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Resuscitation from hemorrhagic shock with fresh and stored blood and polymerized hemoglobin.

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Abstract

BACKGROUND: Hemoglobin (Hb) based oxygen carriers (HBOCs) have been proposed as alternatives to blood for decades. Previous studies demonstrated that large molecular diameter HBOCs based on polymerized bovine Hb (PolybHb) attenuate Hb side-effects and toxicity. The objective of this study was to test the safety and efficacy of tense state PolybHb after long-term storage.

METHODS AND RESULTS: PolybHb was subjected to diafiltration to remove low molecular weight (< 500 kDa) species and stored for 2 years. PolybHb was studied in parallel with blood, collected from rats and stored leukodepleted under blood bank conditions for 3 weeks. Rats were hemorrhaged and resuscitated to 90% of the blood pressure before the hemorrhage with fresh blood, stored blood, fresh PolybHb, or two-year-stored PolybHb. Hemorrhagic shock impaired oxygen delivery and cardiac function. Resuscitation restored blood pressure and cardiac function, but stored blood required a significantly larger transfusion volume to recover from shock compared to fresh blood and PolybHb (fresh and stored). Stored blood transfusion elevated markers of organ damage compared to all other groups.

CONCLUSIONS: These studies indicate that large molecular diameter PolybHb is as efficacious as fresh blood in restoring cardiac function and confirm the lack of degradation of PolybHb's safety or efficacy during long-term storage.

Keywords

Trauma; transfusion; shock; blood storage; hemoglobin based oxygen carrier

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Author contributions: ATW and PC designed the experimental studies; ATW performed experiments and acquired data. CBR and AFP synthesized and characterized PolybHb. ATW, AL, CRM, and CM analyzed data. All authors contributed to writing, and approved the final manuscript.

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INTRODUCTION

Allogenic red blood cell (RBC) transfusion is the gold standard treatment for reduced oxygen (O_2) delivery capacity that is induced by severe anemia and hemorrhagic trauma (1). A large industry has developed around extending the time between blood donation and its transfusion, since fresh blood is rarely available. Despite recent advances in RBC storage, blood shortages are still common and the frequency of these shortages is expected to increase in the future based on current donation and rate of blood usage trends (2). Elective surgeries can be delayed during blood shortages, but there is always an immediate need for RBC transfusions in anemia and trauma. Blood availability is even more severe and critical during emergency situations, such as natural disasters or in active war zones. Blood shortages can be mitigated by extending blood's shelf life, but a safe and reliable O_2-carrying alternative to blood for transfusion medicine remains a necessity for situations when blood is not available, or blood is not an option due to ethical or religious reasons.

The United States Food and Drug Administration (FDA) has approved the storage of human RBCs for up to 42 days at 4°C in appropriate additive solutions. This maximum *ex vivo* storage period was established based on two parameters: hemolysis during storage (<0.8%), and the 24 hour post transfusion survival of transfused RBCs (>75%) (3). While neither of these parameters demonstrate the capacity of RBCs to transport and deliver O_2, they are the only standards currently used to assess stored blood efficacy. Recent evidence suggests that blood quality (and thus O_2 delivery capability) decreases with age due to RBC storage lesions (4). Recent clinical trials have failed to find differences between standard of care blood (typically 3 weeks old) and fresh blood (less than 1 week old), but these trials do not account for the underlying cause of hospital admission or the quality of the transfused RBCs (5,6). Extending the *ex vivo* storage period of blood based on current FDA standards could be detrimental, as current standards do not accurately reflect the function of RBCs after storage.

Hemoglobin (Hb) based oxygen carriers (HBOCs) have been tested as an alternative to RBC transfusion in a multitude of studies. Some commercially developed HBOCs restored blood pressure and decreased the number of RBC units transfused in clinical trials, but caused more adverse effects than the control group (7,8). The side effects induced by early-generation HBOCs were likely due to their small molecular size (9,10). The small molecular diameter of early generations of HBOCs increased facilitated diffusion, favoring vascular hyperoxygenation and scavenging of endothelial cell derived nitric oxide (NO), and extravasated into the extravascular space, which caused vasoconstriction, hypertension, and oxidative tissue injury (11). Additionally, small molecular diameter HBOCs can be filtered by the kidneys, affecting renal filtration rates and impacting renal proximal tubules, which are especially vulnerable to heme and iron toxicity (12).

A new generation of HBOCs with increased molecular weight (MW) and concomitantly increased molecular size compared to previous generations of HBOCs is being developed to mitigate previously observed side-effects associated with low MW HBOCs. The increase in molecular size reduces vascular hyperoxygenation and NO scavenging by slowing the HBOC's diffusion (13). Large molecular diameter polymerized Hb (PolyHb) also prevents

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extravasation and kidney damage by size exclusion, as well as promoting clearance of PolyHb via normal Hb scavenging pathways (14). Increases in MW have no effect on HBOCs' benefits over blood, such as a lack of immunogenicity, universal blood type compatibility, and potentially indefinite storage. Lastly, polymerization of Hb remains the only valid scalable and cost-effective method of increasing HBOC MW/size. This study was designed to test the hypothesis that extended storage does not impact the safety and efficacy of high MW polymerized bovine Hb (PolybHb). To achieve this objective, in this study we compared the ability to resuscitate from hemorrhagic shock (HS) and assessed the degree of organ damage following transfusion of fresh and stored rat blood, and fresh and stored high MW PolybHb.

METHODS

Synthesis of PolybHb.

PolybHb was synthesized in the low oxygen affinity tense (T) quaternary state at a 35:1 molar ratio of glutaraldehyde to bovine Hb, and then subjected to 8–9 cycles of diafiltration on a 500 kDa tangential flow filtration (TFF) module using a modified lactated Ringer's solution as described in the literature (15). This resulted in a PolybHb solution containing only polymerized Hb molecules bracketed between 0.2 μm and 500 kDa. Stored PolybHb was stored at −80°C until usage more than 2 years after it was first synthesized. Fresh PolybHb was stored at −80°C and was used within 3 months of generation. PolybHb was adjusted to a concentration of 10 g/dL with lactated Ringer's solution before transfusion.

Biophysical Properties of PolybHb.

PolybHb equilibrium curves were measured using a Hemox Analyzer (TCS Scientific, Southampton, PA) at 37°C as previously described (15). PolybHb viscosity was measured at 37°C using a computerized cone-plate (4 cm diameter, 2° cone angle) rheometer (Discovery HR-2, TA Instruments, New Castle, DE, USA).

