

injury was 34.8% whereas that of 88 children without inhalation injury was 12.9%. The large %BSA in the patients with inhalation injury may have resulted in inhalation injury as an exceptionally high risk factor in this age group.

The accuracy to predict mortality was 65.5% in model 1 and 66.9% in model 2, indicating that BI was a stronger indicator than %BSA to predict mortality of the burn patients. Although it is troublesome to accurately assess the depth of the burns in each patient, it gives us valuable information not only for burn wound management but also for calculating BI for assessing outcome.

Longitudinal changes of BI resulting in 50% mortality were plotted in 4 years span. BI resulting in 50% mortality was significantly higher in the non-inhalation injury group than that in the inhalation group. Although statistically not significant, BI resulting in 50% mortality has increased since 1995 in both inhalation and non-inhalation groups. It was assumed that skin banking system of Tokyo implemented in 1994 may have had a role in this improvement. The analysis was tried by taking up the use of skin bank as an independent variable, which showed the assumption was right in that the use of skin bank allograft significantly decreased the risk of mortality with the odds ratio of 0.444. In the treatment of extensive burns, skin allograft with skin banking system is indispensable. It is reported that there are 250–300 skin banks in the US, and annual need for allograft skin is estimated to be 32,000 square feet [14]. Transplantation of any organ has not been widely performed in Japan mainly because brain death is not accepted by the people. Although the skin may be harvested from cadavers, skin banking and allograft are not yet popular in Japan because of small donation of the skin in spite of big effort by the burn surgeons. Our data will support a campaign to encourage skin donation and to establish a skin banking system in Japan to improve survival of the severely burned patients.

Cultured skin and artificial dermis are used in trial in some institutions, but not commonly used in many burn care facilities in Japan. Although the clinical trial of Integra has been completed in 2000, it is not yet approved in Japan. Our data include some cases using those new techniques and materials, but they are too small in number to make any influence to statistical patient outcome.

Burn shock used to be a leading cause of death of major burns. However, sepsis and multiple organ failure (MOF) are the main causes of severe burn in recent years. Our data demonstrated that 90% of severely burned patients who died after 30 days of admission died of either sepsis or MOF. Early burn surgery has been advocated to improve survival of severely burned patients by reducing septic complications, and has been introduced in many burn care facilities [15]. As TBUA data base does not include detailed therapeutic modalities, efficacy of early burn surgery for reducing septic complications remains to be unknown.

This is the first report on area-based epidemiological and outcome characteristics of major burns in Tokyo. However, there are some points to be cautious in interpreting the data.

Firstly, the data represent only a portion of burns in Tokyo, about 15% of ambulance transported burn patients by the Tokyo Fire Department. As TBUA burn units accept only severely burned patients, age distribution, causes of burn, and other characteristics are quite different from burns at large in Tokyo.

Secondly, the volume of burn patients and management of burn care such as fluid resuscitation, timing for burn surgery, use of antibiotics, and nutritional support are different from one burn unit to another. However, quality of burn care may be not much different as TBUA has scientific review meetings four times a year. When mortality was analyzed using burn unit as an independent variable, there was no significant difference found in any of 13 burn units.

Thirdly, this is a retrospective study based on data base for the past 20 years. Thus, the data may not reflect the present status of burn care in Tokyo. There has been some technical as well as instrumental improvement for burn care during the past 20-year period. On the other hand percentage of elderly people in the total burn patients has been increasing influencing the mortality of major burn. As elderly burn patients are expected to keep increasing in Japan, establishing effective skin banking system and burn registry system is keenly needed to improve survival of major burns in Japan.

The list of 13 burn units of the Tokyo Burn Unit Association

- Tokyo Women's Medical University Hospital (Dr. Motohiro Nozaki)
- Nippon Medical School Hospital (Dr. Yasuhiro Yamamoto)
- Teikyo University Hospital (Dr. Kunio Kobayashi, Dr. Hiroto Ikeda)
- Koto Hospital (Dr. Motomichi Urabe)
- Kyorin University Hospital (Dr. Shuji Shimazaki)
- Tokyo Medical University Hospital (Dr. Akira Sugamata)
- Keio University Hospital (Dr. Naoki Aikawa)
- Nippon Medical School Tamanagayama Hospital (Dr. Norifumi Ninomiya)
- Tokyo Metropolitan Hiroo Hospital (Dr. Hiroyuki Sakurai)
- Tokyo Metropolitan Bokutoh Hospital (Dr. Yuichi Hamabe)
- Tokyo Metropolitan Fuchu Hospital (Dr. Ryouhei Higuchi)
- National Hospital Organization Disaster Medical Center (Dr. Hiroaki Nakazawa)
- The University of Tokyo Hospital (Dr. Naoki Yahagi)

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References

- [1] Pruitt BA, Mason AD. Epidemiological, demographic and outcome characteristics of burn injury. In: Herndon DN, editor. *Total Burn Care*. London: Saunders; 1996. p. 5–15.
- [2] Vidal-Trecan G, Tchery-Lessenot S, Grossin C, et al. Differences between burns in rural and in urban areas: implication for prevention. *Burns* 2000;26:351–8.
- [3] Saffle JR, Davis B, Williams P. ABA Registry Participant Group. Recent outcomes in the treatment of burn injury in the United States: a report from the American Burn Association Patient Registry. *J Burn Care Rehabil* 1995;16:219–32.
- [4] Wibbenmeyer LA, Amelon MG, Morgan LJ, et al. Predicting survival in an elderly burn patient population. *Burns* 2001;27:583–90.
- [5] Jie X, Baoren C. Mortality rates among 5321 patients with burns admitted to a burn unit in China. *Burns* 2003;29:239–45.
- [6] Vico P, Papillon J. Factors involved in burn mortality: a multivariate statistical approach based on discriminant analysis. *Burns* 1992;18:212–5.
- [7] Ryan CM, Schoenfeld DA, Thorpe WP, et al. Objective estimates of the probability of death from burn injuries. *New Eng J Med* 1998;338:362–6.
- [8] Barrow RE, Spies M, Barrow LN, Herndon DN. Influence of demographics and inhalation injury on burn mortality in children. *Burns* 2004;30:72–7.
- [9] Shroeder J, Kahlke V, Staubach KH, et al. Gender difference in human sepsis. *Arch Surg* 1998;133:1200–5.
- [10] Eachempati SR, Hydo L, Barie PS. Gender-based differences in outcome in patients with sepsis. *Arch Surg* 1999;134:1342–7.
- [11] Muller MJ, Pegg SP, Rule MR. Determinants of death following burn injury. *Brit J Surg* 2001;88:583–7.
- [12] O'Keefe GE, Hunt JL, Purdue GF. An evaluation of risk factors for mortality after burn trauma and the identification of gender-dependent differences in outcomes. *J Am Coll Surg* 2001;192:153–60.
- [13] Takuma K, Suzuki M, Aikawa N, Kobayashi K, Higuchi R. The impact of reproductive aged female sex on the mortality of burn patients. Presented at the 12th Congress of ISBI, Yokohama, 2004.
- [14] McCauley RL. The skin bank. In: Herndon DN, editor. *Total Burn Care*. London: Saunders; 1996. p. 159–63.
- [15] Deitch EA. A policy of early excision and grafting in elderly burn patients shortens the hospital stay and improves survival. *Burns* 1985;12:109–14.

