



## Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution

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<sup>1</sup>Department of Bioengineering, University of California-San Diego, and <sup>2</sup>La Jolla Bioengineering Institute, La Jolla, California; and <sup>3</sup>Advanced Research Institute for Science and Engineering, Waseda University, Tokyo, Japan

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Cabrales, Pedro, Hiromi Sakai, Amy G. Tsai, Shinji Takeoka, Eishun Tsuchida, and Marcos Intaglietta. Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution. *Am J Physiol Heart Circ Physiol* 288: H1885–H1892, 2005. First published November 24, 2004; doi:10.1152/ajpheart.01004.2004.—The oxygen transport capacity of phospholipid vesicles encapsulating purified Hb (HbV) produced with a  $P_{50}$  at which Hb is 50% saturated ( $P_{50}$ ) of 8 (HbV<sub>8</sub>) and 29 mmHg (HbV<sub>29</sub>) was investigated in the hamster chamber window model by using microvascular measurements to determine oxygen delivery during extreme hemodilution. Two isovolemic hemodilution steps were performed with 5% recombinant albumin (rHSA) until Hct was 35% of baseline. Isovolemic exchange was continued using HbV suspended in rHSA solution to a total [Hb] of 5.7 g/dl in blood.  $P_{50}$  was modified by coencapsulating pyridoxal 5'-phosphate. Final Hct was 11% for the HbV groups, with a plasma [Hb] of  $2.1 \pm 0.1$  g/dl after exchange with HbV<sub>8</sub> or HbV<sub>29</sub>. A reference group was hemodiluted to Hct 11% with only rHSA. All groups showed stable blood pressure and heart rate. Arterial oxygen tensions were significantly higher than baseline for the HbV groups and the rHSA group and significantly lower for the HbV groups compared with the rHSA group. Blood pressure was significantly higher for the HbV<sub>8</sub> group compared with the HbV<sub>29</sub> group. Arteriolar and venular blood flows were significantly higher than baseline for the HbV groups. Microvascular oxygen delivery and extraction were similar for the HbV groups but lower for the rHSA group ( $P < 0.05$ ). Venular and tissue  $P_{O_2}$  were statistically higher for the HbV<sub>8</sub> vs. the HbV<sub>29</sub> and rHSA groups ( $P < 0.05$ ). Improved tissue  $P_{O_2}$  is obtained when red blood cells deliver oxygen in combination with a high- rather than low-affinity oxygen carrier.

oxygen-carrying capacity; blood substitutes; tissue oxygen; hemoglobin oxygen affinity

PHOSPHOLIPID VESICLES encapsulating concentrated hemoglobin (Hb) solution [Hb vesicles (HbV) or liposome-encapsulated Hb] provide oxygen-carrying capacity to plasma expanders, reproducing several of the characteristics of red blood cells (RBC) suspended in plasma. HbV contain Hb at a high concentration within a cell membrane-like structure. Their oxygen dissociation curve can be adjusted by varying the concentration of pyridoxal 5'-phosphate (PLP). A widely accepted premise for designing a blood substitute is that its Hb should have an oxygen dissociation curve like that of RBC or one that is right shifted, i.e., having a high  $P_{50}$  to facilitate the unloading of oxygen ( $P_{50}$  is the partial pressure of oxygen at which the Hb molecule is 50% saturated). In a previous study by Sakai et al. (16), vesicles were formulated with  $P_{50}$  values set at 9, 16, and

30 mmHg. The study showed that optimal tissue oxygen conditions were obtained when 80% of the circulating blood was substituted with HbV whose  $P_{50}$  was 16 mmHg, a value considerably lower than the usual value of 28 mmHg for normal blood (16). Oxygen-carrying capacity was found to be well above the oxygen supply limitation.

Recent developments in the field of oxygen-carrying plasma expanders (OCPE) based on molecular Hb solutions reported by Tsai et al. (22) show that the addition of comparatively small amounts of a significantly left-shifted polyethylene glycol-conjugated oxygen carrier ( $P_{50} \sim 5$  mmHg) to blood in extreme hemodilution leads to baseline microvascular and systemic conditions. This result could not be obtained in identical extreme hemodilution experiments with the use of a right-shifted molecular Hb solution at a considerably higher concentration (19).

Extreme hemodilution in the hamster window chamber model to a hematocrit (Hct) level of  $\sim 11\%$  is a powerful tool to test the efficacy of OCPEs in restoring microvascular function and systemic conditions. This Hct is below the threshold at which the organism becomes oxygen supply limited (5, 22, 23). In this scenario, the effects of a blood substitute became magnified upon introduction into the circulation. Furthermore, by encapsulating Hb, a phospholipid vesicle eliminates the problem of Hb extravasation and provides a setting in which the biophysical properties of the infusion solution can be rigorously controlled while allowing for the change in  $P_{50}$ . Therefore, experimenting with vesicles that encapsulate Hb formulated with different  $P_{50}$  values provides the unique opportunity to investigate how oxygen affinity regulates oxygen delivery to the tissue by the microcirculation, a value not attainable by lowering RBC Hb  $P_{50}$  by the administration of sodium cyanate, which may introduce changes in tissue metabolism (7). In addition, RBC and HbV are different in size, flow pattern, homogeneous distribution in the plasma phase, and the mechanism of oxygen unloading in capillaries, and direct comparison between RBC and HbV is impossible. All these conditions indicate that the optimal  $P_{50}$  should be different in HbV and RBC.

In the present study, we investigated the microvascular effects of restoring oxygen-carrying capacity in conditions of extreme hemodilution, introducing by exchange transfusion identical amounts of Hb-carrying vesicles in which oxygen affinity was specifically controlled so that  $P_{50}$  was either 8 or 29 mmHg. The  $P_{50}$  value of 8 mmHg was chosen because it is

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similar to that of a recently developed oxygen carrier that is effective at a low concentration (2–4, 22). In these experiments, the hemodilution protocols were performed using a recombinant albumin solution (13) as the plasma expander.

#### METHODS

Investigations were performed in male golden Syrian hamsters (55–65 g body wt) fitted with a dorsal skinfold chamber window (6). This model has been used extensively for investigations of the intact microvasculature of adipose and subcutaneous tissue and skeletal muscle in conscious animals for extended periods. Pentobarbital sodium (50 mg/kg ip) was used for window implantation and for carotid artery and jugular vein catheterization. The microvasculature was examined 4–5 days after the initial surgery, and only animals passing an established systemic and microcirculatory inclusion criteria, which included having tissue void of low perfusion, inflammation, and edema (21), were entered into the study. Animal handling and care followed the NIH *Guide for the Care and Use of Laboratory Animals*. The experimental protocol was approved by the local animal care committee.

**Preparation of HbV with different P<sub>50</sub>.** HbV were prepared under sterile conditions as previously reported (12, 15). Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Japanese Red Cross Society (Tokyo, Japan). The encapsulated purified Hb (38 g/dl) contained 0 or 14.7 mM PLP (Sigma Chemical, St. Louis, MO) as an allosteric effector at a molar ratio of [PLP]/[Hb] = 0 or 2.5, respectively. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-*L*-glutamate at a molar ratio of 5:5:1 (Nippon Fine Chemical, Osaka, Japan) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (0.3 mol% of the total lipid; NOF, Tokyo, Japan) (17). HbV with a 250-nm diameter were suspended in a physiological saline solution in which [Hb] = 10 g/dl, sterilized with filters (Dismic, pore size 0.45 μm; Toyo Roshi, Tokyo, Japan), and deoxygenated with N<sub>2</sub> bubbling for storage (14). The content of lipopolysaccharide was <0.1 EU/ml.

Before use, the HbV suspension ([Hb] = 10 g/dl, 8.6 ml) was mixed with a solution of recombinant human serum albumin (rHSA 25%, 1.4 ml; Nipro, Osaka, Japan) to regulate the rHSA concentration in the suspending medium of the vesicles to 5 g/dl. Under this condition, the colloid osmotic pressure of the suspension is ~20 mmHg (Wescor 4420 colloid osmometer; Wescor, Logan, UT) (12). As a result, the Hb concentration of the suspension was 8.6 g/dl.

In a previous study (16), HbV were suspended in 8 g/dl HSA. However, we changed to 5 g/dl rHSA because it showed better microvascular perfusion in the hamster window model (i.e., increased red cell velocity and functional capillary density) than 8 g/dl HSA. The suspension was filtered through sterile filters (pore size 0.45 μm; Millipore, Billerica, MA). The characteristics of HbV are listed in Table 1, with all parameters being almost identical except oxygen affinity (HbV<sub>8</sub>, P<sub>50</sub> = 8 mmHg; HbV<sub>29</sub>, P<sub>50</sub> = 29 mmHg).

Table 1. Physical characteristics of solutions

Fluid	Viscosity, cp	COP, mmHg	P <sub>50</sub> , mmHg
rHSA (5%)	0.98	20	
HbV <sub>8</sub> (10 g Hb/dl)	2.92		8
HbV <sub>29</sub> (10 g Hb/dl)	2.96		29
HbV <sub>8</sub> /rHSA (8.6 g Hb/dl)	2.87	20	8
HbV <sub>29</sub> /rHSA (8.6 g Hb/dl)	2.90	20	29

Viscosity was measured at a shear rate of 160 s<sup>-1</sup> at 37°C. COP, colloid osmotic pressure measured at 27°C; P<sub>50</sub>, partial pressure of oxygen at which Hb is 50% saturated; rHSA, recombinant human serum albumin; HbV<sub>8</sub> and HbV<sub>29</sub>, Hb vesicles with a P<sub>50</sub> of 8 and 29 mmHg, respectively.

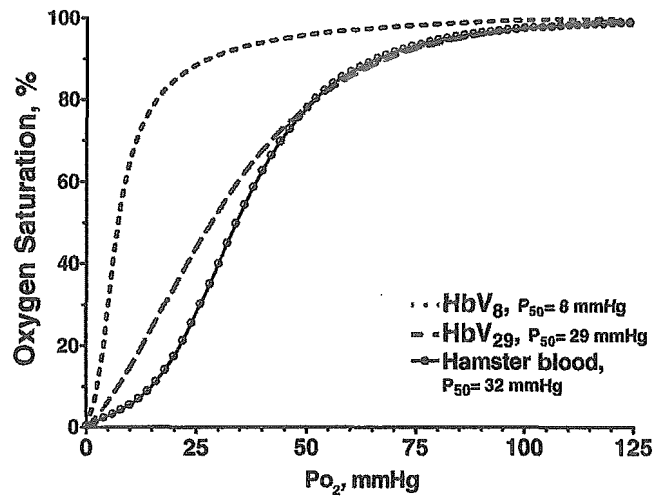


Fig. 1. Oxygen dissociation curves for phospholipid vesicles encapsulating purified Hb (HbV) produced with a P<sub>50</sub> at which Hb is 50% saturated (P<sub>50</sub>) of 8 (HbV<sub>8</sub>) and 29 mmHg (HbV<sub>29</sub>) vs. the dissociation curve for hamster blood (P<sub>50</sub> = 32 mmHg).

**Measurements of P<sub>50</sub> and rate of oxygen release from HbV.** The P<sub>50</sub> and Hill number of each HbV and Hb solution were calculated from oxygen dissociation curves measured with a Hemox analyzer (TCS-Medical Products) at 37°C (Fig. 1).

**Acute isovolemic exchange-transfusion (hemodilution) protocol.** Progressive hemodilution to a final systemic Hct level of 11% was accomplished with three isovolemic exchange steps. This protocol, leading to extreme hemodilution while maintaining stable hemodynamic conditions, is described in detail in a previous report by Tsai (19). Briefly, the volume of each exchange-transfusion step was calculated as a percentage of the blood volume, estimated as 7% of the body weight. An acute anemic state was induced by lowering systemic Hct by 60% with two steps of progressive isovolemic hemodilution using 5% rHSA, referred to as exchange levels 1 and 2. Level 1 exchange was 40% of blood volume, and level 2 and 3 exchanges were 35% of blood volume, respectively.

After level 2, the animals were randomly divided into three experimental groups by being assigned to an experimental group according to a sorting scheme based on a list of random numbers (1). Level 2 exchange was followed by level 3 exchange. Hemodilution with 5% rHSA solution was continued with one group of the level 2 hemodiluted animals, the experimental group rHSA, until Hct was decreased to 11% of baseline (Fig. 2). The test materials were studied by assigning the remainder of the level 2 animals to groups labeled HbV<sub>8</sub> (P<sub>50</sub> = 8 mmHg) and HbV<sub>29</sub> (P<sub>50</sub> = 29 mmHg) and were hemodiluted using these materials, reducing Hct to 11%. Plasma Hb concentrations derived for HbV<sub>8</sub> and HbV<sub>29</sub> after exchange of 35% blood volume are estimated around 2.0–2.3 g/dl for both groups (35% of estimated total Hb content) (21).

Because mixed blood is withdrawn during the exchanges, a 110% blood volume exchange was needed to reduce Hct to 25% of baseline (11% Hct). Test solutions were infused into the jugular vein catheter after passing through an in-line, 13-mm-diameter, 0.2-μm syringe filter at a rate of 100 μl/min. Blood was simultaneously withdrawn using a dual syringe pump (“33” syringe pump; Harvard Apparatus, Holliston, MA) at the same (isovolemic-normovolemic) rate from the carotid artery catheter (4, 5, 19). This slow rate of exchange provided for a stable mean arterial pressure immediately after the exchange. Each animal was allowed a 10-min stabilization period before data acquisition.

**Blood chemistry and biophysical properties.** Arterial blood was collected in heparinized glass capillaries (0.05 ml) and immediately

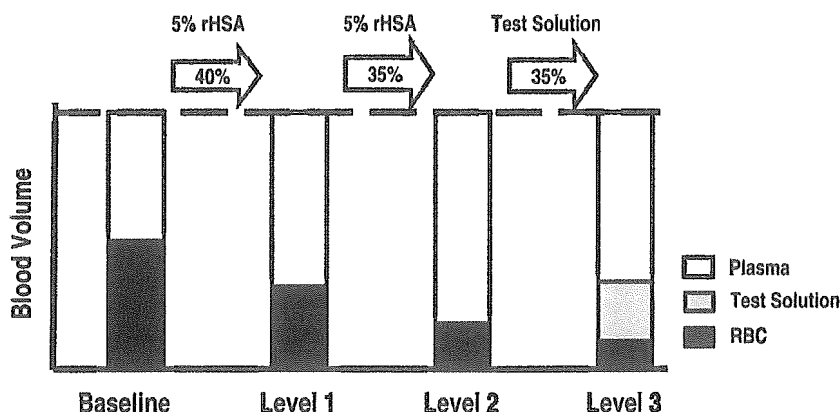


Fig. 2. Hemodilution was attained by means of a progressive, stepwise, isovolemic blood exchange-transfusion protocol. Volume of each exchange-transfusion step was calculated as a percentage of the blood volume, estimated as 7% of body weight. An acute anemic state was induced by lowering systemic Hct, using a 5% recombinant human serum albumin (rHSA) solution, in 2 progressive steps of isovolemic hemodilution labeled level 1 and level 2 exchanges. Level 3 exchange was achieved by a third hemodilution that continued using rHSA or the vesicle solutions HbV<sub>8</sub> or HbV<sub>29</sub> suspended in 5% rHSA (test solutions). RBC, red blood cells.

analyzed for arterial PO<sub>2</sub> (PaO<sub>2</sub>), arterial PCO<sub>2</sub> (PaCO<sub>2</sub>), base excess (BE), and pH (Blood Chemistry Analyzer 248; Bayer, Norwood, MA). The comparatively low PaO<sub>2</sub> and high PaCO<sub>2</sub> values of these animals is a consequence of their adaptation to a fossorial environment. Blood samples for viscosity and colloid osmotic pressure measurements were quickly withdrawn from the animal with a heparinized 5-ml syringe at the end of the experiment for immediate analysis.