Blood Collection and Preparation.

Fresh blood. Blood removed from the animal during hemorrhage was centrifuged at 1500 g for 7 minutes, and the plasma and buffy coat were removed. The plasma removed was then added to the packed RBC pellet to target a Hct of 50%. Stored blood. Briefly, male Sprague-Dawley Rats (Harlan Laboratories, Indianapolis, IN) weighing 300–450 g were anesthetized with isoflurane (5%). Blood was collected via cardiac puncture into citrate phosphate double dextrose (CP2D, which was taken from an Additive Solution 3 [AS-3] blood preparation kit, Haemonetics Corporation, Braintree, MA). Donor blood was then pooled, and the CP2D concentration was adjusted to 14%. Pooled blood was centrifuged at 1000 g for 7 minutes, and the supernatant was removed. AS-3 (22%/whole blood volume) was then added, and blood was mixed gently by inverting the bag for 1 minute. Pooled blood was then passed through a neonatal leukocyte reduction filter (Haemonetics Corporation, Braintree, MA). Leukodepleted rat blood was stored at 4°C for 3 weeks, which was previously shown to pass current FDA standards for stored blood (16).

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Animal Preparation.

Studies were performed in 32 male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 200–250 g. Animal handling and care followed the NIH Guide for Care and Use of Laboratory Animals. The experimental protocol was approved by the UCSD Institutional Animal Care and Use Committee. Briefly, animals were anesthetized using isoflurane (5%/vol for initial induction, 2.5%/vol for maintenance, Drägerwerk AG, Lübeck, Germany) and prepared with: (i) left jugular vein and left femoral artery catheterization, and (ii) left ventricular (LV) conductance catheter introduced through the right carotid artery. Animals were placed in the supine position on a heating pad to maintain core body temperature at 37 °C and allowed to freely breathe from a nosecone. Isoflurane was reduced to 1.5%/vol after surgical preparation. The depth of anesthesia was continually verified via toe pinch and the isoflurane concentration was increased by 0.1%/vol to prevent animal discomfort if needed (Figure S1A).

Inclusion Criteria.

Animals were suitable for experiments if: (i) mean arterial blood pressure (MAP) was above 85 mm Hg at baseline, (ii) stroke volume (SV) was above 90 μL at baseline, (iii) systemic Hb was above 12 g/dL at baseline, and (iv) animals survived the shock period.

Hemorrhagic Shock Resuscitation Protocol.

After surgical instrumentation, animals were allowed to rest for 30 minutes to stabilize before baseline measurements were taken. Then the anesthetized rats were hemorrhaged by withdrawing 50% of the animal's blood volume (BV), estimated as 7% body weight, via the femoral artery catheter over 30 minutes into heparinized syringes. Hypovolemic shock was maintained for 30 minutes. Resuscitation was implemented by infusion of room temperature resuscitation solution through the jugular vein catheter until the animal recovered to 90% of the MAP at baseline. If the animal's MAP fell below 80% of the MAP at baseline, additional resuscitation solution was infused. This resuscitation phase lasted for one hour (Figure S1B).

Volume Infused.

Blood and PolybHb was infused via a flow-controlled syringe pump at 300 μL/min. Total blood volume infused was calculated as $V = Q * t$, where V is the volume infused, Q is the flow rate of the syringe pump, and t is the time that the syringe pump was active, as measured by a stop-watch. Resuscitation % was calculated as $R = \frac{V}{TBV}$ where R is the resuscitation % and TBV is the total blood volume of the rat, estimated as 7% body weight.

Cardiac Function.

A 2F pressure-volume (PV) conductance catheter (SPR-858, Millar Instruments, TX) was inserted into the left ventricle (LV) using the closed chested method (17). Briefly, the PV catheter was inserted through the exposed right carotid artery and slowly advanced into the LV. Pressure and volume signals were acquired continuously (MPVS300, Millar Instruments, Houston, TX, and PowerLab 8/30, AD Instruments, Colorado Springs, CO). LV volume was measured in conductance units (relative volume unit, RVU) and converted to absolute blood volume (μL) at the end of the experiment (17). Parallel volume (the

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contribution of the myocardium to the measured LV volume) was calibrated via IV injection of 35 μL hypertonic saline (15% NaCl w/v) (17).

Systemic Hemodynamics Parameters.

MAP and heart rate (HR) were recorded continuously from the femoral artery (PowerLab 8/30, AD Instruments, CO). Hematocrit (Hct) was measured via centrifugation from arterial blood collected into heparinized capillary tubes. Hb content was determined spectrophotometrically (B-Hemoglobin; Hemocue, Stockholm, Sweden). Arterial and venous blood were collected in heparinized glass capillary tubes (65 μL) and immediately analyzed for oxygen partial pressure (pO_2), carbon dioxide partial pressure (pCO_2), and pH (Siemens 248, Munich, Germany).

Cardiac Pressure-Volume Indices.

Cardiac function was analyzed using PowerLab software (PowerLab 7.8, AD Instruments, Colorado Springs, CO). Cardiac function parameters were calculated from 15 to 20 cardiac cycles at each time point. Stroke volume (SV), stroke work (SW), cardiac output (CO), and cardiac contraction and relaxation rate (dP/dt_{max} and $dP/dt_{min,}$ respectively), were directly calculated in the PowerLab software. Systemic vascular resistance (SVR) was calculated as: $SVR = \frac{MAP}{CO}$. The internal energy utilization (IEU) was used as a measure of internal metabolism of the LV and was calculated as previously described (18).

Oxygen Delivery.

Oxygen delivery (DO_2) was calculated by multiplying CO and the arterial oxygen content. Oxygen consumption (VO_2) was calculated by multiplying CO and the arterio-venous difference in oxygen content.

Markers of Organ Damage and Inflammation.

Aspartate transaminase (AST), alanine transaminase (ALT), and IL-6 levels were determined in serum samples using ELISA kits (KA1625 and KT-6104 from Abnova Corp, Taiwan, and BMS625 from Thermo Fisher, Waltham, MA). Neutrophil gelatinase associated lipocalin (NGAL) was determined in the urine using ELISA kits (ERLCN2, Thermo Fisher). Serum creatinine and blood urea nitrogen (BUN) were measured using colorimetric detection kits (KB02-H2 and K024-H5, Arbor Assays Inc., Ann Arbor, MI). Liver, lung and spleen CXCL1 were measured on whole tissue homogenates by ELISA (ERCXCL1, Thermo Fisher, Waltham) and corrected for protein concentration using the Pierce™ BCA assay kit (Thermo Fisher).

