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Resuscitation from hemorrhagic shock using polymerized hemoglobin compared to blood

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Abstract

The development of an alternative to blood transfusion to treat severe hemorrhage remains a challenge, especially in far forward scenarios when blood is not available. Hemoglobin (Hb) based oxygen (O₂) carriers (HBOCs) were developed to address this need. Hemopure® (HBOC-201, bovine Hb glutamer-250, OPK Biotech, Cambridge, MA, USA), one such HBOC, has been approved for clinical use in South Africa and Russia. At the time of its approval, however, few studies aimed to understand Hemopure's function, administration, and adverse effects compared to blood. We used intravital microscopy to study the microcirculation hemodynamics (arteriolar and venular diameters and blood flow and functional capillary density (FCD)) and oxygenation implications of Hemopure® administration at different Hb concentrations —4, 8, and 12 gHb/dL — compared to fresh blood transfusion during resuscitation from hemorrhagic shock. Experiments were performed in unanesthetized hamsters instrumented with a skinfold window chamber, subjected to hemorrhage (50% of the blood volume, BV), followed by 1 hour hypovolemic shock and fluid resuscitation (50% of the shed volume). Our results show that fluid resuscitation with Hemopure® or blood restored systemic and microvascular parameters. Microcirculation O₂ delivery was directly correlated with Hemopure® concentration, though increased vasoconstriction was as well. FCD reflected the balance between enhanced O₂ transport and reduced blood flow: 12 gHb/dL of Hemopure® and blood decreased FCD compared to the lower concentrations of Hemopure® (p<0.05). The balance between O₂ transport and tissue perfusion can provide superior resuscitation from hemorrhagic shock compared to blood transfusion by using a low Hb concentration of HBOCs relative to blood.

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Author Contributions

D.O analyzed the data and wrote the manuscript and **M.B** analyzed the data. **S. Y** performed the experiments and gathered the data. **P.C** designed the study, performed the experiments, analyzed the data and wrote the manuscript.

Disclosures

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Keywords

resuscitation; hemorrhagic shock; hemoglobin; blood substitutes

Background

In the United States, allogeneic RBC transfusion has long been considered an important treatment option for patients suffering from blood losses (1). However, the supply of blood is particularly vulnerable to pandemics (2, 3) and blood transfusion-related adverse events, both short- and long-term is among the costliest contributor to health care expenditures (4). In addition, the availability of blood is limited in emergency situations such as in war zones or after natural disasters (5). Therefore, the development of a therapeutic product that can suitably replace blood transfusion in cases of severe hemorrhage or during major cardiovascular surgery has been a goal of scientific and commercial efforts (6). Hemoglobin, the protein responsible for the transport of O₂ in the RBC, has served as the precursor for the formulation of blood substitutes. Hb based oxygen (O₂) carriers (HBOCs) were developed to restore intravascular volume and O₂ carrying capacity (7). Previous HBOCs were based on stroma-free Hb (8, 9), the tetramer structure of which would dissociate upon infusion, producing several adverse reactions including kidney failure (10, 11).

Those that produced the first generation of HBOCs tried to replicate blood O₂ transport characteristics by designing HBOCs with similar Hb concentrations to normal blood (12). However, most of these products were not further developed as studies showed that they were more harmful than beneficial. From the first generation of HBOCs, we learned that acellular Hb depletes endothelial nitric oxide (NO) via a NO dioxygenase reaction that produces vasoconstriction and hypertension (13, 14). Other negative reactions partially responsible for vasoconstriction and hypertension exerted by first generation HBOCs were the extravasation of acellular Hb due to its small molecular size, as well as metabolic regulation of blood flow in response to hyper-oxygenation due to facilitated O₂ transport by the acellular Hb (15, 16). To overcome these issues, different strategies have been developed, including Hb polymerization and Hb surface decoration with polyethylene glycol (PEG) (17). These materials represent the second generation of HBOCs. They have demonstrated reduced vasoactivity, longer intravascular retention, minimal toxicity, and superior oxygenation (18, 19). Unfortunately, none of these products are currently approved for human use in the US or Europe, mainly due to adverse events observed in clinical trials (20).

Microvascular function, in terms of blood flow and functional capillaries, is essential for the maintenance of tissue viability because the microcirculation is where O₂ is delivered and metabolic byproducts are washed out (21). HBOC biophysical characteristics determine the mechanisms required to restore volume, microvascular blood flow, and O₂ delivery. Hemopure® (OPK Biotech, Cambridge, MA) is a HBOC developed originally by Biopure Corp (Cambridge, MA) that consists of polymerized bovine Hb. In 2004, the FDA suspended Hemopure®'s clinical trial involving surgical patients after safety concerns (22, 23). Regardless, Hemopure® has been approved for clinical use in South Africa since 2006 and in Russia since 2012 (24). Because of its long shelf life (up to 36 months) at room

temperature and lack of compatibility concerns, Hemopure® presents as solution to banked blood shortages in emergencies situations, natural disasters, and far forward sites where blood is not available. Therefore, more comprehensive research is necessary to define the appropriate administration, applicability, and strategies to mitigate adverse effects.

