Immunohistochemical studies showed that P450aldo was be expressed almost equally among adrenal cortices from the three Na^+-deficient groups (1′–3′).

In this study, OMb was shown for the first time to be present more abundantly in zG than in zFR of rat adrenal cortex. OMb seems to participate in aldosterone formation in zG of rat under angiotensin II-stimulation through regeneration of Asc. The detailed molecular mechanisms on these events await further investigation.

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

Pedro Cabrales,[1,2] Hiromi Sakai,[3] Amy G. Tsai,[1,2] Shinji Takeoka,[3] Eishun Tsuchida,[3] and Marcos Intaglietta[1,2]

[1]*Department of Bioengineering, University of California-San Diego, and* [2]*La Jolla Bioengineering Institute, La Jolla, California; and* [3]*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_{O_2} at which Hb is 50% saturated (P_{50}) of 8 (HbV_8) and 29 mmHg (HbV_{29}) 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 ± 0.1 g/dl after exchange with HbV_8 or HbV_{29}. 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_8 group compared with the HbV_{29} 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_8 vs. the HbV_{29} 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} ~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 ~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

Address for reprint requests and other correspondence: P. Cabrales, Dept. of Bioengineering, 0412, 9500 Gilman Dr., Univ. of California-San Diego, La Jolla, CA 92093-0412 (E-mail: pcabrales@ucsd.edu).

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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_{50}. 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_2 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_8, P_{50} = 8 mmHg; HbV_{29}, P_{50} = 29 mmHg).

Table 1. *Physical characteristics of solutions*

Fluid	Viscosity, cp	COP, mmHg	P_{50}, mmHg
rHSA (5%)	0.98	20	
HbV_8 (10 g Hb/dl)	2.92		8
HbV_{29} (10 g Hb/dl)	2.96		29
HbV_8/rHSA (8.6 g Hb/dl)	2.87	20	8
HbV_{29}/rHSA (8.6 g Hb/dl)	2.90	20	29

Viscosity was measured at a shear rate of 160 s^{-1} at 37°C. COP, colloid osmotic pressure measured at 27°C; P_{50}; partial pressure of oxygen at which Hb is 50% saturated; rHSA, recombinant human serum albumin; HbV_8 and HbV_{29}, Hb vesicles with a P_{50} of 8 and 29 mmHg, respectively.

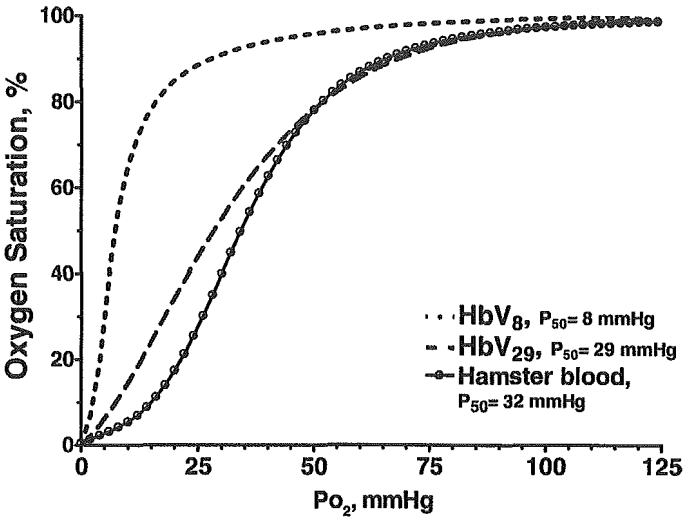

Fig. 1. Oxygen dissociation curves for phospholipid vesicles encapsulating purified Hb (HbV) produced with a P_{O_2} at which Hb is 50% saturated (P_{50}) of 8 (HbV_8) and 29 mmHg (HbV_{29}) vs. the dissociation curve for hamster blood (P_{50} = 32 mmHg).

Measurements of P_{50} and rate of oxygen release from HbV. The P_{50} 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_8 (P_{50} = 8 mmHg) and HbV_{29} (P_{50} = 29 mmHg) and were hemodiluted using these materials, reducing Hct to 11%. Plasma Hb concentrations derived for HbV_8 and HbV_{29} 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

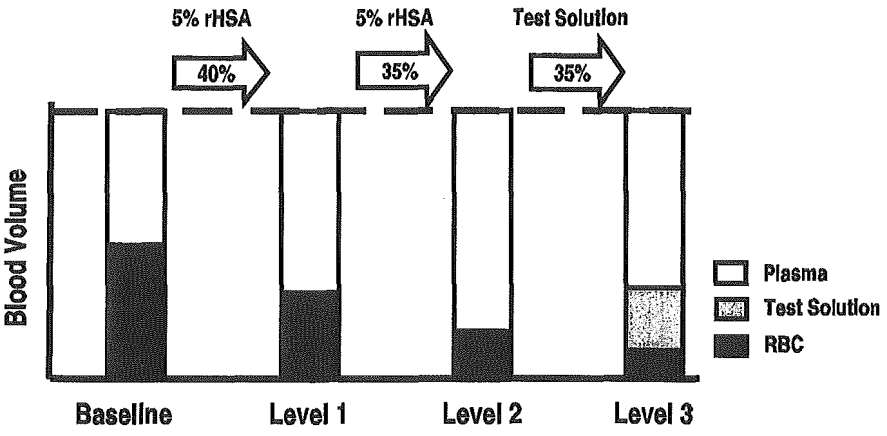

Fig. 2. Hemodilution was attained by means of a progressive, stepwise, isovolemic blood exchange-transfusion protocol. Volume of each exchange-transfusion step was calculated as a percentage of the blood volume, estimated as 7% of body weight. An acute anemic state was induced by lowering systemic Hct, 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_8 or HbV_{29} suspended in 5% rHSA (test solutions). RBC, red blood cells.

analyzed for arterial P_{O_2} (Pa_{O_2}), arterial P_{CO_2} (Pa_{CO_2}), base excess (BE), and pH (Blood Chemistry Analyzer 248; Bayer, Norwood, MA). The comparatively low Pa_{O_2} and high Pa_{CO_2} 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^{-1}. Colloid osmotic pressure (COP) was measured using the Wescor 4420 colloid osmometer (23).

Functional capillary density. Functional capillary density (FCD; in cm^{-1}) 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 centerline 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 P_{O_2} distribution. High-resolution microvascular P_{O_2} 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 P_{O_2} 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). P_{O_2} 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 P_{O_2} 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 P_{O_2} measurements in arterioles, venules, and the interstitium (20). The phosphorescence decay due to quenching at a specific P_{O_2} yields a single decay constant, and in vitro calibration has been demonstrated to be valid for in vivo measurements. Intravascular and perivascular P_{O_2} measurements were made in the arterioles studied, and intravascular P_{O_2} measurements were made in venules. Interstitial tissue P_{O_2} 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_2 delivery, defined as the amount of oxygen delivered by the arterioles to the microcirculation per unit time normalized relative to baseline, and O_2 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*:

$$O_2 \text{ delivery} = \{(RBC_{Hb} \times \gamma \times Sa_{RBC}\%) + (HbV_{Hb} \times \gamma \times Sa_{HbV}\%) + (1 - Hct) \times \alpha \times Pa_{O_2}\} \times Q \quad (1)$$

$$O_2 \text{ extraction} = \{[RBC_{Hb} \times \gamma \times S(a - v)_{RBC}\%] + [HbV_{Hb} \times \gamma \times S(a - v)_{HbV}\%] + (1 - Hct) \times \alpha \times P(a - v)o_2\} \times Q \quad (2)$$

where RBC_{Hb} is the [Hb] in RBC (expressed in g/dl of blood), HbV_{Hb} 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_2/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^{-3} ml O_2/dl plasma mmHg, Pa_{O_2} is the arteriolar partial pressure of oxygen, $P(a\text{-}v)o_2$ is the arteriovenous difference in P_{O_2}, 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 P_{O_2} distribution was determined using phosphorescence-quenching microscopy (9). The duration of the experiment was 3–4 h.

Data analysis. Results are presented as means ± 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_8, and HbV_{29}. 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 ± 0.5 to 11.4 ± 0.6%. The HbV_8 and HbV_{29} groups had a final plasma Hb concentration of 2.1 ± 0.1 g/dl, which increased the total Hb concentration in blood (RBC + Hb in plasma) to 5.7 ± 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 ± 0.3 g/dl (Hct 18.1 ± 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_{29} group and attained the highest value with HbV_8 viscosity. Heart rate after hemodilution followed by exchange transfusion with the HbV solutions was ~10% higher than baseline at the *level 3* exchange. The slight increase in heart rate was not statistically different.