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

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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 (HbV₈) and 29 mmHg (HbV₂₉) 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 HbV₈ or HbV₂₉. 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 HbV₈ group compared with the HbV₂₉ 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 HbV₈ vs. the HbV₂₉ and rHSA groups ($P < 0.05$). Improved tissue P_{O_2} is obtained when red blood cells deliver oxygen in combination with a high- rather than low-affinity oxygen carrier.

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

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

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

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

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

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

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

METHODS

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

Preparation of HbV with different P₅₀. 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 N₂ 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 (HbV₈, P₅₀ = 8 mmHg; HbV₂₉, P₅₀ = 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; P₅₀, partial pressure of oxygen at which Hb is 50% saturated; rHSA, recombinant human serum albumin; HbV₈ and HbV₂₉, Hb vesicles with a P₅₀ of 8 and 29 mmHg, respectively.

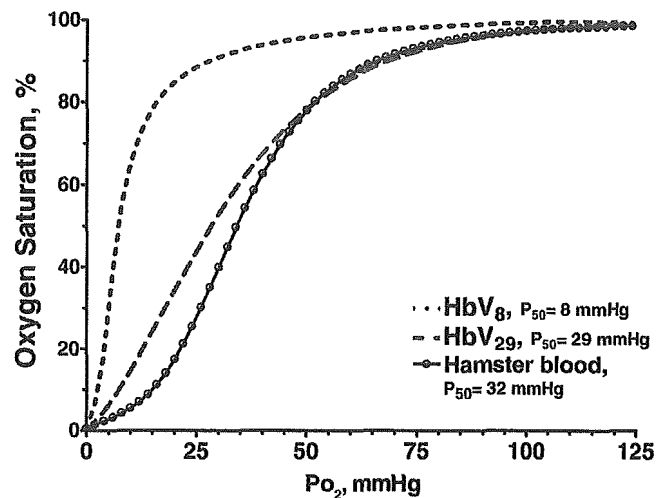


Fig. 1. Oxygen dissociation curves for phospholipid vesicles encapsulating purified Hb (HbV) produced with a P₅₀ at which Hb is 50% saturated (P₅₀) of 8 (HbV₈) and 29 mmHg (HbV₂₉) vs. the dissociation curve for hamster blood (P₅₀ = 32 mmHg).

Measurements of P₅₀ and rate of oxygen release from HbV. The P₅₀ 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 HbV₈ (P₅₀ = 8 mmHg) and HbV₂₉ (P₅₀ = 29 mmHg) and were hemodiluted using these materials, reducing Hct to 11%. Plasma Hb concentrations derived for HbV₈ and HbV₂₉ 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

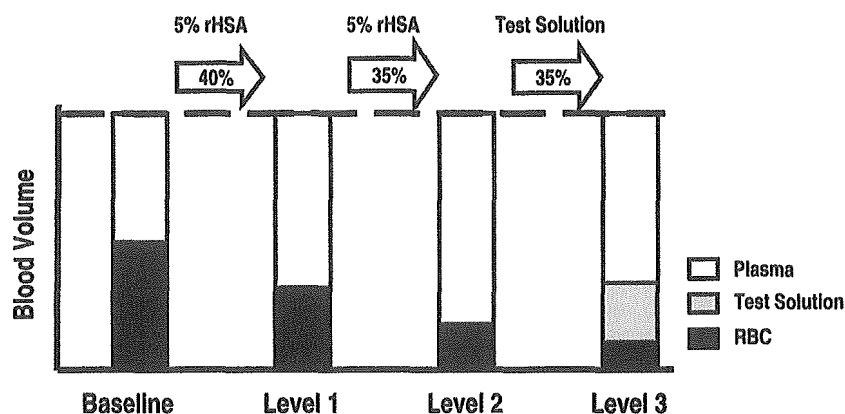


Fig. 2. Hemodilution was attained by means of a progressive, stepwise, isovolemic blood exchange-transfusion protocol. Volume of each exchange-transfusion step was calculated as a percentage of the blood volume, estimated as 7% of body weight. An acute anemic state was induced by lowering systemic Hct, 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 HbV₈ or HbV₂₉ suspended in 5% rHSA (test solutions). RBC, red blood cells.

analyzed for arterial PO₂ (PaO₂), arterial PCO₂ (PaCO₂), base excess (BE), and pH (Blood Chemistry Analyzer 248; Bayer, Norwood, MA). The comparatively low PaO₂ and high PaCO₂ 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 center-line velocity (*V*) was corrected according to vessel size to obtain the mean RBC velocity (11). The video image shearing technique was used to measure vessel diameter (*D*) online. Blood flow was calculated from the measured parameters as $Q = V\pi(D/2)^2$.

Microvascular PO₂ distribution. High-resolution microvascular PO₂ 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 PO₂ 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). PO₂ 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 PO₂ 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 PO₂ measurements in arterioles, venules, and the interstitium (20). The phosphorescence decay due to quenching at a specific PO₂ yields a single decay constant, and *in vitro* calibration has been demonstrated to be valid for *in vivo* measurements. Intravascular and perivascular PO₂ measurements were made in the arterioles studied, and intravascular PO₂ measurements were made in venules. Interstitial tissue PO₂ 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 O₂ delivery, defined as the amount of oxygen delivered by the arterioles to the microcirculation per unit time normalized relative to baseline, and O₂ 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 O₂/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 O₂/dl plasma mmHg, PaO₂ is the arteriolar partial pressure of oxygen, P(a-v)_{O₂} is the arteriovenous difference in PO₂, 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 PO₂ 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, HbV₈, and HbV₂₉. 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 $11.4 \pm 0.6\%$. The HbV₈ and HbV₂₉ 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 HbV₂₉ group and attained the highest value with HbV₈ 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.