Viscosity was measured in a cone/plate viscometer (DV-II+) with a cone spindle (CPE-40; both from Brookfield Engineering Laboratories, Middleboro, MA) at a shear rate of 160 s<sup>-1</sup>. Colloid osmotic pressure (COP) was measured using the Wescor 4420 colloid osmometer (23).

**Functional capillary density.** Functional capillary density (FCD; in cm<sup>-1</sup>) is the total length of RBC-perfused capillaries divided by the area of the microscopic field of view (21). Capillary segments were considered functional if RBC were observed to transit over a 30-s period. FCD was tabulated from the capillary lengths with RBC flow in an area comprising 10 successive microscopic fields (420 × 320 μm). Detailed mappings were made of the chamber vasculature to study the same microvessels throughout the experiment.

**Microhemodynamic parameters.** Arteriolar and venular blood flow velocities were measured online using the photodiode cross-correlation technique (8) (Fiber Optic Photo Diode and Velocity Tracker Correlator model 102B; Vista Electronics, Ramona, CA). The center-line velocity (*V*) was corrected according to vessel size to obtain the mean RBC velocity (11). The video image shearing technique was used to measure vessel diameter (*D*) online. Blood flow was calculated from the measured parameters as  $Q = V\pi(D/2)^2$ .

**Microvascular Po<sub>2</sub> distribution.** High-resolution microvascular Po<sub>2</sub> measurements were made using phosphorescence-quenching microscopy (18), a method based on the oxygen-dependent quenching of phosphorescence emitted by albumin-bound metalloporphyrin complex after pulsed light excitation. Phosphorescence microscopy is not dependent on the level of dye within the tissue, and the decay time is inversely proportional to the Po<sub>2</sub> level. The phosphorescence decay curves were converted to oxygen tensions by using a fluorescence decay curve fitter (model 802; Vista Electronics) (9). This technique has been used in this animal preparation and others for both intravascular and extravascular oxygen tension measurements, because albumin exchange between plasma and tissue allows for sufficient concentrations of albumin-bound dye within the interstitium to achieve an adequate signal-to-noise ratio. Animals received a slow intravenous injection of 15 mg/kg body wt at a concentration of 10.1 mg/ml of a palladium-*meso*-tetra(4-carboxyphenyl)porphyrin (Porphyrin Products, Logan, UT). Po<sub>2</sub> measurements were made 20 min after porphyrin injection, allowing it to be distributed to all the tissues.

In our system, intravascular measurements are made by placing an optical rectangular window (5 × 40 μm) within the vessel of interest,

with the longest side of the rectangular slit positioned parallel to the vessel wall. Tissue Po<sub>2</sub> is measured in regions void of large vessels within intercapillary spaces with an optical window size of ~10 × 10 μm, which allows us to precisely establish the localization of the Po<sub>2</sub> measurements in arterioles, venules, and the interstitium (20). The phosphorescence decay due to quenching at a specific Po<sub>2</sub> yields a single decay constant, and in vitro calibration has been demonstrated to be valid for in vivo measurements. Intravascular and perivascular Po<sub>2</sub> measurements were made in the arterioles studied, and intravascular Po<sub>2</sub> measurements were made in venules. Interstitial tissue Po<sub>2</sub> was measured in regions distant from visible underlying and adjacent vessels.

**Tissue oxygen delivery and extraction.** The microvascular methodology used in our studies allows a detailed analysis of oxygen supply in the tissue. Calculations of O<sub>2</sub> delivery, defined as the amount of oxygen delivered by the arterioles to the microcirculation per unit time normalized relative to baseline, and O<sub>2</sub> extraction, defined as the amount of oxygen released by blood to the tissue by the microcirculation per unit time normalized relative to baseline, were made using Eqs. 1 and 2:

$$O_2 \text{ delivery} = \{(\text{RBC}_{\text{Hb}} \times \gamma \times \text{Sa}_{\text{RBC}}\%) + (\text{HbV}_{\text{Hb}} \times \gamma \times \text{Sa}_{\text{HbV}}\%) + (1 - \text{Hct}) \times \alpha \times \text{Pa}_{\text{O}_2}\} \times Q \quad (1)$$

$$O_2 \text{ extraction} = \{[\text{RBC}_{\text{Hb}} \times \gamma \times S(a - v)_{\text{RBC}}\%] + [\text{HbV}_{\text{Hb}} \times \gamma \times S(a - v)_{\text{HbV}}\%] + (1 - \text{Hct}) \times \alpha \times P(a - v)_{\text{O}_2}\} \times Q \quad (2)$$

where RBC<sub>Hb</sub> is the [Hb] in RBC (expressed in g/dl of blood), HbV<sub>Hb</sub> is the [Hb] in HbV (expressed in g/dl of blood), γ is the oxygen-carrying capacity of Hb at 100% saturation (or 1.34 ml O<sub>2</sub>/g Hb), Sa% indicates the arteriolar oxygen saturation of RBC or HbV. S(a-v)% indicates the arteriovenous difference in oxygen saturation of RBC or HbV. (1 - Hct) is the fractional plasma volume (and converts the equation from units per dl of plasma to per dl of blood), α is the solubility of oxygen in plasma and is equal to 3.14 × 10<sup>-3</sup> ml O<sub>2</sub>/dl plasma mmHg, PaO<sub>2</sub> is the arteriolar partial pressure of oxygen, P(a-v)<sub>O<sub>2</sub></sub> is the arteriovenous difference in Po<sub>2</sub>, and Q is the microvascular flow for each microvessel as a percentage of baseline. The oxygen dissociation curves were determined as described before. In this analysis, microvascular Hct was corrected according to the findings of Lipowsky and Firrell (10).

**Experimental procedure.** Baseline systemic, microvascular, and hemodynamic characterizations were performed before the start of the exchange. After each exchange and a stabilization period of 10 min, systemic and/or microvascular measurements were performed. Exchanges began every hour. After the level 3 exchange transfusion, the same measurements were repeated, and then the Po<sub>2</sub> distribution was determined using phosphorescence-quenching microscopy (9). The duration of the experiment was 3–4 h.

**Data analysis.** Results are presented as means  $\pm$  SD unless otherwise noted. All data are presented as absolute values and ratios relative to baseline values. A ratio of 1.0 signifies no change from baseline, whereas lower and higher ratios are indicative of changes proportionally higher or lower than baseline. The same vessels and functional capillary fields were followed so that direct comparisons to their baseline levels could be performed, allowing for more robust statistics for small sample populations. For repeated measurements, time-related changes were assessed by analysis of variance (ANOVA). Data within each group were analyzed using ANOVA for nonparametric repeated measurement, and when appropriate, post hoc analyses were performed with the Dunn's multiple comparison tests. For level 3 exchange, groups were analyzed using one-way ANOVA, and post hoc analyses were performed with the Bonferroni post tests. All statistics were calculated using GraphPad Prism 4.01 (GraphPad Software, San Diego, CA). Changes were considered statistically significant if  $P < 0.05$ .

## RESULTS

**Exchange transfusion.** Twenty-four animals (55–65 g body wt) entered into the exchange-transfusion (hemodilution) protocol, and all tolerated the experiment without any visible discomfort. Microvascular studies were completed in six preparations for each test material, namely, the level 2 rHSA, HbV<sub>8</sub>, and HbV<sub>29</sub>. The data were analyzed using a model for computing oxygen delivery to the tissue at the microscopic level.

**Hematological changes.** The exchange-transfusion protocol resulted in a final Hct ranging from  $11.0 \pm 0.5$  to  $11.4 \pm 0.6\%$ . The HbV<sub>8</sub> and HbV<sub>29</sub> groups had a final plasma Hb concentration of  $2.1 \pm 0.1$  g/dl, which increased the total Hb concentration in blood (RBC + Hb in plasma) to  $5.7 \pm 0.2$ – $0.3$  g/dl after completion of the level 3 exchange transfusion. Thus oxygen-carrying capacities at this level were similar to those found at level 2, where total blood Hb concentration was  $5.7 \pm 0.3$  g/dl (Hct  $18.1 \pm 0.7$ ) (Table 2).

**Systemic and blood gas parameters.** Changes in the systemic parameters are presented in Fig. 3. Mean arterial pressure was statistically lower for the extreme hemodilution tests with rHSA and the HbV<sub>29</sub> group and attained the highest value with HbV<sub>8</sub> viscosity. Heart rate after hemodilution followed by exchange transfusion with the HbV solutions was  $\sim 10\%$  higher than baseline at the level 3 exchange. The slight increase in heart rate was not statistically different.

Table 2. Laboratory parameters during exchange protocol

	Baseline	Level 1 Hemodilution		Level 2 Hemodilution		Level 3 Hemodilution		
		rHSA	rHSA	rHSA	rHSA	HbV <sub>8</sub>	HbV <sub>29</sub>	
<i>n</i>	24	24	24	24	6	6	6	
Hct, %	$48.8 \pm 1.2$	$28.8 \pm 0.8^*$	$18.1 \pm 0.7^*$	$11.1 \pm 0.8^*$	$11.0 \pm 0.5^*$	$11.4 \pm 0.6^*$		
Hb, g/dl								
Whole blood	$14.8 \pm 0.4$	$9.0 \pm 0.5^*$	$5.7 \pm 0.3^*$	$3.7 \pm 0.4^*$	$5.7 \pm 0.2^{\dagger\dagger}$	$5.7 \pm 0.3^{\dagger\dagger}$		
Plasma					$2.1 \pm 0.1$	$2.1 \pm 0.1$		
PaO <sub>2</sub> , mmHg	$59.2 \pm 4.6$	$68.7 \pm 5.2$	$73.5 \pm 3.7^*$	$87.5 \pm 7.0^*$	$77.1 \pm 4.3^{\dagger\dagger}$	$76.4 \pm 4.4^{\dagger\dagger}$		
PaCO <sub>2</sub> , mmHg	$49.2 \pm 3.6$	$52.4 \pm 6.7$	$49.0 \pm 3.5$	$42.0 \pm 3.2^*$	$53.0 \pm 3.9^{\dagger\dagger}$	$46.8 \pm 4.3$		
Arterial pH	$7.35 \pm 0.02$	$7.35 \pm 0.03$	$7.37 \pm 0.03$	$7.38 \pm 0.04$	$7.35 \pm 0.03$	$7.36 \pm 0.03$		
HCO <sub>3</sub> , mM	$27.9 \pm 2.3$	$28.5 \pm 3.5$	$27.6 \pm 2.2$	$24.8 \pm 2.5$	$28.2 \pm 2.6$	$25.8 \pm 2.1$		
BE, mM	$3.2 \pm 2.0$	$3.4 \pm 2.4$	$2.9 \pm 2.1$	$-0.2 \pm 1.9^*$	$3.1 \pm 1.7^{\dagger}$	$1.0 \pm 2.0$		

Values are means  $\pm$  SD. Baseline values include all animals in the study. No significant differences were detected between the baseline values of each group or between the values after level 1 and level 2 exchange before the exchange with test solutions. Hct, systemic hematocrit; Hb, hemoglobin content of blood; PaO<sub>2</sub>, arterial partial O<sub>2</sub> pressure; PaCO<sub>2</sub>, arterial partial pressure of CO<sub>2</sub>; BE, base excess. \* $P < 0.05$  compared with baseline;  $\dagger P < 0.05$  compared with level 3 rHSA;  $\dagger\dagger P < 0.05$  compared with level 3 HbV<sub>8</sub> to level 3 HbV<sub>29</sub>.

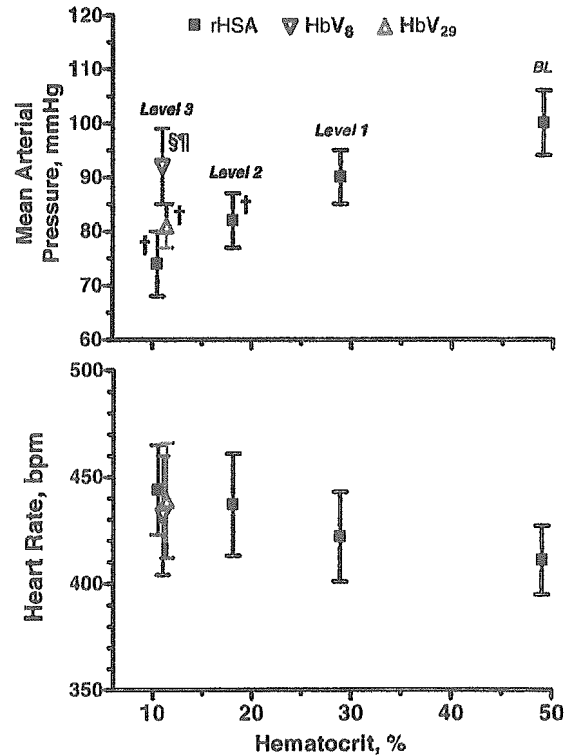


Fig. 3. Mean arterial blood pressure and heart rate [in beats/min (bpm)] at baseline (BL) (Hct 49%) and level 1 (Hct 29%), level 2 (Hct 18%), and level 3 (Hct, 11%) hemodilutions. Level 1 and level 2 exchanges were performed with 5% rHSA as diluent. Level 3 exchange was used to evaluate the oxygen transport of HbV<sub>8</sub> vs. HbV<sub>29</sub> and rHSA.  $\dagger P < 0.05$  relative to baseline;  $\S P < 0.05$  relative to level 3 rHSA;  $\P P < 0.05$ , level 3 with HbV<sub>8</sub> vs. level 3 with HbV<sub>29</sub>.

Analysis of arterial blood gases (Table 2) showed a statistical increase in PO<sub>2</sub> after hemodilution and exchange transfusion. PaCO<sub>2</sub> was unchanged from baseline after hemodilution. Blood pH was not statistically changed. At level 3 exchange, BE was positive and not statistically different between HbV groups, but it was negative and statistically different from baseline for the rHSA group ( $P < 0.05$ ).

**Colligative properties.** Blood viscosities and COP after level 3 exchange were sampled at 1 h and 10 min after completion

Table 3. Rheological properties and COP

Fluid	Blood Viscosity, cp	Plasma Viscosity, cp	COP, mmHg	n
Blood	4.2±0.7	1.2±0.1	17.6±0.7	6
Level 2 rHSA	2.0±0.2*	0.9±0.1	17.2±0.8	4
Level 3 rHSA	1.6±0.2*	0.9±0.1	17.4±1.1	5
Level 3 HbV <sub>8</sub>	1.9±0.3*	1.0±0.1	17.3±0.8	5
Level 3 HbV <sub>29</sub>	2.0±0.4*	1.0±0.1	17.8±1.0	5

Values are means ± SD; n = no. of animals studied. Viscosity was measured at a shear rate of 160 s<sup>-1</sup> at 37°C. COP was measured at 27°C. Hct are presented in Table 2. \*P < 0.05 compared with nondiluted blood.

of the exchange. Table 3 shows that blood viscosity ranges from 1.6 cp (plasma 0.9 cp) for rHSA to 2.0 cP (plasma 1.0 cp) for the HbV groups.

All test materials caused COP to maintain the value for normal blood for this species (5), namely, 17.6 ± 0.7 mmHg at 1 h after the last exchange, showing that introduction of bulk solutions into the circulation caused minor fluid shifts.

**Microhemodynamics.** After level 3 exchange, arteriolar and venular diameters were not statistically different from baseline for any of the groups. Arteriolar flow velocities attained the highest value for the HbV<sub>8</sub> group, being 1.90 relative to baseline, which was statistically significant. The same effect was found in the venular microcirculation, where blood flow velocity was 2.20 relative to baseline. HbV<sub>29</sub> exchange transfusion lowered both arteriolar and venular velocities relative to the values attained at the level 2 exchange. However, venular velocity in this group was statistically significantly higher than in baseline. Notably, the level 2 hemodilution with rHSA caused significantly higher blood flow velocities in the arteriolar and venular microcirculation (Fig. 4).

Combining data for the RBC flow velocity and diameter allowed calculation of the arteriolar and venular blood flows (Fig. 5). The results of this calculation showed that all exchanges caused blood flow to increase. Arteriolar and venular blood flows at level 2 exchange with the use of rHSA were

significantly higher than those at baseline. However, continuing hemodilution with this material to level 3 exchange did not sustain the increase, and arteriolar and venular blood flow, although showing a tendency to remain elevated, were not statistically different from baseline values.

