Vascular resistance and the efficacy of red cell substitutes in a rat hemorrhage model

ROBERT M. WINSLOW, ARMANDO GONZALES, MARIA L. GONZALES, MICHAEL MAGDE, MICHAEL MCCARTHY, RONALD J. ROHLFS, AND KIM D. VANDEGRIFF
Department of Medicine, School of Medicine, University of California, San Diego, and Department of Veterans Affairs Medical Center, San Diego, California 92161

Winslow, Robert M., Armando Gonzales, Maria L. Gonzales, Michael Magde, Michael McCarthy, Ronald J. Rohlfs, and Kim D. Vandegriff. Vascular resistance and the efficacy of red cell substitutes in a rat hemorrhage model. J. Appl. Physiol. 85(3): 993–1003, 1998.—We have compared polyethylene glycol-modified bovine hemoglobin (PEG-Hb; high O2 affinity, high viscosity, high oncotic pressure) and human hemoglobin cross-linked between the α-chains (αα-Hb; low O2 affinity, low viscosity, low oncotic pressure) with a non-O2-carrying plasma expander (pentastarch, high viscosity and oncotic pressure) after a 50% (by volume) exchange transfusion followed by a severe (60% of blood volume) hemorrhage. Mean arterial pressure and systemic vascular resistance rose significantly in the αα-Hb but not in the PEG-Hb animals. Two-hour survival was greater in the PEG-Hb animals (93%) than in control (35%), pentastarch (8%), or αα-Hb (6%) animals. The experimental model we have chosen to evaluate hemodilution; hemorrhage; blood substitutes; hemoglobin; αα-hemoglobin

hemoglobin-based O2 carriers have been proposed as temporary alternatives to red blood cells in certain clinical settings (reviewed in Ref. 29). However, elevation of systemic and pulmonary blood pressure has been observed with some candidate solutions (9, 14), and because of the known reactivity of hemoglobin with NO (6) and the identity of NO as the endothelium-derived relaxing factor (21) it is often assumed that vasoconstriction is a general property of cell-free hemoglobin solutions that might limit their clinical potential. Therefore, an understanding of the hemoglobin-induced vasoactivity is important not only for the development of O2 carriers as therapeutics but also for a deeper understanding of the mechanisms of O2 delivery (D˙O2) to tissue.

On the basis of our observation that hemoglobin derivatives with different vasopressor effects have nearly equal NO-binding properties (23), we recently developed an alternative hypothesis to explain hemoglobin-induced vasoactivity. Theoretical (5, 12) and in vitro data (20) provide strong arguments that cell-free hemoglobin would be expected to facilitate diffusive O2 transport to tissues, but attempts to confirm this prediction in vivo have failed (1, 10, 11). Our hypothesis is that demonstration of diffusive O2 transport by cell-free hemoglobin is prevented by local autoregulatory mechanisms that lead to vasoconstriction as a counterbalance to increased O2 supply (27). Because autoregulation is multifactorial, an important consequence of this hypothesis is that alteration of one or more of the rheological (13) and O2 transport properties (35) of hemoglobin solutions might overcome vasoconstriction.

It is now possible to examine this proposition more closely because of the availability of cell-free O2 carriers with properties that differ in critical ways. Among the properties of hemoglobin solutions that can be varied are viscosity, oncotic pressure, and O2 affinity. In the experiments to be reported here, we explore some of these properties using two well-characterized hemoglobins and a nonhemoglobin plasma expander. The first is a human hemoglobin derivative modified by cross-linking between αα-Hb exchange transfusion offsets the additional O2 transport provided by the cell-free hemoglobin. When resistance does not rise, as with PEG-Hb, even relatively small amounts of cell-free hemoglobin appear to be a very effective blood replacement.

The experimental model we have chosen to evaluate these solutions is 50% isovolemic hemodilution with the test solution followed by an exponential 60% blood volume hemorrhage (7). This hemorrhage is severe enough so that ~50% of control animals die within 1 h after conclusion of hemorrhage. Thus the effectiveness of perturbations such as hemodilution is readily apparent not only in overall survival but also in the physiological changes that occur during and after the hemorrhage. An additional reason to choose this model is that perioperative hemodilution is a clinical application for which “blood substitutes” might be ideally suited (31).

Our results show that exchange transfusion with PEG-Hb does not raise blood pressure, and after hemorrhage, animals show many features of improved tissue oxygenation, i.e., better survival, normal acid-base status, lower lactic acid production, and higher cardiac output (Q), than non-exchange-transfused animals. In contrast, exchange transfusion with αα-Hb raises blood pressure significantly, but after hemorrhage, animals have shortened survival, higher lactic acid production, and lower Q than non-exchange-transfused animals. Control experiments with PS indicate that the favor-
able results with PEG-Hb are not due merely to its increased viscosity and/or oncotic pressure. This study is an important first step toward the goal of a better understanding of the role of viscosity, oncotic pressure, and O₂ affinity in D¨O₂ by cell-free O₂ carriers.