In this study, we investigate the effect of Hemopure® Hb concentrations on systemic and microvascular parameters, including microhemodynamics and O₂ delivery during resuscitation from severe hemorrhagic shock compared to blood. Our results indicate that Hemopure® favored the recovery of systemic parameters; however microvascular function was compromised with higher concentrations of Hemopure® compared to blood. Resuscitation with low doses of Hemopure® was as effective as blood, suggesting that Hemopure® or acellular HBOCs should be administered at a lower Hb dose relative to blood during resuscitation from hemorrhage to achieve a balance between enhanced O₂ delivery and tissue perfusion.

METHODS

Materials

Hemopure® was received as a donation from OPK Biotech (Cambridge, MA).

Animal preparation—Male Syrian Golden hamsters weighting 55 to 70 grams (Charles River Laboratories, Boston, MA) were fitted with a window chamber model. Animal handling and care followed the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The local animal care committee further approved the experimental protocol. The window chamber preparation presents a microvascular network of intact tissue that can be studied without anesthesia (25). The tissues to be studied are comprised of skeletal muscle and subcutaneous connective tissue, and have been thoroughly described in the literature. Briefly, the animals were prepared for chamber implantation under anesthesia. The window chamber consists of two titanium frames with a 15-mm diameter circular observation window. Sutures were used to lift the dorsal skin away from the animal and one frame of the titanium chamber was positioned in contact with the animal skin. One side of the skinfold was removed following the outline of the window until only a thin monolayer of retractor muscle and subcutaneous skin of the opposing side remained. Then, a cover glass was placed on the exposed tissue and held in place by the other frame of the chamber under a drop of saline. The animals were allowed 2 days for recovery; then each animal's chamber was assessed under the microscope for any signs of edema, bleeding, or unusual neovascularization. Barring these complications, the animal was anesthetized again and arterial (carotid) and venous (jugular) catheters were implanted. The catheters were tunneled under the skin and exteriorized at the dorsal side of the neck where they were attached to the chamber frame for easy access. The animals were then allowed another day for recovery before the hemorrhagic shock experiments.

Inclusion criteria—Animals were considered suitable for experiments if (1) systemic parameters were within the normal range, namely: heart rate (HR) greater than 340 bpm, mean arterial pressure (MAP) greater than 80 mmHg, systemic Hct greater than 45% and arterial oxygen partial pressure (PaO₂) greater than 50 mmHg; and (2) microscopic

examination of the tissue in the chamber observed under 20X magnification did not reveal signs of edema, bleeding or unusual neovascularization. Hamsters, fossorial animals that have adapted to an underground environment, have lower arterial PO₂ than other rodents. However, microvascular PO₂ distribution in the chamber window model is similar to other rodents (26).

Hemorrhage and resuscitation protocol—Acute hemorrhage was produced by withdrawal of 50% of the blood volume (BV) via the carotid artery catheter within 10 min. The animal's blood volume was estimated as 7% of the animal's body weight. One hour after the hemorrhage, the animals received a single bolus infusion of either an estimated 25% BV of Hemopure® at different Hb concentrations or the shed blood. Hemopure® Hb concentration was adjusted by dilution with lactated Ringer's solution. The parameters being analyzed were measured before hemorrhage induction (baseline), after hemorrhage (shock), and up to 90 min post volume replacement (resuscitation). A schematic timeline of the protocol is shown in Figure 1B.

Resuscitation fluids and experimental groups—A total of 24 animals were included in the study. They were randomly assigned to each experimental group depending on the fluid used for resuscitation. The fluids used were *i*) Blood, shed blood from the same animal (Group labeled **Blood**, *n*=6) and *ii*) Hemopure® (OPK Biotech Cambridge, MA) at three different Hb concentrations—4 g/dL (Group labeled **HBOC4**, *n*=6), 8 g/dL (Group labeled **HBOC8**, *n*=6) and 12 g/dL (Group labeled **HBOC12**, *n*=6).

Systemic hemodynamic and blood gas parameter—MAP and HR were continuously recorded (MP150; Biopac Systems, Santa Barbara, Ca). Arterial blood collected in a heparinized capillary tube was immediately analyzed for PaO₂, PaCO₂, base excess, and pH (Blood Chemistry Analyzer 248; Bayer, Norwood, MA). The hematocrit (Hct) was measured from centrifuged arterial blood taken in heparinized capillary tubes. The Hb concentrations were measured spectrophotometrically using the B-Hemoglobin (Hemocue, Stockholm, Sweden). Hemocue's cuvettes hemolyze the RBC, converts the Hb to azide-metahemoglobin and corrects for cell debris to measure Hb concentration (27). Total Hb was measured directly from a drop of arterial blood and plasma Hb was measured using the plasma collected after Hct measurements. Hemocue has been validated for plasma Hb concentrations between 0.2 and 5 g/dL with unencapsulated HBOCs (28).