Positive CD45 neutrophils were quantified in the bronchoalveolar lavage (BAL) fluid collected by instilling sterile PBS into the lung. The percentage fraction of neutrophils in BAL was determined by flow cytometry (FACSCalibur; BD, Franklin Lakes, NJ). Neutrophils were identified by their typical appearance in the forward/side scatter and their expression of CD45 (554875, BD Biosciences).

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Preparation and Immunoprecipitation of Rat Tissue Extracts for Ferritin Determination.

Rat tissues were frozen and stored at −80°C until analyzed. Lysis buffer containing 1% deionized Triton X-100 and 0.1% sodium azide in 50 mM Tris-HCl pH 7.5 with Chelex-100 and Phenyl-methyl-sulfonyl-fluoride (0.25 mM) was added to the homogenized tissue, which was vortexed, sonicated for 1 min, and incubated on ice for 30 min, vortexing every 5–10 min. Aliquots were taken, centrifuged at 3,000 rpm for 15 min, and the supernatant analyzed for total protein (BCA, Pierce) and ferritin. The remaining suspension of homogenized tissue was incubated at 70°C for 10 min, cooled on ice and centrifuged at 10,000 g for 20 min. The supernatant was collected, and the pellet discarded. The total amount of ferritin in each extract was calculated using the results of the ELISA (Cat. no.MCA-155, Serotec, Oxford, UK). Saturating amounts of immune serum against rat tissue ferritin was then added to the extracts.

Epinephrine/Norepinephrine.

Catecholamine plasma concentrations were determined using commercially available ELISA kits for catecholamines (Catecholamines ELISA kit - Ref: BA-E-6600, ImmuSmol, France). All ELISA samples were run in accordance with the manufacturer's instructions.

Statistical Analysis.

Results are presented as the mean ± SEM, as Tukey box-and-whisker plots, or as the median and 95% confidence interval. The values are presented as absolute values and relative to the baseline. A ratio of 1.0 signifies no change from baseline, whereas lower or higher ratios are indicative of changes proportionally lower or higher compared to baseline, respectively. Outliers are shown in the box-and-whisker plots as filled circles of the group's respective colors. The Grubbs' method was used to assess closeness for all measured parameters at baseline and shock. Before experiments were initiated, sample size was determined based on $\alpha = 0.05$ and a power of 0.9 to detect differences in primary end points (MAP, contractility, and percent resuscitation) greater than 10%. Statistically significant changes between solutions and time points were analyzed using two-way analysis of variance (ANOVA), followed by *post-hoc* analyses using Tukey's multiple comparisons test when appropriate. All statistics were calculated using the Python Library StatsModels (ver. 0.9.0) (19). Results were considered statistically significant if $P < 0.05$.

RESULTS

PolybHb Characterization:

Fresh PolybHb had lower Hb-O_2 affinity than stored PolybHb at the time of production (P_{50} = 34 mmHg and 30 mmHg, respectively), but similar Hb cooperativity, as indicated by the Hill coefficient of the Hb equilibrium curve (~1). Fresh and stored PolybHb both had high viscosity compared to rat blood (16.1, 16.6, and 4.2 cP at 100 s^{-1}, respectively).

In Vivo Studies.

Thirty-two (n = 32) animals were entered into the hemorrhagic shock/resuscitation study. Animals were randomly assigned to the following groups: Fresh blood (**fBlood**, n = 8),

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stored blood (**sBlood**, n = 8), fresh PolybHb (**fPHB**, n = 8), and stored PolybHb (**sPHB**, n = 8). All animals survived the protocol and there were no statistical differences between animals at baseline.

Systemic Hemodynamics.

All animals had similar blood pressure at baseline and at the end of shock (Figure 1A). Restoration of blood pressure at 10 mins into resuscitation was similar for fBlood, fPHB, and sPHB, but statistically significantly lower for sBlood. With continued transfusion, all animals achieved the transfusion goal of recovering blood pressure and there were no statistically significant differences in MAP between groups 60 mins into resuscitation. Shock resulted in bradycardia relative to baseline, and the reduced HR was not resolved with transfusion of any test solution (Figure 1B). CO decreased from baseline during shock and remained statistically different from baseline during resuscitation for fBlood and sBlood, but CO was not significantly different than baseline level during resuscitation for fPHB and sPHB (Figure 1C). Resuscitation with fBlood and sBlood decreased CO 2% and 14% relative to shock, whereas fPHB and sPHB increased CO 14% and 22% relative to shock. SVR decreased during shock and was elevated relative to baseline for all groups after resuscitation (Figure 1D).

Cardiac Function.

SV decreased from baseline during shock. However, since resuscitation was implemented until 90% of the baseline MAP was reached, most hemodynamic and cardiac function parameters did not show statistical differences between groups after resuscitation. During resuscitation, sBlood, fPHB, and sPHB presented higher SV compared to fBlood, but differences between groups were not statistically significant (Figure S2A). Shock decreased SW and resuscitation partially restored SW in all groups, but fPHB and sPHB showed higher SW than fBlood and sBlood, likely due to their higher viscosity. (Figure S2B). Isovolemic contraction rate (dp/dt_{max}) decreased during shock and improved upon resuscitation but did not reach baseline values in any of the groups (Figure S2C). When normalized to end diastolic volume (V_{ed}), the isovolemic contraction rate ($dp/dt_{max}/V_{ed}$) did not change significantly from baseline, other than the sPHB group, whose contractility was statistically significantly lower than baseline (Figure 1E). The isovolemic relaxation rate (dp/dt_{min}) decreased during shock and was restored upon resuscitation, but no group recovered to their baseline isovolemic relaxation rate (Figure 1F). IEU decreased during shock and increased upon resuscitation, and fPHB and sPHB showed slightly higher IEU than fBlood and sBlood (Figure S2D).

Hematology.

A summary of hematological changes is presented in Table 1. The sBlood group required a statistically significantly greater transfusion volume for resuscitation than the fBlood, fPHB, and sPHB groups (Figure 2). The fBlood, fPHB, and sPHB groups all required similar transfusion volumes to resuscitate from HS. As a result of the higher transfusion volume, sBlood showed significantly higher Hct than fBlood. Resuscitation increased Hb (and oxygen carrying capacity), but the lower Hb concentration of PolybHb compared to

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transfused (fresh or stored) blood resulted in a lower overall total Hb level for fPHB and sPHB than fBlood and sBlood.