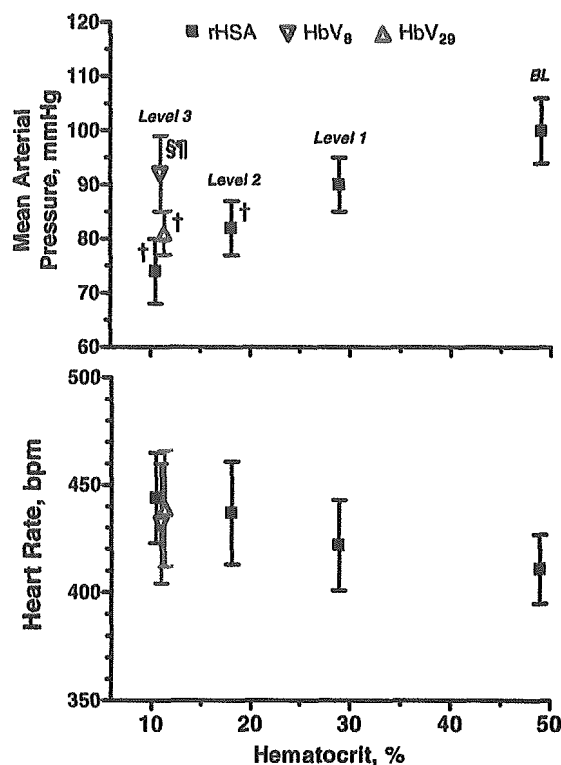


Fig. 3. Mean arterial blood pressure and heart rate [in beats/min (bpm)] at baseline (BL) (Hct 49%) and level 1 (Hct 29%), level 2 (Hct 18%), and level 3 (Hct, 11%) hemodilutions. Level 1 and level 2 exchanges were performed with 5% rHSA as diluent. Level 3 exchange was used to evaluate the oxygen transport of HbV₈ vs. HbV₂₉ and rHSA. † $P < 0.05$ relative to baseline; § $P < 0.05$ relative to level 3 rHSA; ¶ $P < 0.05$, level 3 with HbV₈ vs. level 3 with HbV₂₉.

Analysis of arterial blood gases (Table 2) showed a statistical increase in P_{O_2} after hemodilution and exchange transfusion. P_{aCO_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

	Baseline	Level 1 Hemodilution		Level 3 Hemodilution		
		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
PaO 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*†
P _{aCO} 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; PaO₂, arterial partial O₂ pressure; P_{aCO}₂, arterial partial pressure of CO₂; BE, base excess. * $P < 0.05$ compared with baseline; † $P < 0.05$ compared with level 3 rHSA; ‡ $P < 0.05$ compared with level 3 HbV₈ to level 3 HbV₂₉.

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 HbV₈ 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. HbV₂₉ 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 HbV₈ and HbV₂₉ caused blood flow to be significantly higher than baseline. Furthermore, the HbV₈ group showed consistently higher blood flows than the HbV₂₉ 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, HbV₈, and HbV₂₉ 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, HbV₈, and HbV₂₉ groups showed that these materials produced virtually identical distributions of arteriolar microvascular Po₂ (arterioles averaged 49.5 mmHg), although HbV₈ tended to be higher (Fig. 7). The decrease of RBC from level 2 to level 3 did not decrease the arteriolar Po₂. Venular Po₂ after level 3 was significantly lower than at level 2 exchange in all cases (rHSA, 7.2 ± 3.2 mmHg; HbV₈, 15.1 ± 3.7 mmHg; HbV₂₉, 9.6 ± 4.2 mmHg).

Tissue Po₂ 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 HbV₈ group, being 14.0 ± 2.2 mmHg. By comparison, tissue Po₂ for the HbV₂₉ group was 9.2 ± 2.7 mmHg and for the rHSA group, 2.6 ± 1.4 mmHg, which was significantly lower compared with the HbV₈ and HbV₂₉ groups (Fig. 7).

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

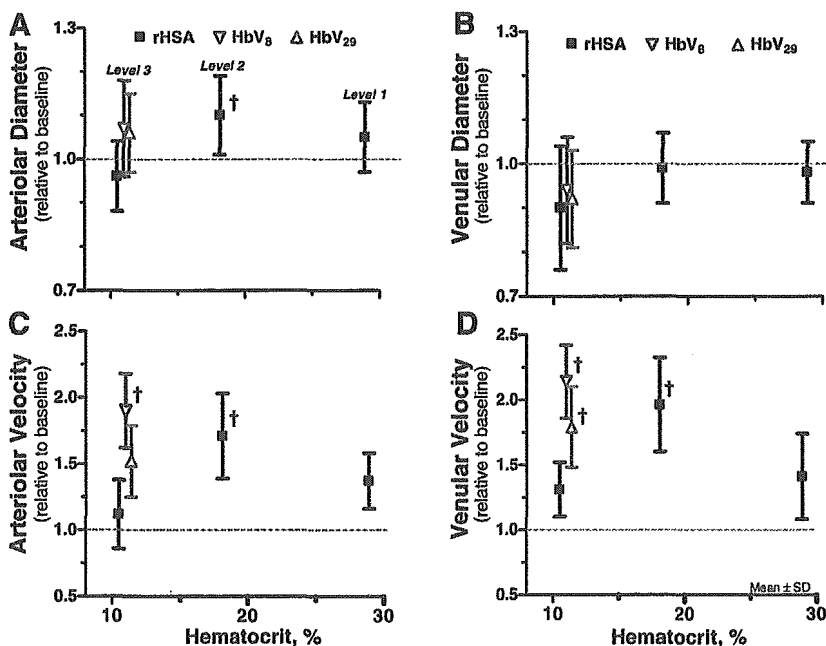
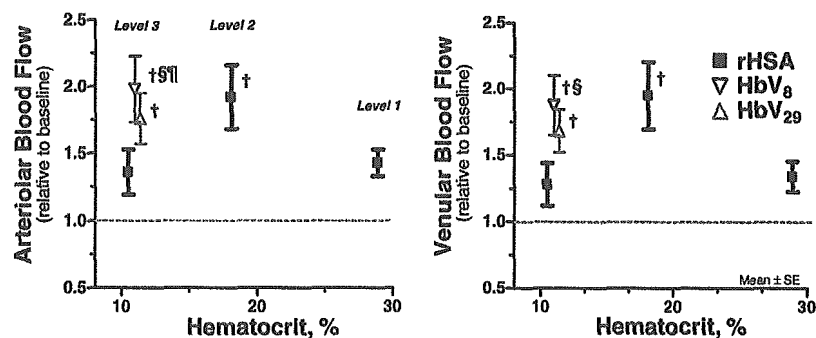


Fig. 4. Changes relative to baseline in arteriolar and venular hemodynamics at the level 1, level 2, and level 3 exchanges. Dashed lines represent baseline level. †P < 0.05 relative to baseline. Arteriolar (A) and venular (B) diameters (μm, means ± SD, n = no. of vessels studied) in each animal group 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 HbV₈: A, 64.9 ± 15.7, n = 20; V, 64.6 ± 18.2, n = 20. Level 3 with HbV₂₉: 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 HbV₈: A, 7.2 ± 1.8, n = 20; V, 3.3 ± 1.0, n = 20. Level 3 with HbV₂₉: A, 7.0 ± 1.7, n = 20; V, 3.0 ± 0.9, n = 20.