Microvascular experimental setup—The awake animals were placed in a restraining tube with a longitudinal slit from which the window chamber protruded and then fixed to the microscopic stage of a transillumination intravital microscope (BX51WI, Olympus, New Hyde Park, NY). The animals were then given 20 min to adjust to the change in the tube environment before measurements were performed. Measurements were done using a 40X (LUMPFL-WIR, numerical aperture of 0.8, Olympus) water immersion objective.

Microhemodynamics—Detailed mappings were made of the chamber vasculature so that the same vessels studied in control could be followed throughout the experiment. Arteriolar and venular blood flow velocities were measured online utilizing the photodiode cross-correlation method (Photo-Diode/Velocity, Vista Electronics, San Diego, CA) (29). The

measured centerline velocity (V) was corrected according to the blood vessel size to obtain the mean RBC velocity. A video image-shearing method was used to measure vessel diameter (D). Blood flow (Q) was computed from the aforementioned measured values as $Q = \pi * V * (D/2)^2$.

Functional capillary density (FCD)—Functional capillaries, defined as those capillary segments with RBC transit of at least a single RBC in a period of 15 s. They were assessed in 10 successive microscopic fields, totaling a region of 0.46 mm^2 (see schematic Figure 1A), each field was observed for a period of 60s. The fields were chosen at baseline (before hemorrhage) by a distinctive anatomic landmark (i.e., large vascular bifurcation) to easily and go back to the same fields and vessels at each observation time point. Observation of the fields was done systematically by displacing the microscopic field of view by a field width in 10 successive steps in the lateral direction (relative to the observer). Each step was $240 \text{ }\mu\text{m}$ long when referred to the tissue. Each field had between five and 10 capillary segments with RBC flow at baseline. Functional capillary density (FCD) is measured on cm^{-1} , it is calculated by measuring and adding the length of capillaries that had RBC transit in the field of view and divided by the area of the microscopic field of view. The relative change in FCD from baseline levels after each intervention is indicative of the extent of capillary perfusion (30).

Microvascular and tissue PO_2 distribution— PO_2 was measured 90 minutes after resuscitation using palladium-porphyrin phosphorescence quenching microscopy (PQM) (see schematic Figure 1A). This method uses the relationship between the phosphorescence decay rate of excited Palladium-mesotetra-(4-carboxyphenyl) porphyrin (Frontier Scientific Porphyrin Products, Logan, UT) and the O_2 concentration to calculate the PO_2 according to the Stern-Volmer equation. PQM has a high spatial resolution, as it is independent of the concentration of the dye. Animals received a $100 \text{ }\mu\text{L}$ intravenous injection of the phosphorescence complex dye (100 mg/kg) 10 min before PO_2 measurements. Intravascular measurements were made overlapping a rectangular optical window to the vessel of interest, with the longest side of the rectangle parallel to the vessel longitudinal axis. These measurements were made in large feeding arterioles, smaller arcading arterioles, large venules, and smaller collecting venules. Tissue PO_2 measurements were made in interstitial regions (at least 20 measurements per animal) avoiding large blood vessels and capillaries with a $20 \text{ }\mu\text{m}$ by $20 \text{ }\mu\text{m}$ optical window to obtain an estimate of the lowest oxygen level within the tissue (31).

Oxygen delivery/extraction and extraction ratio—The microvascular methodology used in this study allows for detailed analysis of O_2 delivery and extraction to the tissue. Calculations take into account the differences in RBC and plasma Hb O_2 transport properties for oxygen delivery (DO_2) and extraction (VO_2), respectively,

$$\begin{aligned}\text{DO}_2 &= [(RBC_{Hb} \times \gamma \times S_A) + (Plasma_{Hb} \times \gamma \times S_{HBOC})] \times Q \\ \text{VO}_2 &= [(RBC_{Hb} \times \gamma \times S_A) + (Plasma_{Hb} \times \gamma \times S_{HBOC A-V})] \times Q\end{aligned}$$