Level 3 exchange transfusion with HbV<sub>8</sub> and HbV<sub>29</sub> caused blood flow to be significantly higher than baseline. Furthermore, the HbV<sub>8</sub> group showed consistently higher blood flows than the HbV<sub>29</sub> group; however, the trend was not statistically significant.

**Functional capillary density.** The number of capillaries with RBC passage upon level 3 hemodilution in the rHSA, HbV<sub>8</sub>, and HbV<sub>29</sub> groups was 62 ± 9, 76 ± 12, and 72 ± 13% of baseline, respectively. These values were statistically different from baseline but not statistically different with respect to each other (Fig. 6).

**Microvascular oxygen distribution.** Oxygen tension measured using phosphorescence microscopy after level 3 exchange transfusion in the rHSA, HbV<sub>8</sub>, and HbV<sub>29</sub> groups showed that these materials produced virtually identical distributions of arteriolar microvascular Po<sub>2</sub> (arterioles averaged 49.5 mmHg), although HbV<sub>8</sub> tended to be higher (Fig. 7). The decrease of RBC from level 2 to level 3 did not decrease the arteriolar Po<sub>2</sub>. Venular Po<sub>2</sub> after level 3 was significantly lower than at level 2 exchange in all cases (rHSA, 7.2 ± 3.2 mmHg; HbV<sub>8</sub>, 15.1 ± 3.7 mmHg; HbV<sub>29</sub>, 9.6 ± 4.2 mmHg).

Tissue Po<sub>2</sub> values at level 3 exchange were consistently lower than those at level 2 exchange (20.1 ± 2.2 mmHg), with the difference being statistically significant. The highest was attained by the HbV<sub>8</sub> group, being 14.0 ± 2.2 mmHg. By comparison, tissue Po<sub>2</sub> for the HbV<sub>29</sub> group was 9.2 ± 2.7 mmHg and for the rHSA group, 2.6 ± 1.4 mmHg, which was significantly lower compared with the HbV<sub>8</sub> and HbV<sub>29</sub> groups (Fig. 7).

**Oxygen delivery and extraction.** Figure 8 shows the results of the analysis for delivery and release of oxygen by the

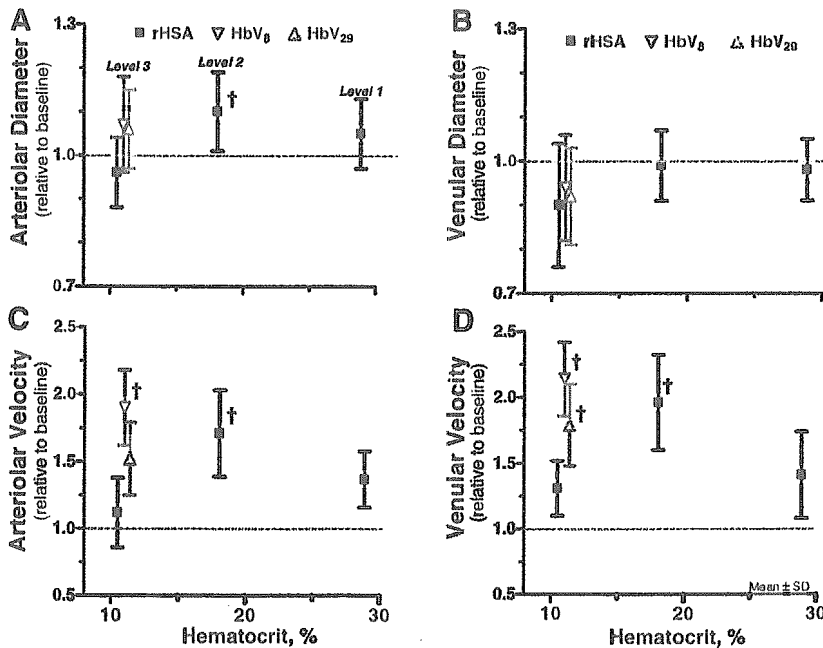
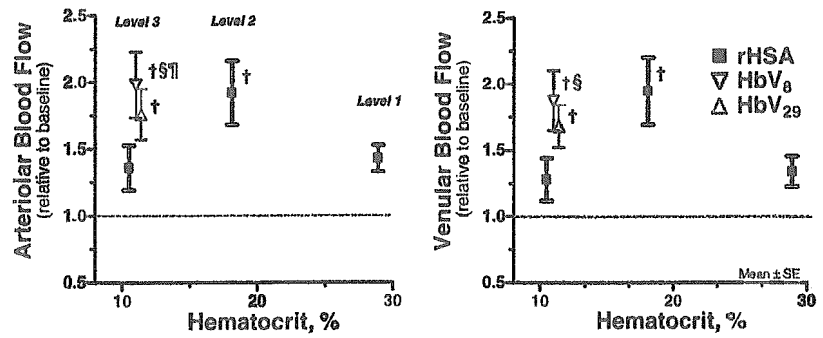


Fig. 4. Changes relative to baseline in arteriolar and venular hemodynamics at the level 1, level 2, and level 3 exchanges. Dashed lines represent baseline level. †P < 0.05 relative to baseline. Arteriolar (A) and venular (B) diameters (μm, means ± SD, n = no. of vessels studied) in each animal group were as follows. Baseline: arterioles (A), 61.2 ± 10.2, n = 80; venules (V), 62.0 ± 12.3, n = 80. Level 1 with rHSA: A, 65.6 ± 12.4; V, 61.1 ± 12.4. Level 2 with rHSA: A, 66.1 ± 14.6; V, 64.3 ± 15.2. Level 3 with rHSA: A, 63.7 ± 14.4, n = 20; V, 61.0 ± 16.7, n = 20. Level 3 with HbV<sub>8</sub>: A, 64.9 ± 15.7, n = 20; V, 64.6 ± 18.2, n = 20. Level 3 with HbV<sub>29</sub>: A, 66.1 ± 16.2, n = 20; V, 63.1 ± 18.0, n = 20. Arteriolar (C) and venular (D) RBC velocities (mm/s, means ± SD) in each animal group were as follows. Baseline: A, 4.9 ± 1.3; V, 1.7 ± 0.5. Level 1 with rHSA: A, 6.3 ± 1.4; V, 2.0 ± 0.7. Level 2 with rHSA: A, 7.9 ± 1.5; V, 2.6 ± 0.9. Level 3 rHSA: A, 5.2 ± 2.0, n = 20; V, 1.9 ± 1.1, n = 20. Level 3 with HbV<sub>8</sub>: A, 7.2 ± 1.8, n = 20; V, 3.3 ± 1.0, n = 20. Level 3 with HbV<sub>29</sub>: A, 7.0 ± 1.7, n = 20; V, 3.0 ± 0.9, n = 20.

Fig. 5. Arteriolar and venular flow (nl/s, means  $\pm$  SD,  $n$  = no. of vessels studied) in each animal group were as follows. Baseline: arterioles (A),  $14.8 \pm 7.1$ ,  $n = 76$ ; venules (V),  $5.0 \pm 2.9$ ,  $n = 76$ . Level 1 with rHSA: A,  $21.9 \pm 9.7$ ; V,  $5.8 \pm 3.6$ . Level 2 with rHSA: A,  $27.2 \pm 16.1$ ; V,  $8.3 \pm 4.2$ . Level 3 with rHSA: A,  $16.9 \pm 6.8$ ,  $n = 20$ ; V,  $5.4 \pm 4.8$ ,  $n = 20$ . Level 3 with HbV<sub>8</sub>: A,  $23.4 \pm 8.1$ ,  $n = 18$ ; V,  $9.9 \pm 5.1$ ,  $n = 18$ . Level 3 with HbV<sub>29</sub>: A,  $21.0 \pm 8.0$ ,  $n = 18$ ; V,  $8.3 \pm 5.2$ ,  $n = 18$ .



microcirculation. It is apparent that exchanging RBC for HbV<sub>8</sub> maintains oxygen delivery to the tissue, whereas HbV<sub>29</sub> reduces this by  $\sim 20\%$ , and continued hemodilution with a non-oxygen-carrying material significantly depresses oxygen delivery to the tissue, reducing this to half of that attained at the level 2 hemodilution.

#### DISCUSSION

The principal finding of this study is that under identical extreme hemodilution conditions, with the use of vesicles encapsulating Hb with normal  $P_{50}$  (HbV<sub>29</sub> = 29 mmHg) and low  $P_{50}$  (HbV<sub>8</sub> = 8 mmHg), tissue  $P_{O_2}$  is statistically significantly higher when the high oxygen affinity material is used, namely,  $14.0 \pm 2.2$  vs.  $9.2 \pm 2.7$  mmHg. The significantly increased tissue  $P_{O_2}$  attained with HbV<sub>8</sub> appears to be due to a series of incremental improvements in microvascular and macrovascular hemodynamics comprising the increase of arteriolar blood flow and mean arterial blood pressure, which was significantly higher ( $P < 0.05$ ) for HbV<sub>8</sub> than for HbV<sub>29</sub>.

In the hemodilution procedures of this study, blood was exchanged with a rHSA solution as a colloidal plasma expander, which was the same suspending medium used for the Hb vesicles. Therefore, in these experiments, we can make a direct comparison between an oxygen-carrying and non-oxy-

gen-carrying blood substitute, uncomplicated by the presence of additional materials. Our results show that the level 2 hemodilution with rHSA leads to maintained functional capillary density and significantly improved arteriolar and venular blood flow, although somewhat lowered central blood pressure. The latter finding is not necessarily negative and may reflect a lowered overall peripheral vascular resistance due to the decrease of blood viscosity after hemodilution. The fact that microvascular flow is significantly increased indicates that the level 2 hemodilution with rHSA provides the tissue with adequate microvascular perfusion and that this colloid is an adequate plasma expander.

Average oxygen delivery and extraction were somewhat greater for HbV<sub>8</sub> than for HbV<sub>29</sub>. These are calculated values and are not statistically significantly different; however, the same difference was found in all micro and macro parameters measured in this study.

The level 2 hemodilution and the succeeding level 3 hemodilution with either HbV<sub>8</sub> and HbV<sub>29</sub> resulted in the same total Hb concentration in the circulation (5.7 and 5.8 g Hb/dl); however, oxygen delivery was lower with HbV<sub>29</sub> and lowest with rHSA, as might be expected due to the low Hb content (3.7 g Hb/dl) in the absence of plasma Hb for the rHSA group. Therefore, because all groups had the same Hct at the level 3 hemodilution, the sustained oxygen consumption and tissue  $P_{O_2}$  relative to the rHSA group clearly demonstrate that Hb vesicles release oxygen. However, the vesicles with the lowest  $P_{50}$  provide an oxygen delivery capacity identical to that of blood at level 2 hemodilution, whereas vesicles with a high  $P_{50}$  lower oxygen delivery at the microcirculatory level, an effect probably caused by the decreased blood flow associated with HbV<sub>29</sub>.

The differences in tissue  $P_{O_2}$ , mean arterial blood pressure, and arteriolar blood flow between HbV<sub>8</sub> and HbV<sub>29</sub> show that in designing a blood substitute, it is not sufficient to provide adequate oxygen-carrying capacity. Once a suitable oxygen carrier is available, it also must be able to maintain or enhance other circulatory transport parameters, particularly flow. The Hb vesicles used in this study are vasoinactive, and the difference in  $P_{50}$  appears to be a factor in improving flow condition that is not related to vasoactivity. An explanation for this may be related to the inherent variability of tissue  $P_{O_2}$  shown in this and other studies (4, 22), which may be enhanced in extreme hemodilution. This variability determines that if average tissue  $P_{O_2}$  is low, portions of the tissue may become anoxic. Introducing a small quantity of a low- $P_{50}$  Hb oxygen carrier into the circulation will deliver oxygen only to those parts of the tissue

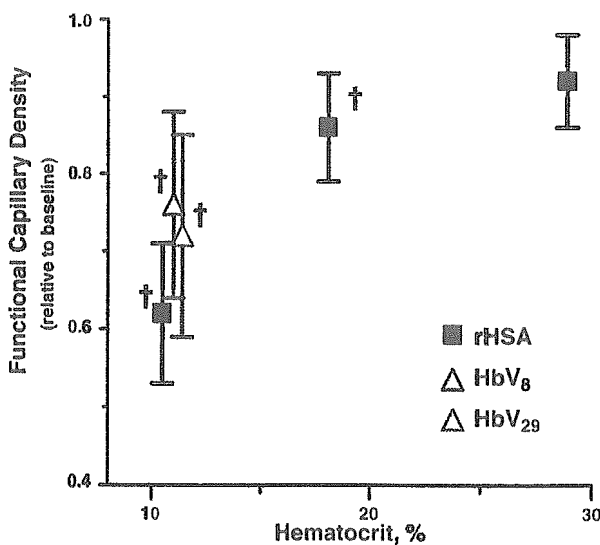


Fig. 6. Functional capillary density after the level 1, level 2, and level 3 exchanges for the different test fluids. All values are relative to baseline levels. † $P < 0.05$  relative to baseline.

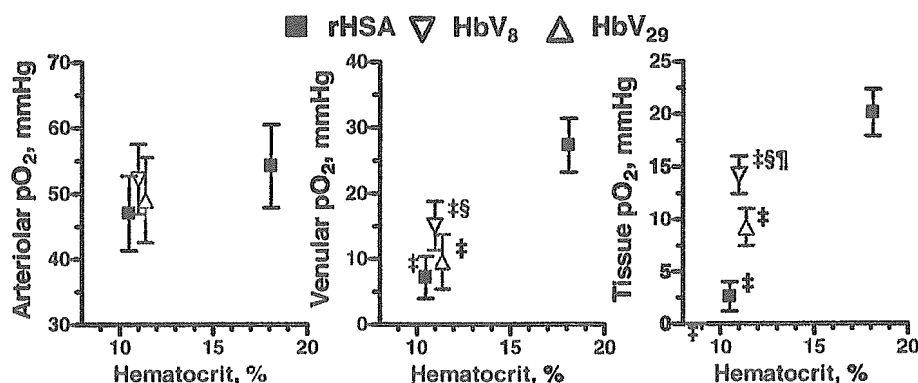


Fig. 7. Intravascular PO<sub>2</sub> after the level 2 and level 3 hemodilutions. Values are presented as means  $\pm$  SD. ‡*P* < 0.05 compared with level 2 with rHSA; §*P* < 0.05 compared with level 3 with rHSA; ¶*P* < 0.05, level 3 with HbV<sub>8</sub> vs. level 3 with HbV<sub>29</sub>.

where the anoxic threshold is passed, thus eliminating the inherent variability of oxygen delivery shown by the variability of tissue PO<sub>2</sub>.

Considering the significantly improved blood pressure and the trend toward higher flow for HbV<sub>8</sub> (in the absence of vasoconstriction and changes in the rheological properties of blood), it is possible that in conditions of extreme hemodilution the cardiac function should be improved because of the proposed more homogenous heart tissue oxygenation using HbV<sub>8</sub> vs. HbV<sub>29</sub>.

In summary, the present results show that either HbV<sub>8</sub> or HbV<sub>29</sub> are efficient oxygen carriers that do not cause vasoactivity. The experiments were carried out using rHSA as a hemodiluent, and this material was adequate as a plasma volume substitute. Oxygen extraction was similar for both oxygen carriers; however, HbV<sub>8</sub> appeared to be beneficial at the systemic level, because base excess remained at baseline levels, whereas it was decreased for HbV<sub>29</sub>. This finding suggests that improved tissue PO<sub>2</sub> and microcirculatory oxygen delivery may be efficient in other tissues. The improvement obtained may be specific to the conditions of these experiments

in which the vesicles were tested for their capacity to restore tissue PO<sub>2</sub>, FCD, and oxygen extraction in the microcirculation during extreme hemodilution. The significant differences in the tissue oxygen parameters produced by the presence of low-P<sub>50</sub> Hbs vs. an identical oxygen carrier with normal P<sub>50</sub> suggests that small amounts of Hbs with high oxygen affinity may have therapeutic effects in the treatment of ischemic conditions (6).