**MATERIALS AND METHODS**

Test solutions. PEG-Hb is bovine hemoglobin conjugated to 5,000-mol-wt methoxypolyoxylethylene (PEG) chains (36). The PEG chains are covalently attached to ε-amino groups of surface lysine residues such that 10–12 PEG chains are attached to each tetramer. It was supplied as a gift from Enzon. αα-Hb was prepared as described previously (32) and supplied as a gift from the US Army Blood Research Detachment, Walter Reed Army Institute for Research. PS is the commercial product Pentaspan obtained from DuPont Merck. The O₂ affinity of rat blood was determined using an apparatus described previously (34) in which pH and PCO₂ are held constant at 7.4 and 40 Torr, respectively. O₂ affinity of αα-Hb and PEG-Hb were determined by simultaneous measurements of absorbance and D¨O₂ using a diode array spectrophotometer (model 3000, Milton Roy) and a micro-O₂ electrode (Microelectrodes) and amplifier. Saturation was determined as a function of PO₂ by analysis of the optical spectrum collected at every PO₂ data point (26). Colloidal osmotic (oncotic) pressure was measured using a colloid osmometer (model 4420, Wescor, Logan, UT). Average molecular weight was calculated according to general thermodynamic analysis, as reported elsewhere (25). Viscosity was measured at 37°C using a capillary viscometer (22). According to the specifications of the products, PEG-Hb and αα-Hb contain 0.3 endotoxin units per milliliter of endotoxin. Starting methemoglobin levels were <5.0% in each of the solutions.

Autodiation kinetics for αα-Hb and PEG-Hb were measured by following the absorbance changes that accompany the conversion of oxyhemoglobin to methemoglobin for air-equilibrated samples in 0.1 M bis-Tris propane-0.1 M NaCl (pH 7.4, 37°C). Data were analyzed as single-wavelength absorbance changes at 401 and 420 nm and by multicomponent analysis of the visible spectrum between 480 and 650 nm for the fraction of methemoglobin. Time courses were fitted to single-exponential expressions, and results were similar at both wavelengths or by multicomponent analysis.

Rates of hemolysis were determined using fully oxidized hemoglobin. Rates were measured using a double mutated sperm whale apomyoglobin (H64Y/V68F, a kind gift from J. S. Olson, Rice University) as a heme acceptor (8). The rate-limiting step in this reaction is heme dissociation, and the time course of heme exchange between methemoglobin and the apomyoglobin reflects the rate of heme loss from methemoglobin. Rates were measured in 0.15 M phosphate-0.45 M sucrose (pH 7.0, 37°C). All proteins exhibited biphasic kinetic behavior, with the kinetic phases displaying equal amplitudes due to chain differences. According to Hargrove et al. (8), the rapid and slow kinetic phases for unmodified HbA0 correspond to αα- and β-subunits, respectively.

Experimental animals. Groups of male Sprague-Dawley rats (210–350 g; Charles River) were studied. Control animals were treated exactly as the experimental animals, except no exchange transfusion was performed before the hemorrhage period. In this test animals, exchange transfusion was carried out according to the protocol described below with PS, PEG-Hb, or αα-Hb. Because of the small size of these animals, the amount of blood taken for analysis was strictly controlled. Therefore, separate animals were used for 1) blood pressure and acid-base measurements, 2) Q, 3) blood volume determinations, or 4) arterial and mixed venous blood-gas content.

Surgical preparation of animals. All animal protocols were approved by the Animal Care Committee of the San Diego Veterans Affairs Medical Center. Animals were fed ad libitum before all experiments. Rats were anesthetized with 250 µl of a mixture of ketamine (71 mg/ml; Aveco, Fort Dodge, IA), acepromazine (2.85 mg/ml; Fermenta, Kansas City, MO), and xylazine (2.85 mg/ml; Lloyd Laboratories, Shenandoah, IA). The area of the left femoral artery and vein was exposed by blunt dissection, and a polyethylene catheter (PE-50) was placed into the abdominal aorta via the femoral artery to allow rapid withdrawal of arterial blood. An identical catheter was placed in the inferior vena cava via the femoral vein to allow injection of the Evans blue dye in blood volume experiments. Catheters were tunneled subcutaneously, exteriorized through the tail, and flushed with ~100 µl of normal saline. Animals were allowed to recover from the procedure and remained in their cages for an additional 24 h before being used in experiments.

Blood volume. The method used to measure plasma and blood volume has been described previously (15). Briefly, a small amount of Evans blue dye is administered to the test animal by injection into a femoral vein catheter, and blood is sampled from the contralateral femoral artery at 5, 10, 15, and 20 min after the injection. Plasma is separated by centrifugation, and the visible optical spectrum is recorded. After a baseline correction of the absorbance at 620 nm for the presence of plasma hemoglobin, linear regression of the absorbance values is done to obtain the extrapolated zero-time value. This absorbance value is used to calculate the dilution relative to the starting concentration of Evans blue dye, and with the measured hematocrit and appropriate corrections for plasma trapping and the “Fcell” ratio, the total body-to-venous hematocrit ratio, total blood volume is calculated.