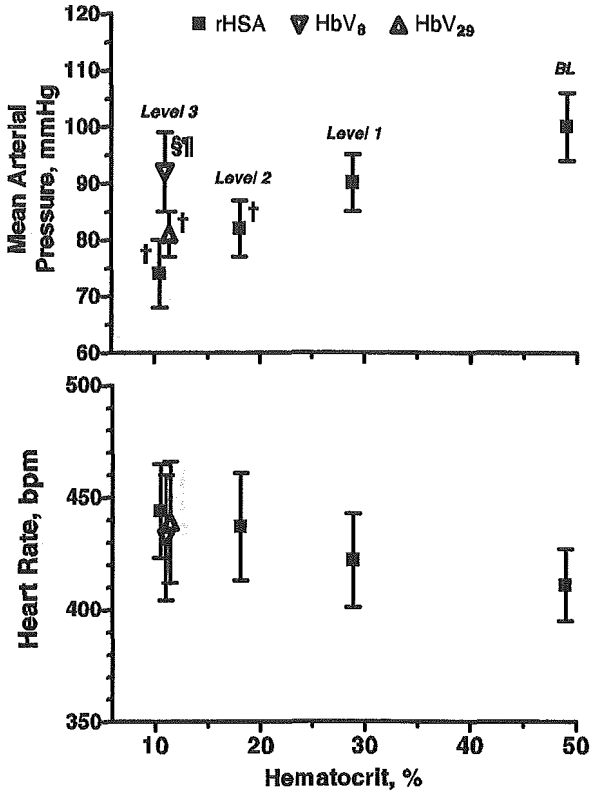

Fig. 3. Mean arterial blood pressure and heart rate [in beats/min (bpm)] at baseline (BL) (Hct 49%) and *level 1* (Hct 29%), *level 2* (Hct 18%), and *level 3* (Hct, 11%) hemodilutions. *Level 1* and *level* 2 exchanges were performed with 5% rHSA as diluent. *Level 3* exchange was used to evaluate the oxygen transport of HbV_8 vs. HbV_{29} and rHSA. †$P < 0.05$ relative to baseline; §$P < 0.05$ relative to *level 3* rHSA; ¶$P < 0.05$, *level 3* with HbV_8 vs. *level 3* with HbV_{29}.

Analysis of arterial blood gases (Table 2) showed a statistical increase in P_{O_2} after hemodilution and exchange transfusion. Pa_{CO_2} 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 2. *Laboratory parameters during exchange protocol*

		Level 1 Hemodilution	*Level 2* Hemodilution	*Level 3* Hemodilution		
	Baseline	rHSA	rHSA	rHSA	HbV_8	HbV_{29}
n	24	24	24	6	6	6
Hct, %	48.8±1.2	28.8±0.8*	18.1±0.7*	11.1±0.8*	11.0±0.5*	11.4±0.6*
Hb, g/dl						
Whole blood	14.8±0.4	9.0±0.5*	5.7±0.3*	3.7±0.4*	5.7±0.2*†	5.7±0.3*†
Plasma					2.1±0.1	2.1±0.1
Pa_{O_2}, mmHg	59.2±4.6	68.7±5.2	73.5±3.7*	87.5±7.0*	77.1±4.3*†	76.4±4.4*†
Pa_{CO_2}, mmHg	49.2±3.6	52.4±6.7	49.0±3.5	42.0±3.2*	53.0±3.9†‡	46.8±4.3
Arterial pH	7.35±0.02	7.35±0.03	7.37±0.03	7.38±0.04	7.35±0.03	7.36±0.03
HCO_3, mM	27.9±2.3	28.5±3.5	27.6±2.2	24.8±2.5	28.2±2.6	25.8±2.1
BE, mM	3.2±2.0	3.4±2.4	2.9±2.1	−0.2±1.9*	3.1±1.7†	1.0±2.0

Values are means ± 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; Pa_{o_2}, arterial partial O_2 pressure; Pa_{CO_2}, arterial partial pressure of CO_2; BE, base excess. *$P < 0.05$ compared with baseline; †$P < 0.05$ compared with *level 3* rHSA; ‡$P < 0.05$ compared with *level 3* HbV_8 to *level 3* HbV_{29}.

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_8	1.9±0.3*	1.0±0.1	17.3±0.8	6
Level 3 HbV_{29}	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^{-1} 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_8 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_{29} 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_8 and HbV_{29} caused blood flow to be significantly higher than baseline. Furthermore, the HbV_8 group showed consistently higher blood flows than the HbV_{29} 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_8, and HbV_{29} 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_8, and HbV_{29} groups showed that these materials produced virtually identical distributions of arteriolar microvascular P_{O_2} (arterioles averaged 49.5 mmHg), although HbV_8 tended to be higher (Fig. 7). The decrease of RBC from *level 2* to *level 3* did not decrease the arteriolar P_{O_2}. Venular P_{O_2} after *level 3* was significantly lower than at *level 2* exchange in all cases (rHSA, 7.2 ± 3.2 mmHg; HbV_8, 15.1 ± 3.7 mmHg; HbV_{29}, 9.6 ± 4.2 mmHg).

Tissue P_{O_2} 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_8 group, being 14.0 ± 2.2 mmHg. By comparison, tissue P_{O_2} for the HbV_{29} 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_8 and HbV_{29} groups (Fig. 7).

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

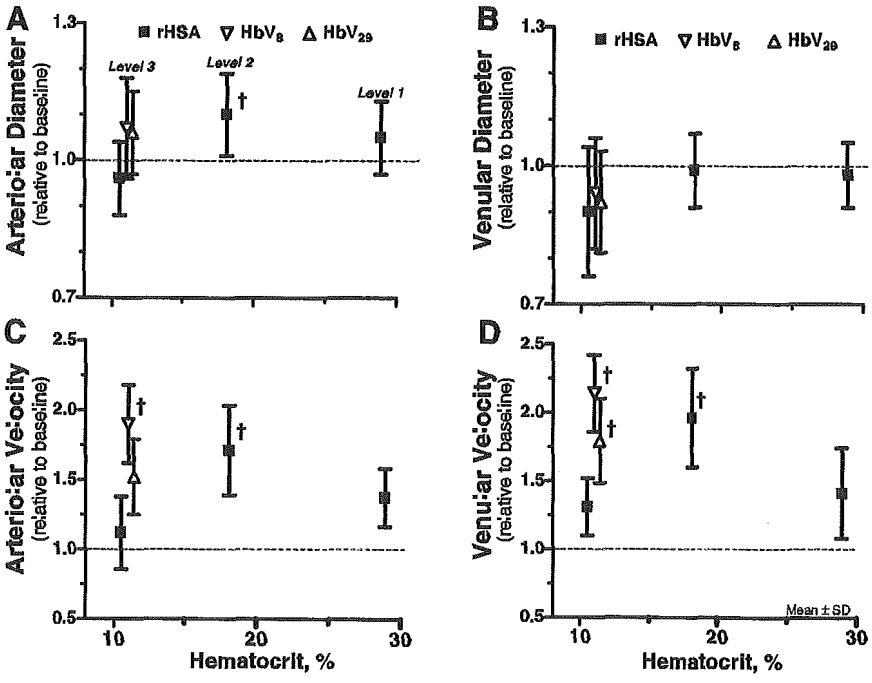

Fig. 4. Changes relative to baseline in arteriolar and venular hemodynamics at the *level 1*, *level 2*, and *level 3* exchanges. Dashed lines represent baseline level. †$P <$ 0.05 relative to baseline. Arteriolar (*A*) and venular (*B*) diameters (μm, means ± SD, *n* = no. of vessels studied) in each 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_8: A, 64.9 ± 15.7, *n* = 20; V, 64.6 ± 18.2, *n* = 20. *Level 3* with HbV_{29}: 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_8: A, 7.2 ± 1.8, *n* = 20; V, 3.3 ± 1.0, *n* = 20. *Level 3* with HbV_{29}: A, 7.0 ± 1.7, *n* = 20; V, 3.0 ± 0.9, *n* = 20.