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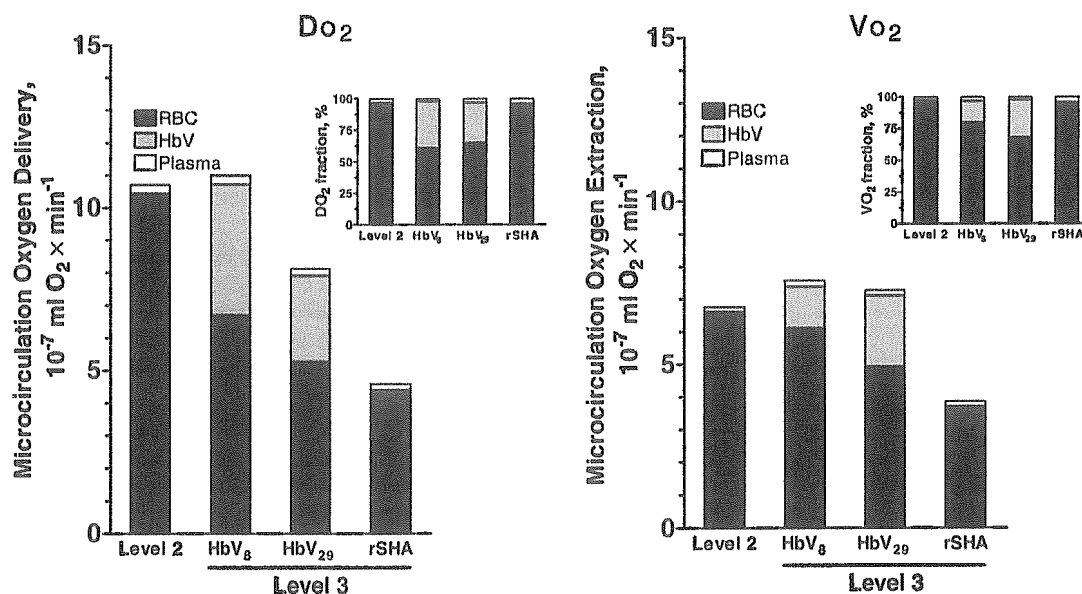


Fig. 8. Arterial oxygen delivery (DO<sub>2</sub>) and extraction (VO<sub>2</sub>) before and after the level 3 hemodilution. Calculations of global oxygen transport are not directly measurable in our model; however, the changes relative to baseline can be calculated using the measured parameters. These calculations can be identified as those presented without standard deviations to focus on their tendencies rather than on the variability of the measurement.

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# New generation of hemoglobin-based oxygen carriers evaluated for oxygenation of critically ischemic hamster flap tissue

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**Objectives:** The aim of this study was to investigate and compare the effects of a traditionally formulated, low-viscosity, right-shifted polymerized bovine hemoglobin solution and a highly viscous, left-shifted hemoglobin vesicle solution (HbV-HES) on the oxygenation of critically ischemic peripheral tissue.

**Design:** Randomized, prospective study.

**Setting:** University laboratory.

**Subject:** A total of 40 male golden Syrian hamsters.

**Interventions:** Island flaps were dissected from the back skin of anesthetized hamsters. The flap included a critically ischemic, hypoxic area that was perfused via a collateralized vasculature. One hour after completion of the preparation, the animals received a 33% blood exchange with 6% hydroxyethyl starch 200/0.5 (HES, n = 9), HbV suspended in HES (HbV-HES, n = 8), or polymerized bovine hemoglobin solution (n = 9).

**Measurements and Main Results:** Three hours after the blood exchange, microcirculatory blood flow (laser-Doppler flowmetry) was increased to 262% of baseline for HbV-HES ( $p < .01$ ) and

197% for polymerized bovine hemoglobin solution ( $p < .05$  vs. baseline and HbV-HES). Partial tissue oxygen tension (bare fiber probes) was only improved after HbV-HES (9.4 torr to 14.2 torr,  $p < .01$  vs. baseline and other groups). The tissue lactate/pyruvate ratio (microdialysis) was elevated to 51 in the untreated control animals, and to  $34 \pm 8$  after HbV-HES ( $p < .05$  vs. control) and  $38 \pm 11$  after polymerized bovine hemoglobin solution (not significant).

**Conclusions:** Our study suggests that in critically ischemic and hypoxic collateralized peripheral tissue, oxygenation may be improved by normovolemic hemodilution with HbV-HES. We attributed this improvement to a better restoration of the microcirculation and oxygen delivery due to the formulation of the solution. (Crit Care Med 2005; 33:806-812)

**KEY WORDS:** arterial occlusive diseases; blood substitutes; collateral circulation; energy metabolism; microcirculation; surgical flaps

Occlusion of the anatomic blood supply may lead to critical ischemia in a variety of organs and tissues. Functionality and survival of these tissues depend on the maintenance of adequate oxygen delivery via a collateral vasculature, which is determined by its perfusion and oxygen content.

Artificial oxygen carriers, first developed >30 yrs ago, were created to increase the oxygen content of arterialized blood without risking the adverse effects associated with blood transfusions (1-3). These products were designed to meet the physicochemical properties of normal blood as closely as possible. However, a number of drawbacks have hindered their introduction into clinical practice. One major problem was the vasoconstrictor effect of cell-free hemoglobins, presumably due to their scavenging of nitric oxide (NO) (1-3). This adverse effect has been circumvented by chemical modification of the hemoglobin molecules. Hemopure (Biopure, Cambridge, MA) is one of two chemically modified hemoglobin products currently awaiting U.S. Food and Drug Administration approval after having been extensively tested in clinical trials as a replacement for blood transfusions (4). Hemopure has been approved for routine clinical use in South Africa. Oxyglobin, its veterinary equivalent, was introduced to the U.S. market in 1998. It is a polyionic colloidal

fluid consisting of glutaraldehyde-polymerized, ultrapurified bovine hemoglobin (PBHb) suspended in a modified Ringer's solution. Oxyglobin contains 13 g/dL hemoglobin (Table 1). Hemoglobin concentration, osmolarity, and viscosity are in a physiologic range, whereas colloid osmotic pressure was set higher and oxygen affinity was right-shifted ( $P_{50} = 54$  torr) (5) with the scope of blood volume expansion and facilitated oxygen release.

In past years, a new concept has emerged in which oxygen carriers are regarded as oxygen therapeutics rather than as blood substitutes (2, 3, 5-8). Still, in an attempt to avoid blood transfusions, the new generation of artificial oxygen carriers are used to improve the microcirculation and redistribute oxygen in favor of the tissues in need without necessarily augmenting total hemoglobin concentration. For this purpose, increased colloid osmotic pressure, viscosity, and oxygen affinity of the oxygen-carrying solutions proved to be advantageous and more relevant than

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Table 1. Physicochemical characteristics of hamster blood and the diluents

	Hamster Blood	Hamster Plasma	HES	HbV-HES	PBHb
Hb concentration, g/dL	18		0	7.5	13
Mean particle diameter, nm	7,000			250	10
Mean molecular mass, kDa				3,000,000	180
P <sub>50</sub> , torr	28			15	54
metHb concentration, %				5.2	5.0
Oncotic pressure, mm Hg		18	36	36	38
Viscosity of solution, cP	4.5	1.2	1.9	11.5	1.8
Viscosity in circulation, cP		1.34	1.31	1.68 <sup>a</sup>	1.42 <sup>a,b</sup>

HES, 6% hydroxyethyl starch; HbV-HES, hemoglobin vesicles suspended in HES; PBHb, polymerized bovine hemoglobin; Hb, hemoglobin; metHb, methemoglobin.

<sup>a</sup>*p* < .01 vs. hamster plasma; <sup>b</sup>*p* < .01 vs. HbV-HES. Viscosity of the solutions was measured at 37°C and at 150 s<sup>-1</sup>; plasma viscosity was measured at 25°C; data are mean values.

raising the hemoglobin concentration in blood, as evidenced by a series of experimental shock studies (5, 7, 8). It seems conceivable that the concept of targeting oxygen delivery to ischemic tissues may be particularly applicable in an acute situation of local tissue ischemia due to peripheral arterial occlusion, serving as a bridge until adequate perfusion can be re-established either by spontaneous neovascularization or surgical revascularization. In previous studies, we were able to demonstrate that oxygenation in collateralized, ischemic hamster flap tissue was improved after isovolemic hemodilution with highly viscous, highly oncotic solutions containing left-shifted, encapsulated human hemoglobins (9, 10). Encapsulation represents a new alternative to chemical modification and was achieved by a phospholipid bilayer membrane coated with polyethylene glycol (11).

The aim of this study was to compare the effects of a traditionally formulated polymerized bovine hemoglobin solution (Oxyglobin) and a highly viscous solution containing a low concentration of left-shifted encapsulated hemoglobins (the new generation of oxygen carriers), both administered in the course of intentional isovolemic hemodilution, on the oxygenation of acutely and critically ischemic peripheral tissues in the well-established

hamster-flap model described previously (9, 10, 12). Due to its widespread clinical use, hydroxyethyl starch (HES) was chosen as the diluent in which the encapsulated hemoglobins (hemoglobin vesicles, HbV) were suspended. Microcirculatory blood flow, partial tissue oxygen tension, tissue lactate concentration, and lactate/pyruvate ratio were taken as end points.

MATERIALS AND METHODS

**Animals and Solutions.** Experiments were performed according to the National Institutes of Health guidelines for the care and use of laboratory animals and with the approval of the local animal ethics committee. A total of 40 male Syrian golden hamsters (Charles River, Sulzfeld, Germany) weighing 75–110 g were included in this study. The animals were randomly assigned and equally distributed to the control group and three groups subjected to normovolemic hemodilution with 6% HES 200/0.5 (Fresenius, Stans, Switzerland), HbV suspended in HES (HbV-HES), and PBHb, respectively. HbV was prepared as described previously (11). PBHb (Oxyglobin) was purchased from Biopure. The physicochemical characteristics of the solutions are described in Table 1.

**Animal and Flap Preparation.** A hamster skin-flap model was used as previously described in detail (9, 10, 12). Anesthesia was induced by pentobarbital injected intraperitoneally (100 mg/kg body weight, Nembutal, Abbott Laboratories, Chicago, IL). The carotid artery and external jugular vein were cannulated for administration of anesthesia, blood exchange, laboratory analysis, and monitoring blood pressure. Catheterization and flap dissection were performed with the aid of an

Table 2. Systemic and laboratory data at baseline and at 1 and 3 hrs after blood exchange

	Baseline	1 hr	3 hrs
MAP, mm Hg			
Control	91 ± 6	90 ± 5	83 ± 11 <sup>a</sup>
HES	93 ± 6	92 ± 6	84 ± 7 <sup>a</sup>
HbV-HES	94 ± 3	92 ± 3	80 ± 7 <sup>b</sup>
PBHb	95 ± 5	92 ± 3	93 ± 6
Hematocrit			
Control	0.55 ± 0.04	0.54 ± 0.03	0.52 ± 0.03
HES	0.59 ± 0.04	0.31 ± 0.04 <sup>b</sup>	0.31 ± 0.04 <sup>b</sup>
HbV-HES	0.59 ± 0.04	0.31 ± 0.02 <sup>b</sup>	0.33 ± 0.04 <sup>b</sup>
PBHb	0.58 ± 0.05	0.32 ± 0.05 <sup>b</sup>	0.31 ± 0.04 <sup>b</sup>
Total Hb concentration, g/dL			
Control	17.8 ± 1.2	17.2 ± 1.0	17.0 ± 1.1
HES	17.8 ± 1.3	9.7 ± 0.9 <sup>b</sup>	10.5 ± 1.0 <sup>b</sup>
HbV-HES	18.8 ± 0.7	11.2 ± 1.3 <sup>b</sup>	12.1 ± 1.0 <sup>b</sup>
PBHb	18.0 ± 1.8	13.6 ± 0.9 <sup>b</sup>	11.9 ± 1.1 <sup>b</sup>
P <sub>O2</sub> , torr			
Control	43 ± 3	44 ± 6	43 ± 4
HES	43 ± 13	56 ± 10	64 ± 13 <sup>b</sup>
HbV-HES	43 ± 4	51 ± 13	68 ± 9 <sup>b</sup>
PBHb	43 ± 8	53 ± 9	61 ± 11 <sup>b</sup>
P <sub>CO2</sub> , torr			
Control	58 ± 8	58 ± 9	56 ± 8
HES	57 ± 3	56 ± 3	47 ± 13 <sup>a</sup>
HbV-HES	53 ± 4	51 ± 4	41 ± 3 <sup>b</sup>
PBHb	57 ± 5	53 ± 4 <sup>a</sup>	47 ± 5 <sup>b</sup>
pH			
Control	7.28 ± 0.06	7.29 ± 0.06	7.29 ± 0.06
HES	7.23 ± 0.03	7.27 ± 0.03 <sup>a</sup>	7.31 ± 0.07 <sup>b</sup>
HbV-HES	7.27 ± 0.03	7.30 ± 0.03 <sup>a</sup>	7.34 ± 0.02 <sup>b</sup>
PBHb	7.25 ± 0.02	7.30 ± 0.05 <sup>a</sup>	7.34 ± 0.06 <sup>b</sup>

MAP, mean arterial pressure; HES, 6% hydroxyethyl starch; HbV-HES, hemoglobin vesicles suspended in HES; PBHb, polymerized bovine hemoglobin.

<sup>a</sup>*p* < .05 and <sup>b</sup>*p* < .01 vs. baseline. Values displayed as mean ± sd.

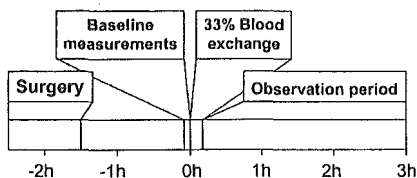


Figure 1. Diagram illustrating sequence of interventions.

Table 3. Microvascular diameters (in micrometers) at baseline

	Control	HES	HbV-HES	PBHb
Flap artery	112 ± 15	122 ± 18	114 ± 8	118 ± 17
Anatomically perfused tissue				
Conduit arterioles	57 ± 8	58 ± 14	54 ± 12	56 ± 15
End arterioles	8.4 ± 3.2	8.9 ± 3.4	7.7 ± 2.7	7.8 ± 2.3
Ischemic tissue				
Conduit arterioles	56 ± 9	54 ± 15	60 ± 10	54 ± 17
End arterioles	8.4 ± 3.2	8.9 ± 3.4	7.7 ± 2.7	7.8 ± 2.3

HES, 6% hydroxyethyl starch; HbV-HES, hemoglobin vesicles suspended in HES; PBHb, polymerized bovine hemoglobin.  
Values are mean ± SD.