Hemodynamic measurements. For the hemodynamic measurements, the femoral artery catheter was connected, through a stopcock, to a pressure transducer (model 1050, UFI, Monro, CA), and arterial pressure was sampled continuously at 100 Hz using a data-collections system (model MP100WSW, BIOPAC Systems, Goleta, CA). The data were stored in digital form for subsequent off-line analysis. The arterial pressure signal was analyzed using a program written in FORTRAN. Heart rate for each beat was calculated as the reciprocal of the interval between successive pressure peaks. Systolic and diastolic pressures were the maximum and minimum pressures, respectively, and the mean arterial pressure (MAP) was diastolic + ½(systolic – diastolic) pressure. Myocardial contractility (dp/dt) was calculated from the maximum positive slope for each pressure cycle. Mean values of heart rate, systolic and diastolic pressures, MAP, pulse pressure, and dp/dt were averaged for each minute of data. Q was measured by injection of cold saline via the jugular vein. Mixing occurs in the pulmonary capillary beds, and Q was measured by a thermodilution catheter and Q computer (Columbia Instruments, Columbus, OH). Systemic vascular resistance (SVR) was calculated as MAP/Q. O₂ transport. Because of the volume of blood needed to analyze O₂ and CO₂ content, a separate group of animals was used for measurements of O₂ transport. In these experiments, hemodilution and subsequent hemorrhage were carried out as with the other animals, but at intervals blood samples were removed simultaneously from the femoral artery and the inferior vena cava. These were analyzed for blood gases (pH, PO₂, PCO₂), hematologic quantities (hematocrit, hemoglobin, plasma hemoglobin), and O₂ content (Lex-O₂-Con, Hospex).
Blood-gas, hematologic, and lactate measurements. Arterial pH, PCO₂, and PO₂ were measured in a blood-gas analyzer (model ABL5, Radiometer) using 100-µl samples of heparinized blood. Lactic acid was measured in femoral artery blood using a lactate analyzer (model 1500-L, Yellow Springs Instruments). Total CO₂ and base excess were calculated using a lactate analyzer (model 1500-L, Yellow Springs Instruments). Fully conscious animals were placed in Plexiglas restrainers. The arterial and venous cannulas were flushed with 200 and 100 µl, respectively, of heparinized saline (100 U/ml). The arterial and venous catheters were connected to an infusion pump (model 4262000, Labconco, Kansas City, MO), and exchange transfusion was carried out at a rate of ~2 ml/min to a total volume of solution that equaled 50% of estimated blood volume. The peristaltic pump was operated so that blood was removed at exactly the same rate at which test material was infused. Test solutions were filtered through a 0.22-µm filter immediately before infusion, and the infusate tubing passed through a 37°C water bath. Flow rate at which test material was infused. Test solutions were filtered through a 0.22-µm filter immediately before infusion, and the infusate tubing passed through a 37°C water bath.

Hemorrhage. The hemorrhage protocol was begun ~10 min after completion of the exchange transfusion by pumping out arterial blood from the femoral artery at a rate of 0.5 ml/min. The pump was started at the beginning of each 10-min period and run for a time calculated to complete the removal of 60% of the blood volume by the end of 60 min. The total blood volume (TBV) at the end of each 10-min period is

\[ TBV = TBV_0 \exp(-0.01526t) \]  

where TBV₀ is the initial blood volume, assumed to be 60 ml/kg (15), and t is time (minutes). Removal of the required quantities of blood was accomplished by running the pump for a period of time corresponding to the amount of blood to be removed divided by the pump rate, i.e., 0.5 ml/min. At the end of the 60-min hemorrhage period, the animals were monitored for an additional 1 h before euthanasia. Blood samples were taken every 10 min, and the volume of blood removed was added to that removed by the pump to adhere strictly to the hemorrhage protocol.

Statistical and survival analysis. Statistical and survival analyses were done using PROPHET (Bolt, Baranek and Newman, Cambridge, MA). Unless otherwise noted, values are means ± SE. Differences between means were considered significant at P < 0.05. For the survival analyses, animals were observed for a minimum of 120 min after the start of the hemorrhage, and survival data were analyzed according to the method of Kaplan and Meier (16). The elapses times from the start of hemorrhage to death or censoring for each animal were recorded. Data were considered censored if the animal was alive at 120 min. The data were grouped into 10-min intervals, and for each interval the cumulative proportion alive and its standard error were calculated. Significance of survival differences between groups was analyzed using the Mantel-Cox test of significance (17).

Because the amount of blood needed for some determinations would significantly influence the hemorrhage experiments, some calculated values (SVR, stroke volume (SV), D₀₂, O₂ consumption (VₐO₂)) were calculated from two measurements in different groups of animals. Thus, when a quantity C (e.g., SVR) is calculated as the product of two sets of data A (e.g., MAP) and B (e.g., 1/Q), standard errors of the quantity C were calculated by analysis of the variances in the quantities A and B, and the t-statistic for the differences between sets of C (i.e., C₁ and C₂) was calculated from the pooled estimate of the standard deviations. These procedures are documented in the PROPHET system.

RESULTS

Test solutions. Table 1 gives some physical properties of the three test solutions and compares them with rat blood. Note that the O₂ half-saturation pressure of hemoglobin (P₅₀) of PEG-Hb and its Hill coefficient are much lower than the values for blood or for ααaa-Hb. The molecular weight given for PS is a mean weight-average value (Pentaspan prescribing information, DuPont Merck). The osmotic pressure of PEG-Hb is much higher than that of rat or human blood and higher than that of ααaa-Hb. The viscosities of PEG-Hb and PS are about equal to that of blood, whereas the viscosity of ααaa-Hb is that of water.

Hematologic measurements. The hematologic measurements are shown in Table 2. Hematocrit, total hemoglobin, and plasma hemoglobin were measured at baseline, immediately after exchange transfusion, and at 30, 60, and 120 min after the start of hemorrhage. The drop in hematocrit after hemodilution is greatest in the animals treated with PEG-Hb, presumably because of its greater oncotic pressure and, therefore, hemodilution (Table 1). As expected, total hemoglobin follows hematocrit closely in all animals. Plasma hemoglobin, however, differs significantly between the two cell-free hemoglobin groups: ααaa-Hb and PEG-Hb animals. In the former, plasma hemoglobin reaches a maximum of 3.9 g/dl after hemodilution; in the latter it reaches only 1.9 g/dl. Also the disappearance of plasma hemoglobin is much faster in the ααaa-Hb than in the PEG-Hb animals (3.6 and 8.9 h, respectively). The lower concentration in the PEG-Hb animals is partly due to the lower hemoglobin concentration of the stock solution (Table 1) and partly to the greater blood volume expansion (15).