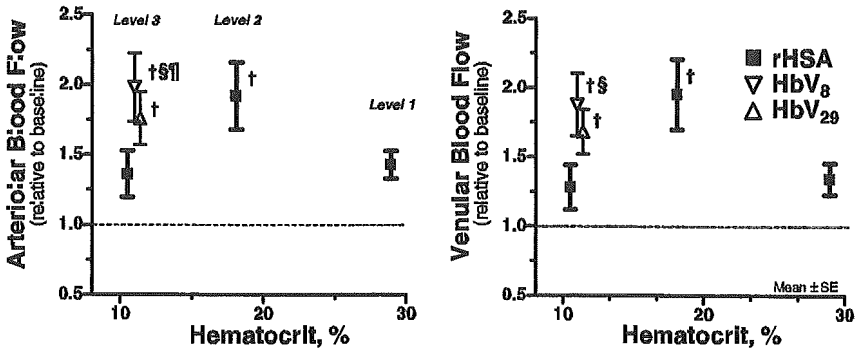

Fig. 5. Arteriolar and venular flow (nl/s, means ± SD, n = no. of vessels studied) in each animal group were as follows. Baseline: arterioles (A), 14.8 ± 7.1, n = 76; venules (V), 5.0 ± 2.9, n = 76. *Level 1* with rHSA: A, 21.9 ± 9.7; V, 5.8 ± 3.6. *Level 2* with rHSA: A, 27.2 ± 16.1; V, 8.3 ± 4.2. *Level 3* with rHSA: A, 16.9 ± 6.8, n = 20; V, 5.4 ± 4.8, n = 20. *Level 3* with HbV_8: A, 23.4 ± 8.1, n = 18; V, 9.9 ± 5.1, n = 18. *Level 3* with HbV_{29}: A, 21.0 ± 8.0, n = 18; V, 8.3 ± 5.2, n = 18.

microcirculation. It is apparent that exchanging RBC for HbV_8 maintains oxygen delivery to the tissue, whereas HbV_{29} reduces this by ~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_{29} = 29 mmHg) and low P_{50} (HbV_8 = 8 mmHg), tissue P_{O_2} is statistically significantly higher when the high oxygen affinity material is used, namely, 14.0 ± 2.2 vs. 9.2 ± 2.7 mmHg. The significantly increased tissue P_{O_2} attained with HbV_8 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_8 than for HbV_{29}.

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-oxygen-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.

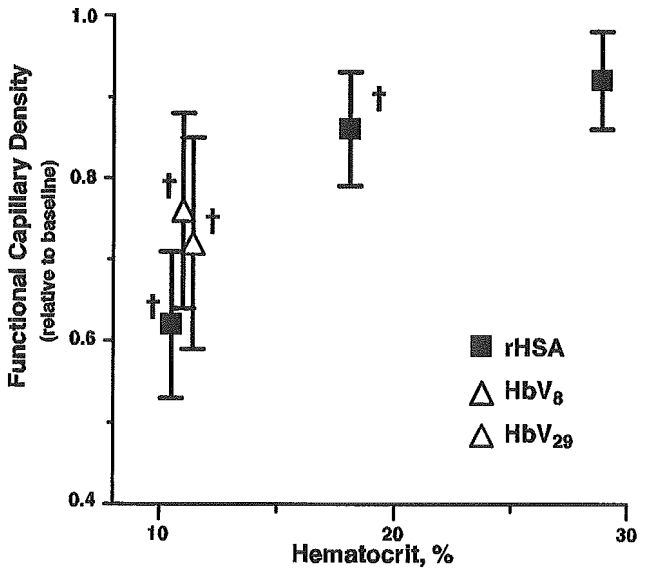

Fig. 6. Functional capillary density after the *level 1*, *level 2*, and *level 3* exchanges for the different test fluids. All values are relative to baseline levels. †$P < 0.05$ relative to baseline.

Average oxygen delivery and extraction were somewhat greater for HbV_8 than for HbV_{29}. 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_8 and HbV_{29} 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_{29} 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_{29}.

The differences in tissue P_{O_2}, mean arterial blood pressure, and arteriolar blood flow between HbV_8 and HbV_{29} 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

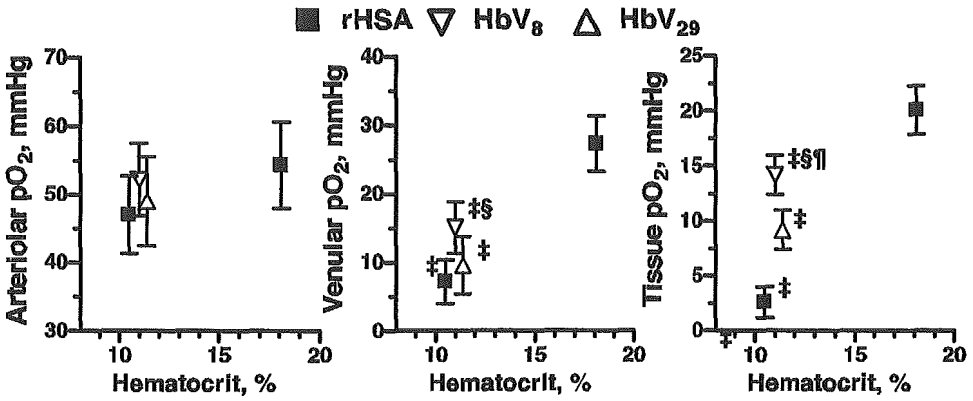

Fig. 7. Intravascular P_{O_2} after the *level 2* and *level 3* hemodilutions. Values are presented as means ± SD. ‡$P < 0.05$ compared with *level 2* with rHSA; §$P < 0.05$ compared with *level 3* with rHSA; ¶$P < 0.05$, *level 3* with HbV_8 vs. *level 3* with HbV_{29}.

where the anoxic threshold is passed, thus eliminating the inherent variability of oxygen delivery shown by the variability of tissue P_{O_2}.

Considering the significantly improved blood pressure and the trend toward higher flow for HbV_8 (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_8 vs. HbV_{29}.

In summary, the present results show that either HbV_8 or HbV_{29} 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_8 appeared to be beneficial at the systemic level, because base excess remained at baseline levels, whereas it was decreased for HbV_{29}. This finding suggests that improved tissue P_{O_2} 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 P_{O_2}, 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_{50} Hbs vs. an identical oxygen carrier with normal P_{50} 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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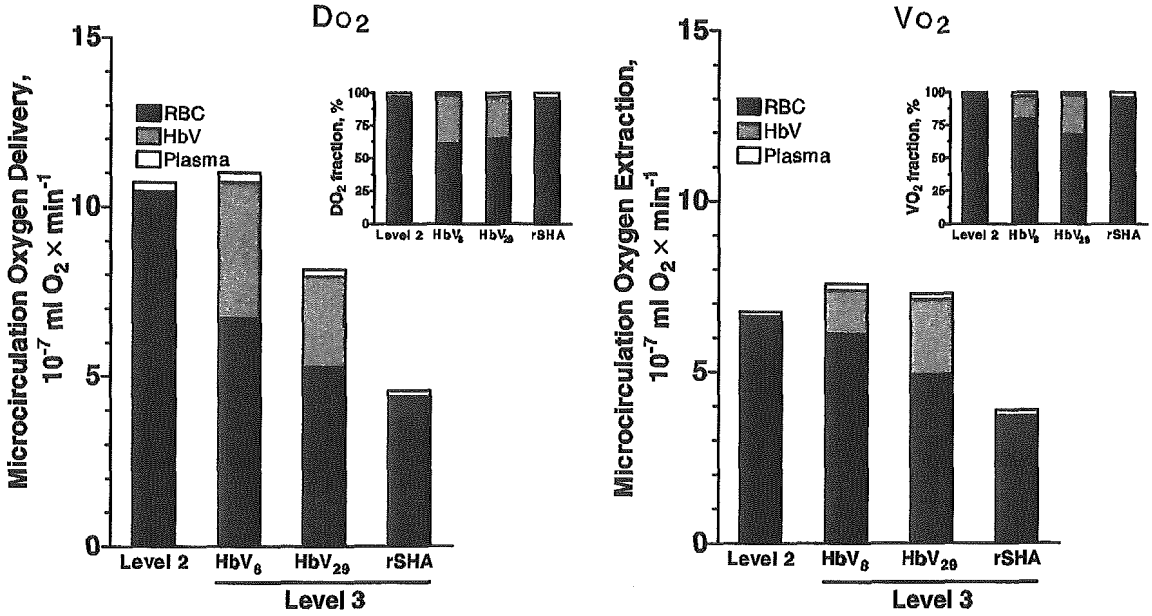

Fig. 8. Arterial oxygen delivery (D_{O_2}) and extraction (V_{O_2}) before and after the *level 3* hemodilution. Calculations of global oxygen transport are not directly measurable in our model; however, the changes relative to baseline can be calculated using the measured parameters. These calculations 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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Oxygen release from low and normal P_{50} Hb vesicles in transiently occluded arterioles of the hamster window model

Hiromi Sakai,[1] Pedro Cabrales,[2,3] Amy G. Tsai,[2,3] Eishun Tsuchida,[1] and Marcos Intaglietta[2,3]

[1]*Advanced Research Institute for Science and Engineering, Waseda University, Tokyo, Japan; and* [2]*Department of Bioengineering, University of California-San Diego, and* [3]*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_8 and HbV_{29}, respectively) and observed their O_2-releasing behavior from an occluded arteriole in a hamster skinfold window model. Conscious hamsters received HbV_8 or HbV_{29} at a dose rate of 7 ml/kg. In the microscopic view, an arteriole (diameter: 53.0 ± 6.6 μm) was occluded transcutaneously by a glass pipette on a manipulator, and the reduction of the intra-arteriolar P_{O_2} 100 μm 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_8 infusion showed a slightly slower rate of P_{O_2} reduction compared with that after HbV_{29} 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_8 showed a significantly slower rate of O_2 release compared with HbV_{29} 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 ~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 μm 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.