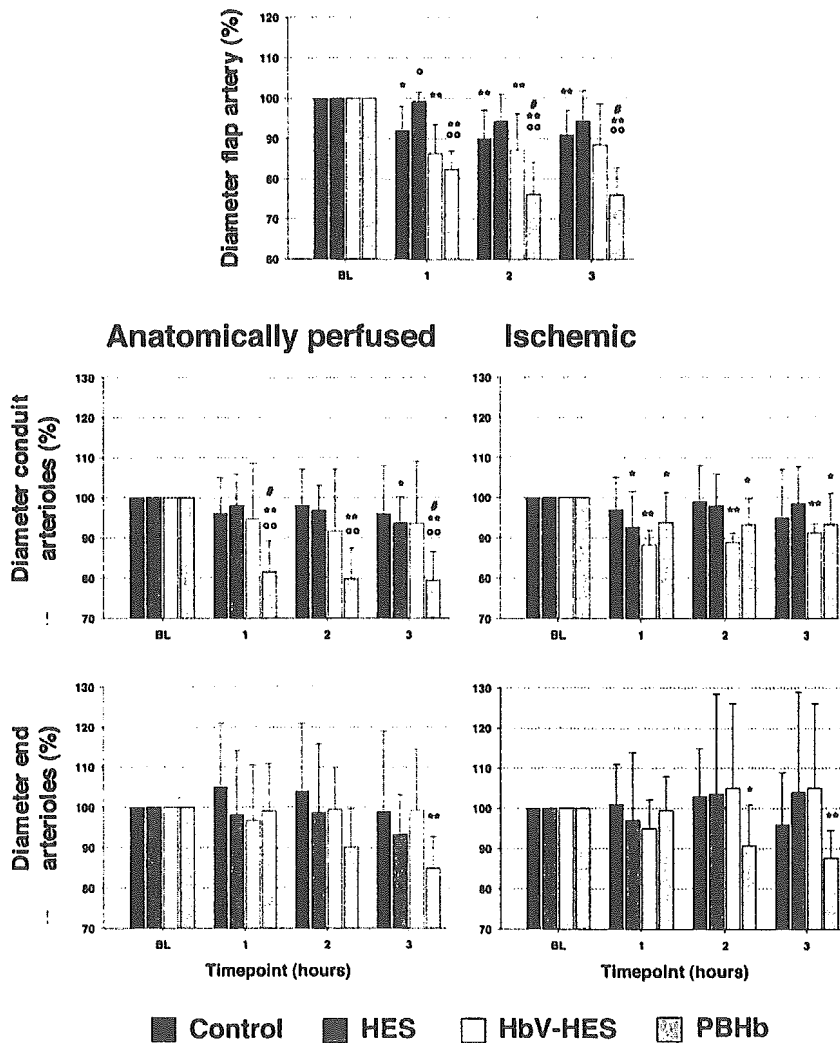


Figure 2. Microvascular diameters in flap artery, conduit arterioles, and end arterioles in the anatomically perfused and ischemic tissues at baseline (BL) and after hemodilution with 6% hydroxyethyl starch (HES), hemoglobin vesicles suspended in HES (HbV-HES), and polymerized bovine hemoglobin (PBHb). Data are given as a percentage of baseline and represent mean values and SD. \* $p < .05$ , \*\* $p < .01$  vs. baseline; ° $p < .05$ , °° $p < .01$  vs. control; # $p < .05$  vs. HbV-HES.

operating microscope at  $\times 10$  magnification (Wild, Heerbrugg, Switzerland). An island flap measuring  $3 \times 2$  cm was dissected from the

shaved and epilated back skin of the animal. The flap consisted of skin and a thin layer of panniculus carnosus muscle and was perfused

by one vascular axis that bifurcated into two equal-sized branches within the flap, each of them supplying a separate vascular territory. One of the branches was transected after being secured with microsurgical ligatures, thus rendering the corresponding vascular territory ischemic. This tissue was perfused by a collateral vasculature connecting the two vascular networks. During surgery, the flap was irrigated with 0.9% NaCl to prevent the flap from drying out. The animal was placed on a specially designed Plexiglas stage including a platform for fixation of the flap.

**Laboratory Analysis.** Blood samples were taken from the carotid artery catheter and collected in heparin-washed microtubes for immediate measurements of total hemoglobin concentration, pH and systemic arterial  $P_{O_2}$  and  $P_{CO_2}$  (ABL 625, Radiometer, Copenhagen, Denmark). Hematocrit was determined by centrifugation. The colloid osmotic pressure of the diluents was measured with a colloid osmometer (model 4420, Wescor, Logan, UT) with a 30,000 d cutoff membrane. The viscosity was measured with a cone-plate viscometer (PVII+, Brookfield Engineering, Middleboro, MA) or a capillary rheometer (Anton Parr DCS 300, Parr Physica, Graz, Austria) at 37°C. Viscosity of blood and plasma were measured 3 hrs after hemodilution with a Höppler-type viscosimeter (HAAKE Messtechnik, Karlsruhe, Germany).

**Microhemodynamics and Partial Tissue Oxygen Tension.** Investigations were performed using an intravital microscope (Axio-plan 1, Zeiss, Jena, Germany). Microscopic images were captured by a television camera (Intensified CCD camera, Kappa Messtechnik, Gleichen, Germany), recorded on video (50 Hz, Panasonic, Osaka, Japan), and displayed on a television screen (Trinitron PVM-1454QM, Sony, Tokyo, Japan). The preparation was observed visually with a  $\times 40$  objective resulting in a total optical magnification of  $\times 909$  on the video monitor. Transilluminating with a green filter produced a well-defined image of the width of the erythrocyte column, which could then be measured manually on the television screen. A mapping of the vasculature was made before the baseline measurements were taken to allow for repeated measurements of diameters on exactly the same vessel and location. The arterioles were classified according to physiologic and anatomic features into conduit arterioles and end arterioles (9, 10, 12).

We used combined bare fiber probes (Oxy-lite probes, Oxford Optronix, Oxford, UK) to measure tissue oxygen tension, temperature, and microvascular blood flow continuously. Microvascular blood flow was measured with two 230- $\mu$ m fibers. The sensitive tip of the oxygen probe (100  $\mu$ m in diameter) consisted of Ruthenium-III-(tris)-chloride, which measured  $P_{O_2}$  by fluorescence quenching of the dye. A T-type thermocouple was attached to the probe, which was coated with a biocompatible sleeve of polyurethane. According to

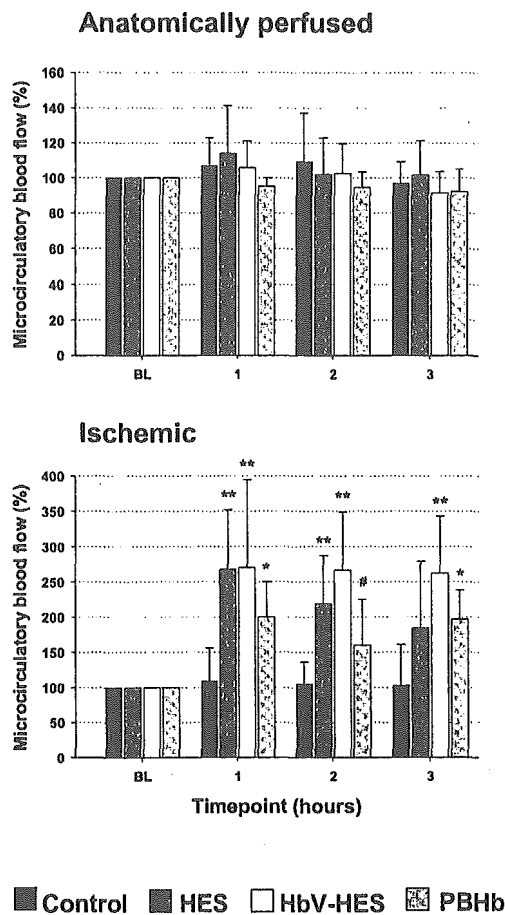


Figure 3. Microcirculatory blood flow in the anatomically perfused and ischemic tissues at baseline (BL) and after hemodilution with 6% hydroxyethyl starch (HES), hemoglobin vesicles suspended in HES (HbV-HES), and polymerized bovine hemoglobin (PBHb). Data are given as a percentage of baseline and represent mean values and sd. \* $p < .05$ , \*\* $p < .01$  vs. baseline and control; # $p < .05$  vs. HbV-HES.

the manufacturer, the bare fiber probe provides resolutions of  $<1$  torr and  $0.1^{\circ}\text{C}$  for partial oxygen tension and temperature, respectively. The data on blood flow were displayed in arbitrary perfusion units and further processed into percentages of the baseline.

**Carbohydrate Metabolite Concentrations.** The interstitial concentrations of glucose, pyruvate, and lactate were assessed by microdialysis, as previously described (13, 14). The system used in our study included microprobes (CMA/20, CMA Microdialysis AB, Stockholm, Sweden) carrying a microcell that was perfused by a microinjection pump (CMA/100, CMA Microdialysis AB). The molecular cutoff of the membrane was 20,000 d. This pore size does not allow the tested Hb compounds to penetrate into the microcell, as confirmed by preliminary pilot experiments. The outlet tube was connected to a refrigerated fraction collector (CMA/200 F, CMA Microdialysis AB) in which the dialysates were collected in microvials, stored at  $4^{\circ}\text{C}$ , and further processed for laboratory analysis (CMA 600, CMA Microdialysis AB). The microcell was continuously

perfused with isotonic Ringer's solution at a flow rate of  $0.75 \mu\text{L}/\text{min}$ , which resulted in a time delay from the membrane to the microvial of 7 mins. The sampling time was set at 60 mins. Before each experiment, the probes were equilibrated according to the guidelines of the supplier.

**Protocol.** The animals were kept under light anesthesia with a continuous infusion of  $50 \text{ mg}/\text{mL}$  pentobarbital given at a rate of approximately  $0.5 \text{ mg}\cdot\text{min}^{-1}\cdot\text{kg}$  body weight $^{-1}$  throughout the experiment. The depth of anesthesia was regulated by tolerating a noxious reflex due to pinching of the hind paw, but no nonaversive reflexes (palpebral, corneal, and jaw reflex) (12). With a heating pad and the room's temperature at  $28^{\circ}\text{C}$ , the temperatures of the animals were kept constant at  $32^{\circ}\text{C}$ , which was verified with a microthermometer placed on the abdominal skin.

Microdialysis and Oxylite probes were inserted subcutaneously in the middle of each vascular territory of the flap. The timing of the interventions is illustrated in Figure 1. The

collection of the microdialysates started after a 30-min stabilization period. Another hour later, the baseline values were obtained. Thereafter, one third of the total blood volume was exchanged with HES or the oxygen-carrying solutions. This was achieved by simultaneous blood withdrawal via the carotid catheter and infusion via the jugular catheter over 15 mins. Exclusion criteria were abnormalities of the vascular anatomy, insufficient optical clarity, mean arterial pressure of  $<60$  mm Hg, and systemic arterial pH,  $\text{Po}_2$ , and  $\text{PCo}_2$  outside the normal ranges at baseline (7.19–7.29, 35–55 torr, and 45–65 torr, respectively). The animals were killed with an overdose of pentobarbital at the end of the experiment.

**Statistical Analysis.** The InStat version 3 program (Graph Pad Software, San Diego, CA) was used for statistical analysis. The data were presented as mean  $\pm$  sd. The time-related differences between repeat measurements were assessed by the paired analysis of variance, followed by the Dunnett's posttest. Differences between the groups were assessed by unpaired analysis of variance and Tukey's posttest. A value of  $p < .05$  was taken to represent statistical significance.

## RESULTS

Six animals (two control animals, one in the HES group, two in the HbV-HES group, and one in the PBHb group) did not fulfill the inclusion criteria and were excluded from this study. The systemic data are presented in Table 2. Mean arterial pressure gradually declined during the experiment in all groups ( $p < .05$ ) except the Oxyglobin group, in which mean arterial pressure remained virtually unchanged ( $p < .05$  vs. other groups). Similar hematocrits were obtained in all hemodiluted animals. After the 33% blood exchange with HES, Hb concentration was reduced to  $9.7 \pm 0.9 \text{ g}/\text{dL}$ , whereas the addition of HbV to the diluent enhanced the total Hb concentration to  $11.2 \pm 1.3 \text{ g}/\text{dL}$  ( $p < .05$  vs. HES), and hemodilution with PBHb resulted in a total Hb concentration of  $13.6 \pm 0.9 \text{ g}/\text{dL}$  ( $p < .05$  vs. HbV-HES). However, this difference became less pronounced over time. Hemodilution increased mean  $\text{Po}_2$  from 43 torr to mean values of  $>60$  torr and mean pH from 7.23–7.27 to mean values of 7.31–7.34, whereas mean  $\text{PCo}_2$  decreased from 53–57 torr to 41–47 torr (all  $p < .01$  vs. baseline).

At baseline, the microvascular diameters were similar in all groups (Table 3). The behaviors of the microvascular diameters are shown in Figure 2. The diameters were gradually reduced over time in

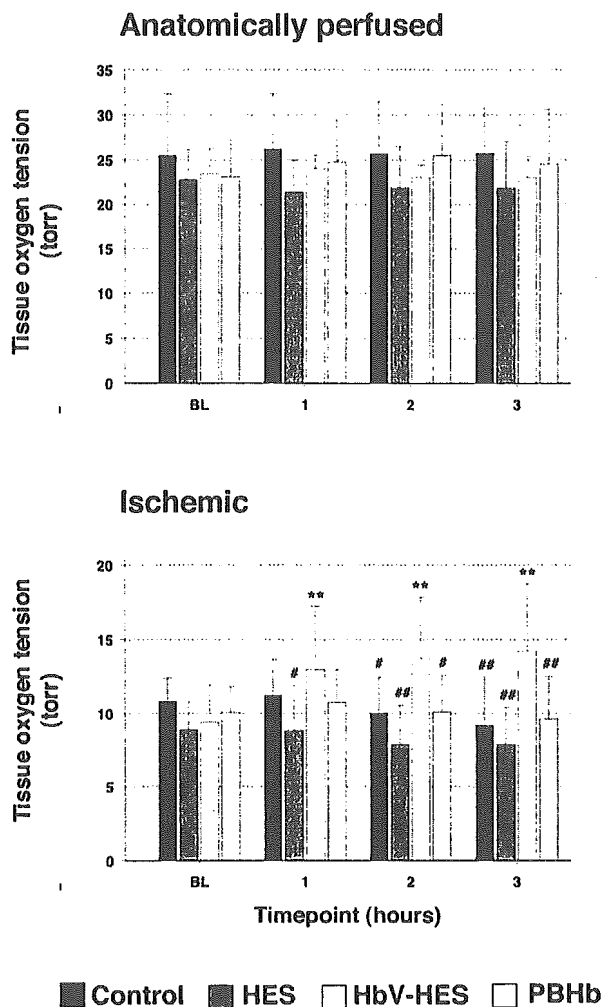


Figure 4.  $P_{O_2}$  in the anatomically perfused and ischemic tissues at baseline (BL) and after hemodilution with 6% hydroxyethyl starch (HES), hemoglobin vesicles suspended in HES (HbV-HES), and polymerized bovine hemoglobin (PBHb). Data represent mean values and SD. \*\* $p < .01$  vs. baseline and control; # $p < .05$ , ## $p < .01$  vs. HbV-HES.

the flap artery in all animals (not significant for HES,  $p < .01$  for other groups) but most substantially in the PBHb group, where they reached  $76\% \pm 7\%$  of baseline ( $p < .01$  vs. control,  $p < .05$  vs. HbV-HES). In the anatomically perfused tissue, diameters were decreased to  $79\% \pm 7\%$  in the conduit arterioles ( $p < .01$  vs. baseline and control,  $p < .05$  vs. HbV-HES) and  $85\% \pm 8\%$  ( $p < .01$  vs. baseline) in the end arterioles after receiving PBHb, whereas no significant differences were observed between the other groups. A similar yet less pronounced pattern was seen in the collateralized arterioles ( $p < .01$  vs. baseline for PBHb).

The mean baseline laser-Doppler signals ranged between 61 and 114 perfusion units in the anatomically perfused

tissue and between 3 and 17 perfusion units in the ischemic part. Microcirculatory blood flow remained virtually unchanged in the anatomically perfused tissue in all groups (Fig. 3), whereas mean blood flow was maximally increased after hemodilution by 168% for HES ( $p < .01$ ), 170% for HbV-HES ( $p < .01$ ), and 100% for PBHb ( $p < .05$  vs. baseline and HbV-HES).

Oxygen tension was significantly reduced in the ischemic tissue compared with the anatomically perfused part ( $p < .01$ ) (Fig. 4). It remained at baseline levels in both parts of the flap and in all groups except for HbV-HES, which showed a  $P_{O_2}$  increase from  $9.4 \pm 2.5$  torr to  $14.2 \pm 4.5$  torr in the ischemic tissue ( $p < .01$  vs. baseline and other groups).