Blood volume. The effect of exchange transfusion on blood volume is presented in Table 3. PS and PEG-Hb

<table>
<thead>
<tr>
<th>Table 1. Properties of the test solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Blood</td>
</tr>
<tr>
<td>Concentration, g/dl</td>
</tr>
<tr>
<td>P₅₀ (37°C), Torr</td>
</tr>
<tr>
<td>Hill coefficient</td>
</tr>
<tr>
<td>Mol wt</td>
</tr>
<tr>
<td>Viscosity, cP</td>
</tr>
<tr>
<td>COP, mmHg</td>
</tr>
<tr>
<td>Autoxidation</td>
</tr>
<tr>
<td>Heme loss, h⁻¹</td>
</tr>
<tr>
<td>kₛₚₐₚ</td>
</tr>
</tbody>
</table>

ααaa-Hb, human hemoglobin cross-linked between α-chains; PEG-Hb, polyethylene glycol-modified bovine hemoglobin; PS, pentastarch; P₅₀, O₂ half-saturation pressure of hemoglobin; COP, colloid osmotic pressure; kₛₚₚₚ, rapid; kₛₚₚₚ, observed rate constant and rate constants for rapid and slow phases of 2-phased reaction, respectively. *Molecular weight determined by osmotic pressure measurements (25). †From Pentaspan prescribing information (DuPont Merck).
expanded the blood volume to almost the same extent, whereas exchange transfusion with αα-Hb contracted the blood volume significantly. The difference in volume expansion caused by exchange transfusion with PS compared with PEG-Hb is not statistically significant (P = 0.625); however, the contraction after hemodilution with αα-Hb compared with both PS and PEG-Hb is highly significant (P < 0.001 in both cases). As has been reported previously (15), the volume expansion consequent to exchange transfusion with PEG-Hb, or PS in this experiment, is temporary, lasting only ~2 h after the exchange. The plasma volume is a dynamic quantity (15); after an initial expansion it gradually contracts after administration of PEG-Hb, as the hemoglobin is cleared from the circulation.

Survival. Overall survival in the various groups of animals is shown graphically in Fig. 1, and the mean survival times are given in Table 4. In untreated controls the survival was 46% at 2 h after the hemorrhage was started. The overall survival is worsened after 50% exchange transfusion with PS (8%) and αα-Hb (6%) but markedly improved in the PEG-Hb animals (93%; Fig. 1). Differences in survival between groups are all statistically significant, except for the αα-Hb-PS comparison, which is not significant (P = 0.15). Note in Table 4 the lack of relationship between survival and postexchange hematocrit or hemoglobin concentration. In particular, the PEG-Hb animals had markedly improved survival even compared with the control group, with a mean hemoglobin concentration only about one-half that of the controls.

Hemodynamics. MAP values are shown in Fig. 2. Exchange transfusion with αα-Hb produces a mean rise from 110.8 ± 2.9 to 125.6 ± 4.2 mmHg (difference, P < 0.02). A mean drop from 114.1 ± 4.6 to 100.5 ± 5.0 mmHg was observed during exchange transfusion in the PS animals, but the difference was not statistically significant (P = 0.1). No significant change was observed between baseline and postexchange periods in the PEG-Hb animals, although Fig. 2 shows a small rise immediately after the beginning of the exchange. After initiation of hemorrhage, MAP fell immediately in the control and αα-Hb animals, possibly because of their contracted blood volumes (Table 3). The fall in MAP is much slower in the PS animals and insignificant in those receiving PEG-Hb. The immediate fall in pressure in the control animals was followed by a gradual increase, but values never returned to baseline levels.

Over the course of the hemorrhage and subsequent observation period, the MAP was very sensitive to the withdrawal of blood (each 10 min) in the control animals but was more stable in the PS and PEG-Hb animals. Again, this could be due to the greater initial

### Table 2. Hematologic measurements

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Baseline</th>
<th>Post-ET</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>38.6 ± 0.9</td>
<td>38.6 ± 0.9</td>
<td>27.7 ± 0.7</td>
<td>24.8 ± 0.9</td>
<td>23.1 ± 0.6</td>
</tr>
<tr>
<td>PS</td>
<td>4</td>
<td>42.4 ± 1.3</td>
<td>18.4 ± 1.0</td>
<td>14.6 ± 0.6</td>
<td>15.0 ± 0.7</td>
<td>16.8 ± 1.8</td>
</tr>
<tr>
<td>αα-Hb</td>
<td>6</td>
<td>39.5 ± 0.7</td>
<td>18.5 ± 0.4</td>
<td>13.1 ± 0.3</td>
<td>13.4 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>PEG-Hb</td>
<td>5</td>
<td>40.5 ± 1.2</td>
<td>15.8 ± 0.4</td>
<td>13.4 ± 0.3</td>
<td>12.9 ± 0.2</td>
<td>13.3 ± 0.3</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>13.8 ± 0.3</td>
<td>13.8 ± 0.3</td>
<td>9.2 ± 0.3</td>
<td>8.8 ± 0.3</td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td>PS</td>
<td>4</td>
<td>15.2 ± 0.4</td>
<td>6.8 ± 0.4</td>
<td>5.5 ± 0.3</td>
<td>5.4 ± 0.3</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td>αα-Hb</td>
<td>6</td>
<td>14.0 ± 0.3</td>
<td>10.2 ± 0.2</td>
<td>6.8 ± 0.2</td>
<td>7.2 ± 0.4</td>
<td>*</td>
</tr>
<tr>
<td>PEG-Hb</td>
<td>5</td>
<td>14.5 ± 0.6</td>
<td>7.6 ± 0.1</td>
<td>6.5 ± 0.1</td>
<td>6.1 ± 0.1</td>
<td>5.8 ± 1.0</td>
</tr>
<tr>
<td>Plasma Hb, g/dl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αα-Hb</td>
<td>6</td>
<td>3.9 ± 0.1</td>
<td>2.4 ± 0.07</td>
<td>2.3 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>*</td>
</tr>
<tr>
<td>PEG-Hb</td>
<td>5</td>
<td>1.9 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats; Hct, hematocrit; ET, exchange transfusion. *Too few animals alive to permit statistical analysis. Standard errors are not given when only 1 animal is alive.