Blood Chemistry.

Shock resulted in acidosis, decreased arterial pCO_2, and increased arterial pO_2 for all groups (Table 2). Resuscitation did not correct systemic acidosis in any of the groups; however, arterial pO_2 was restored to baseline levels by the end of the protocol. fBlood and sBlood showed elevated arterial pCO_2 relative to baseline, while fPHB and sPHB returned to baseline pCO_2 levels.

Oxygen Delivery.

Oxygen delivery (DO_2) decreased during shock and was never fully restored during resuscitation with either blood or PolybHb. DO_2 in the sBlood group was marginally higher than the DO_2 for the fBlood, fPHB, and sPHB groups, primarily due to a significantly larger transfused volume, and concurrently higher Hct and total Hb (Figure 3A). Oxygen extraction (VO_2) was increased 10 mins into resuscitation in all groups, but VO_2 was slightly lower at the end of resuscitation for the fBlood and sBlood groups compared to the fPHB and sPHB groups (Figure 3B). The O_2 extraction to delivery ratio (VO_2/DO_2) was significantly higher than baseline for fPHB and sPHB, but not for fBlood or sBlood (Figure 3C).

Markers of Organ Damage or Organ Function.

The sBlood group showed significantly higher markers of organ injury compared to the fBlood, fPHB, and sPHB groups. Organs like the kidney and liver appear to be more affected by resuscitation with stored blood than PolybHb. This can be observed by differences in u-NGAL, serum creatinine, and BUN, markers of kidney injury, and by the differences in AST and ALT, markers of liver injury. Stress hormones were significantly lower for fBlood than sBlood, fPHB, and sPHB; however, norepinephrine was higher for the fPHB and sPHB groups than the sBlood group (Figure 4). CXCL1, a tissue inflammatory marker, was increased in sBlood, fPHB, and sPHB compared to fBlood in the lungs, spleen and liver. Additionally, lung neutrophil recruitment was significantly higher for fPHB and sPHB compared to sBlood. While the proinflammatory cytokine interleukin-6 was higher for sBlood than fPHB and sPHB, the levels of the anti-inflammatory cytokine interleukin-10 was not different among these 3 groups (Figure 5). Furthermore, ferritin levels increased for the sBlood, fPHB, and sPHB groups compared to the fBlood group. However, bilirubin was elevated only in animals transfused with stored blood (Figure 6). The fPHB and sPHB groups showed a significantly higher ratio of heavy-chain (H) ferritin to light-chain (L) ferritin compared to the sBlood group.

DISCUSSION

The principal finding of this study is that resuscitation of rats from HS with fresh and stored PolybHb restores systemic hemodynamics, markers of cardiac function, and O_2 delivery to a similar extent as fresh blood. These results also demonstrate that PolybHb's capacity to resuscitate from HS does not change after 2 years of storage, while 3 weeks of storage alters blood's ability to resuscitate from HS. Resuscitation by means of transfusions of fresh

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blood, and PolybHb, both fresh and stored, met the resuscitation goal of recovering MAP to 90% of baseline, within 10 minutes into resuscitation and maintained MAP during the observation period. On the other hand, animals receiving stored blood only reached the resuscitation goal after infusion of 140% and 65% additional transfusion volume than fresh blood and PolybHb, respectively (Figure 2), and delayed reaching the resuscitation goal to the end of the protocol (60 mins from the beginning of the infusion, Figure 1A). All groups recovered vascular resistance to similar degrees, suggesting that resuscitation with PolybHb does not result in vasoconstriction relative to resuscitation with blood.

Other studies have used total Hb to guide resuscitation from HS, but total Hb is not a positive indicator of recovery nor a direct indicator of tissue oxygenation (20,21). In this study, resuscitation was directed to achieve a target blood pressure, and the volume transfused was a primary metric to measure the efficacy of the resuscitation solution. The volume transfused to recover pressure accounts for the response of the cardiovascular system to correct effective blood volume, restore blood flow, and reinstate oxygen delivery. Systemic parameters, such as MAP and HR, suggest that the experimental protocol was followed closely. All animals reached the experimental goal, but animals transfused with stored blood experienced a slower recovery and required a larger infusion volume.

During shock, limited blood flow and oxygen delivery causes cells to shift toward anaerobic metabolism, resulting in a buildup of lactic acid and creating metabolic acidosis. Through the Bohr effect, acidosis decreases RBC Hb's oxygen affinity, and increases oxygen offloading to tissues (22). The intrinsic low oxygen affinity of T-state PolybHb could promote oxygen offloading to tissues, which would help to re-establish oxygenation and minimize the metabolic sequelae associated with HS. In the case of stored blood transfusion, metabolic acidosis may be exacerbated due to depletion of 2,3-bisphosphoglycerate (BPG) in the RBCs during blood storage (23), which decreases the p50 and limits the Bohr effect. Since 2,3-BPG driven regulation of Hb O_2 affinity was first observed, the importance of RBC metabolism during blood storage has been pointed out under both physiological and pathological conditions. 2,3-BPG concentration in stored RBCs recovers slowly, and decreases O_2 extraction immediately following resuscitation (24). Previous *in vivo* studies have demonstrated that increased Hb O_2 affinity impairs tissue oxygenation, thus preventing restoration of aerobic metabolism after resuscitation from HS with stored blood (16).

One of the most significant results of this study, is the large transfusion volume required to resuscitate from HS of stored blood compared to both fresh blood and PolybHb during resuscitation. To reach the blood pressure goal, sBlood required reinfusion of the entire hemorrhaged volume (50% of the total blood volume), whereas fBlood required a substantially lower volume to be reinfused (21% of the total blood volume), and both fPHB and sPHB required a similar volume as fBlood (31% and 29%, respectively). Thus, blood usage can be potentially decreased by using fresh blood when possible. Moreover, the increased transfusion volume necessary to achieve hemodynamic stability with stored blood favors different types of transfusion-related adverse reactions that may occur (25,26). Animals in the sBlood group experienced systemic inflammation, as indicated by a significantly elevated IL-6 concentration in plasma, higher levels of markers of neutrophil recruitment in the spleen and liver, and neutrophil activation in the lungs compared to the

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fBlood group and PolybHb groups. These parameters indicate that animals in the fPHB and sPHB also experienced systemic inflammation compared to fBlood, but to a lesser extent than sBlood. Elevated spleen inflammatory markers in the sBlood group is consistent with the spleen's function in clearing damaged or aged RBCs from the circulation. The RBC storage lesions and reduced deformability are likely responsible for increased in splenic inflammatory markers, as neutrophils are necessary for splenic hemolysis of stored RBCs through phagocytosis (27). This hypothesis is further supported by the increased splenic ferritin and bilirubin for the sBlood group, both of which are markers of hemolysis (28). Since PolybHb is acellular, the effect on the spleen is less pronounced for fPHB and sPHB than for the sBlood group. On the other hand, in the lungs, the acellular nature of PolybHb allows for direct interaction of the Hb with the lung resident macrophages and dendritic cells, upregulating recruitment of lung neutrophils (as indicated by lung CXCL1) in the fPHB and sPHB groups, which could be detrimental (29). However, lung neutrophils are more active (as indicated by MPO activity and neutrophil activation) for the sBlood group than the fBlood and PolybHb groups, likely due to increased oxidized Hb (metHb) in stored blood (29).