Fig. 5. Arteriolar and venular flow (ml/s, means \pm 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 HbV₈: A, 23.4 ± 8.1 , $n = 18$; V, 9.9 ± 5.1 , $n = 18$. Level 3 with HbV₂₉: A, 21.0 ± 8.0 , $n = 18$; V, 8.3 ± 5.2 , $n = 18$.



microcirculation. It is apparent that exchanging RBC for HbV₈ maintains oxygen delivery to the tissue, whereas HbV₂₉ reduces this by $\sim 20\%$, and continued hemodilution with a non-oxygen-carrying material significantly depresses oxygen delivery to the tissue, reducing this to half of that attained at the level 2 hemodilution.

DISCUSSION

The principal finding of this study is that under identical extreme hemodilution conditions, with the use of vesicles encapsulating Hb with normal P_{50} (HbV₂₉ = 29 mmHg) and low P_{50} (HbV₈ = 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 HbV₈ 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 HbV₈ than for HbV₂₉.

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

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

Average oxygen delivery and extraction were somewhat greater for HbV₈ than for HbV₂₉. 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 HbV₈ and HbV₂₉ resulted in the same total Hb concentration in the circulation (5.7 and 5.8 g Hb/dl); however, oxygen delivery was lower with HbV₂₉ 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 HbV₂₉.

The differences in tissue P_{O_2} , mean arterial blood pressure, and arteriolar blood flow between HbV₈ and HbV₂₉ show that in designing a blood substitute, it is not sufficient to provide adequate oxygen-carrying capacity. Once a suitable oxygen carrier is available, it also must be able to maintain or enhance other circulatory transport parameters, particularly flow. The Hb vesicles used in this study are vasoinactive, and the difference in P_{50} appears to be a factor in improving flow condition that is not related to vasoactivity. An explanation for this may be related to the inherent variability of tissue P_{O_2} shown in this and other studies (4, 22), which may be enhanced in extreme hemodilution. This variability determines that if average tissue P_{O_2} is low, portions of the tissue may become anoxic. Introducing a small quantity of a low- P_{50} Hb oxygen carrier into the circulation will deliver oxygen only to those parts of the tissue

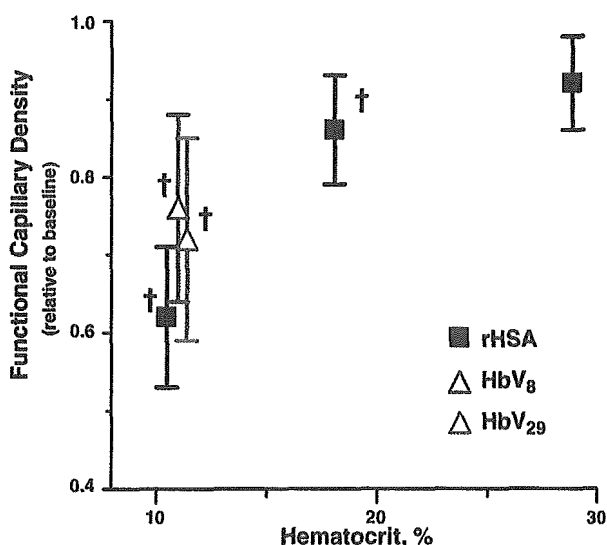


Fig. 6. Functional capillary density after the level 1, level 2, and level 3 exchanges for the different test fluids. All values are relative to baseline levels. $\dagger P < 0.05$ relative to baseline.

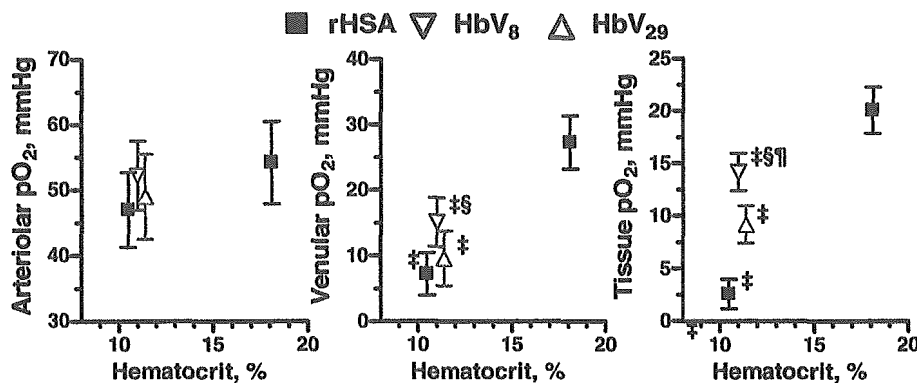


Fig. 7. Intravascular PO₂ after the level 2 and level 3 hemodilutions. Values are presented as means ± SD. ‡P < 0.05 compared with level 2 with rHSA; §P < 0.05 compared with level 3 with rHSA; ¶P < 0.05, level 3 with HbV₈ vs. level 3 with HbV₂₉.

where the anoxic threshold is passed, thus eliminating the inherent variability of oxygen delivery shown by the variability of tissue PO₂.

Considering the significantly improved blood pressure and the trend toward higher flow for HbV₈ (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 HbV₈ vs. HbV₂₉.