### Table 3. Blood volume after 50% exchange transfusion

<table>
<thead>
<tr>
<th>Solution</th>
<th>n</th>
<th>Volume, ml/kg</th>
<th>P (baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>58.3 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>5</td>
<td>71.7 ± 4.4</td>
<td>0.017</td>
</tr>
<tr>
<td>αα-Hb</td>
<td>5</td>
<td>49.0 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PEG-Hb</td>
<td>5</td>
<td>74.0 ± 1.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats.
blood volume in those two groups of animals. After an initial fall in the \(\alpha\alpha\)-Hb animals, a prompt rise was observed, reaching a maximum of 129.1 \(\pm\) 4.1 mmHg at 50 min of hemorrhage. In fact, the MAP in the \(\alpha\alpha\)-Hb animals did not fall much below the baseline, showing that maintenance of the MAP is not a good correlate with overall survival. MAP values in the PEG-Hb animals did not fall after initiation of the hemorrhage. Rather, a gradual decrease was observed over the entire duration of the experiment, reaching a minimum MAP of 89.1 \(\pm\) 5.2 mmHg at 105 min after the start of hemorrhage.

Heart rate. The heart rate changes are shown in Fig. 3. The baseline rate in the control animals was 425 \(\pm\) 2.9 (SE) beats/min. At the start of hemorrhage, the rate dropped slightly, but by the end of the hemorrhage the rate had increased to 495 \(\pm\) 2.0 beats/min, significantly faster than the baseline rate (\(P < 0.001\)). At the end of the hemorrhage, the PS animals had a mean heart rate of 495 \(\pm\) 2.0 beats/min, a rate significantly faster than the PS baseline of 408 \(\pm\) 5.3 beats/min (\(P < 0.001\)) but not significantly different from the controls at the end of hemorrhage. In contrast, exchange transfusion with \(\alpha\alpha\)-Hb led to a significant drop in the heart rate (434 \(\pm\) 3.9 to 345 \(\pm\) 1.8 beats/min, \(P < 0.001\)). By the end of the exchange transfusion the heart rate in the \(\alpha\alpha\)-Hb animals had returned to a value not significantly different from baseline and then increased slightly during the hemorrhage and subsequent observation period. Heart rate was fairly stable in the PEG-Hb animals, despite a slight but significant fall after exchange transfusion (from 427 \(\pm\) 1.6 to 383 \(\pm\) 1.0 beats/min, \(P < 0.001\)) and a slightly lower rate (412 \(\pm\) 1.1 beats/min, \(P < 0.001\)) at the end of hemorrhage.

dP/ dt. \(dP/ dt\), the maximum positive slope of the pulse contour, is shown in Fig. 4. In the control animals, this value increases within \(-15\) min after the beginning of the hemorrhage, rising significantly (\(P < 0.001\)) from a baseline of 1,336 \(\pm\) 8.2 to 1,513 \(\pm\) 6.7 mmHg/s before returning to baseline shortly before death of the animals. In the PS animals, exchange transfusion produces a slight but significant fall after exchange transfusion (from 427 \(\pm\) 1.6 to 383 \(\pm\) 1.0 beats/min, \(P < 0.001\)) and a slightly lower rate (412 \(\pm\) 1.1 beats/min, \(P < 0.001\)) at the end of hemorrhage.

Table 4. Overall survival

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Post-ET Hct, %</th>
<th>Post-ET Hb, g/dl</th>
<th>120-min Survival, %</th>
<th>Mean Survival Time, min</th>
<th>P (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22</td>
<td>38.6 (\pm) 0.9</td>
<td>13.8 (\pm) 0.3</td>
<td>46</td>
<td>105 (\pm) 7</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>13</td>
<td>18.4 (\pm) 1.0</td>
<td>6.8 (\pm) 0.4</td>
<td>8</td>
<td>74 (\pm) 7</td>
<td>0.0277</td>
</tr>
<tr>
<td>(\alpha\alpha)-Hb</td>
<td>18</td>
<td>18.5 (\pm) 0.4</td>
<td>10.2 (\pm) 0.2</td>
<td>6</td>
<td>52 (\pm) 6</td>
<td>0.0005*</td>
</tr>
<tr>
<td>PEG-Hb</td>
<td>15</td>
<td>15.8 (\pm) 0.4</td>
<td>7.6 (\pm) 0.1</td>
<td>93</td>
<td>&gt;120</td>
<td>0.0046</td>
</tr>
</tbody>
</table>