Where RBC_{Hb} is the Hb from RBCs, Total Hb – plasma Hb (g_{Hb}/dL_{blood}), $Plasma_{Hb}$ is the HBOC Hb (g_{Hb}/dL_{blood}), γ is the O_2 -carrying capacity of saturated Hb ($1.34 \text{ mL } O_2/g_{Hb}$), $SA\%$ is the arteriolar Hb O_2 saturation in the RBC, S_{HBOC} is the arteriolar Hb O_2 saturation of the Hemopure®, the $A-V$ suffix indicates the difference between arteriolar and venular saturations, and Q is the microvascular flow. Hamster blood and Hemopure®, Hb O_2 saturation were calculated using the intravascular PO_2 s measured using the PQM and assuming equilibrium between PO_2 and Hb O_2 saturation. Oxygen equilibrium curves for blood and Hemopure® were obtained by deoxygenation of O_2 -equilibrated samples in a Hemox buffer at 37°C , using a Hemox Analyzer (TCS Scientific Corporation, New Hope, PA). The oxygen extraction ratio (OER) was defined as the ration between O_2 extraction and delivery (VO_2/DO_2), which reflects the changes in arterial and venular O_2 saturations weighted by the volumetric flows. Oxygen delivery and extraction were calculated using the blood flow and intravascular PO_2 of all blood vessels studied from each animal included in a group. Typically 4 to 5 arterioles and 4 to 5 venules were measured in each animal. Previous studies have shown that VO_2 remained relatively constant and independent of DO_2 , as DO_2 was sustained above VO_2 in the same experimental model (32).

Data analysis—Results are presented as mean $\pm$ SD. Data within same groups were analyzed using ANOVA for repeated measurements, comprising the Kruskal-Wallis test. When appropriate, post hoc tests were performed with the Dunn multiple comparison test. The data between the groups was analyzed using Two-Way ANOVA tests, followed by appropriate Bonferroni tests. Microhemodynamic measurements were compared against baseline levels obtained prior to experimentation. Data are presented as absolute values or as ratios relative to baseline. A ratio of 1.0 translates into no alteration from baseline, whereas lower or higher ratios are indicative of proportional changes from baseline levels (i.e. 1.5 would mean a 50% increase). The same vessels and functional capillary fields were followed so that direct comparisons with their baseline levels could be performed, yielding more robust statistics for small sample populations. All statistical analyses were performed using GraphPad Prism software (GraphPad Software 4.01, Inc, San Diego, CA). Statistical significance was attributed if $P < 0.05$.

RESULTS

A total of 24 animals entered the study. All animals completed the hemorrhage shock resuscitation protocol without visible signs of discomfort or distress.

Physical properties of HBOCs

The physical properties of Hemopure® are presented in Table 1. The colloid osmotic pressure (COP) increased with the Hb concentration. Viscosity and the Hb affinity for O_2 were not significantly affected by the Hb concentration. The P50 of hamster blood is slightly higher than that of human blood (27 mmHg), and the P50 of Hemopure® is considerably higher (40 mmHg) and independent of the Hb concentration. The degree of right shift in the Hemopure® O_2 dissociation curve favors O_2 delivery at higher PO_2 s compared to the blood.

Systemic Parameters

Systemic and blood gas parameters during the shock resuscitation protocol are presented in Table 1 upper panel. Hct and Hb decreased after hemorrhage compared to baseline. After resuscitation, the Hct level in groups HBOC4, HBOC8 and HBOC12 decreased, while it remained constant ($31\% \pm 1\%$) in the Blood group. HBOC4 and HBOC8 groups had significantly decreased total Hb at 60 and 90 minutes after resuscitation, whereas HBOC12 maintained total Hb concentration during the post resuscitation period. The Blood group had significantly higher total Hb post resuscitation compared to the HBOC groups. In the Hemopure® groups, plasma Hb underwent an increase proportional to Hemopure® Hb concentration.

Changes in MAP during the protocol are presented in Figure 2. The hemorrhage significantly reduced MAP. Resuscitation restored MAP in all groups following the shock. The HBOC4 group had lower MAP at 60 and 90 minutes after resuscitation compared to baseline, while the other groups recovered MAP to baseline levels. At the end of protocol, the MAP of the HBOC4 group was 90% of baseline, whereas the MAP of the HBOC8, HBOC12 and the blood groups showed increases in MAP by 4.1%, 5.6% and 1.2% above baseline, respectively. Throughout the protocol, HR was not significantly different for the groups HBOC8, HBOC12 and Blood. HBOC4 group exhibited significantly higher HR at 60 min after resuscitation compared to the others groups.

Blood Gas and Chemistry parameters

Blood gas parameters are presented in Table 1 mid panel. Arterial PO_2 was significantly increased during shock compared to baseline. PCO_2 was reduced following shock and partially recovered after resuscitation, but remained lower than baseline. Resuscitation with Blood produced a significant increase in arterial PCO_2 compared to the remaining groups. All HBOC groups exhibited the same blood gas parameters (PO_2 and PCO_2). The pH was significantly reduced following hemorrhage and recovered after resuscitation in all groups. Base excess (BE) was significantly lower than baseline after shock. After resuscitation, all groups showed significant recovery of BE to near baseline values.