In summary, the present results show that either HbV₈ or HbV₂₉ 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, HbV₈ appeared to be beneficial at the systemic level, because base excess remained at baseline levels, whereas it was decreased for HbV₂₉. This finding suggests that improved tissue PO₂ 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 PO₂, FCD, and oxygen extraction in the microcirculation during extreme hemodilution. The significant differences in the tissue oxygen parameters produced by the presence of low-P₅₀ Hbs vs. an identical oxygen carrier with normal P₅₀ 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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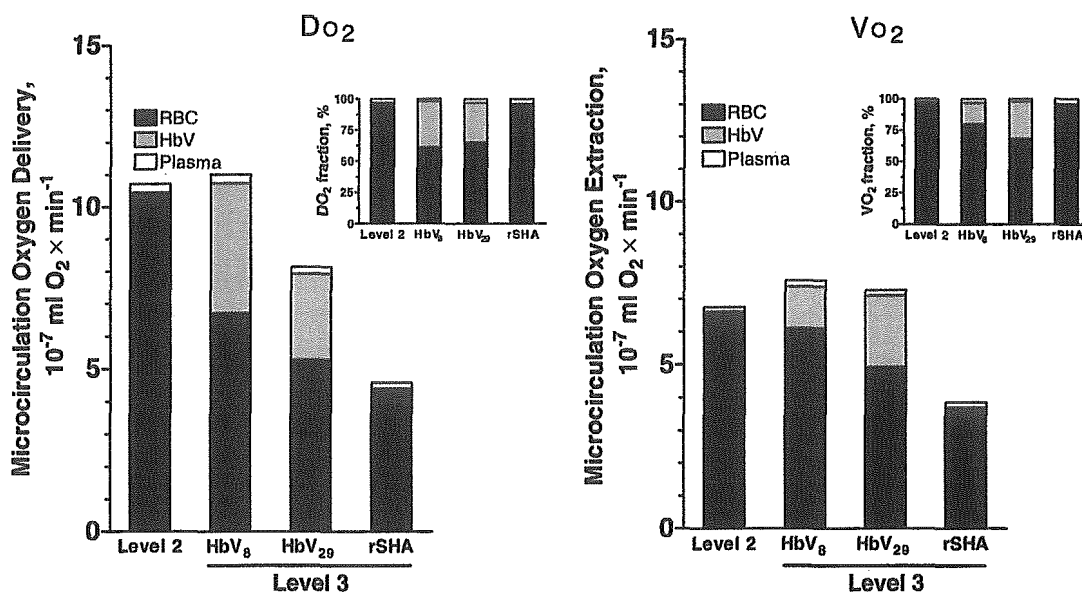


Fig. 8. Arterial oxygen delivery (DO₂) and extraction (VO₂) before and after the level 3 hemodilution. Calculations of global oxygen transport are not directly measurable in our model; however, the changes relative to baseline can be calculated using the measured parameters. These calculations can be identified as those presented without standard deviations to focus on their tendencies rather than on the variability of the measurement.

REFERENCES

1. Altman DG and Bland JM. Statistics notes: how to randomise. *BMJ* 319: 703–704, 1999.
2. Baines AD and Drangova R. Does dopamine use several signal pathways to inhibit Na-Pi transport in OK cells? *J Am Soc Nephrol* 9: 1604–1612, 1998.
3. Baines AD and Ho P. O₂ affinity of cross-linked hemoglobins modifies O₂ metabolism in proximal tubules. *J Appl Physiol* 95: 563–570, 2003.
4. Cabrales P, Kanika ND, Manjula BN, Tsai AG, Acharya SA, and Intaglietta M. Microvascular P_{O₂} during extreme hemodilution with hemoglobin site specifically PEGylated at Cys-93(β) in hamster window chamber. *Am J Physiol Heart Circ Physiol* 287: H1609–H1617, 2004.
5. Cabrales P, Tsai AG, and Intaglietta M. Microvascular pressure and functional capillary density in extreme hemodilution with low and high plasma viscosity expanders. *Am J Physiol Heart Circ Physiol* 287: H363–H373, 2004.
6. Endrich B, Asaishi K, Götz A, and Messmer K. Technical report: a new chamber technique for microvascular studies in unanaesthetized hamsters. *Res Exp Med (Berl)* 177: 125–134, 1980.
7. Hogan MC, Behout DE, and Wagner PD. Effect of increased Hb-O₂ affinity on V_{O₂ max} at constant O₂ delivery in dog muscle in situ. *J Appl Physiol* 70: 2656–2662, 1991.
8. Intaglietta M, Silverman NR, and Tompkins WR. Capillary flow velocity measurements in vivo and in situ by television methods. *Microvasc Res* 10: 165–179, 1975.
9. Kerger H, Groth G, Kalenka A, Vajkoczy P, Tsai AG, and Intaglietta M. pO₂ measurements by phosphorescence quenching: characteristics and applications of an automated system. *Microvasc Res* 65: 32–38, 2003.
10. Lipowsky HH and Firrell JC. Microvascular hemodynamics during systemic hemodilution and hemoconcentration. *Am J Physiol Heart Circ Physiol* 250: H908–H922, 1986.
11. Lipowsky HH and Zweifach BW. Application of the “two-slit” photometric technique to the measurement of microvascular volumetric flow rates. *Microvasc Res* 15: 93–101, 1978.
12. Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, and Intaglietta M. Molecular dimensions of Hb-based O₂ carriers determine constriction of resistance arteries and hypertension. *Am J Physiol Heart Circ Physiol* 279: H908–H915, 2000.
13. Sakai H, Masada Y, Horinouchi H, Yamamoto M, Ikeda E, Takeoka S, Kobayashi K, and Tsuchida E. Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit Care Med* 32: 539–545, 2004.
14. Sakai H, Tomiyama KI, Sou K, Takeoka S, and Tsuchida E. Polyethyleneglycol-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. *Bioconjug Chem* 11: 425–432, 2000.
15. Sakai H, Tsai AG, Intaglietta M, and Tsuchida E. Hemoglobin encapsulation with polyethylene glycol-modified and unmodified vesicles: systemic and microvascular hemodynamics at 80% blood substitution. In: *Advances in Blood Substitutes. Industrial Opportunities and Medical Challenges*, edited by Winslow RM, Vandegriff KD, and Intaglietta M. Boston, MA: Birkhäuser, 1997, p. 151–166.
16. Sakai H, Tsai AG, Rohlfis RJ, Hara H, Takeoka S, Tsuchida E, and Intaglietta M. Microvascular responses to hemodilution with Hb vesicles as RBC substitutes: influence of O₂ affinity. *Am J Physiol Heart Circ Physiol* 276: H553–H562, 1999.
17. Sou K, Endo T, Takeoka S, and Tsuchida E. Poly(ethylene glycol)-modification of the phospholipid vesicles by using the spontaneous incorporation of poly(ethylene glycol)-lipid into the vesicles. *Bioconjug Chem* 11: 372–379, 2000.
18. Torres Filho IP and Intaglietta M. Microvessel P_{O₂} measurements by phosphorescence decay method. *Am J Physiol Heart Circ Physiol* 265: H1434–H1438, 1993.
19. Tsai AG. Influence of cell-free hemoglobin on local tissue perfusion and oxygenation after acute anemia after isovolemic hemodilution. *Transfusion* 41: 1290–1298, 2001.
20. Tsai AG, Friesenecker B, Mazzoni MC, Kerger H, Buerk DG, Johnson PC, and Intaglietta M. Microvascular and tissue oxygen gradients in the rat mesentery. *Proc Natl Acad Sci USA* 95: 6590–6595, 1998.
21. Tsai AG, Friesenecker B, McCarthy M, Sakai H, and Intaglietta M. Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skin fold model. *Am J Physiol Heart Circ Physiol* 275: H2170–H2180, 1998.
22. Tsai AG, Vandegriff KD, Intaglietta M, and Winslow RM. Targeted O₂ delivery by low-P₅₀ hemoglobin: a new basis for O₂ therapeutics. *Am J Physiol Heart Circ Physiol* 285: H1411–H1419, 2003.
23. Webb AR, Barclay SA, and Bennett ED. In vitro colloid osmotic pressure of commonly used plasma expanders and substitutes: a study of the diffusibility of colloid molecules. *Intensive Care Med* 15: 116–120, 1989.