Address for reprint requests and other correspondence: E. Tsuchida, Advanced Research Institute for Science and Engineering, Waseda Univ., Tokyo 169-8555, Japan (E-mail: eishun@waseda.jp).

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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_{29}) 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_8) 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 μm) and deoxygenated with N_2 bubbling for storage. The endotoxin content was measured with a modified Limulus amebocyte lysate assay, and the level was less than 0.2 EU/ml (27). The O_2 equilibrium curves (OECs) of HbV_{29} and HbV_8 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 ± 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 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).

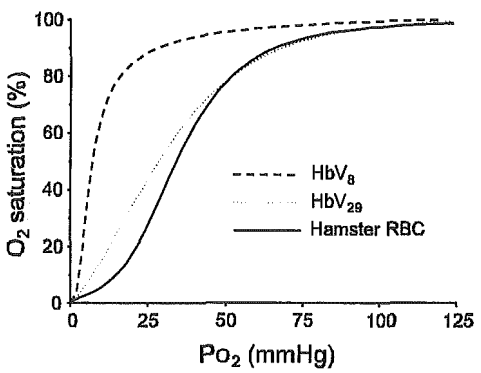

Fig. 1. Oxygen equilibrium curves (OECs) of Hb vesicles (HbVs) at a Po_2 where Hb is half-saturated (P_{50}) of 8 mmHg (HbV_8) and 29 mmHg (HbV_{29}) measured with a Hemox Analyzer (TCS Medical Products) at 37°C compared with hamster blood. RBC, red blood cells.

Table 1. *Physicochemical properties of HbV_8 and HbV_{29} compared with hamster blood*

Parameters	HbV_8	HbV_{29}	Hamster Blood
Hb concentration, g/dl	10	10	14.8 ± 0.5
Particle diameter, nm	250 ± 64	247 ± 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_8 and HbV_{29}, Hb vesicles (HbVs) at 8- and 29-mmHg Po_2 at which Hb is 50% saturated (P_{50}); PLP, pyridoxal 5′-phosphate. *Size of hamster red blood cells (RBCs) (39).

Infusion of HbV_8 and HbV_{29} 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 Po_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 Po_2 (see below) were performed before the infusion of HbV_8 or HbV_{29} 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 ± 6.6 μm) 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-

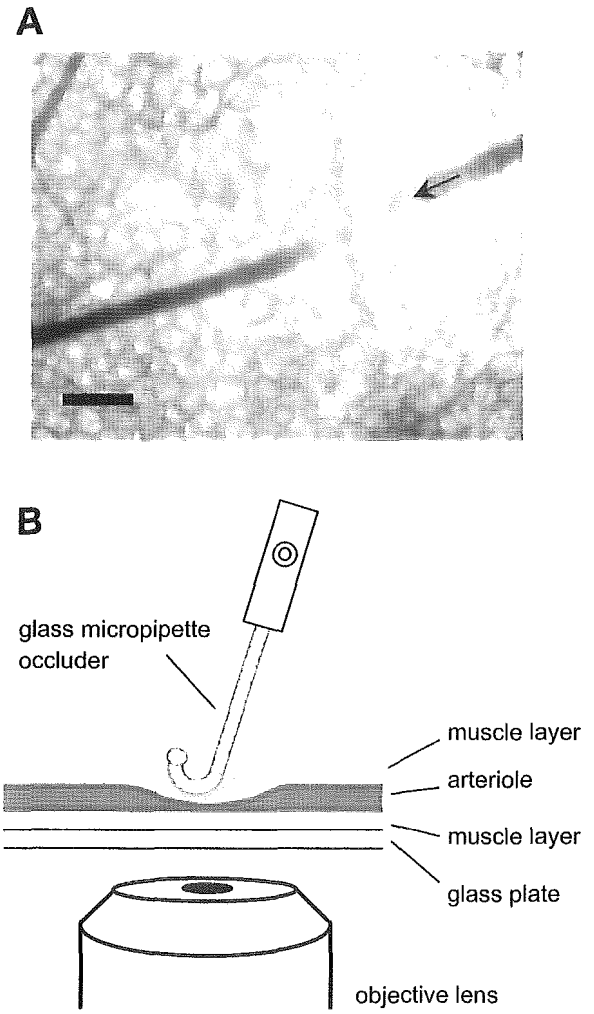

Fig. 2. *A*: microscopic image of an occluded arteriole in the hamster window chamber. The glass fiber lies across the arteriole. Scale bar = 100 μm. *B*: schematic representation of occlusion of *A* showing the different tissue layers of the skin (not to scale).

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:

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

where τ_o and τ 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 × 5 μm 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 ± 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°C) according to Boyle-Charle'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 atm·l·K^{-1}·mol^{-1}), and T is absolute temperature [23.6 (ml) = 9.3×10^{-4} (mol) × 0.082 × (273 + 37) × 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°C was calculated to be 2.42 ml in 100 ml water. Sa_{O_2}(RBC) and Sa_{O_2}(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 ([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 ± 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 ± 1.23 ml O_2/dl blood according to *Eq. 3*. After the infusion of HbV_8 and HbV_{29}, the O_2 content increased to 20.30 ± 1.18 and 20.17 ± 1.54 ml O_2/dl blood, respectively, due to the O_2 bound to HbVs. The contributions of HbV_8 and HbV_{29} to whole O_2 content were 1.51 ± 0.01 and 1.25 ± 0.07 ml O_2/dl blood, respectively. The HbV_8 group showed higher O_2 content than the HbV_{29} group due to the higher $Sa_{O_2}(HbV_8)$, which was 95.9 ± 0.6% compared with the $Sa_{O_2}(HbV_{29})$ of 79.6 ± 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_8	HbV_{29}
Arteriolar diameter, μm	53.0±6.6	56.2±6.8	55.8±6.9
Centerline flow velocity, mm/s	3.1±0.5	3.4±0.7	3.5±0.5
Blood flow rate, nl/s	6.8±1.6	8.7±3.1	8.5±2.1
Arteriolar P_{O_2}, mmHg	50.7±4.7	51.4±4.8	52.1±5.3
Sa_{O_2}(RBC), %	78.1±5.1	76.0±7.7	77.9±6.5
Sa_{O_2}(HbV), %		95.9±0.6†	79.6±4.7
O_2 content in whole blood, ml O_2/dl blood	18.61±1.23	20.30±1.18*	20.17±1.54*
O_2 content in HbV, ml O_2/dl blood		1.51±0.01	1.25±0.07

Values are means ± SD. Arteriolar P_{O_2}, O_2 saturation (Sa_{O_2}) and O_2 contents were obtained during 6 s before occlusion. *$P < 0.05$ vs. before infusion; †$P < 0.05$ vs. RBCs and HbV_{29}.

30-s occlusion, respectively. When the P_{O_2} values were expressed as relative to the baseline values (before occlusion), infusion of HbV_8 tended to show a slower rate of reduction of P_{O_2} compared with the infusion of HbV_{29} and without infusion (Fig. 4). There was a significant difference between the HbV_8 infusion and before infusion groups only at 7 s ($P = 0.035$).