Resuscitation from HS with PolybHb and stored blood appears to induce acute kidney injury, as detected by the increase in markers of kidney function (creatinine and BUN). Previous generations of HBOCs caused significant kidney damage (12), but the fPHB and sPHB groups showed significantly less acute kidney injury (as reflected by low u-NAGL and serum creatinine) compared to sBlood, but more acute kidney injury than fBlood. In addition, the sBlood group showed a statistically significant increase in markers of liver injury (AST and ALT) compared to the fBlood, fPHB, and sPHB groups, though these markers were also elevated for fPHB and sPHB compared to fBlood. These elevated markers of organ injury in the sBlood group and PolybHb groups are likely due to storage lesions in RBCs, as well as the acellular nature of PolybHb, ultimately resulting in free Hb and heme exposure. When the innate Hb scavenging system becomes saturated after transfusion of stored blood (30), free Hb can be filtered through the kidneys, causing kidney damage (30). Similarly, in the liver, extravasation of acellular Hb and release of heme and iron causes oxidative stress, promoting acute liver injury (31). The Hb scavenging system also binds and degrades PolybHb, but at a slower rate than acellular Hb (14). Differences in bilirubin, a byproduct of heme catabolism, suggest that a significant amount of the iron stored following PolybHb transfusion was due to free iron present after transfusion, whereas the iron stored following stored blood transfusion was derived from heme. Without management of free iron post-transfusion, PolybHb could lead to iron toxicity, but previous studies have shown that supplementing HBOC transfusion with an iron chelator (deferoxamine) significantly reduced tissue toxicity (32). The higher ratio of H to L ferritin seen after transfusion of PolybHb compared to stored blood is indicative of low levels of oxidized heme in the transfused PolybHb (33). Furthermore, the lower H-ferritin levels observed after transfusion of stored blood suggests that the acellular Hb released upon transfusion of stored RBCs oxidizes rapidly in circulation. This is also supported by aforementioned markers of lung neutrophil activation, as neutrophils are activated by metHb, but not non-oxidized Hb (29).

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Limitations.

Studies in rats are not directly translatable to clinical scenarios, but these initial results show promise regarding the efficacy of high MW PolybHb in resuscitation from HS. Furthermore, rats do not mimic the human response to oxidative stress since they innately produce ascorbic acid and upregulate ascorbic acid production in times of oxidative stress. Further testing in guinea pigs (a non-ascorbic acid producing animal species) is planned to assess the safety of this class of HBOCs. Finally, these experiments were designed to examine what happens during the 'golden hour' of resuscitation from HS and long- term studies with PolybHb are needed. To ensure there are no sequelae due to transfusion of PolybHb, and to examine whether the acute markers of injury continue to chronic injury, primary outcomes need to be observed over longer periods of time, and other functional outcomes should be assessed.

In summary, these results show that PolybHb possesses similar efficacy as fresh blood during resuscitation from HS in terms of functional parameters. Furthermore, rat blood stored for 3 weeks required a significantly higher transfusion volume, slowed recovery of MAP, and resulted in increased markers of liver injury, kidney injury, and lung inflammation compared to fresh blood. fPHB, and sPHB also showed increased markers of organ injury and inflammation compared to fBlood, and many of these markers were similar to the injury from sBlood. This injury, and the efficacy of PolybHb was consistent following 2 years of storage, which was clearly not true of rat blood after 3 weeks of storage. Although further studies are needed to ensure safety, these data support the use of high MW PolybHb as an initial oxygen bridge until blood is available for resuscitation in HS scenarios. In conclusion this study indicates that high MW PolybHb is efficacious in restoring markers of cardiac function after HS, and confirms PolybHb's efficacy after long-term storage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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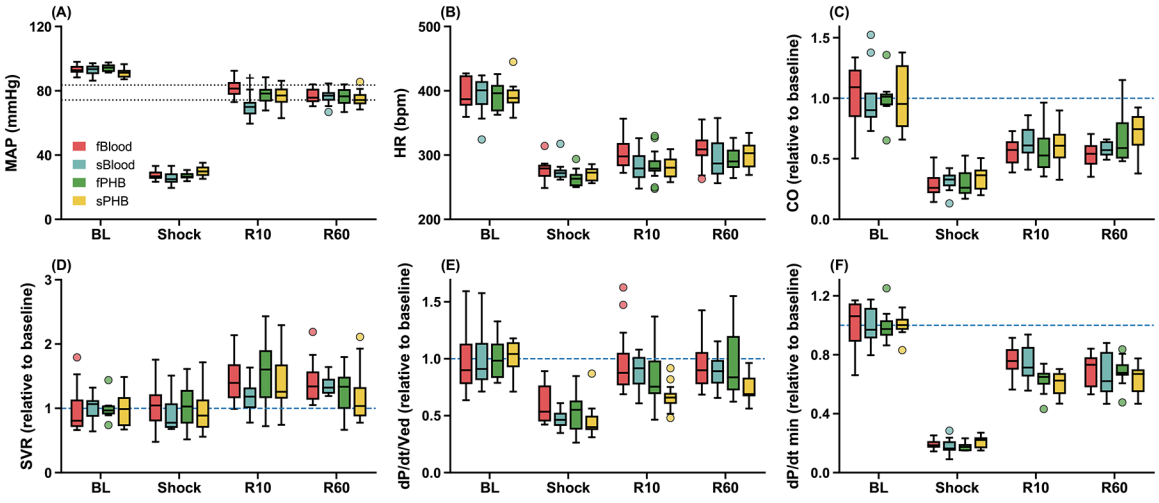

Figure 1.