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

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

Design: Randomized, prospective study.

Setting: University laboratory.

Subject: A total of 40 male golden Syrian hamsters.

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

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

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

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

Key Words: arterial occlusive diseases; blood substitutes; collateral circulation; energy metabolism; microcirculation; surgical flaps

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

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

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

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

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Table 1. Physicochemical characteristics of hamster blood and the diluents

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

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

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

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

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

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

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

	Baseline	1 hr	3 hrs
MAP, mm Hg			
Control	91 ± 6	90 ± 5	83 ± 11 ^a
HES	93 ± 6	92 ± 6	84 ± 7 ^a
HbV-HES	94 ± 3	92 ± 3	80 ± 7 ^b
PBHb	95 ± 5	92 ± 3	93 ± 6
Hematocrit			
Control	0.55 ± 0.04	0.54 ± 0.03	0.52 ± 0.03
HES	0.59 ± 0.04	0.31 ± 0.04 ^b	0.31 ± 0.04 ^b
HbV-HES	0.59 ± 0.04	0.31 ± 0.02 ^b	0.33 ± 0.04 ^b
PBHb	0.58 ± 0.05	0.32 ± 0.05 ^b	0.31 ± 0.04 ^b
Total Hb concentration, g/dL			
Control	17.8 ± 1.2	17.2 ± 1.0	17.0 ± 1.1
HES	17.8 ± 1.3	9.7 ± 0.9 ^b	10.5 ± 1.0 ^b
HbV-HES	18.8 ± 0.7	11.2 ± 1.3 ^b	12.1 ± 1.0 ^b
PBHb	18.0 ± 1.8	13.6 ± 0.9 ^b	11.9 ± 1.1 ^b
PO ₂ , torr			
Control	43 ± 3	44 ± 6	43 ± 4
HES	43 ± 13	56 ± 10	64 ± 13 ^b
HbV-HES	43 ± 4	51 ± 13	68 ± 9 ^b
PBHb	43 ± 8	53 ± 9	61 ± 11 ^b
PCO ₂ , torr			
Control	58 ± 8	58 ± 9	56 ± 8
HES	57 ± 3	56 ± 3	47 ± 13 ^a
HbV-HES	53 ± 4	51 ± 4	41 ± 3 ^b
PBHb	57 ± 5	53 ± 4 ^a	47 ± 5 ^b
pH			
Control	7.28 ± 0.06	7.29 ± 0.06	7.29 ± 0.06
HES	7.23 ± 0.03	7.27 ± 0.03 ^a	7.31 ± 0.07 ^b
HbV-HES	7.27 ± 0.03	7.30 ± 0.03 ^a	7.34 ± 0.02 ^b
PBHb	7.25 ± 0.02	7.30 ± 0.05 ^a	7.34 ± 0.06 ^b

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

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

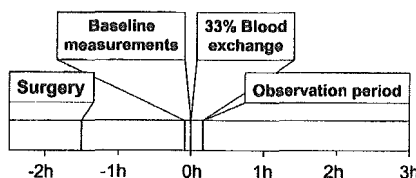


Figure 1. Diagram illustrating sequence of interventions.

MATERIALS AND METHODS

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

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

Table 3. Microvascular diameters (in micrometers) at baseline

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

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

Values are mean ± SD.

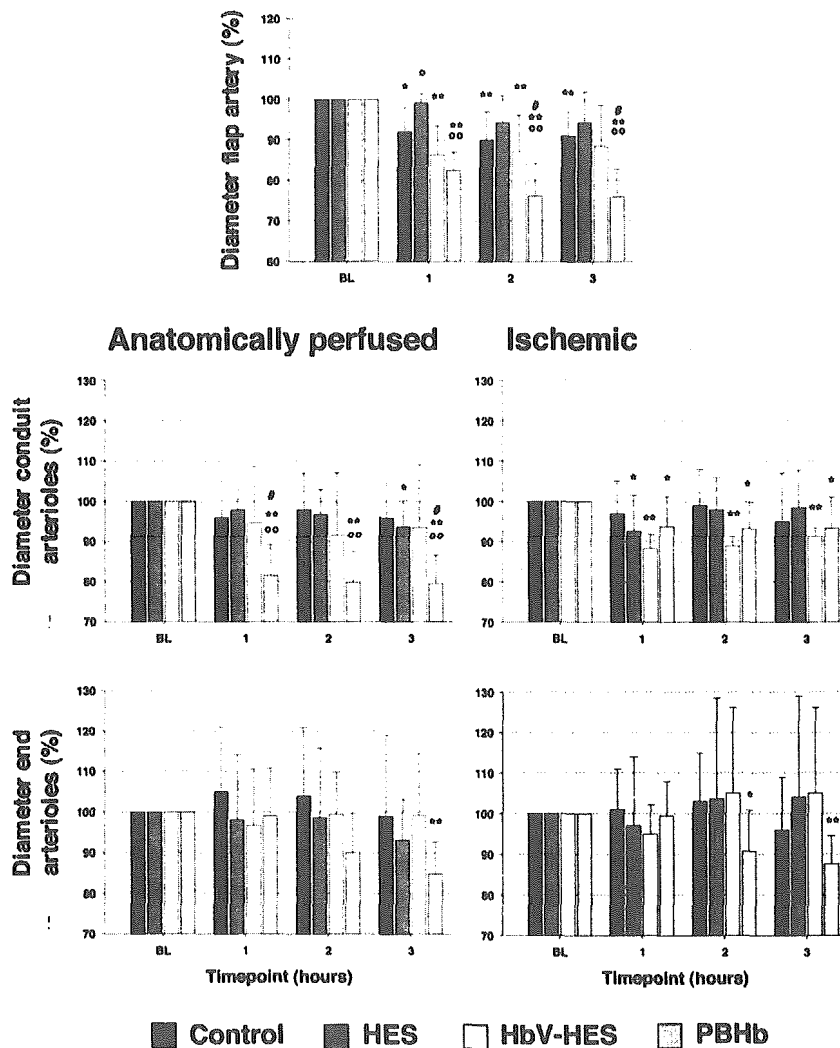


Figure 2. Microvascular diameters in flap artery, conduit arterioles, and end arterioles in the anatomically perfused and ischemic tissues at baseline (BL) and after hemodilution with 6% hydroxyethyl starch (HES), hemoglobin vesicles suspended in HES (HbV-HES), and polymerized bovine hemoglobin (PBHb).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