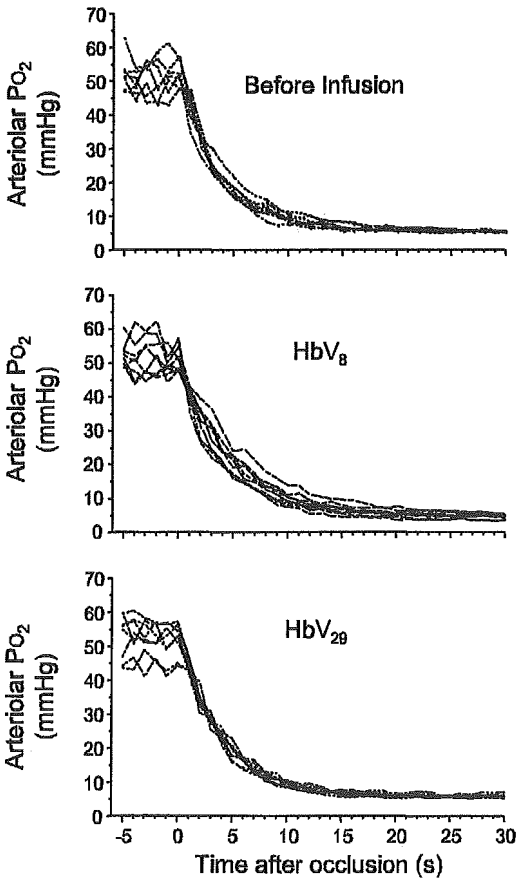

Fig. 3. Time course of P_{O_2} in the blood of an occluded arteriole (diameter, 53.0 ± 6.6 μm) before and after infusion of 7 ml/kg HbV_8 or HbV_{29} into hamsters. Measurements were made in blood at a distance of 50 μm from the point of occlusion. Most vessels equilibrate to intravascular partial pressure in the range of 4–6 mmHg about 15–20 s after occlusion.

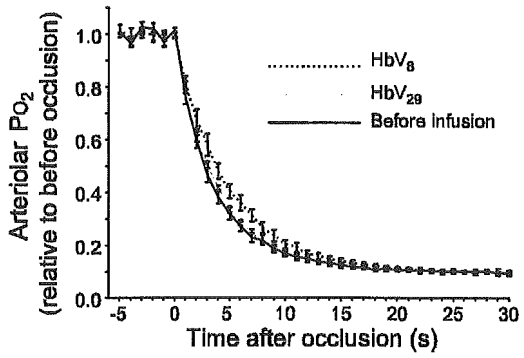

Fig. 4. Changes in P_{O_2} relative to before occlusion. The data in Fig. 3 were averaged. Baseline values before occlusion were obtained as the average of 6 values before occlusion and fixed as 1.0. There was a significant difference between the HbV_8 infusion and before infusion groups only at 7 s ($P = 0.035$).

Sa_{O_2}(RBC) and Sa_{O_2}(HbV) at every arteriolar P_{O_2} 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 5*A* shows the changes in the whole arteriolar O_2 content

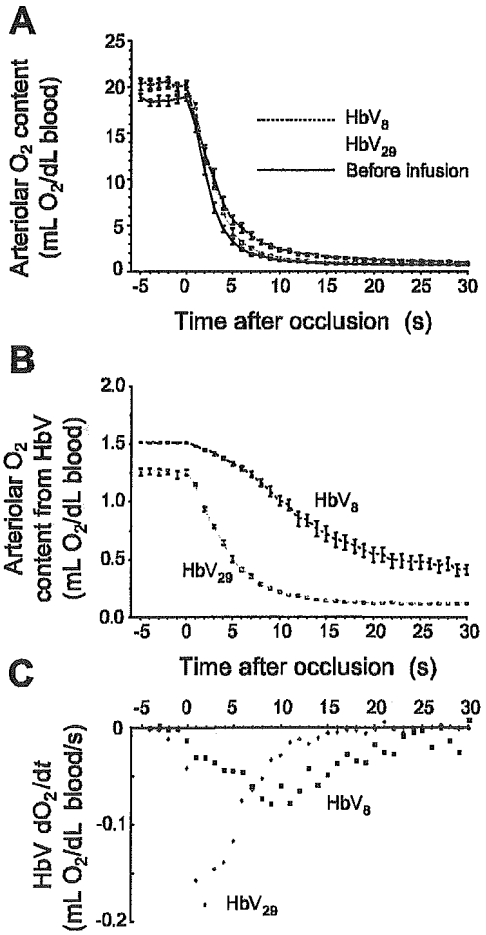

Fig. 5. *A*: time course of the arteriolar O_2 content in whole blood of an occluded arteriole before and after infusion of 7 ml/kg HbV_8 or HbV_{29} into hamsters. The O_2 contents were calculated using *Eq. 2* and the data of OECs (Fig. 1) and P_{O_2} changes (Fig. 3). *B*: time course of the O_2 content 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_2 loss dO_2/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_8 group showed a slower rate of reduction compared with the HbV_{29} 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. 5*B*. HbV_8 showed a very slow rate of O_2 release. After 30 s of occlusion, the arteriolar P_{O_2} decreased to 5.2 ± 0.7 mmHg. However, $Sa_{O_2}(HbV_8)$ was 26.1 ± 7.3% and did not reach steady state but continued O_2 release. HbV_{29} showed almost no change after 15 s, and $Sa_{O_2}(HbV_{29})$ was 7.4 ± 1.0% after 30 s. Figure 5*C* shows the rate of O_2 loss from HbVs obtained by the differentiation of the graphs in Fig. 5B. HbV_{29} 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_8 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_8 and HbV_{29} 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_8 increased significantly and was about 0.55 after 10 s. This indicated that HbV_8, 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_8 (P_{50} = 8 mmHg) with a high O_2 affinity (low P_{50}) releases O_2 at a slower rate than does HbV_{29} in occluded arterioles of the hamster dorsal skinfold model. Furthermore, we found that HbV_8, and not HbV_{29}, 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 ± 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. $Sa_{O_2}(HbV_8)$ at 5 mmHg is estimated to be about 26% according to the OECs (Fig. 1), which is higher than that for HbV_{29} (6%) and RBCs (2%); thus HbV_8 remains a source of O_2 for a longer period in a prolonged occlusion, because the fraction of O_2 from HbV_8 was 0.5 or higher, overwhelming the contribution from RBCs, as shown in Fig. 6.

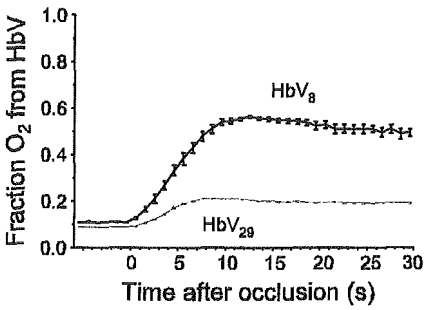

Fig. 6. Time course of the fraction of O_2 content from HbVs in whole blood. The extended time of occlusion induced hypoxic conditions and the fraction of O_2 content from HbV_8 increased significantly compared with HbV_{29}.

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 μm) 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_8 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_8 (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 collaterized 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,

is that they are homogeneously dispersed in the plasma phase and therefore can deliver O_2 more homogeneously to the periphery than RBCs because microvascular hematocrit is heterogeneous particularly in pathological states. In such conditions, HbVs with a higher O_2 affinity should show a slower O_2 unloading that would be effective for oxygenating ischemic tissues.

In conclusion, HbVs provide the unique feature of allowing for the regulation of P_{50} by modulating the amount of coencapsulated PLP (33, 45). Recent studies showed the effectiveness of HBOCs with a lower P_{50} (higher O_2 affinity) as a means of implementing O_2 delivery targeted to ischemic tissue (2, 3, 41, 43). Thus this experimental method provides data useful for the design and optimization of O_2 carriers and suggests the possible utilization of HbVs for therapeutic approaches aimed at remedying ischemic conditions.

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Is hemoglobin in hemoglobin vesicles infused for isovolemic hemodilution necessary to improve oxygenation in critically ischemic hamster skin?