Microhemodynamics and functional capillary density

Microvascular diameter and blood flows for arterioles and venules relative to baseline during hemorrhage and resuscitation protocol are presented in Figure 3; absolute values are reported in the figure legend. Shock reduced arteriolar diameter, flow velocity, and blood flow. The HBOC4 and Blood groups showed significantly higher restoration of arteriolar diameter compared to HBOC8 and HBOC12 groups. The HBOC12 group did not significantly recover diameter after resuscitation. None of the fluids tested produced full restoration of microvascular blood flows, only reaching 60% of baseline in the best case. HBOC4 had significantly higher blood flows than the other groups. The Blood group had lower venular diameters and flows after resuscitation compared to all of the HBOC groups. The FCD changes are presented in Figure 4. The FCD was dramatically reduced after hemorrhage. Resuscitation restored FCD in all groups, without reaching baseline level. The HBOC8 and HBOC4 groups presented significantly higher FCD levels compared to HBOC12 and Blood groups.

Oxygen delivery and extraction and tissue PO₂

Oxygen delivery, extraction, and extraction ratio during resuscitation are presented in Figure 5. HBOC12 group had increased DO₂ compared to HBOC8 and HBOC4 groups (Figure 5A). DO₂ for the HBOC8, HBOC4 and Blood groups were not statistically different from each other. Oxygen extraction (VO₂) correlated with DO₂. The HBOC12 group had increased VO₂ compared to HBOC8 and HBOC4 groups (Figure 5B). All HBOCs groups showed greater VO₂ values compared to the Blood group. The oxygen extraction ratio (OER) was significantly higher in all HBOC groups compared to blood. Tissue PO₂ (Figure 5D) was significantly higher in the HBOC8 and Blood groups compared to HBOC4 and HBOC12 groups, and was not significantly different between them.

DISCUSSION

The principal finding of this study is that the Hb concentration of a HBOC (Hemopure®) had minor influence on systemic parameters when used as resuscitation fluid from hemorrhagic shock. However, the amount of Hb in the plasma affected microcirculation parameters—it produced vasoconstriction, reduced blood flow, and reduced FCD—thus indicating that during fluid resuscitation from hemorrhagic shock with HBOCs, the systemic hemodynamic parameters do not entirely reflect the actual tissue perfusion state. Moreover, resuscitation from hemorrhagic shock with HBOCs increased O₂ delivery proportional to Hb concentration, but O₂ uptake by the tissue was determined by tissue metabolic demand.

The vasoconstrictor effects associated with the intravascular use of HBOCs have been extensively described (33), and three theories have been proposed. First, acellular Hb scavenges NO, a potent smooth muscle tone relaxing factor, producing vasoconstriction and therefore hypertension (34). Second, acellular Hb enhances hyper-oxygenation of the tissues as O₂ is both dissolved and bound to Hb in the plasma, triggering autoregulatory metabolic mechanisms that induce vasoconstriction to limit blood flow (35). Third, acellular Hb extravasates through the endothelial lining, deposits in the interstitial space and undergoes uncontrolled autoxidation processes, forming methemoglobin (metHb), reactive O₂ species, and inflammation and vascular damage, hence affecting vascular function and diameter (36, 37). Independent of whatever underlying mechanism is ultimately responsible, it is clear that an increase in HBOC concentration results in increased vasoconstriction. The inconsistency patterns on vessel diameter, blood flow and FCD observed in the study are due to changes in perfusion pressure. The HBOC stimulates arteriolar vasoconstriction, elevating vascular resistance proximal to the constriction but reducing pressure distal to it. This would result in depressurization of capillaries, leading to reduced RBC flow (i.e., loss of FCD), lowered microvascular hematocrit, and consequent reduced O₂ delivery to the tissues served by the capillary network. Therefore, the circulatory effects of HBOC infusion appear to represent a balance of positive (e.g., increased O₂ carrying capacity and perfusion pressure) and negative (e.g. vasoconstriction) stimuli. In this context, the moderate vasoactivity of small amounts of HBOC may have beneficial effects increasing perfusion pressure without compromising microvascular blood flow and FCD.

The O₂ carrying capacity and O₂ delivered to the tissues by the HBOCs were both a function of the Hb concentration. However, O₂ delivered was mostly a function of blood flow and O₂