Values are means \(\pm\) SE. n, No. of rats. *\(\alpha\alpha\)-Hb vs. PS, P = NS.
tion with αα-Hb produces a small but significant (P < 0.001) rise in dP/dt from 1,211 ± 9.0 to 1,257 ± 8.9 mmHg/s and then a significant (P < 0.02) fall in dP/dt to 825 ± 21.0 mmHg/s during hemorrhage. Finally, exchange transfusion with PEG-Hb does not produce a significant change in dP/dt from the baseline value of 1,094 ± 9.0 mmHg/s, but there is a gradual but significant (P < 0.001) rise during and after hemorrhage to 1,405 ± 51.1 mmHg/s. In terms of the absolute change from baseline, the control, PS, and PEG-Hb treatments produce about the same rise, but the rise is delayed and prolonged in the PEG-Hb animals compared with the other two groups.

Q. Q measurements by the direct thermodilution technique are shown in Fig. 5. After exchange transfusion with both PEG-Hb and PS, Q increases in parallel with the blood volume expansion shown in Table 3. However, because of the large variation in individual measurements, only the rise in PEG-Hb animals was significant when subjected to a paired t-test (P = 0.031). The fall in Q in the control and αα-Hb animals is prompt and significant at all time points (P < 0.001). Q also falls in the PS animals relative to baseline at ≥20 min after hemorrhage (P < 0.002). In the PEG-Hb animals, Q is never significantly different from the baseline values (except immediately after the exchange, as noted) even at 120 min after the start of the hemorrhage. The principal component of the changes in

Q is the SV, (Fig. 6). The changes follow closely the changes in Q (Fig. 5). Of particular interest, the SV in the PEG-Hb animals is essentially the same as baseline after the end of the hemorrhage.

Figure 7 shows the SVR. Some variation in the data is caused by the need to use data from different groups of animals for the Q and MAP values. Even with this limitation, the difference between the PEG-Hb, control, and αα-Hb experiments is striking. During the course of the hemorrhage, the SVR more than quadruples in the controls as the MAP drops significantly. The SVR in the PEG-Hb animals remains at prehemorrhage levels and doubles in the PS animals.

O2 transport. Figure 8 presents D˙O2, the product of Q and arterial O2 content (CaO2). D˙O2 falls for all three dilution groups after exchange transfusion. D˙O2 continues to fall markedly in the PS, αα-Hb, and control groups after the initiation of the hemorrhage, whereas it remains stable at ~50% of baseline in the PEG-Hb animals. This is explained by the increased Q in the
latter group (Fig. 5). In the control, \(\alpha\alpha\)-Hb, and PS animals, the \(\dot{D}O_2\) values are virtually identical after the first 10 min of hemorrhage.

Although plasma methemoglobin was not measured directly in these studies, the \(O_2\) transport data also permit the calculation of the amount of \(O_2\) transported by the plasma hemoglobin fraction. Thus, from the measured \(P_{O_2}\), \(P_{CO_2}\), and \(pH\), red cell saturation can be calculated (30), and red cell \(O_2\) content can then be obtained as the product of red cell saturation and red cell \(O_2\) content. The difference between red cell \(O_2\) content and the measured \(Ca\ O_2\) is plasma hemoglobin \(O_2\) content and, when divided by plasma hemoglobin \(O_2\) capacity (plasma Hb \(\times 1.34\)), gives plasma hemoglobin saturation. This calculation yields \(\sim 75\%\) plasma hemoglobin saturation for the \(\alpha\alpha\)-Hb and PEG-Hb animals and provides evidence that there can be no more than 25\% methemoglobin in either of the plasma hemoglobin fractions.

The \(O_2\) extraction ratio \([OER, (Ca_{O_2} - Cv_{O_2})/Ca_{O_2}, where Cv_{O_2} is venous O_2 content]\) is shown in Fig. 9. This index is apparently sensitive to changes in overall \(O_2\) transport, since there is a significant rise in the PS and \(\alpha\alpha\)-Hb animals with exchange transfusion alone. There is no significant change in the PEG-Hb animals. In the \(\alpha\alpha\)-Hb animals the ratio rises to \(\sim 100\%\) almost immediately after the start of the hemorrhage, whereas the rise, while still marked, is slightly less in the PS and control animals. The rise in the ratio is more gradual in the PEG-Hb animals and does not exceed \(\sim 75\%\) of \(\dot{D}O_2\) at any time during the hemorrhage observation period.

\(V_2\) (Fig. 10) falls significantly and rapidly after the start of the hemorrhage in the control, PS, and \(\alpha\alpha\)-Hb animals. A small drop in \(V_2\) is seen after the exchange transfusion in the PEG-Hb animals, but recovery is complete by the end of the first 10-min hemorrhage period. Only the PEG-Hb animals are able to maintain stable \(V_2\) values during the entire experiment.

\(V_2\) as a function of \(\dot{D}O_2\) is shown in Fig. 11. It is striking that none of the PEG-Hb \(V_2\) values falls below the “critical” \(\dot{D}O_2\) of \(\sim 20\) ml·min\(^{-1}\)·kg\(^{-1}\), whereas in each of the other groups of animals it declines below this point during the hemorrhage.