Jan A. Plock,[1] Claudio Contaldo,[1] Hiromi Sakai,[2] Eishun Tsuchida,[2] Michael Leunig,[1] Andrej Banic,[1] Michael D. Menger,[3] and Dominique Erni[1]

[1]*Department of Orthopedic, Plastic and Hand Surgery, Inselspital University Hospital, Berne, Switzerland;*
[2]*Advanced Research Institute for Science and Engineering, Waseda University, Tokyo, Japan; and*
[3]*Institute for Clinical and Experimental Surgery, University of Saarland, Homburg/Saar, Germany*

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Plock, Jan A., Claudio Contaldo, Hiromi Sakai, Eishun Tsuchida, Michael Leunig, Andrej Banic, Michael D. Menger, and Dominique Erni. Is hemoglobin in hemoglobin vesicles infused for isovolemic hemodilution necessary to improve oxygenation in critically ischemic hamster skin? *Am J Physiol Heart Circ Physiol* 289: H2624–H2631, 2005. First published August 5, 2005; doi:10.1152/ajpheart.00308.2005.—The aim of this study was to test the influence of hemoglobin, encapsulated in phospholipid vesicles as an oxygen carrier, given in the course of isovolemic hemodilution to improve oxygenation in critically ischemic hamster flap tissue. Capillary hemodynamics and macromolecular leakage were investigated with intravital microscopy and analyzed off-line with the CapImage software. Partial tissue oxygen tension was measured with fluorescence quenching electrodes. The occurrence of apoptosis was assessed with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. Vesicles with (HbV) or without (V) encapsulated Hb were suspended in 6% hydroxyethyl starch (HES) used for the 33% blood exchange. In the ischemic tissue, hemodilution led to an increase in functional capillary density by 31% for HES ($P < 0.01$ vs. other groups), 66% for V-HES, and 62% for HbV-HES (all $P < 0.01$ vs. control). Capillary diameters behaved inversely proportional to capillary microhemodynamics. The 20% increase in macromolecular leakage found over time in control animals was completely abolished in the vesicles groups ($P < 0.01$) but not with HES. Oxygen tension was improved from 10.7 to 16.0 mmHg after HbV-HES ($P < 0.01$ vs. baseline and other groups). Compared with the other groups, apoptosis was significantly reduced after HbV-HES ($P < 0.01$). We conclude that the encapsulation of Hb was essential to attenuate hypoxia and subsequent cell death in the critically ischemic tissue. However, the effect was partly attributed to the rheological changes exerted by the vesicles.

blood substitutes; capillary hemodynamics; hypoxia; capillary leakage; apoptosis

CRITICAL ISCHEMIA is characterized by a reduction of nutrient blood flow, thus causing hypoxia that may eventually lead to apoptosis and cell death. One of the most frequent etiologies of critical ischemia is the acute peripheral arterial obstruction. Oxygenation and survival of ischemic myocardial (13, 24), cerebral (23, 32), and peripheral (6) tissues could successfully be improved after the infusion of solutions containing artificial oxygen carriers, such as perfluorocarbons and chemically modified Hbs.

In recent studies (8, 12), we were able to demonstrate that hypoxia in ischemic hamster flap tissue was attenuated by isovolemic hemodilution with colloid solutions supplemented with phospholipid vesicles containing isolated, purified human Hb. The effect was ascribed to the combination of an improvement of the impaired microcirculation and the presence of the Hb vesicles (HbVs) (12), and it correlated with the degree of blood exchange (8). However, it was not possible to outline the extent to which either the rheological changes or the presence of Hb contributed to this benefit. In other words, it could not be excluded that similar success could have been achieved with the use of phospholipid vesicles void of oxygen carriers, which in turn would have a significant impact on their clinical application, because the manufacturing of the vesicles could be simplified and possible adverse effects related to the encapsulated Hb could be avoided. Furthermore, it may be postulated that the presence of cell-free Hbs may lead to arteriolar vasoconstriction with (4, 26) or without (14) scavenging of nitric oxide, which may further deteriorate microvascular perfusion and oxygen delivery in the ischemic tissue.

In this context, the viscosity of the diluent appears to play a pivotal role. Because of the large size of the vesicles, the viscosity of HbV solutions is manyfold higher than that of hamster plasma (12, 26). Raising the viscosity in the plasma phase of the circulating blood led to shear stress-induced, nitric oxide-mediated arteriolar vasodilation (2, 9), which was made responsible for increasing microcirculatory blood flow (2), microvascular pressure (3), and functional capillary density (FCD) (2, 3) in healthy tissue in hamsters. Furthermore, according to the Stokes-Einstein equation, the diffusivity of oxygen through the plasma is inversely proportional to its viscosity, an effect that may contribute to the distribution of oxygen release in favor of hypoxic tissues, in which oxygen diffusion is ensured by the high gradient of partial oxygen tension.

The hypothesis to be tested in this study was whether the presence of Hb in the HbV is needed to obtain the previously reported benefit of isovolemic hemodilution with HbV on the oxygenation of the ischemic hamster flap tissue (8, 12) or whether similar effects could be obtained with a suspension of vesicles void of Hb due to their viscosity-related effect on arteriolar and capillary hemodynamics and on tissue oxygenation.

MATERIALS AND METHODS

Experiments were performed according to the National Institutes of Health guidelines for the care and use of laboratory animals and with

Address for reprint requests and other correspondence: D. Erni, Division of Plastic and Reconstructive Surgery, Inselspital Univ. Hospital, CH-3010 Berne, Switzerland (e-mail: dominique.erni@insel.ch).

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the approval of the local Animal Ethics Committee. Forty-eight male Syrian golden hamsters weighing 65–85 g were used in this study. The animals were randomly assigned to the control group or to one of three groups subjected to normovolemic hemodilution with 6% hydroxyethyl starch 200–0.5 (HES; Fresenius, Stans, Switzerland) or vesicles with or without encapsulated Hb suspended in hydroxyethyl starch (HbV-HES and V-HES, respectively).

Animal and flap preparation. A hamster skin flap model was used as previously described in detail (7, 8, 10–12). Anesthesia was induced by pentobarbital sodium (Nembutal) injected intraperitoneally (100 mg/kg body wt; Abbott Laboratories, Chicago, IL). The carotid artery and external jugular vein were cannulated for administration of anesthesia, blood exchange, laboratory analysis, and monitoring arterial blood pressure (Type514; Spacelabs, Hillsboro, OR). Catheterization and flap dissection were performed with the aid of an operating microscope at ×10 magnification (Wild; Heerbrugg, Switzerland). An island flap measuring 3 × 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 it was perfused by one vascular axis, which bifurcates 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 merely perfused by a collateral vasculature connecting the two vascular networks. During surgery, the flap was irrigated with 0.9% NaCl solution 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. During surgery, 4 mg papaverine hydrochloride (Sigma Chemical, St. Louis, MO) dissolved in 1 ml physiological saline solution were applied to the pedicle by a soaked cotton tip to prevent vascular spasm.

Vesicle solutions. The vesicles were prepared as previously reported (27, 28). They consisted of a phospholipid bilayer membrane coated with polyethylene glycol encapsulating either physiological saline solution (V) or isolated and purified human hemoglobin (HbV). The sizes of V and HbV were 274 ± 32 and 253 ± 63 nm, respectively. The Hb concentration inside the HbV was ~35 g/dl, and its P_{50} was 9 mmHg, which was calculated from the O_2 equilibrium curve measured with a Hemox Analyzer (TCS Medical Products) at 37°C (33). The vesicles were suspended in a solution with a final HES concentration of 6%.

Laboratory analysis. Blood samples were collected in 40-μl heparin-washed microtubes for measurement of total Hb concentration and arterial blood gases with the use of the Radiometer ABL 625 system (Radiometer; Copenhagen, Denmark). By validating this system, we have found that the vesicle-bound hemoglobin concentration may be overestimated by maximally 10%, whereas the results were not affected by the lipid concentrations present in our study. 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-kDa 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. Viscosities of blood and plasma were measured 4 h after hemodilution with a Höppler-type viscosimeter (HAAKE Messtechnik, Karlsruhe, Germany). The physicochemical characteristics of the solutions are summarized in Table 1. Oxygen content (ml/dl) in the carotid artery was calculated according to the equation

$$[O_2] = 1.34 \cdot \{([Hb_{RBC}] \cdot So_{2RBC}) + ([Hb_{HbV}] \cdot So_{2HbV})\}, \quad (1)$$

where 1.34 corresponds to the amount of oxygen (given in milliliters) bound to 1 g of Hb at 100% saturation. So_2 is the fractional oxygen saturation of red blood cells (RBCs) and HbV, which was derived from Po_2 by using the oxygen dissociation curves of the two hemoglobins (29).