Hemodynamics and cardiac function pre-and-post HS/resuscitation with fresh and stored blood and T-state PolybHb. **(A)** MAP is not initially restored with sBlood, but fPHB and sPHB restored MAP to the same degree as fBlood. The resuscitation goal range is shown as black dotted lines at 80% and 90% of the baseline MAP. **(B)** HR is suppressed after shock and remained that way. **(C)** CO is decreased during shock and partially recovers for fPHB and sPHB. **(D)** SVR increases to a similar degree upon transfusion of all materials. **(E)** Isovolemic contraction relative to Ved is slightly lower upon transfusion of fPHB and sPHB. **(F)** Isovolemic relaxation is initially suppressed with fPHB and sPHB. Outliers are shown in the box-and-whisker plots as filled circles of the group's respective colors. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

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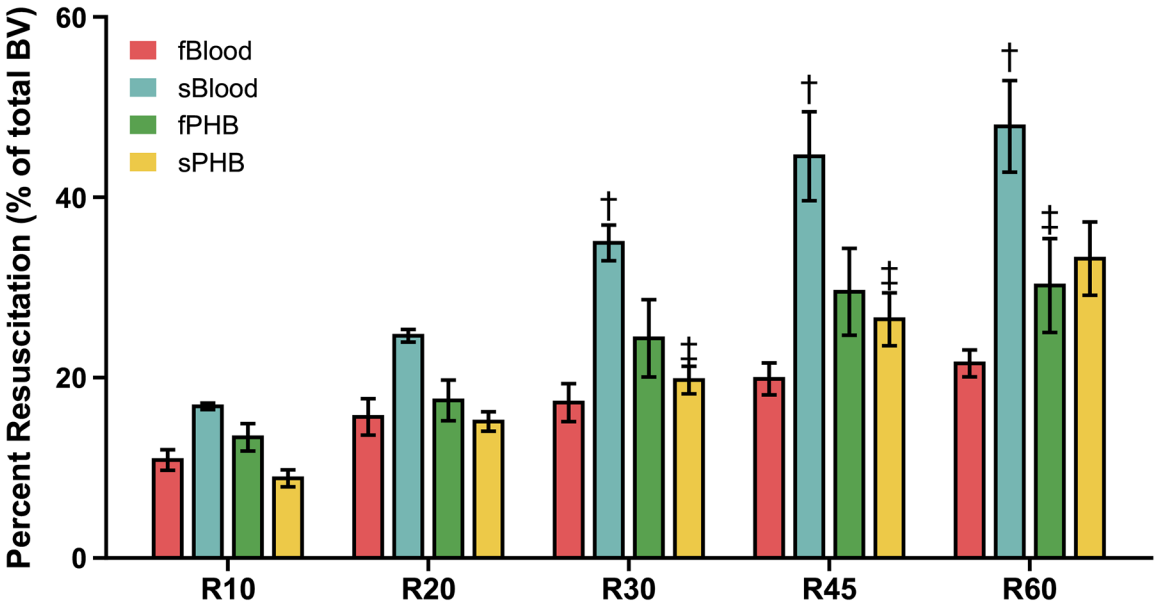

Figure 2.

The same volume of fBlood, fPHB, and sPHB were needed for resuscitation. N = 8 per group. Data are presented as mean ± SEM. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

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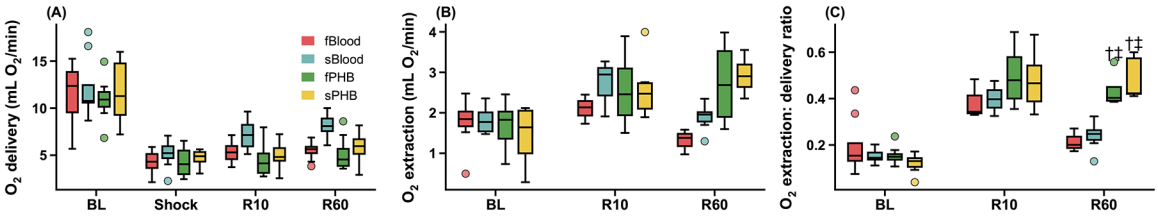

Figure 3.

Systemic oxygen delivery parameters pre-and-post HS/resuscitation with fresh and stored blood and T-state PolybHb. **(A)** Oxygen delivery decreases with shock and stays suppressed, but oxygen delivery increases for sBlood due to the additional transfusion volume. **(B)** Oxygen extraction post-hemorrhage is increased. Oxygen extraction is slightly increased in the fPHB and sPHB groups due to the low oxygen affinity of T-state PolybHb. **(C)** The oxygen delivery: extraction ratio is increased post-shock but decreases to near-baseline for the fBlood and sBlood groups. Outliers are shown in the box-and-whisker plots as filled circles of the group's respective colors. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