Glucose concentrations were gradually decreased in the anatomically perfused tissue over time (Fig. 5), reaching  $1.5 \pm 1.1$  mmol/L in the control group ( $p < .01$ ),  $2.7 \pm 1.2$  mmol/L for HES (not significant),  $3.1 \pm 1.4$  mmol/L for HbV-HES (not significant,  $p < .05$  vs. control), and  $2.2 \pm 0.9$  mmol/L for PBHb ( $p < .01$ ). The reductions were more accentuated in the ischemic tissue, at  $0.8 \pm 0.6$  mmol/L in the control group ( $p < .01$ ),  $1.4 \pm 0.9$  mmol/L in the HES group ( $p < .01$ ),  $1.6 \pm 1.4$  mmol/L in the HbV-HES group (not significant), and  $1.3 \pm 0.7$  mmol/L in the PBHb group ( $p < .01$ ). At baseline, the mean lactate concentrations ranged between 1.7 and 2.2 mmol/L in both parts of the tissue in all groups. The values remained virtually stable in the anatomically perfused tissue. In the ischemic tissue, lactate concentrations were raised to  $2.8 \pm 0.6$  mmol/L in control ( $p < .01$ ) and to  $3.4 \pm 1.4$  mmol/L in HES ( $p < .01$ ) but only to  $2.4 \pm 1.0$  mmol/L in HbV-HES and to  $2.2 \pm 0.7$  mmol/L in PBHb (both not significant). At baseline, the mean lactate/pyruvate ratio was higher in the ischemic tissue (24–27) compared with the anatomic part (19–22). No relevant changes occurred in the anatomic part. Lactate/pyruvate ratio was increased to  $51 \pm 23$  in the control group and  $48 \pm 12$  in the HES group (both  $p < .01$ ) but only to  $34 \pm 8$  and  $38 \pm 11$  for HbV-HES (not significant vs. baseline,  $p < .05$  vs. control) and PBHb ( $p < .05$ ), respectively.

## DISCUSSION

The principal findings of this study were that the elevation in lactate concentration and lactate/pyruvate ratio in the critically ischemic tissue could be attenuated by isovolemic hemodilution with the oxygen-carrying solutions. This suggests that oxidative energy metabolism was improved in the hypoxic cells, which is crucial for their survival and functional outcome. The effect was superior for HbV-HES, which also yielded markedly higher oxygen tension in the ischemic flap tissue. Both tissue oxygen tension and oxidative energy metabolism are dependent on the oxygen supply to this tissue, which is determined by the oxygen content of the blood entering the collateralized tissue and the perfusion of the latter.

As expected, microcirculatory blood flow was substantially improved in all hemodiluted animals due to the hematocrit reduction; however, microcirculatory blood flow was improved to a lesser ex-

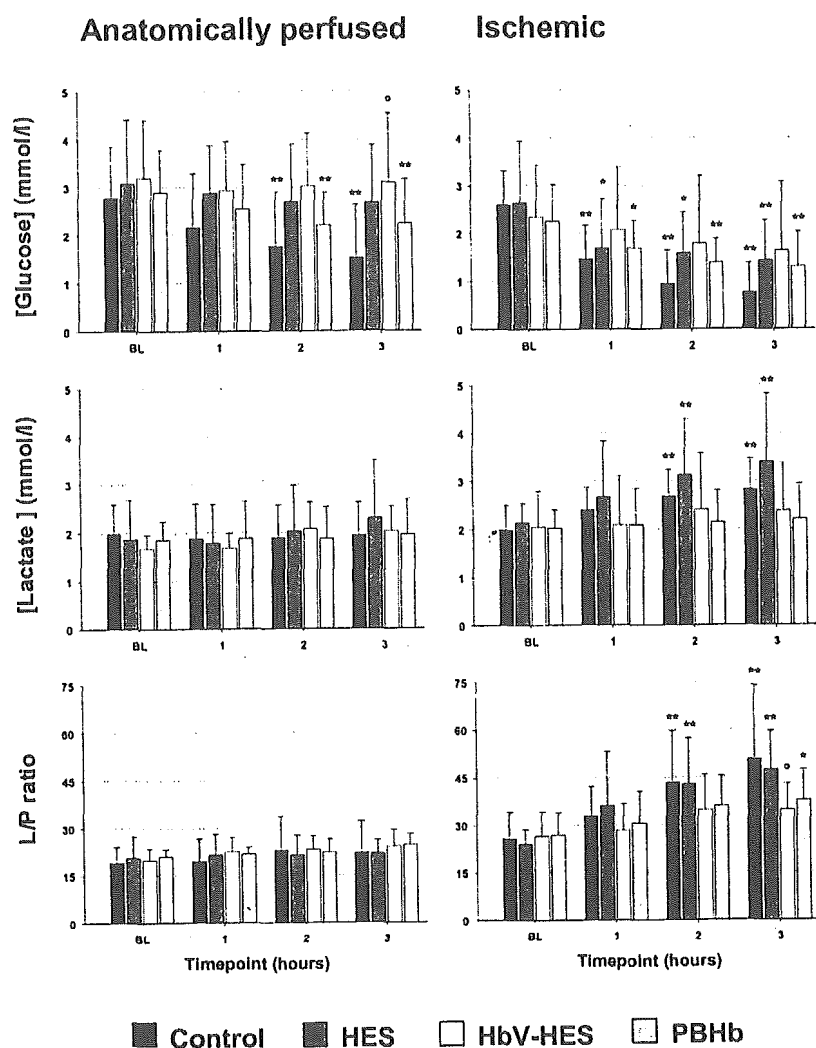


Figure 5. Carbohydrate metabolite concentrations in the anatomically perfused and ischemic tissues at baseline (BL) and after hemodilution with 6% hydroxyethyl starch (HES), hemoglobin vesicles suspended in HES (HbV-HES), and polymerized bovine hemoglobin (PBHb). Data represent mean values and sd. \* $p < .05$ , \*\* $p < .01$  vs. baseline; ° $p < .05$  vs. control.

tent for PBHb, which also yielded diminished microvascular diameters. In previous studies we demonstrated that microvascular blood flow and diameter were closely related to the rheologic formulation of the diluent, thus advocating solutions with both high colloid osmotic pressure and high viscosity (9).

The colloid osmotic pressure of the solutions used in the present study was approximately twice as high as that of hamster plasma, thus causing volume expansion, as evidenced by hematocrit values that were markedly lower than the theoretical value expected after a normovolemic 33% blood exchange. The volume expansion was similar for both oxygen-carrying solutions, whereas he-

modilution with HbV-HES resulted in markedly higher viscosity in the plasma phase. Plasma viscosity affects shear stress on the vascular lining, thus stimulating the NO-mediated relaxation of the vascular tone in small arteries and large arterioles (15). NO may be scavenged by cell-free hemoglobin, which may cause a strong vasopressor effect and subsequent hypertension (1, 16). It has been shown that the degree of NO-scavenging is correlated with the size of the hemoglobin compound (16), the HbV being more than ten times larger than the PBHb. NO-scavenging was most likely the reason for the microvascular narrowing found after the administration of PBHb in our study. This vasoconstriction was

Our study suggests that in critically ischemic and hypoxic collateralized peripheral tissue, oxygenation may be improved by normovolemic hemodilution with hemoglobin vesicles suspended in hydroxyethyl starch.

most pronounced in the flap artery. This vascular segment was considered most sensitive to NO-mediated regulation of vascular tone (17) and vasopressor effect of acellular hemoglobins (16), which may explain the higher mean arterial pressure we obtained in the animals receiving PBHb compared with HbV-HES. Taken together, our data suggest that HbV-HES provides a better balance of oxygen carrier-related viscosity increase and NO-scavenging.

However, the prevention of metabolic deterioration in the ischemic flap tissue after hemodilution with the oxygen-carrying solutions cannot be solely related to the improved microcirculatory blood flow because no metabolic benefit was observed in the animals receiving HES. One possible explanation is that due to their small size, the supplemented hemoglobins may perfuse capillaries that are no longer accessible by red blood cells and that are therefore relieved of their function. Although not measured in this study, impaired functional capillary density, a common feature in the compromised microcirculation (5, 7, 18), must be assumed in the ischemic flap tissue. Indeed, circulating HbVs were observed in capillaries showing a cessation of red blood cell flux (18).

The improved oxygen tension found in the ischemic tissue after hemodilution with HbV-HES is indicative of a larger amount of oxygen being brought to the collateralized vasculature than is the case with the PBHb group. According to the oxygen dissociation curve for hamster blood (5, 19), hemodilution with both oxygen-carrying solutions led to an in-

crease in arterial oxygen saturation of the cellular hemoglobin from approximately 80% at baseline to 90% at 3 hrs after blood exchange, whereas the estimated oxygen saturation was approximately 95% for the HbV but only 60% for PBHb, thus resulting in a slightly higher arterial oxygen content in the HbV-HES animals. Furthermore, it may be postulated that HbVs prevented the transmural diffusion of oxygen during the passage through the upstream vasculature. This mechanism may influence oxygen delivery to collateralized tissues substantially, because up to 40–50% of the systemic arterial oxygen was estimated to be lost from the upstream circulation before reaching the collateral vasculature nourishing the ischemic flap tissue (12). Upstream oxygen loss may be influenced in many ways by the addition of oxygen-carrying solutions. According to the Stokes-Einstein equation, the diffusivity of oxygen through the plasma is inversely proportional to the size of the plasma-bound oxygen carrier and the viscosity of the suspension, which were both greater for HbV-HES. Facilitated diffusion has been reported for PBHbs in static (20) and dynamic (21) *in vitro* models, whereas virtually no such effect has been obtained with HbVs (20, 22). Enhancing the oxygen affinity of the added oxygen carrier shifts transmural oxygen diffusion downstream, as has repeatedly been demonstrated *in vivo* (5, 19), *in vitro* (22), and in mathematical models (23). In a recent study, we were able to show that the oxygenation in the critically ischemic hamster flap tissue could substantially be improved by left-shifting of the HbV (10). Taken together, it may be assumed that with HbV-HES, there was less unwanted oxygen loss in the upstream vasculature before reaching the collateralized, ischemic flap tissue than with PBHb. This was likely due to the larger size of HbV-HES, the higher oxygen affinity of its hemoglobin compound, and the higher viscosity of the solution.

## CONCLUSIONS

From our data, we conclude that hypoxia in the critically ischemic hamster flap tissue was substantially attenuated

after hemodilution with HbV-HES and PBHb due to improved microcirculation and the presence of the supplemented oxygen carriers. It seemed that due to its rheologic formulation and physiochemical properties, HbV-HES provided a superior oxygen supply to the ischemic flap tissue. Therefore, our data suggest that the concept of targeting oxygen delivery to where it is most needed may find a particular, clinically most relevant application in the treatment of hypoxia in critically ischemic peripheral tissues. However, these findings may not be extrapolated to vital organs. Moreover, the beneficial effect obtained with the artificial oxygen carriers may be further enhanced by adding antioxidant enzymes to attenuate any possible ischemia-reperfusion injury (1).

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## Oxygen release from low and normal $P_{50}$ Hb vesicles in transiently occluded arterioles of the hamster window model

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<sup>1</sup>Advanced Research Institute for Science and Engineering, Waseda University, Tokyo, Japan; and <sup>2</sup>Department of Bioengineering, University of California-San Diego, and <sup>3</sup>La Jolla Bioengineering Institute, La Jolla, California

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**Sakai, Hiromi, Pedro Cabrales, Amy G. Tsai, Eishun Tsuchida, and Marcos Intaglietta.** Oxygen release from low and normal  $P_{50}$  Hb vesicles in transiently occluded arterioles of the hamster window model. *Am J Physiol Heart Circ Physiol* 288: H2897–H2903, 2005. First published January 28, 2005; doi:10.1152/ajpheart.01184.2004.—A phospholipid vesicle encapsulating Hb [Hb vesicle (HbV)] has been developed as a transfusion alternative. One characteristic of HbV is that the  $O_2$  affinity [ $P_{O_2}$  at which Hb is 50% saturated ( $P_{50}$ )] of Hb can be easily regulated by the amount of the coencapsulated allosteric effector pyridoxal 5'-phosphate. In this study, we prepared two HbVs with different  $P_{50}$ s (8 and 29 mmHg, termed HbV<sub>8</sub> and HbV<sub>29</sub>, respectively) and observed their  $O_2$ -releasing behavior from an occluded arteriole in a hamster skinfold window model. Conscious hamsters received HbV<sub>8</sub> or HbV<sub>29</sub> at a dose rate of 7 ml/kg. In the microscopic view, an arteriole (diameter:  $53.0 \pm 6.6 \mu\text{m}$ ) was occluded transcatheterally by a glass pipette on a manipulator, and the reduction of the intra-arteriolar  $P_{O_2}$  100  $\mu\text{m}$  down from the occlusion was measured by the phosphorescence quenching of preinfused Pd-porphyrin. The baseline arteriolar  $P_{O_2}$  (50–52 mmHg) decreased to about 5 mmHg for all the groups. Occlusion after HbV<sub>8</sub> infusion showed a slightly slower rate of  $P_{O_2}$  reduction compared with that after HbV<sub>29</sub> infusion. The arteriolar  $O_2$  content was calculated at each reducing  $P_{O_2}$  in combination with the  $O_2$  equilibrium curves of HbVs, and it was clarified that HbV<sub>8</sub> showed a significantly slower rate of  $O_2$  release compared with HbV<sub>29</sub> and was a primary source of  $O_2$  (maximum fraction, 0.55) overwhelming red blood cells when the  $P_{O_2}$  was reduced (e.g., <10 mmHg) despite a small dosage of HbV. This result supports the possible utilization of Hb-based  $O_2$  carriers with lower  $P_{50}$  for oxygenation of ischemic tissues.

blood substitutes; artificial red blood cells; occlusion; microhemodynamics; liposome

PHOSPHOLIPID VESICLES encapsulating concentrated human Hb [Hb vesicles (HbV)] or liposome-encapsulated Hb can serve as a transfusion alternative whose  $O_2$  carrying capacity can be formulated to be comparable to that of blood (1, 5, 8, 16, 24, 30). The capsular structure of HbV (particle diameter  $\sim 250$  nm) has characteristics similar to those of natural red blood cells (RBCs), because both have membranes that prevent direct contact of Hb with the components of blood and the endothelial lining, mitigating cellular injury due to Hb-mediated prooxidative species (4, 38). Furthermore, Hb encapsulation in vesicles prevents a hypertensive response induced by free Hbs that scavenge the endogenous vasorelaxation factors nitric oxide (NO) and carbon monoxide (12, 18, 26). The safety of HbV has been confirmed in rodent models in terms of the prompt metabolism of the components of HbV in the reticuloendothelial

system, which was demonstrated by histopathological analysis and plasma biochemical analysis (28, 29).

One of the characteristics of the capsular HbV is that its physicochemical characteristics such as  $O_2$  affinity [ $O_2$  tension at which Hb is half-saturated with  $O_2$  ( $P_{50}$ )] can be easily regulated by manipulating the amount of an allosteric effector coencapsulated in HbV. This property provides additional flexibility in formulating the  $O_2$  transport properties of HbV by comparison with the chemically modified Hbs whose  $P_{50}$  is modified and fixed by chemical reactions such as cross-linking or polymer conjugation (34). We use pyridoxal 5'-phosphate (PLP) as the allosteric effector (33, 45). For example, coencapsulation of PLP at the molar ratio of PLP to Hb of 2.5:1 yields a  $P_{50}$  of about 29 mmHg. On the other hand, HbVs without PLP have a  $P_{50}$  of 8 mmHg. Historically,  $P_{50}$  was set similar to that of RBCs or about 25–30 mmHg, which theoretically allows sufficient  $O_2$  unloading as blood transits the microcirculation. Decreasing  $O_2$  affinity (increasing  $P_{50}$ ) increases  $O_2$  unloading in the peripheral blood circulation as shown by the enhanced  $O_2$  release and improved exercise capacity in mutant mice that carry high  $P_{50}$  RBCs (36).