Microhemodynamic measurements. Investigations were performed with the use of an intravital microscope (Axioplan 1; Zeiss, Jena, Germany). Microscopic images were captured by a television camera (intensified charge-coupled device camera; Kappa Messtechnik, Gleichen, Germany), recorded on video (50 Hz; Panasonic, Osaka, Japan), and displayed on a television screen for subsequent off-line analysis (Trinitron PVM-1454QM; Sony, Tokyo, Japan). The preparation was observed visually with a ×40 objective with a numerical aperture of 0.75, which resulted in a theoretical resolution of ~300 nm and a total optical magnification of ×909 on the video monitor, where 1 pixel corresponded to 264 nm in the tissue. The microvessels were classified according to physiological and anatomical features into conduit arterioles (connections to each other), end arterioles, and small venules (10, 12). The vessels were chosen for examination according to their optical clarity. The intraluminal microvascular diameters were measured visually on the television screen with the use of 2% fluorescein isothiocyanate-labeled dextran (FITC dextran, molecular mass 150 kDa; Sigma Chemical, Buchs, Switzerland) injected intra-arterially (0.05 ml), an excitation filter (485–505 nm), a dichroic mirror (510 nm), and a barrier filter (530 nm). The capillary hemodynamics and macromolecular leakage were assessed with a computer-assisted image analysis system (CapImage; Zeintl Software, Heidelberg, Germany) (17). Capillary diameters were obtained from the averages of five consecutive measurements. Because the capillary diameters measured with the present technique may possibly be underestimated because of the use of fluorescence microscopy and the optical properties of the microscope (22), the values were given in percentages of the mean obtained in the anatomically perfused tissue of the control group at baseline. FCD was defined as the length of RBC-perfused capillaries per observation field and expressed in centimeters per square centimeters. The product of RBC velocity and FCD was taken as an index reflecting the perfusion of the tissue with RBCs. The endothelial integrity was assessed by measuring macromolecular leakage (18). This was achieved by densitometric analysis of the fluorescence of FITC dextran 10 min after its injection. Macromolecular leakage was expressed by the ratio of fluorescence obtained in the interstitial space versus capillary fluorescence.

Table 1. *Physicochemical characteristics of hamster blood and diluents*

	Hamster Blood	Hamster Plasma	HES	V-HES	HbV-HES
[Hb], g/dl	18	0	0	0	7.5
[metHb], %					<3
[Lipid], g/dl				4.4	4.2
Oncotic pressure, mmHg		18	36	36	36
Viscosity of solution, cP	4.5	1.2	1.9	11.5	11.5
Plasma viscosity 4 h after exchange transfusion, cP		1.34 ± 0.03	1.31 ± 0.06	1.74 ± 0.13*	1.67 ± 0.12*

Values are means ± SD. HES, 6% hydroxyethyl starch; V-HES and HbV-HES, vesicles with and without hemoglobin suspended in HES, respectively; [Hb], hemoglobin concentration; [metHb], methemoglobin concentration. [Hb] was measured by a cyanomethemoglobin method, and [lipid] was measured with enzymatic method with use of phospholipase D. Viscosity of solutions was measured at 37°C and at 150 s^{-1}; plasma viscosity was measured at 25°C. *$P <$ 0.01 vs. hamster plasma and HES.

Tissue oxygen tension. Partial tissue oxygen tension was assessed with combined bare fiber probes with a tip diameter of 450 μm (Oxylite probes; Oxford Optronix, Oxford, UK). The sensitive tip of the oxygen probe (100-μm diameter) consists of ruthenium-III-(Tris)-chloride, which measures 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 the manufacturer, the bare fiber probe provides resolutions of <1 mmHg and 0.1°C for partial oxygen tension and temperature, respectively, and the sampling area of the oxygen sensors is 0.25–0.35 mm^3. The probes were inserted into the subcutaneous tissue in the middle of each vascular territory under visual microscopic control. Care was taken to place the probes in such a way that no arterioles or large venules lay within the sampling area.

Tissue viability. The occurrence of apoptosis was assessed with the transferase-mediated dUTP nick end-labeling (TUNEL) assay (In Situ Cell Death Detection Kit, tetramethylrhodamine red; Roche Diagnostics, Rotkreuz, Switzerland) (1). All steps were performed according to the supplier's instructions. Tissue samples were obtained from the middle of each vascular territory. The samples were transferred to gelatinized microslides and air-dried overnight at 37°C. The sections were dewaxed in xylene (three changes), rehydrated in ethanol, and rinsed in Tris-buffered saline [50 mM Tris·NaCl, pH 7.4, containing 100 mM sodium chloride (two changes)], and then incubated in 20 μg/ml proteinase K for 15 min at room temperature. Endogenous peroxidase activity was suppressed by treatment with 0.3% hydrogen peroxide for 10 min. The sections were then incubated with terminal deoxynucleotidyl transferase enzyme for 1 h at 37°C followed by peroxidase-conjugated anti-digoxigenin antibody for 30 min at room temperature. The reaction was visualized by diaminobenzidine substrate for 8 min at room temperature. Thereafter, the sections were washed three times with Tris-buffered saline. The labeled DNA fragments were visualized by incubating the sections with tetramethylrhodamine used as a fluorescence marker, and the sections were examined with a fluorescence microscope (Leica DM/RB; Leica Microsystems, Wetzlar, Germany). Data were given as the averages of fluorescent cells counted in five randomly selected visual fields (0.5 × 0.5 mm) for the dermis and epidermis separately. Sebaceous glands and hair follicles were identified and excluded from the cell counts because of their consistently high apoptosis rate.

Protocol. The animals were kept under light anesthesia with a continuous infusion of 50 mg/ml pentobarbital sodium given at a rate of ~0.5 $mg \cdot min^{-1} \cdot kg$ body wt^{-1} throughout the experiment. The depth of anesthesia was regulated by tolerance of a noxious reflex due to pinching of the hind paw but no nonaversive reflexes (palpebral, corneal, and jaw reflex) (10). A constant temperature in the animal and flap preparation was maintained by means of a heating pad and by keeping room temperature at 28°C.

Baseline values were obtained after a postoperative period of 1 h had elapsed for stabilization. Thereafter, one-third of the total blood volume was exchanged with HES or the vesicle solutions. This was achieved by simultaneous blood withdrawal via the carotid catheter and infusion via the jugular catheter over 15 min. Measurements were taken hourly until 4 h after hemodilution, and tissue samples for immunohistochemical analysis were taken after 5 h.

Exclusion criteria were abnormalities of the vascular anatomy, insufficient optical clarity, mean arterial pressure <60 mmHg, and systemic arterial pH, P_{O_2}, and P_{CO_2} outside the normal ranges at baseline (7.19–7.29, 35–55, and 45–65 mmHg, respectively).

The animals were euthanized with an overdose of pentobarbital sodium at the end of the experiment.

Statistical analysis. The InStat version 3 program (Graph Pad Software; San Diego, CA) was utilized for statistical analysis. The data were presented as means ± SD. The time-related differences between repeat measurements were assessed by the paired ANOVA, followed by Dunnett's posttest. The differences between groups were assessed by the unpaired ANOVA, followed by Tukey's posttest. If only two sets of data were to be compared, paired (repeat measurements) and unpaired (differences between groups) *t*-tests were used. A value of $P < 0.05$ was taken to represent statistical significance.

RESULTS

Six animals did not fulfill the inclusion criteria and were excluded from this study, thus resulting in sample sizes of $n = 11$ for control, $n = 11$ for HES, $n = 9$ for V-HES, and $n = 11$ for HbV-HES.

The systemic data are summarized in Table 2. Similar hematocrits were obtained in all hemodiluted animals. The blood exchange reduced mean total Hb concentration to 10.4 and 10.1 g/dl for HES and V-HES, respectively, but only to 13.0 g/dl if HbV was added ($P < 0.01$ vs. other groups). Hemodilution increased arterial P_{O_2} to mean values of 58–61 mmHg ($P < 0.01$ vs. baseline) and decreased P_{CO_2} to 40–41 mmHg ($P < 0.05$), whereas pH remained virtually unchanged. Compared with the control animals, plasma viscosity was increased from 1.34 to ~1.7 cP after hemodilution with both vesicle solutions ($P < 0.01$ vs. control) but not with HES (Table 1).

Hemodilution resulted in an arterial oxygen content decrease from ~18 to 12.8 ± 1.5 ml/dl for HES and 12.6 ± 1.3 ml/dl for V-HES (both $P < 0.01$) after 4 h, whereas this reduction of oxygen-carrying capacity was significantly attenuated by adding HbV to the diluent (15.7 ± 1.2 ml/dl; $P < 0.01$ vs. baseline and other groups) (Fig. 1).