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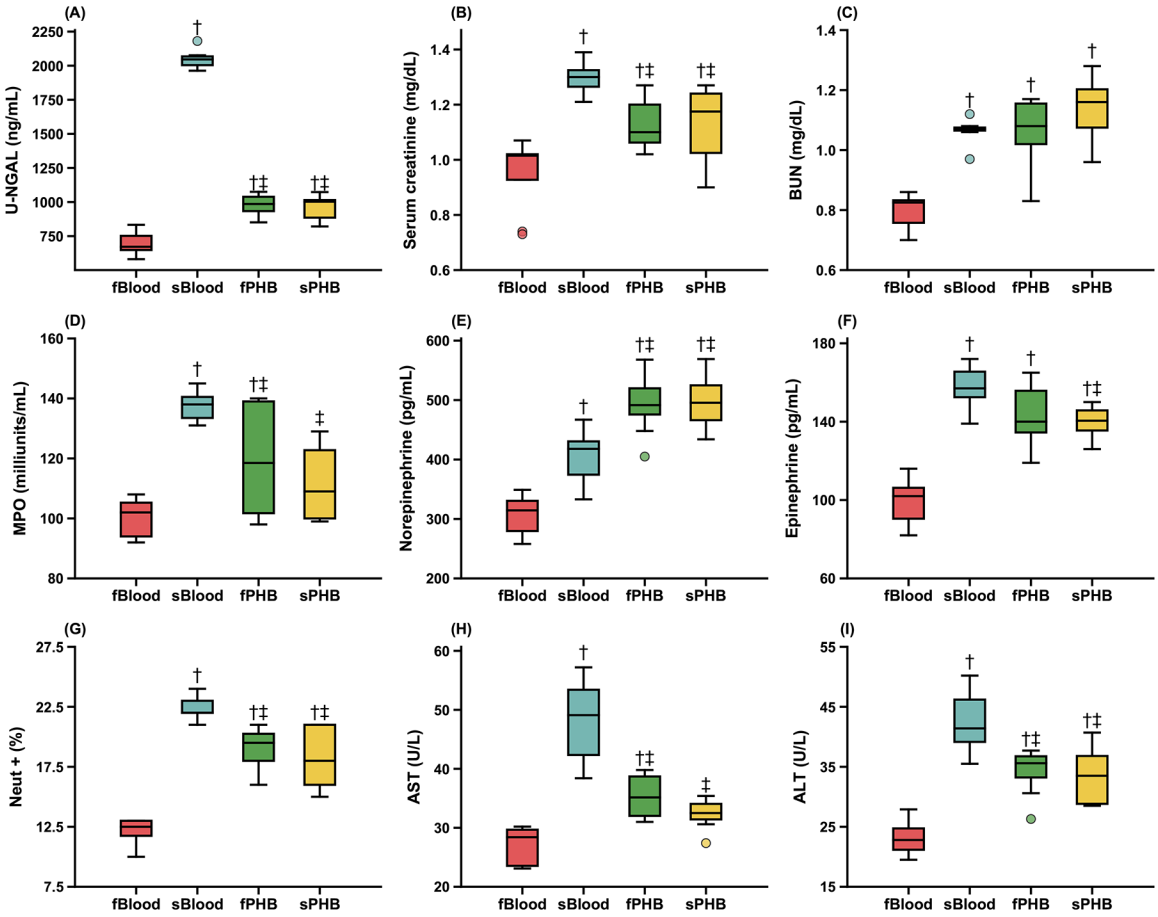

Figure 4.

Markers of tissue damage and stress. **(A)** Significantly more kidney damage is observed in sBlood than fBlood, fPHB, or sPHB. **(B),(C)** Kidney function is suppressed for sBlood, fPHB, and sPHB. **(D)** Myeloperoxidase is most active in lungs for the sBlood group. **(E),(F)** sBlood, fPHB, and sPHB show higher levels of acute stress hormones. **(G)** Significantly more neutrophils are activated in the lungs for the sBlood group. **(H),(I)** More liver damage is observed in the sBlood group compared to fBlood, fPHB, sPHB. Outliers are shown in the box-and-whisker plots as filled circles of the group's respective colors. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

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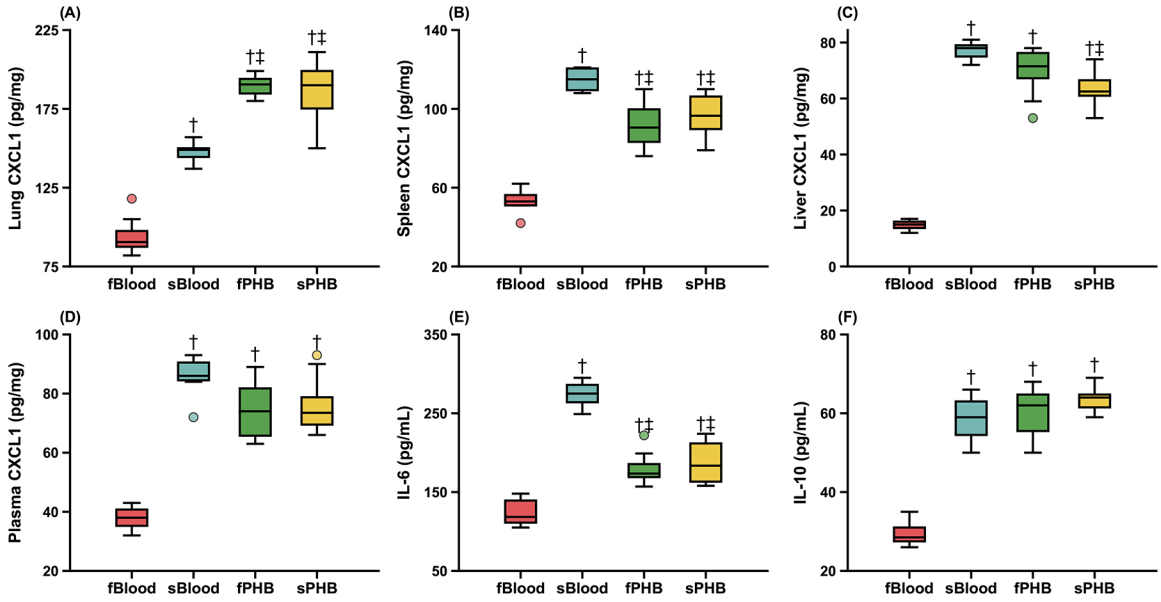

Figure 5.

Cytokines post-hemorrhagic shock/resuscitation. **(A)** fPHB and sPHB show significantly higher lung neutrophil recruitment than fBlood. **(B),(C)** Significantly more reticuloendothelial inflammation is observed in sBlood than fBlood, fPHB, or sPHB. **(D)** Plasma CXCL1 is elevated at the end of resuscitation for sBlood, fPHB, and sPHB. **(E)** Significantly higher levels of the proinflammatory cytokine IL-6 are present in the sBlood group, but **(F)** similar levels of the anti-inflammatory cytokine IL-10 are present for the sBlood, fPHB, and sPHB groups at the end of resuscitation. Outliers are shown in the box-and-whisker plots as filled circles of the group's respective colors. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