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

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

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

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

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

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

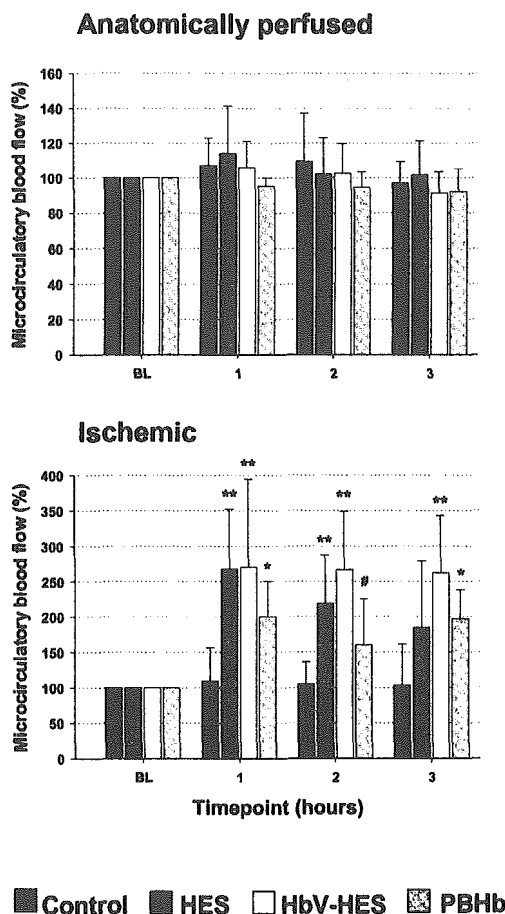


Figure 3. Microcirculatory blood flow in the anatomically perfused and ischemic tissues at baseline (BL) and after hemodilution with 6% hydroxyethyl starch (HES), hemoglobin vesicles suspended in HES (HbV-HES), and polymerized bovine hemoglobin (PBHb). Data are given as a percentage of baseline and represent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

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

Carbohydrate Metabolite Concentrations.

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

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

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

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

collection of the microdialysates started after a 30-min stabilization period. Another hour later, the baseline values were obtained. Thereafter, one third of the total blood volume was exchanged with HES or the oxygen-carrying solutions. This was achieved by simultaneous blood withdrawal via the carotid catheter and infusion via the jugular catheter over 15 mins. Exclusion criteria were abnormalities of the vascular anatomy, insufficient optical clarity, mean arterial pressure of <60 mm Hg, and systemic arterial pH, P_{O_2} , and P_{CO_2} outside the normal ranges at baseline (7.19–7.29, 35–55 torr, and 45–65 torr, respectively). The animals were killed with an overdose of pentobarbital at the end of the experiment.

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

RESULTS

Six animals (two control animals, one in the HES group, two in the HbV-HES group, and one in the PBHb group) did not fulfill the inclusion criteria and were excluded from this study. The systemic data are presented in Table 2. Mean arterial pressure gradually declined during the experiment in all groups ($p < .05$) except the Oxyglobin group, in which mean arterial pressure remained virtually unchanged ($p < .05$ vs. other groups). Similar hematocrits were obtained in all hemodiluted animals. After the 33% blood exchange with HES, Hb concentration was reduced to $9.7 \pm 0.9 \text{ g}/\text{dL}$, whereas the addition of HbV to the diluent enhanced the total Hb concentration to $11.2 \pm 1.3 \text{ g}/\text{dL}$ ($p < .05$ vs. HES), and hemodilution with PBHb resulted in a total Hb concentration of $13.6 \pm 0.9 \text{ g}/\text{dL}$ ($p < .05$ vs. HbV-HES). However, this difference became less pronounced over time. Hemodilution increased mean P_{O_2} from 43 torr to mean values of >60 torr and mean pH from 7.23–7.27 to mean values of 7.31–7.34, whereas mean P_{CO_2} decreased from 53–57 torr to 41–47 torr (all $p < .01$ vs. baseline).

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

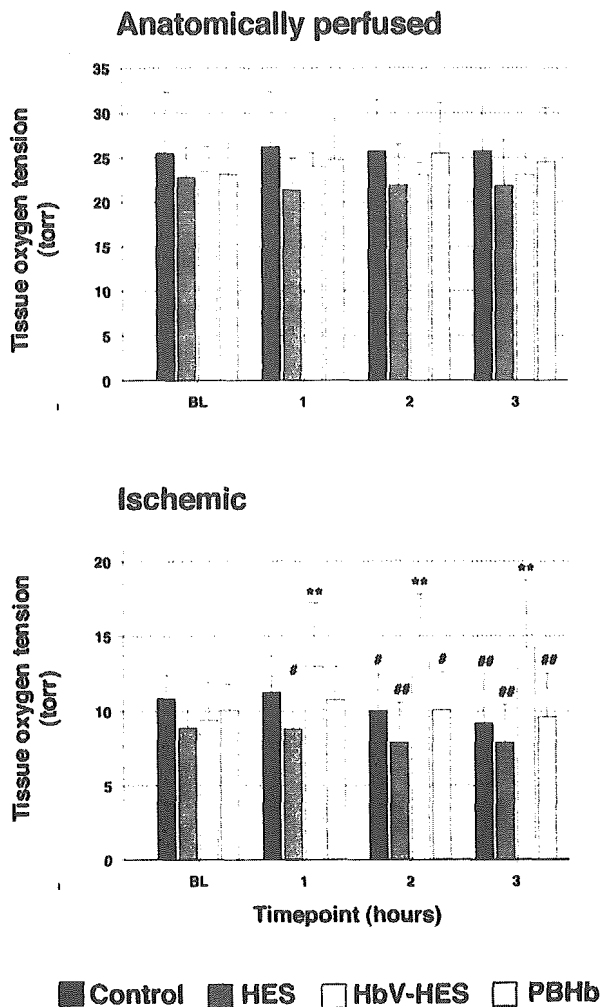


Figure 4. P_{O_2} in the anatomically perfused and ischemic tissues at baseline (BL) and after hemodilution with 6% hydroxyethyl starch (HES), hemoglobin vesicles suspended in HES (HbV-HES), and polymerized bovine hemoglobin (PBHb). Data represent mean values and SD. ** $p < .01$ vs. baseline and control;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