Table 2. *Systemic and laboratory data at baseline and 1 and 4 h after blood exchange*

	Baseline	1 h	4 h
MAP, mmHg			
Control	109±5	104±8	101±7
HES	105±8	107±5	99±2
V-HES	107±5	109±5	102±6
HbV-HES	105±5	107±5	103±3
Hematocrit			
Control	0.55±0.03	0.55±0.03	0.53±0.03
HES	0.57±0.03	0.33±0.03[b,d]	0.33±0.03[b,d]
V-HES	0.57±0.02	0.32±0.02[b,d]	0.32±0.01[b,d]
HbV-HES	0.56±0.02	0.33±0.02[b,d]	0.33±0.02[b,d]
Total Hb concentration, g/dl			
Control	18.0±1.1	18.0±1.4	17.2±1.1
HES	17.7±1.2	10.4±0.8[b,d]	11.2±0.8[b,d]
V-HES	17.8±1.3	10.1±0.3[b,d]	10.7±0.5[b,d]
HbV-HES	17.9±0.9	13.0±0.4[b,e]	13.2±0.7[b,e]
P_{O_2}, mmHg			
Control	43±3	44±6	49±8
HES	42±5	52±9[a]	59±12[b]
V-HES	40±8	52±8[a]	61±15[b]
HbV-HES	44±6	57±8[b,c]	58±10[b]
P_{CO_2}, mmHg			
Control	53±6	52±3	48±6
HES	52±4	48±5	41±7[a]
V-HES	51±6	43±8[a,c]	40±11[a]
HbV-HES	51±7	43±8[a,c]	41±6[a]
pH			
Control	7.34±0.04	7.34±0.05	7.36±0.05
HES	7.35±0.05	7.39±0.05	7.39±0.07
V-HES	7.33±0.05	7.38±0.06	7.37±0.08
HbV-HES	7.34±0.06	7.37±0.06	7.34±0.04

Values are means ± SD. MAP, mean arterial pressure. [a]$P < 0.05$ and [b]$P < 0.01$ vs. baseline; [c]$P < 0.05$ and [d]$P < 0.01$ vs. control; [e]$P < 0.01$ vs. other groups.

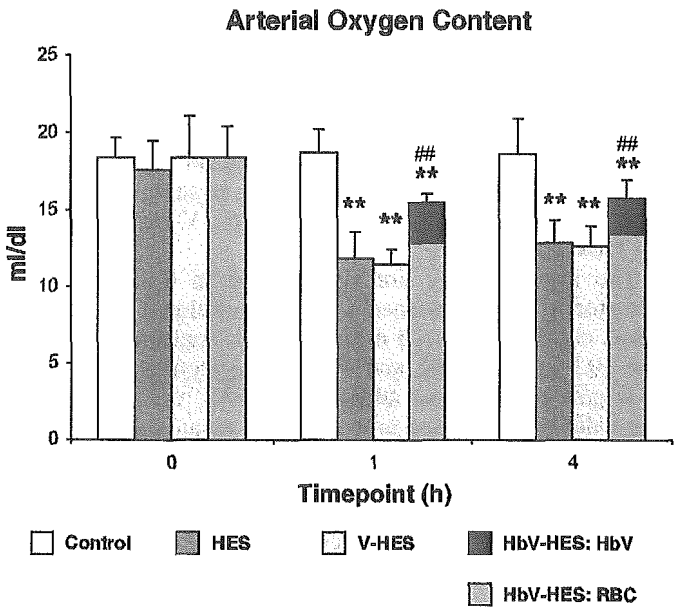

Fig. 1. Oxygen content in carotid artery at baseline and 1 and 4 h after hemodilution with 6% hydroxyethyl starch (HES) and vesicles with (HbV-HES) and without (V-HES) Hb suspended in HES, including relative contribution of red blood cells (RBCs) and HbV. Data are given as percentages of baseline and represent means ± SD. $^{**}P < 0.01$ vs. baseline; $\#\#P < 0.01$ vs. other groups.

At baseline, the microvascular diameters were 42 ± 17 μm for conduit arterioles, 10.6 ± 3.5 μm for end arterioles, and 88 ± 14 μm for venules. In both flap areas and in all groups, the diameters were similar at baseline and they remained virtually unchanged throughout the experiments.

The behavior of the capillary hemodynamics in both parts of the flap is shown in Fig. 2. At baseline, the capillaries in the ischemic tissue were significantly wider than the anatomically perfused capillaries (means of 3.31–3.33 vs. 2.79–2.82 μm; $P < 0.01$). In the control group, the capillaries further dilated over time in both the anatomically perfused and the ischemic tissue by 25% and 9%, respectively (both $P < 0.01$). This time-related dilation was significantly attenuated in all hemodiluted animals ($P < 0.01$ vs. control), the most pronounced after HbV-HES, which resulted in a reduction of capillary diameter in the ischemic tissue to values close to baseline values obtained in the anatomically perfused tissue (2.85 ± 0.03 μm; $P < 0.01$ vs. baseline and other groups). The induction of ischemia reduced capillary RBC velocity by ~60% ($P < 0.01$). Hemodilution increased RBC velocity by ~50% in the anatomically perfused tissue and ~150% in the ischemic tissue (both $P < 0.01$ vs. baseline and control) for all diluents, whereas RBC velocity further declined in the ischemic tissue of the control animals over time by 67% ($P < 0.01$). In the ischemic tissue, baseline FCD was ~50% lower than in the anatomically perfused tissue ($P < 0.01$). In the control group, FCD decreased to 85% of baseline in the anatomically perfused tissue and to 69% in the ischemic tissue over time (both $P < 0.01$), whereas hemodilution kept FCD at baseline levels in the anatomically perfused tissue ($P < 0.01$ vs. control) and increased FCD in the ischemic tissue by 31% after HES ($P < 0.01$ vs. other groups), 66% after V-HES, and 62% after HbV-HES (all $P < 0.01$ vs. baseline). At baseline, the calculated RBC perfusion index in the ischemic tissue was reduced to ~20% of the value obtained in the anatomically perfused tissue ($P < 0.01$), and it was further decreased in both tissues of the control animals over time ($P < 0.01$). Hemodilution raised the RBC perfusion index by ~50% in the anatomically perfused tissue, independently of the diluent given ($P < 0.01$ vs. baseline and control), and by 186% after HES ($P < 0.01$ vs. other groups), 330% after V-HES, and 316% after HbV-HES in the ischemic tissue (all $P < 0.01$ vs. baseline and control; P = not significant between vesicle groups).

The baseline macromolecular leakage was slightly increased in the ischemic tissue compared with the anatomically perfused part (not significant; Fig. 3). In the control and HES groups, macromolecular leakage was increased by 20–30% in both parts of the flap over time ($P < 0.01$ for anatomical; $P < 0.05$ for ischemic), whereas it remained virtually unchanged after hemodilution with the vesicle solutions ($P < 0.01$ vs. control and HES).

The baseline mean Po_2 ranged from 22.7 to 25.2 mmHg in the anatomically perfused tissue and was significantly reduced in the ischemic tissue to 10.2–10.8 mmHg ($P < 0.01$; Fig. 4). The values remained at baseline levels in both parts of the flap and in all groups except for HbV-HES, which led to a significant Po_2 increase to 16.0 ± 1.8 mmHg in the ischemic tissue ($P < 0.01$ vs. baseline and other groups).

A massive accumulation of TUNEL-positive nuclei was observed in the ischemic tissue of untreated animals (Fig. 5). Compared with the anatomically perfused tissue, a 2-fold increase was counted in the dermis and a 1.5-fold increase in the epidermis (Fig. 6; both $P < 0.01$), which were both partly attenuated by diluting the animals with HES and V-HES (both $P < 0.01$ vs. control) and completely abolished after HbV-HES, which also revealed significantly lower counts in the anatomically perfused tissue ($P < 0.01$ vs. other groups).

DISCUSSION

This study was designed to determine the relevance of Hb supplemented as an oxygen carrier to a solution used for isovolemic hemodilution with the scope of improving oxygenation in critically ischemic tissue, as previously described (8, 12). This was made possible by direct comparison of the oxygen-carrying solution with a solution void of oxygen carriers but with otherwise absolutely identical physicochemical properties, a constellation that, to our knowledge, has not yet been investigated. Our findings revealed that the presence of Hb in the vesicles administered in the course of isovolemic hemodilution was essential to significantly attenuate both hypoxia and subsequent cell death in the critically ischemic tissue, which were restored to values in the range of those found in the anatomically perfused tissue.

However, some benefit in tissue survival could also be obtained with the diluents void of oxygen carriers, which was related to a substantial improvement in all capillary hemodynamic parameters, and which was more pronounced in the compromised microcirculation in the ischemic tissue. The level of hemodilution we chose is considered to yield the maximal RBC flux at the capillary level (20). However, compared with HES, the improvement in capillary hemodynamics in the ischemic tissue was further enhanced by adding vesicles to the solution, which resulted in a significant increase in plasma