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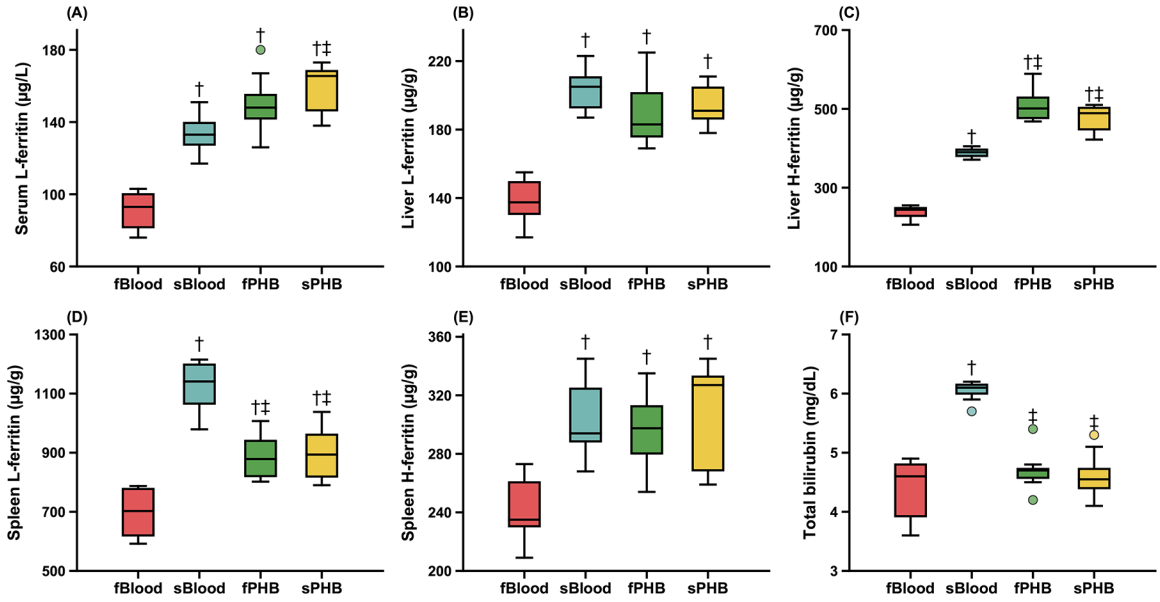

Figure 6.

Iron transport post-transfusion. Ferritin is elevated in the reticuloendothelial system for sBlood, fPHB, and sPHB at the end of resuscitation. **(A)** Significantly higher levels of L-ferritin are present in the serum. **(B),(C)** Ferritin levels in the liver suggest that PolybHb is primarily metabolized in the liver. **(D),(E)** Ferritin levels in the spleen suggest that the spleen removes significantly more senescent RBCs than PolybHb. **(F)** Bilirubin is increased in the sBlood group. Outliers are shown in the box-and-whisker plots as filled circles of the group's respective colors. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

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Table 1.

Hematological parameters during the hemorrhagic shock/resuscitation protocol. pHb = plasma Hb. Data are presented as median [95% CI]. N=8 per group.

Time point	Group	Hct, %	Hb, g/dL	pHb, g/dL
Baseline	fBlood	42 [41 – 44]	13.1 [12.7 – 13.5]	
	sBlood	43 [42 – 44]	13.6 [13.2 – 14.1]	
	fPHB	43 [41 – 45]	13.6 [13.0 – 14.2]	
	sPHB	42 [41 – 43]	13.3 [12.9 – 13.7]	
Shock	fBlood	28 [27 – 28]	8.8 [8.5 – 9.1]	
	sBlood	29 [28 – 30]	9.1 [8.7 – 9.4]	
	fPHB	29 [28 – 30]	9.4 [8.8 – 9.9]	
	sPHB	28 [27 – 29]	8.9 [8.7 – 9.0]	
R10	fBlood	35 [33 – 37]	11.0 [10.6 – 11.4]	0 [0 – 0]
	sBlood	38 [37 – 38]†	12.4 [12.2 – 12.7]†	0 [0 – 0]
	fPHB	26 [25 – 28]†‡	10.4 [9.8 – 11.0]‡	3.3 [2.6 – 4.0]†‡
	sPHB	26 [24 – 28]†‡	9.7 [9.4 – 10.0]†‡	2.0 [1.4 – 2.6]†‡
R60	fBlood	39 [37 – 41]	12.2 [11.7 – 12.7]	0 [0 – 0]
	sBlood	50 [46 – 53]†	16.3 [14.8 – 17.8]†	0.1 [0.1 – 0.1]
	fPHB	22 [19 – 24]†‡	10.9 [10.1 – 11.7]†‡	5.3 [3.7 – 7.0]†‡
	sPHB	20 [19 – 21]†‡	10.4 [9.7 – 11.1]†‡	4.8 [3.7 – 5.8]†‡

†P<0.05 vs fBlood,

‡P<0.05 vs sBlood.

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Table 2.

Blood gasses during the hemorrhagic shock/resuscitation protocol. Data are presented as median [95% CI]. N=8 per group.

Time point	Group	pH	pCO_2, mmHg	pO_2, mmHg
Baseline	fBlood	7.40 [7.38 – 7.43]	44.5 [41.0 – 47.9]	83.2 [78.0 – 88.5]
	sBlood	7.42 [7.40 – 7.43]	40.2 [37.6 – 42.9]	88.4 [82.8 – 94.0]
	fPHB	7.42 [7.39 – 7.44]	42.3 [38.0 – 46.6]	81.1 [75.1 – 87.1]
	sPHB	7.40 [7.38 – 7.43]	40.1 [35.7 – 44.6]	84.5 [75.2 – 93.7]
Shock	fBlood	7.28 [7.23 – 7.32]	38.3 [34.8 – 41.8]	105.8 [95.4 – 116.2]
	sBlood	7.28 [7.21 – 7.34]	32.3 [27.5 – 37.1]	119.5 [112.8 – 126.1]
	fPHB	7.30 [7.28 – 7.33]	37.2 [35.0 – 39.5]	109.2 [104.1 – 114.3]
	sPHB	7.30 [7.27 – 7.34]	35.0 [31.9 – 38.2]	106.8 [99.4 – 114.2]
R10	fBlood	7.27 [7.22 – 7.32]	42.0 [38.9 – 45.1]	89.0 [81.3 – 96.8]
	sBlood	7.26 [7.20 – 7.32]	39.1 [35.0 – 43.2]	102.6 [91.4 – 113.8]
	fPHB	7.29 [7.27 – 7.31]	42.1 [39.1 – 45.2]	90.8 [85.6 – 95.9]
	sPHB	7.31 [7.28 – 7.35]	37.8 [34.8 – 40.8]	96.7 [86.0 – 107.4]
R60	fBlood	7.31 [7.28 – 7.34]	48.9 [44.7 – 53.1]	81.5 [72.8 – 90.2]
	sBlood	7.31 [7.26 – 7.37]	46.4 [42.6 – 50.2]	82.6 [75.1 – 90.1]
	fPHB	7.32 [7.29 – 7.34]	45.2 [42.7 – 47.8]	85.0 [80.0 – 90.0]
	sPHB	7.31 [7.26 – 7.36]	42.2 [39.8 – 44.6]	83.9 [69.9 – 97.9]

†P<0.05 vs fBlood,

‡P<0.05 vs sBlood.