Respiratory and acid-base response to hemorrhage. The respiratory and acid-base response to hemorrhage is in proportion to overall survival. Thus the PEG-Hb animals demonstrate little rise in \(P_{O_2}\) or fall in \(P_{CO_2}\) or base excess and maintain their arterial \(pH\) essentially constant throughout the experiments (Fig. 12). In contrast, the \(\alpha\alpha\)-Hb animals hyperventilated significantly, as demonstrated by the rise in \(P_{O_2}\) and fall in \(P_{CO_2}\). This response did not compensate for the restricted \(\dot{D}O_2\), however, and these animals showed a dramatic fall in base excess and eventually unstable
PH regulation. The control and the PS animals were intermediate with regard to these blood-gas values, with the PS animals showing less disturbance than controls.

The greatest perturbation of respiratory and acid-base regulation is seen in the animals exchanged transfused with \( \alpha \alpha \)-Hb, despite the fact that the \( \alpha \alpha \)-Hb animals have the same hematocrit as the PEG-Hb animals (Table 2) and a higher total hemoglobin concentration than the PS or PEG-Hb animals (Table 2).

A separate measure of the effectiveness of \( O_2 \) transport and tissue ischemia is demonstrated by the arterial lactate measurements shown in Fig. 13. The PS animals demonstrate higher lactate levels than the controls, presumably because of their lower hemoglobin concentrations. The \( \alpha \alpha \)-Hb animals show the highest accumulation, despite their higher total hemoglobin concentration. The PEG-Hb animals produce less lactate than the controls, despite a significantly lower total hemoglobin concentration.

DISCUSSION

We have described a severe hemorrhage model, which has been chosen because only such a model has the potential to demonstrate the efficacy of cell-free hemoglobin. With no intervention, our 60% exponential hemorrhage protocol is lethal in 58% of rats 1 h after completion of the hemorrhage. Thus improvement in survival to 93% after a 50% (by volume) hemodilution with PEG-Hb is especially noteworthy, considering that the PEG-Hb animals began the hemorrhage with a total hemoglobin concentration of only 7.6 g/dl compared with 13.8 g/dl in the controls (Table 2). This is the first demonstration of which we are aware in which cell-free hemoglobin is more effective in preventing the consequences of severe hemorrhage than red blood cells alone. This model is also extremely relevant to the clinical development of red cell substitutes, because to be efficacious the effects of any proposed new agent will have to be clearly tied to some positive clinical outcome, such as survival. Moreover, removal of autologous blood before surgery and replacement with a substitute represent one principally planned application for these solutions.

Solution properties. It is not possible to obtain solutions for study as red cell substitutes that differ from each other such that the influence of isolated variables can be clearly understood. The solutions we have chosen in the present study do allow some simple comparisons, however. For example, \( \alpha \alpha \)-Hb and PEG-Hb have different solution and functional properties. The comparison between PEG-Hb and PS is of interest, because both of these solutions have equivalent oncotic pressures and viscosities (Table 1). They differ primarily in that only PEG-Hb carries bound \( O_2 \). That the effects of PEG-Hb are not due only to its high oncotic pressure and viscosity is shown clearly by its effect on survival, lactic acid concentration, \( Q \dot{O}_2 \), and \( V \dot{O}_2 \) compared with PS. \( \alpha \alpha \)-Hb and PEG-Hb carry \( O_2 \), yet they differ strikingly in their \( O_2 \)-binding properties and physiological effects. More favorable results were obtained with PEG-Hb, even though its \( O_2 \) affinity is higher than that of \( \alpha \alpha \)-Hb and its plasma concentration was lower in our animals (Table 2).

The PEG-Hb and PS solutions have increased oncotic pressure and viscosity. The extent to which increased viscosity of the solution relative to \( \alpha \alpha \)-Hb plays a role is not completely clear. A theoretical examination of this question by Intaglietta and co-workers (13) suggests that viscosity may be a significant factor, but PEG-Hb and PS are matched for viscosity and yet have markedly different effects on survival, lactic acid production, and global \( V \dot{O}_2 \).

Physiological response. Hyperventilation is a normal response to hemorrhage (7). This is presumed to be mediated by protons produced in tissues as a consequence of reduced \( D \dot{O}_2 \). Although the initial effect of this \( H^+ \) production is not detectable as a change in arterial pH, the respiratory center in the brain is highly sensi-
tive to it, and hyperventilation occurs almost immediately after the onset of hemorrhage.

PEG-Hb is the most effective blood replacement in this model, as demonstrated by the virtual lack of response in pH, PCO2, PO2, and base excess. In other words, animals maintain essentially perfect acid-base homeostasis, despite the loss of an estimated 60% of their total blood volume by hemorrhage. However, most striking is the fact that animals exchange transfused with PEG-Hb maintain their acid-base balance and survive as well as untreated controls, even though the total hemoglobin concentration in these animals is much less.

It appears that the best indicator of mortality in our animals is their inability to compensate for the acidosis resulting from insufficient D˙O2 to tissues. Hannon and co-workers (7), working with the US Army, found that disordered acid-base balance (as measured by base excess and PCO2, for example) is the best predictor of death in hemorrhagic pigs. Our data clearly show that PEG-Hb improves the acid-base balance, as judged by arterial PCO2, pH, base excess, and lactic acid accumulation, relative to control, PS, and αα-Hb animals. A significant part of this effect may be due to the plasma expansion properties of PS and PEG-Hb, but it cannot be the total explanation, because the two are matched for oncotic pressure, and yet their effects on blood volume are the same.

The significantly reduced effect on arterial PCO2 in the PEG-Hb animals indicates that the drive to hyperventilate in these animals is lower than in the control or the PS animals. Although this could conceivably be due to a central depressive effect, it is more likely due to improved D˙O2. This conclusion is supported by the better survival and lactic acid response in the PEG-Hb animals than in the control or the PS group.