Hemoglobin-based  $O_2$  carriers (HBOCs) of molecular dimensions as well as HbV could be effective for the targeted oxygenation of ischemic tissues (6, 43) because the small particle dimension would allow their passage through constricted or partially occluded vessels that do not allow the passage of RBCs (19). Blood flow in these vessels and in collateral vessels is usually slow, thus increasing RBC transit times (7, 11). As a result, tissue  $P_{O_2}$  is low and RBCs release most of their  $O_2$  before reaching the capillary circulation. As an example, if tissue  $P_{O_2}$  is below 5 mmHg,  $O_2$  saturation ( $Sa_{O_2}$ ) of RBCs would be around 5%, and RBCs will have released most of their  $O_2$  before they reach the ischemic tissue. Thus an HBOC with a normal  $P_{50}$  similar to RBCs would not be effective for carrying  $O_2$  to the ischemic tissue.

In this study, we evaluate the rate of  $O_2$  release from HbVs with high and low  $P_{50}$ s from arterioles immediately after their occlusion. We selected arterioles with diameters of about 50  $\mu\text{m}$  because this size of arterioles contributes significantly to tissue oxygenation in normal conditions (13). This model was selected to determine the ability of HbVs to retain or release  $O_2$  in hypoxic conditions and establish their suitability for oxygenating ischemic tissues.

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## MATERIALS AND METHODS

**Preparation of HbVs.** HbVs with different  $P_{50}$ s were prepared under sterile conditions as previously reported (32, 34, 37). Hb was purified from outdated donated human blood provided by the Japanese Red Cross Society (Tokyo, Japan). HbVs with a  $P_{50} = 29$  mmHg (HbV<sub>29</sub>) was prepared by adding the allosteric effector pyridoxal 5'-phosphate (PLP; 14.7 mM, Sigma Chemical; St. Louis, MO) to Hb (38 g/dl) at a molar ratio of PLP to Hb = 2.5. HbVs with a  $P_{50} = 8$  mmHg (HbV<sub>8</sub>) were prepared by adding no allosteric effector to the Hb solution. The Hb solution was encapsulated within vesicles composed of Presome PPG-I [a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-di-*O*-octadecyl-*N*-succinyl-L-glutamate at a molar ratio of 5:5:1 (Nippon Fine Chemicals; Osaka, Japan)], and the particle size of HbVs was regulated by an extrusion method. The surface of the HbVs was modified with polyethylene glycol (molecular mass: 5 kDa, 0.3 mol% of the lipids in the outer surface of vesicles) using 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-polyethylene glycol (Sunbright DSPE-50H, H-form, NOF; Tokyo, Japan). HbVs were suspended in a physiological salt solution and sterilized with filters (Dismic, Toyo Roshi; Tokyo, Japan; pore size: 0.45  $\mu$ m) and deoxygenated with N<sub>2</sub> bubbling for storage. The endotoxin content was measured with a modified Limulus amoebocyte lysate assay, and the level was less than 0.2 EU/ml (27). The O<sub>2</sub> equilibrium curves (OECs) of HbV<sub>29</sub> and HbV<sub>8</sub> were obtained by a Hemox Analyzer (TCS-Medical Products; Philadelphia, PA), as shown in Fig. 1. The physicochemical parameters of the HbVs are listed in Table 1.

**Animal model and preparation.** Experiments were carried out in 12 male Syrian golden hamsters (59  $\pm$  12 g body wt, Charles Rivers; Worcester, MA). The dorsal skinfold consisting of two layers of skin and muscle was fitted with two titanium frames with a 15-mm circular opening and surgically installed under intraperitoneal pentobarbital sodium anesthesia (~50 mg/kg body wt, Abbott Laboratory; North Chicago, IL). After the hair on the back skin of the hamster was removed, layers of skin muscle were separated from the subcutaneous tissue and removed until a thin monolayer of muscle including the small artery and vein and one layer of intact skin remained. A coverglass (diameter 12 mm) held by one frame covered the exposed tissue allowing intravital observation of the microcirculation (20, 22, 25).

Polyethylene (PE) tubes (PE-10, Becton-Dickinson; Parsippany, NJ; ~1 cm) were connected to PE-50 tubing (~25 cm) via silicone elastomer medical tubes (~4 cm, Technical Products; Decatur, GA) and were implanted in the jugular vein and the carotid artery. They were passed from the ventral to the dorsal side of the neck and exteriorized through the skin at the base of the chamber. Patency of the catheters was ensured by filling them with heparinized saline (40

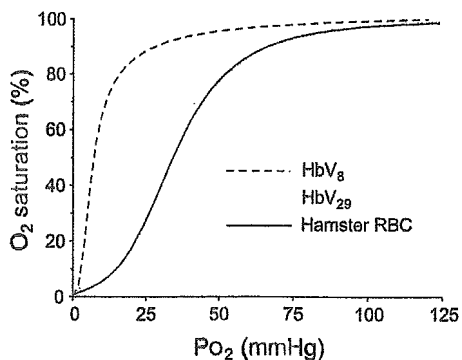


Fig. 1. Oxygen equilibrium curves (OECs) of Hb vesicles (HbVs) at a  $P_{O_2}$  where Hb is half-saturated ( $P_{50}$ ) of 8 mmHg (HbV<sub>8</sub>) and 29 mmHg (HbV<sub>29</sub>) measured with a Hemox Analyzer (TCS Medical Products) at 37°C compared with hamster blood. RBC, red blood cells.

Table 1. Physicochemical properties of HbV<sub>8</sub> and HbV<sub>29</sub> compared with hamster blood

Parameters	HbV <sub>8</sub>	HbV <sub>29</sub>	Hamster Blood
Hb concentration, g/dl	10	10	14.8 $\pm$ 0.5
Particle diameter, nm	250 $\pm$ 64	247 $\pm$ 44	5,000–7,000*
$P_{50}$ , mmHg	8	29	28
Molar ratio of PLP to Hb	0	2.5	
MetHb, %	<3	<1	
HbCO, %	<2	<2	

HbV<sub>8</sub> and HbV<sub>29</sub>. Hb vesicles (HbVs) at 8- and 29-mmHg  $P_{O_2}$  at which Hb is 50% saturated ( $P_{50}$ ); PLP, pyridoxal 5'-phosphate. \*Size of hamster red blood cells (RBCs) (39).

IU/ml). Microvascular observations of the awake and unanesthetized hamsters were performed 5 days after chamber implantation to mitigate the effects of surgery. The hamster was placed in a perforated plastic tube from which the window chamber protruded to minimize animal movement without impeding respiration. All animal studies were approved by the Animal Care and Use Committee of University of California-San Diego and performed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Washington, DC: National Academy Press, 1996).

**Infusion of HbV<sub>8</sub> and HbV<sub>29</sub> and occlusion of an arteriole.** The unanesthetized animal was placed in a perforated plastic tube and stabilized under the microscope. Animals were suitable for the experiments if systemic variables were within normal range, namely, heart rate >340 beats/min, mean arterial pressure >80 mmHg, systemic hematocrit >45%, and arterial  $P_{O_2}$  >50 mmHg, and microscopic examination of the tissue in the chamber did not reveal signs of edema or bleeding. Baseline measurements of microvascular parameters and  $P_{O_2}$  (see below) were performed before the infusion of HbV<sub>8</sub> or HbV<sub>29</sub> suspended in physiological saline solution into the venous line at 7 ml/kg. Systemic blood volume was estimated as 70 ml/kg. In our previous reports of resuscitation from hemorrhagic shock or hemodilution, HbVs were suspended in an albumin solution to regulate colloid osmotic pressure (30, 33). However, in the present study, we did not use albumin to minimize the hypervolemic effect. For the same reason, the infusion amount was minimized to equal 10% blood volume (7 ml/kg).

After we stabilized the condition and measured the systemic parameters for 20 min, diameter and blood flow of the selected arterioles were measured. Large feeding arterioles or small arcading arterioles (diameter  $53.0 \pm 6.6$   $\mu$ m) were selected for observation. The arterioles were occluded by means of a glass micropipette whose end was drawn into a long fiber by a pipette puller (Fig. 2). The fiber was bent over a flame, and the knee of the bend was used to press on the intact skin of the preparation mounted in an inverted microscope that allowed observation of the opposite side, i.e., the intact microcirculation. Once an arteriole was selected for measurement, the microoccluder is moved to the skin side, between the intact skin and the optics of the substage illumination. The tip of the occluder was placed near the center of the optical field of view of the microscope, and the vessel was similarly placed using the stage micrometric position control. This arrangement allowed for direct microscopic observation of the occluded vessel and the stopped flow as shown in Fig. 2. The duration of occlusion was 30 s.

**Measurement of microhemodynamic parameters.** Microvessels were observed by transillumination with an inverted microscope (IMT-2, Olympus; Tokyo, Japan). Microscopic images were video recorded (Cohu 4815-2000; San Diego, CA) and transferred to a television videocassette recorder (Sony Trinitron PVM-1271Q monitor; Tokyo, Japan) and Panasonic AG-7355 video recorder (Tokyo, Japan). Arterioles were classified according to their position within the microvascular network according to the previously reported scheme (33). Microvascular diameter and RBC velocity before occlu-

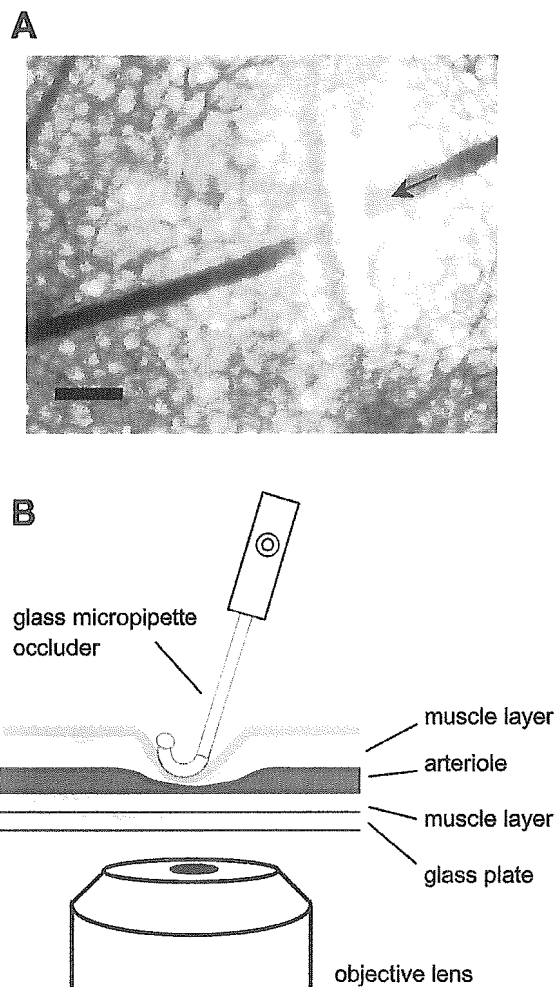


Fig. 2. A: microscopic image of an occluded arteriole in the hamster window chamber. The glass fiber lies across the arteriole. Scale bar = 100  $\mu\text{m}$ . B: schematic representation of occlusion of A showing the different tissue layers of the skin (not to scale).

sion were analyzed on-line in the arterioles (14, 15). Vessel diameter was measured with an image-shearing system (Digital Video Image Shearing Monitor 908, I.P.M.; San Diego, CA), whereas RBC velocity was analyzed by photodiodes and the cross-correlation technique (Velocity Tracker Mod-102 B, I.P.M.). The blood flow rate ( $Q$ ) was calculated using the following equation:

$$Q = (\text{RBC velocity}/R_v) \times (\text{diameter}/2)^2 \quad (1)$$

where  $R_v = 1.6$  and is the ratio of the centerline velocity to average blood velocity according to data from glass tubes (20).

Palladium-porphyrin bound to bovine albumin solution (7.6 wt%, 0.1 ml) was injected intravenously 20 min before the infusion of HbVs. Arteriolar blood  $P_{O_2}$  was noninvasively determined by measuring the rate of decay of phosphorescence emitted by the metalloporphyrin complex after pulsed light excitation, which is a function of the local  $O_2$  concentration (17, 40, 44). The relationship between phosphorescence lifetime and  $P_{O_2}$  is given by the following Stern-Volmer equation:

$$\tau_0/\tau = 1 + k_q \times \tau_0 \times P_{O_2} \quad (2)$$

where  $\tau_0$  and  $\tau$  are the phosphorescence lifetimes in the absence of molecular  $O_2$  and at a given  $P_{O_2}$ , respectively, and  $k_q$  is the quenching constant, with both factors being pH and temperature dependent.

Light was gathered from an optical window of  $20 \times 5 \mu\text{m}$  placed longitudinally along the blood vessels. Measurements in the blood compartment were made every second using a single flash.

The  $P_{O_2}$  decay curves induced by the occlusion were obtained before the infusion of HbVs and 20 min after the infusion of HbVs. The  $Sa_{O_2}$  of HbVs at every  $P_{O_2}$  were obtained from the OECs (Fig. 1), and the total  $O_2$  content in blood (ml  $O_2$  in 1 dl blood) can be estimated using the following equation:

$$O_2 \text{ content} = 23.6 \times \frac{[Sa_{O_2}(\text{RBC}) + 0.0667 \times Sa_{O_2}(\text{HbV})]}{100} + 2.42 \times \frac{P_{O_2}}{713} \quad (3)$$

In this calculation, we used 15 g/dl as the average Hb concentration in arterial blood ( $14.8 \pm 0.5$  g/dl, heme concentration 9.3 mM), which was measured with a handheld photometer (B-Hemoglobin Photometer, Hemocue). One hundred milliliters of blood contain 23.6 ml  $O_2$  bound to Hb when  $Sa_{O_2}$  is 100% (volume of an ideal gas at  $37^\circ\text{C}$ ) according to Boyle-Charles's gas law,  $PV = nRT$ , where  $P$  (in atm) is atmospheric pressure,  $V$  (in liters) is gas volume,  $n$  is mole number,  $R$  is the gas constant ( $0.082 \text{ atm} \cdot \text{L} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ ), and  $T$  is absolute temperature [ $23.6 \text{ (ml)} = 9.3 \times 10^{-4} \text{ (mol)} \times 0.082 \times (273 + 37) \times 1,000$ ]. The physically dissolved  $O_2$  content at 1 atm  $O_2$  (713 mmHg) after subtracting the vapor pressure of water = 47 mmHg) at  $37^\circ\text{C}$  was calculated to be 2.42 ml in 100 ml water.  $Sa_{O_2}(\text{RBC})$  and  $Sa_{O_2}(\text{HbV})$  are  $Sa_{O_2}$ s of RBCs and HbVs, respectively, at each arteriolar  $P_{O_2}$  during the experiments.

HbVs were suspended in physiological saline solution ( $[\text{Hb}] = 10$  g/dl); therefore, their infusion lowered colloid osmotic pressure, causing the extravasation of plasma fluid. To account for this, we carried out our measurements 20 min after HbV infusion and assumed that this interval was sufficient for normalizing blood volume through the release of extra fluid to the interstitium, thus increasing plasma Hb concentration by 6.7%.

**Data analysis.** Data are given as means  $\pm$  SD for the indicated number of animals. Data were analyzed using ANOVA followed by Fisher's protected least-significant difference test between groups according to the previous studies. Student's  $t$ -test was used for comparisons within each group. All statistics were calculated using GraphPad Prism 4.01 (Graph Pad Software; San Diego, CA). Changes were considered statistically significant if  $P < 0.05$ .

## RESULTS

**Hemodynamic properties of arterioles.** The profiles of the selected arterioles, diameters, centerline RBC velocities, blood flow rates, and intra-arteriolar  $P_{O_2}$  values before and after infusion of HbVs are listed in Table 2. There was no significant difference between the groups. The  $O_2$  content in blood attributed to hamster RBCs and physically dissolved  $O_2$  at the observed arteriolar  $P_{O_2}$  was estimated as  $18.61 \pm 1.23$  ml  $O_2$ /dl blood according to Eq. 3. After the infusion of HbV<sub>8</sub> and HbV<sub>29</sub>, the  $O_2$  content increased to  $20.30 \pm 1.18$  and  $20.17 \pm 1.54$  ml  $O_2$ /dl blood, respectively, due to the  $O_2$  bound to HbVs. The contributions of HbV<sub>8</sub> and HbV<sub>29</sub> to whole  $O_2$  content were  $1.51 \pm 0.01$  and  $1.25 \pm 0.07$  ml  $O_2$ /dl blood, respectively. The HbV<sub>8</sub> group showed higher  $O_2$  content than the HbV<sub>29</sub> group due to the higher  $Sa_{O_2}(\text{HbV}_8)$ , which was  $95.9 \pm 0.6\%$  compared with the  $Sa_{O_2}(\text{HbV}_{29})$  of  $79.6 \pm 4.7\%$ .