affinity for the acellular Hb. O₂ delivery to the tissues following a blood transfusion is significantly inferior to that of an HBOC transfusion, as acellular Hb enhances O₂ transport via facilitated O₂ diffusion of oxygenated-Hb (38, 39). The participation of an HBOC in O₂ delivery depends on the rate of diffusion of O₂ through the Hb solution, which is eightfold compared with plasma. This rate is modulated by Hb concentration and O₂ affinity (40). HBOCs can readily release O₂ bounded to the Hb, as O₂ has less physical diffusion barriers and shorter diffusion paths. Thus, the use of HBOCs to increase O₂ delivery to tissues results from the balance between O₂ carrying capacity, HBOCs' O₂ affinity, and tissue perfusion (19). It is important to notice that O₂ delivery is a function of Hb concentration, the O₂ extraction, and the extraction ratio, all of which were determined by the perfusion and FCD. Hemopure®'s low O₂ affinity (P50 of 40 mmHg) has been interpreted as negative property to replace or reinstate blood O₂ carrying capacity. However, small amounts of HBOC can augment O₂ transport due to its reduced O₂ affinity and O₂ dissociation kinetics. Therefore, the metabolic demand for O₂ in the tissue was best matched by the group with the lowest Hb concentration (4g_{Hb}/dL), the O₂ extraction of which was no different from the O₂ extraction of the Blood group. These results indicate that with only a small amount of acellular HBOC, it is possible to attain systemic and microvascular benefits similar to those attained with a blood transfusion for the treatment of hemorrhagic shock.

Vasoconstriction induced by HBOCs is the most notable limitation to their use and facilitated O₂ transport is their greatest benefit, though few studies have focused on the specific balance between these two mechanisms. This study compared Hemopure® with autologous transfusion, the best possible case in a clinical setting, demonstrating that the use of an acellular HBOC at low Hb concentration could be equally effective as blood transfusion in terms of O₂ delivery to tissues and avoidance of excessive vasoconstriction. Previous studies using Oxyglobin® (OPK Biotech, Cambridge, MA), the approved veterinarian analog to Hemopure® (OPK Biotech, Cambridge, MA), produced similar outcomes during resuscitation from hemorrhagic shock (41). However, the biochemical differences between Oxyglobin® and Hemopure® indicate that Hemopure® provides greater O₂ transport and reduces vasoconstriction at a similar concentration to Oxyglobin®. Thus, Hemopure® can be used at higher doses compared to Oxyglobin®, mostly due to the elimination of lower molecular mass molecules in Hemopure® that are present in Oxyglobin®. In this study, Hemopure® at 8 g/dL administered at the same volume as blood at 15 g/dL provides significantly higher O₂ delivery and FCD without increasing blood pressure. Clinically, blood transfusions are completed with banked blood, which is subjected to storage lesions that affect the outcome post-transfusion (42, 43). The biochemical changes that occur in blood during storage have been shown to compromise the effectiveness of resuscitation following hemorrhage (44). Therefore, a well-characterized HBOC could provide better resuscitation than blood when administered at the appropriate dose.

Study limitations

This study provides valuable information to understand the mechanisms of action and proper dosage of HBOCs (particularly, polymerized Hb preparations, Hemopure®) in relation to blood transfusions, which is currently considered the gold standard treatment. Our study differs from trauma scenarios since the experimental protocol consisted of a controlled

hemorrhage of 50% of the animal's BV followed by a limited volume resuscitation of 50% of the shed volume. Given the diversity of trauma situations that require volume resuscitation, specific clinical studies to determine optimal administration of HBOCs are needed. As conventional paradigms determined by blood transfusion cannot be applied to HBOCs administration during trauma, these clinical studies would further require the determination of appropriate endpoints. In order to understand the mechanisms exerted by HBOCs, systemic and microcirculatory parameters need to be studied, given that systemic parameters alone do not provide prompt information about tissue perfusion and oxygenation. Clinical analysis of the microcirculation has been used successfully to direct therapies and should be expanded (45). Another limitation of this study is that only healthy animals were included, whereas clinical use of HBOC will be administered to subjects with potentially narrowed cardiovascular homeostasis. Calculated metabolic parameters (DO_2 , VO_2 and OER) assume equilibrium between all the compartments transporting the O_2 , plasma Hb and RBC Hb. As blood does not include the plasma Hb compartment, these calculations may over estimate O_2 transport in the presence of HBOC, and tissue PO_2 measurements may be a more important indicator of oxygenation than calculated parameters. Future studies should aim to establish the relation between calculated metabolic parameters and tissue PO_2 in the presence of HBOC to understand the correct dose and administration of HBOC. Lastly, the small animal size prevented us from obtaining central venous PO_2 measurement to compare systemic and microvascular O_2 delivery and extraction, to increase the clinical relevance of the results.

