance; MRT, mean residence time; V_{ss} volume of distribution at steady-state, terminal k, terminal slope; terminal T_{1/2}, terminal half-life; C_{max}, concentration maximal.