**Changes in  $P_{O_2}$  in arterioles after occlusion in the presence of HbVs.** Arteriolar  $P_{O_2}$  before occlusion was about 50–52 mmHg in average for all groups and started to decrease significantly immediately after occlusion, as shown in Fig. 3. In all groups,  $P_{O_2}$  fell to about 10 and 5 mmHg after 10- and

**Table 2. Profiles of arterioles for occlusion before and after infusion of HbVs**

Parameters	Before Infusion	After HbV Infusion	
		HbV <sub>8</sub>	HbV <sub>29</sub>
Arteriolar diameter, $\mu\text{m}$	53.0 $\pm$ 6.6	56.2 $\pm$ 6.8	55.8 $\pm$ 6.9
Centerline flow velocity, mm/s	3.1 $\pm$ 0.5	3.4 $\pm$ 0.7	3.5 $\pm$ 0.5
Blood flow rate, nl/s	6.8 $\pm$ 1.6	8.7 $\pm$ 3.1	8.5 $\pm$ 2.1
Arteriolar PO <sub>2</sub> , mmHg	50.7 $\pm$ 4.7	51.4 $\pm$ 4.8	52.1 $\pm$ 5.3
SaO <sub>2</sub> (RBC), %	78.1 $\pm$ 5.1	76.0 $\pm$ 7.7	77.9 $\pm$ 6.5
SaO <sub>2</sub> (HbV), %		95.9 $\pm$ 0.6†	79.6 $\pm$ 4.7
O <sub>2</sub> content in whole blood, ml O <sub>2</sub> /dl blood	18.61 $\pm$ 1.23	20.30 $\pm$ 1.18*	20.17 $\pm$ 1.54*
O <sub>2</sub> content in HbV, ml O <sub>2</sub> /dl blood		1.51 $\pm$ 0.01	1.25 $\pm$ 0.07

Values are means  $\pm$  SD. Arteriolar PO<sub>2</sub>, O<sub>2</sub> saturation (SaO<sub>2</sub>) and O<sub>2</sub> contents were obtained during 6 s before occlusion. \* $P < 0.05$  vs. before infusion; † $P < 0.05$  vs. RBCs and HbV<sub>29</sub>.

30-s occlusion, respectively. When the PO<sub>2</sub> values were expressed as relative to the baseline values (before occlusion), infusion of HbV<sub>8</sub> tended to show a slower rate of reduction of PO<sub>2</sub> compared with the infusion of HbV<sub>29</sub> and without infusion (Fig. 4). There was a significant difference between the HbV<sub>8</sub> infusion and before infusion groups only at 7 s ( $P = 0.035$ ).

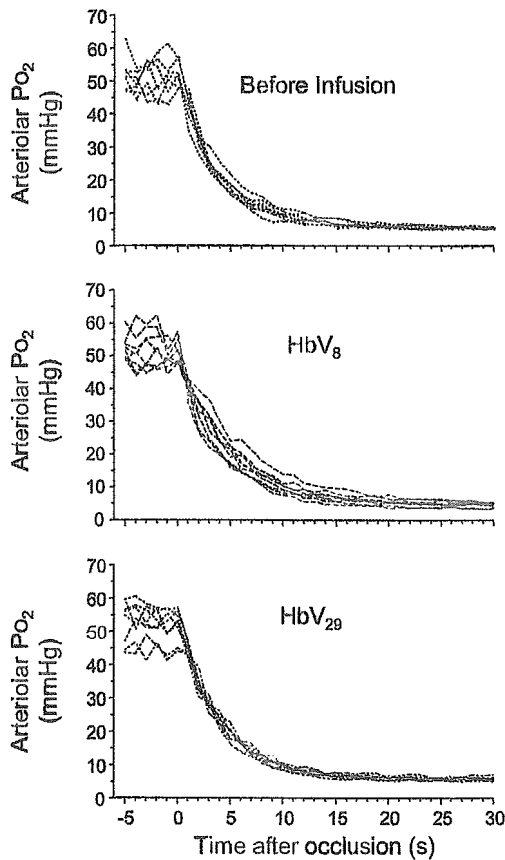


Fig. 3. Time course of PO<sub>2</sub> in the blood of an occluded arteriole (diameter, 53.0  $\pm$  6.6  $\mu\text{m}$ ) before and after infusion of 7 ml/kg HbV<sub>8</sub> or HbV<sub>29</sub> into hamsters. Measurements were made in blood at a distance of 50  $\mu\text{m}$  from the point of occlusion. Most vessels equilibrate to intravascular partial pressure in the range of 4–6 mmHg about 15–20 s after occlusion.

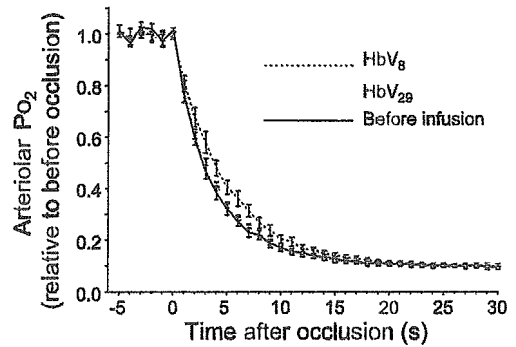


Fig. 4. Changes in PO<sub>2</sub> relative to before occlusion. The data in Fig. 3 were averaged. Baseline values before occlusion were obtained as the average of 6 values before occlusion and fixed as 1.0. There was a significant difference between the HbV<sub>8</sub> infusion and before infusion groups only at 7 s ( $P = 0.035$ ).

SaO<sub>2</sub>(RBC) and SaO<sub>2</sub>(HbV) at every arteriolar PO<sub>2</sub> value can be estimated using the OECs in Fig. 1 assuming that the conditions in the arteriole (such as temperature and pH) do not change significantly from the normal condition (37°C, pH 7.4). Figure 5A shows the changes in the whole arteriolar O<sub>2</sub> content

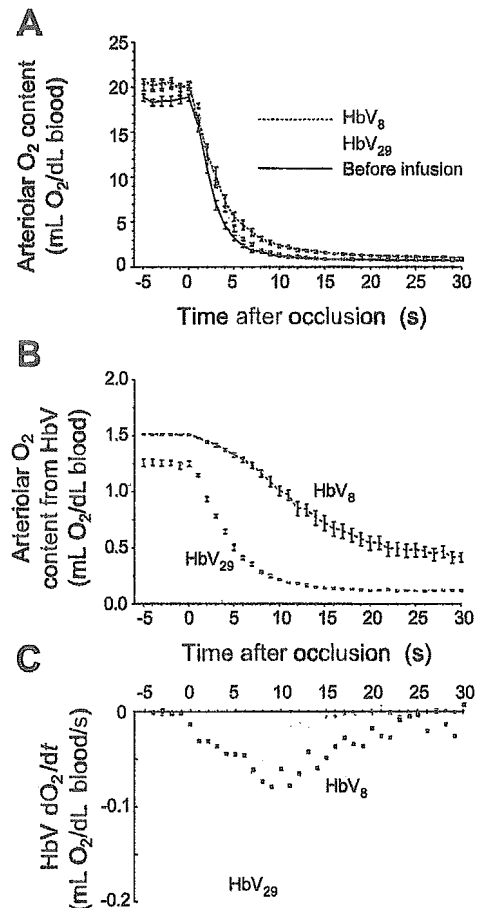


Fig. 5. A: time course of the arteriolar O<sub>2</sub> content in whole blood of an occluded arteriole before and after infusion of 7 ml/kg HbV<sub>8</sub> or HbV<sub>29</sub> into hamsters. The O<sub>2</sub> contents were calculated using Eq. 2 and the data of OECs (Fig. 1) and PO<sub>2</sub> changes (Fig. 3). B: time course of the O<sub>2</sub> content derived from HbVs in the blood. The contributions of HbVs are derived from the data in A and magnified in scale. C: rate of O<sub>2</sub> loss dO<sub>2</sub>/dt from HbVs. The graphs in B were differentiated and plotted.

during the occlusion. Immediately after occlusion, the  $O_2$  content decreased rapidly. The HbV<sub>8</sub> group showed a slower rate of reduction compared with the HbV<sub>29</sub> group and the group before HbV infusion. To demonstrate the contribution of HbVs clearly, only the  $O_2$  content of HbVs is shown in Fig. 5B. HbV<sub>8</sub> showed a very slow rate of  $O_2$  release. After 30 s of occlusion, the arteriolar  $P_{O_2}$  decreased to  $5.2 \pm 0.7$  mmHg. However,  $Sa_{O_2}$ (HbV<sub>8</sub>) was  $26.1 \pm 7.3\%$  and did not reach steady state but continued  $O_2$  release. HbV<sub>29</sub> showed almost no change after 15 s, and  $Sa_{O_2}$ (HbV<sub>29</sub>) was  $7.4 \pm 1.0\%$  after 30 s. Figure 5C shows the rate of  $O_2$  loss from HbVs obtained by the differentiation of the graphs in Fig. 5B. HbV<sub>29</sub> showed the fastest  $O_2$  loss with the maximum of 0.18 ml  $O_2$ /dl blood sec after only 2 s of occlusion and did not supply  $O_2$  after 17 s. On the other hand, HbV<sub>8</sub> showed a moderate  $O_2$  loss and showed the maximum of 0.08 ml  $O_2$ /dl blood after 10 s of occlusion and continued to release  $O_2$  until 30 s.

Figure 6 shows the fraction of  $O_2$  in blood originating from HbVs. Before occlusion of the arterioles, the fractions of HbV<sub>8</sub> and HbV<sub>29</sub> are very small and similar because of the small dosage compared with the originally present RBCs. However, after occlusion, the fraction of  $O_2$  from HbV<sub>8</sub> increased significantly and was about 0.55 after 10 s. This indicated that HbV<sub>8</sub>, and not RBCs, was the main source of the  $O_2$  carrier when  $P_{O_2}$  attained very low values.

#### DISCUSSION

The principal finding of this study is that HbV<sub>8</sub> ( $P_{50} = 8$  mmHg) with a high  $O_2$  affinity (low  $P_{50}$ ) releases  $O_2$  at a slower rate than does HbV<sub>29</sub> in occluded arterioles of the hamster dorsal skinfold model. Furthermore, we found that HbV<sub>8</sub>, and not HbV<sub>29</sub>, is the main  $O_2$  source in ischemic conditions.

The immediate occlusion of blood flow in the arterioles caused a rapid reduction of  $O_2$  content. Similar phenomena have been observed by Richmond et al. (23) in rat spinotrapezius muscle tissue. There is substantial evidence that the arteriolar wall is a significant  $O_2$  sink, consuming  $O_2$  at a rate that is much greater than most tissues (9, 35, 42), which explains in part the significant and rapid drop of  $P_{O_2}$  found in our study. In our experiments, only one arteriole was occluded at a time in the intact subcutaneous tissue, and arteriolar  $P_{O_2}$  decreased to about 5 mmHg, which was higher than the critical  $P_{O_2}$  ( $2.9 \pm 0.5$  mmHg) in the rat spinotrapezius muscle tissue (23). Although the  $O_2$  supply was significantly reduced, diffusion of  $O_2$  from the other surrounding arterioles, venules, and

capillaries near the occlusion should contribute to maintaining tissue  $P_{O_2}$  at a higher value than in the study of Richmond et al. (23), where the supply of blood to the tissue was stopped altogether.  $Sa_{O_2}$ (HbV<sub>8</sub>) at 5 mmHg is estimated to be about 26% according to the OECs (Fig. 1), which is higher than that for HbV<sub>29</sub> (6%) and RBCs (2%); thus HbV<sub>8</sub> remains a source of  $O_2$  for a longer period in a prolonged occlusion, because the fraction of  $O_2$  from HbV<sub>8</sub> was 0.5 or higher, overwhelming the contribution from RBCs, as shown in Fig. 6.

A limitation of our experimental method is that  $Sa_{O_2}$  is estimated under the assumption that conditions in the target arteriole are identical to that of the OEC measurement; however, the  $O_2$  affinity of Hb changes as a function of temperature, pH, electrolyte concentration, and  $CO_2$  content. Local ischemic conditions caused by the occlusion could affect pH and increase  $CO_2$  tension, resulting in a slight decrease in the  $O_2$  affinity (increased  $P_{50}$ ); however, it is unlikely that this would introduce a significant error in the measurement of  $O_2$  release considering the short duration of the occlusion (30 s).

We have previously demonstrated using an artificial narrow polymer tube (inner diameter: 28  $\mu$ m) surrounded by a sodium dithionate solution to consume  $O_2$  that a Hb solution under continuous flow conditions (1 mm/s) facilitates  $O_2$  release when mixed with RBCs. Conversely, HbV did not show this phenomenon (31). This difference is due to the small size of  $O_2$ -bound acellular Hb molecules, which diffuse and therefore contribute to the facilitated  $O_2$  transport (21, 31), whereas HbVs (diameter, about 250 nm) are too large to show sufficient diffusion for the facilitated  $O_2$  transport. In these conditions,  $O_2$  affinity ( $P_{50}$ ) becomes the determining factor for the rate of  $O_2$  release and transport to the vessels wall. Thus, in our present results, the presence of HbVs did not facilitate the reduction of  $P_{O_2}$  or  $O_2$  content but retarded the reduction of  $P_{O_2}$  and  $O_2$  content.

Our experimental model is designed to characterize the  $O_2$  release behavior of blood from an occluded microvessel and does not directly related to clinical ischemic conditions because the occlusion of the small arteriole for 30 s does not induce tissue ischemia other than the transient event in the proximity of the microvessel. However, our data suggest that HbV<sub>8</sub> could be a significant source of  $O_2$  in an ischemic condition with significantly lowered tissue  $P_{O_2}$ . Because of the small dosage of HbV<sub>8</sub> (7 ml/kg), the  $O_2$  content in the blood after occlusion (5 ml  $O_2$ /dl blood at 5 s) is significantly smaller than the baseline value (20 ml  $O_2$ /dl blood at 0 s). To enhance the contribution of HbVs, a larger dosage and sustained blood flow would be required. Contaldo et al. (7) recently demonstrated that inducing hemodilution using up to 50% blood exchange with HbV ( $P_{50} = 15$  mmHg) suspended in dextran effectively oxygenated ischemic collateralized tissue in skin flaps. This phenomenon could be explained by low  $P_{50}$  HbVs retaining  $O_2$  in the upstream vessels and delivering it to the ischemic tissue via collateral arterioles, even when these may have significantly slower blood flow. It has been proposed that small-sized HBOCs oxygenate ischemic tissue by being able to pass through constricted or partially occluded vessels that do not allow the passage of RBCs; however, the results from Contaldo et al. (17) as well as those from our experimental model do not serve to support this concept, because arterioles were completely ligated or occluded. It should be noted, however, that an advantage of small HBOCs, including HbVs,

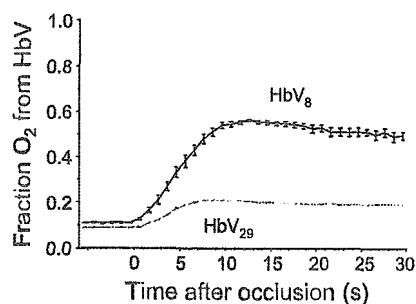


Fig. 6. Time course of the fraction of  $O_2$  content from HbVs in whole blood. The extended time of occlusion induced hypoxic conditions and the fraction of  $O_2$  content from HbV<sub>8</sub> increased significantly compared with HbV<sub>29</sub>.