In conclusion, this study shows that the dose (Hb concentration) of HBOC used for resuscitation from hemorrhagic shock determines the extent of vasoconstriction and O_2 delivery to the tissues. Oxygen transport by acellular HBOC was more effective than the O_2 transported by the Hb encapsulated in RBCs, as superior oxygenation was attained with lower concentrations of Hb with Hemopure®. However, a balance exists between the plasma Hb concentration and the tissue perfusion because acellular Hb causes vasoconstriction and reduces FCD. Blood pressure, HR, and blood gases are important clinical parameters, but they did not reflect the extent of tissue perfusion and oxygenation at the microvascular level. Therefore, previous studies and clinical trials with HBOCs may have failed to establish the appropriate dose of HBOCs due to incomplete information about microcirculatory function. Although the techniques used here and the observations generated by this study cannot be directly translated to clinical scenarios, they nonetheless elucidate the mechanisms by which HBOCs elicit their desired and undesired effects. These results also show that a beneficial effect can be attained using HBOCs at low Hb concentrations without causing severe vasoconstriction or hypertension, by enhancing perfusion, and facilitating O_2 delivery and extraction by tissues. The HBOCs used in this study have been tested in humans and are approved outside the US and Europe. However, few studies have explored the underlying mechanisms by which this product functions, which could ultimately lead to formulation improvements and widespread use of HBOCs in scenarios where blood transfusions are needed, but blood is not an option.

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Figure 1.

A. Model used, measured parameters and PQM set up. Top left image is an example of the window chamber preparation with the carotid and jugular catheters attached to it before the beginning experiments. Bottom left image shows a schematic representation of the PQM set up used for PO_2 measurements. Top right image shows a typical capillary bed for the quantification of functional capillary density (FCD). Bottom right image shows typical arterioles and venules where blood flow is measured. **B. Schematic illustration of hemorrhage shock protocol.** Baseline characterization is performed prior to the hemorrhage. Hypovolemic shock is induced by withdrawing 50% of estimated animal's BV. Hypovolemia is maintained for 60 minutes, and then resuscitation is completed by infusion of 25% of animal's BV with the test fluid. Parameters are measured throughout protocol at 15, 30 and 50 minutes after the onset of hemorrhage shock, followed by measurements taken at 15, 30, 60 and 90 minutes post resuscitation.

Figure 2. Mean Arterial Pressure during protocol experiment using Blood, HBOC4, HBOC8, and HBOC12

Values are presented as means $\pm$ SD. No significant differences were found between the groups HBOC8 and HBOC12 nor between the groups HBOC12 and Blood were. †, $P < 0.05$ compared with Baseline, ‡, $P < 0.05$ compared with Shock.

Figure 3. Microvascular hemodynamic data relative to baseline during shock and resuscitation using Blood, HBOC4, HBOC8, and HBOC12

Values shown are mean $\pm$ SD. †, $P < 0.05$ compared with Baseline, ‡, $P < 0.05$ compared with Shock. Baseline diameters (μm , mean $\pm$ SD), (A) and (B), for each animal group were as follows: HBOC4 (arterioles [A]: 55.4 ± 7.4 , $n = 27$; venules [V]: 59.1 ± 7.3 , $n = 29$); HBOC8 (A: 56.8 ± 6.7 , $n = 30$; V: 60.9 ± 6.8 , $n = 33$); HBOC12 (A: 62.8 ± 7.5 , $n = 26$, V: 64.4 ± 8.0 , $n = 29$); Blood (A: 54.3 ± 5.8 , $n = 26$, V: 53.5 ± 5.4 , $n = 26$). Calculated baseline blood flow, (C) and (D), (nL/s, mean $\pm$ SD) for each animal group were as follows: HBOC4 (arterioles [A]: 10.2 ± 4.9 , $n = 27$; venules [V]: 6.0 ± 2.6 , $n = 29$); HBOC8 (A: 11.4 ± 4.9 , $n = 30$; V: 6.7 ± 2.8 , $n = 33$); HBOC12 (A: 15.0 ± 5.6 , $n = 26$, V: 8.4 ± 4.0 , $n = 29$); Blood (A: 9.9 ± 3.0 , $n = 26$, V: 5.7 ± 1.8 , $n = 26$), with 'n' indicating number of vessels studied. In (C) and (D), the values within the groups were significant when compared to their respective Shock and Baselines for all data points.

Figure 4. Functional Capillary Density relative to baseline during shock and resuscitation phases
 Values shown are mean $\pm$ SD. The groups HBOC4 and HBOC8 were not different throughout protocol. The groups HBOC12 and Blood were not different throughout protocol. The FCD measurements at baseline (cm^{-1} , mean $\pm$ SD) were as follows: HBOC4 (112 ± 8); HBOC8 (124 ± 14); HBOC12 (121 ± 16); Blood (107 ± 10). $\dagger P < 0.05$ compared with Baseline, $\ddagger P < 0.05$ compared with Shock.

Figure 5. Microvascular oxygen delivery (DO₂), extraction (VO₂), extraction ratio (VO₂/DO₂) and Tissue PO₂

All parameters are presented as absolute values and given as mean ± SD. A) The DO₂ of groups HBOC4, HBOC8 and Blood were not significantly different. DO₂ calculations per group included 27 arterioles for HBOC4, 30 arterioles for HBOC8, 26 arterioles for HBOC12 and 26 arterioles for Blood, respectively. B) The VO₂ for the groups HBOC4 and HBOC8 were not significantly different. VO₂ calculations pre group included all the arterioles used for DO₂, and 29 venules for HBOC4, 33 venules for HBOC8, 29 venules for HBOC12 and 26 venules for Blood, respectively. C) The O₂ extraction ratio (OER) (VO₂/DO₂) was higher for all the groups that received HBOC compared to blood. D) Tissue O₂ tension was no different for HBOC8 compared to blood.