Maintained V˙O2 and reduced lactic acid accumulation are striking features of the PEG-Hb animals. Lactic acid accumulation is a measure of O2 debt and has been shown to be related to survival in humans with hypovolemic shock by a number of studies. Broder and Weil (2) showed that only 11% of shock patients

![Fig. 12. Acid-base changes during exchange transfusion, 60% exponential hemorrhage, and for 1 h thereafter. Almost no detectable departure from baseline values is seen in PEG-Hb animals for any of these parameters, whereas control, PS, and αα-Hb animals demonstrate a rise in PO2, a decrease in PCO2, greater base deficit, and acidosis during experimental period. These results are consistent with hyperventilation in latter 3 groups, but not PEG-Hb animals. BE, base excess.](image)

![Fig. 13. Lactic acid accumulation during exchange transfusion, 60% exponential hemorrhage, and for 1 h thereafter. Lactate accumulation is significantly greater in αα-Hb, PS, and control (in that order) than in PEG-Hb animals.](image)
survive when the lactic acid production is >4 meq/l, and Weil and Affi (28) showed that, as lactic acid concentration rises from 2 to 8 meq/l, the survival drops from 90 to 10%. In these cited studies, lactic acid production is presumably due to hypoperfusion and O₂ debt (18) due to decreased D˙O₂.

Vasoconstriction may severely limit O₂ supply. Indeed, the control, αα-Hb, and PS animals demonstrated reduced Q during the hemorrhage, whereas the PEG-Hb animals did not (Fig. 5). Figure 11 shows that, in the later stages of the hemorrhage, D˙O₂ is still not supply limited in the PEG-Hb animals, whereas it clearly is in the other three groups. This is primarily because Q remains high in the PEG-Hb animals (Fig. 7). Figure 2 shows that neither the PEG-Hb nor the PS solution raises the resting MAP in rats during or after the 50% exchange transfusion. In the same model, αα-Hb produces an immediate rise in MAP of ~20 mmHg. Furthermore, there is only a slow, slight drop in MAP during the 60% hemorrhage in the PEG-Hb animals but a significant drop in the PS animals. Thus SVR remains low in the PEG-Hb animals relative to the other three groups (Fig. 7). Our hypothesis is that this lack of vasoactivity, along with the volume-expanding properties of the hyperoncotic colloids (PEG-Hb and PS), permits efficient perfusion of tissues and unloading of O₂ in capillary beds.

The improved survival with PEG-Hb exchange transfusion is all the more striking, since the hematocrit is only 15.8% and plasma PEG-Hb concentration is 1.9 g/dl. Moreover, the small amount of PEG-Hb that is present has a P₅₀ of only 10 Torr. Our results are in contrast to a similar study using red blood cells (4) in which significantly reduced survival was found in animals in which anemia and increased red cell O₂ affinity were observed at the start of the hemorrhage. A key conclusion from our study, therefore, is that critical properties of red cell substitutes, such as O₂ affinity, O₂ capacity, and oncotic pressure, may be different from critical properties of red blood cells. The hierarchy of critical properties of cell-free red cell substitutes in still unclear. For example, P₅₀ seems to be less important than oncotic pressure and, possibly, viscosity. Only further experiments with clearly defined solutions will define the order of importance.

The mechanism of vasoactivity of hemoglobin solutions remains to be clarified. We show here that PEG-Hb has essentially no effect on MAP and vascular resistance, even though its intrinsic reactivity with NO is almost identical to that of αα-Hb (23). Thus the widely held opinion that NO scavenging is responsible for the vasoactivity of hemoglobin solutions (3) may be an oversimplification. We have proposed separately that autoregulation of O₂ supply in the microcirculation may play a role at least as important as NO scavenging (13, 27, 35). The precise biochemical mechanism for such regulation remains a mystery, but one candidate might be ATP-sensitive K⁺ channels, which are known to be sensitive to O₂ supply (19). Alternatively, αα-Hb and PEG-Hb may extravasate at different rates and thus scavenge subluminal NO to different extents.

Implications for design of red cell substitutes. An important observation from our experiments is the lack of correlation between a pressor effect and survival. In fact, the group with the best pressor effect, αα-Hb animals, had the poorest survival. This is in opposition to the reported benefits of rapid restoration of blood pressure with the commercial product DCLHb (24). However, our results may not be strictly comparable, since our protocol is different (hemodilution vs. resuscitation) and since, whether αα-Hb is the same product as DCLHb, even though they contain the same chemical modification, has never been resolved in the literature. However, there is no question that, in our protocol, αα-Hb produces detrimental results, even compared with the controls: lactic acid production is higher, acid-base disturbances are more pronounced, and survival is worse.

It would appear that a major goal of red cell substitute design should be to maintain blood volume and support circulation with low vascular resistance. Our data suggest that this can be accomplished by optimization of the key properties of oncotic pressure, viscosity, and O₂ binding and capacity.

The authors acknowledge with gratitude the expert assistance of René Schad in the preparation of the manuscript.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-48018 and an unrestricted gift by Enzon to the Medicine, Education, and Research Foundation at the University of California, San Diego.

A preliminary report of these results has been presented in abstract form (33).

Address for reprint requests: R. M. Winslow, VA Medical Center (111-E), 3350 La Jolla Village Dr., San Diego, CA 92161.

Received 10 November 1997; accepted in final form 18 May 1998.

REFERENCES


References from: Blood Substitutes: New Challenges