Table 1
Systemic parameters during hemorrhage protocol and physical properties of the HBOCs

	Baseline	Resuscitation 60 min				Resuscitation 90 min				
		Shock 50 min	HBOC4	HBOC8	HBOC12	Blood	HBOC4	HBOC8	HBOC12	Blood
Systemic Parameters										
Hct	49 ± 3	29 ± 2 [†]	27 ± 2 [†]	25 ± 2 [†]	23 ± 2 [†]	31 ± 1	24 ± 2 [†]	23 ± 2 [†]	23 ± 2 [†]	31 ± 1
Total Hb	15.1 ± 0.8	8.9 ± 0.4 [†]	8.7 ± 0.5 [†]	8.6 ± 0.5 [†]	8.5 ± 0.6 [†]	9.5 ± 0.2 [†]	7.8 ± 0.5 [†]	7.9 ± 0.4 [†]	8.3 ± 0.5 [†]	9.5 ± 0.1 [†]
Plasma Hb	-	-	0.6 ± 0.1	1.1 ± 0.1	1.4 ± 0.2	-	0.5 ± 0.1	0.9 ± 0.1	1.5 ± 0.2	-
MAP	105.7 ± 6.3	47.0 ± 3.4 [†]	95.3 ± 5.3 [‡]	105.6 ± 7.9 [‡]	107.9 ± 4.8 [‡]	104.3 ± 6.2 [‡]	94.9 ± 6.7 [‡]	108.9 ± 6.9 [‡]	112.6 ± 5.8 [‡]	106.5 ± 5.7 [‡]
Heart Rate	435 ± 35	398 ± 35	417 ± 31 ^{§, *}	373 ± 20 [†]	367 ± 15 [†]	373 ± 37	407 ± 19	395 ± 22	368 ± 23 [†]	384 ± 47
Gas & Blood Chemistry										
PaO2	57.6 ± 2.9	89.8 ± 5.1 [†]	78.0 ± 4.8 [†]	80.4 ± 3.5 [†]	75.4 ± 3.2 [†]	69.8 ± 1.7 [†]	77.0 ± 3.1 [†]	79.7 ± 0.6 [†]	74.6 ± 3.0 [†]	63.1 ± 1.6 [‡]
PaCO2	54.7 ± 2.6	35.8 ± 1.7 [†]	44.7 ± 1.7 [†]	46.4 ± 2.3 [†]	47.2 ± 1.9 [†]	50.6 ± 1.5	46.7 ± 3.4 [†]	45.2 ± 1.8 [†]	46.2 ± 3.1 [†]	51.0 ± 1.8
Arterial pH	7.336±0.039	7.287±0.023	7.317±0.038	7.332±0.051 [‡]	7.359±0.041	7.339±0.007 [‡]	7.369±0.052	7.339±0.044 [‡]	7.354±0.043	7.346±0.022 [‡]
Base Excess	2.9 ± 3.2	-8.8 ± 1.0 [†]	-3.3 ± 2.2 ^{*, §}	-1.5 ± 3.3 [‡]	0.7 ± 2.8 [‡]	1.5 ± 1.2 [‡]	1.2 ± 3.7 [‡]	-1.7 ± 2.4 [‡]	-0.2 ± 3.0 [‡]	2.2 ± 2.2 [‡]
Perivascular PO2	22.8 ± 2.1	-	-	-	-	-	11.0 ± 1.38 ^{*, §}	15.1 ± 1.6 [*]	12.0 ± 1.4 [†]	16.2 ± 1.9 [‡]

Biophysical properties			
	HBOC4	HBOC8	HBOC12
Hamster Blood			
Hb	4.1 ± 0.1	8.0 ± 0.1	12.1 ± 0.2
COP	8 ± 1	15 ± 1	25 ± 2
Viscosity	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.1
P50	40 ± 1	40 ± 1	41 ± 2

Values are reported as means ± SD. Baseline and shock included data from all the experimental groups.

[†] P < 0.05 compared with baseline;

[‡] P < 0.05 compared with Shock;

[§] P < 0.05 compared with HBOC8;

^{*} P < 0.05 compared with HBOC12;

$p < 0.05$ compared with Blood. Colloidal oncotic pressure (COP) was measured at 37°C. HBOC4 indicates polymerized bovine Hb O₂ carrier diluted to a concentration of 4 g/dL; HBOC8, Hb concentration of 8 g/dL; HBOC12, Hb concentration of 12 g/dL. Hamster blood is fresh collected blood from donor animals.