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

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

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

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

Glucose concentrations were gradually decreased in the anatomically perfused tissue over time (Fig. 5), reaching 1.5 ± 1.1 mmol/L in the control group ($p < .01$), 2.7 ± 1.2 mmol/L for HES (not significant), 3.1 ± 1.4 mmol/L for HbV-HES (not significant, $p < .05$ vs. control), and 2.2 ± 0.9 mmol/L for PBHb ($p < .01$). The reductions were more accentuated in the ischemic tissue, at 0.8 ± 0.6 mmol/L in the control group ($p < .01$), 1.4 ± 0.9 mmol/L in the HES group ($p < .01$), 1.6 ± 1.4 mmol/L in the HbV-HES group (not significant), and 1.3 ± 0.7 mmol/L in the PBHb group ($p < .01$). At baseline, the mean lactate concentrations ranged between 1.7 and 2.2 mmol/L in both parts of the tissue in all groups. The values remained virtually stable in the anatomically perfused tissue. In the ischemic tissue, lactate concentrations were raised to 2.8 ± 0.6 mmol/L in control ($p < .01$) and to 3.4 ± 1.4 mmol/L in HES ($p < .01$) but only to 2.4 ± 1.0 mmol/L in HbV-HES and to 2.2 ± 0.7 mmol/L in PBHb (both not significant). At baseline, the mean lactate/pyruvate ratio was higher in the ischemic tissue (24–27) compared with the anatomic part. Lactate/pyruvate ratio was increased to 51 ± 23 in the control group and 48 ± 12 in the HES group (both $p < .01$) but only to 34 ± 8 and 38 ± 11 for HbV-HES (not significant vs. baseline, $p < .05$ vs. control) and PBHb ($p < .05$), respectively.

DISCUSSION

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

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

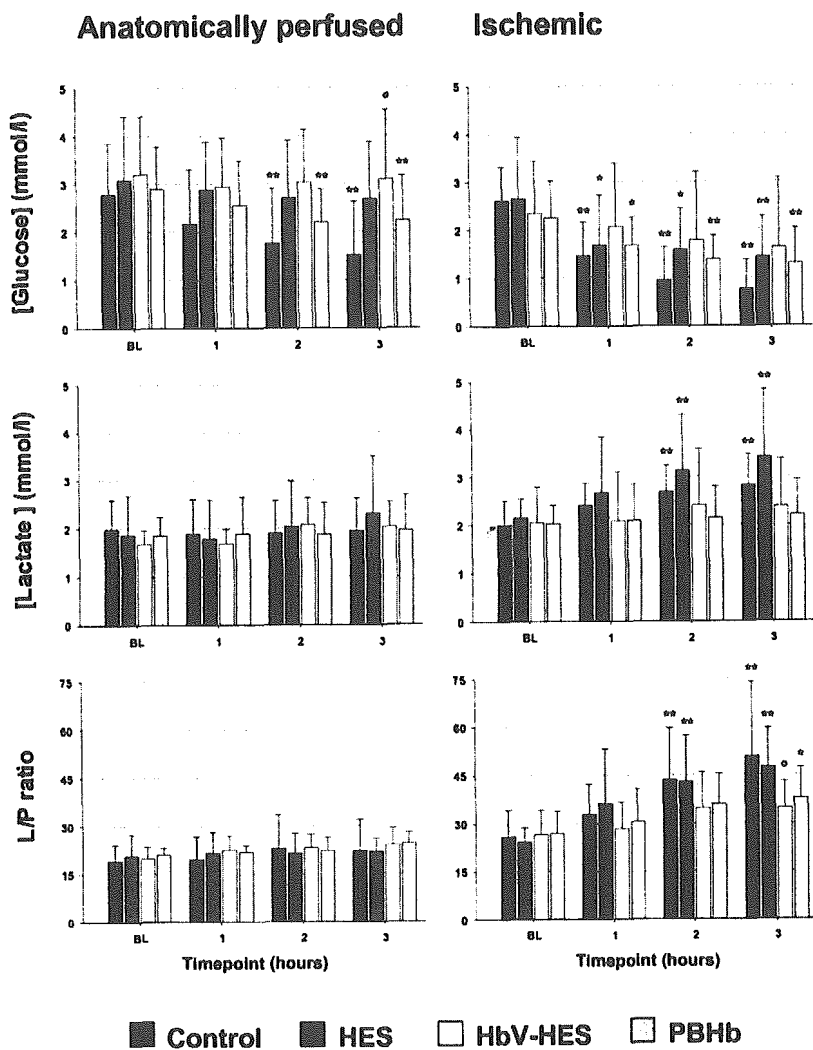


Figure 5. Carbohydrate metabolite concentrations in the anatomically perfused and ischemic tissues at baseline (BL) and after hemodilution with 6% hydroxyethyl starch (HES), hemoglobin vesicles suspended in HES (HbV-HES), and polymerized bovine hemoglobin (PBHb). Data represent mean values and SD. *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

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

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

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

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

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

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

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

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

CONCLUSIONS

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

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

REFERENCES

- Chang TM: Artificial cells for cell and organ replacements. *Artif Organs* 2004; 28:265–270
- Jahr JS, Nesargi SB, Lewis K, et al: Blood substitutes and oxygen therapeutics: An overview and current status. *Am J Ther* 2002; 9:437–443
- Winslow RM: Current status of blood substitute research: Towards a new paradigm. *J Intern Med* 2003; 253:508–517
- Sprung J, Kindscher JD, Wahr JA, et al: The use of bovine hemoglobin glutamer-250 (Hemopure) in surgical patients: Results of a multicenter, randomized, single-blinded trial. *Anesth Analg* 2002; 94:799–808
- Tsai AG, Vandegriff KD, Intaglietta M, et al: Targeted O₂ delivery by low-P50 hemoglobin: A new basis for O₂ therapeutics. *Am J Physiol* 2003; 285:H1411–H1419
- Intaglietta M: Microcirculatory basis for the design of artificial blood. *Microcirculation* 1999; 6:247–258
- Wettstein R, Tsai AG, Erni D, et al: Resuscitation with polyethylene glycol-modified human hemoglobin improves microcirculatory blood flow and tissue oxygenation after hemorrhagic shock in awake hamsters. *Crit Care Med* 2003; 31:1882–1884
- Winslow RM, Gonzales A, Gonzales ML, et al: Vascular resistance and the efficacy of red cell substitutes in a rat hemorrhage model. *J Appl Physiol* 1998; 85:993–1003
- Erni D, Wettstein R, Schramm S, et al: Normovolemic hemodilution with Hb vesicle solution attenuates hypoxia in ischemic hamster flap tissue. *Am J Physiol* 2003; 284: H1702–H1709
- Contaldo C, Schramm S, Wettstein R, et al: Improved oxygenation in ischemic hamster flap tissue is correlated with increasing hemodilution with Hb vesicles and their O₂ affinity. *Am J Physiol* 2003; 285:H1140–H1147
- Sakai H, Takeoka S, Park SI, et al: Surface modification of hemoglobin vesicles with poly(ethyleneglycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. *Bioconjug Chem* 1997; 8:15–22
- Erni D, Sakai H, Tsai AG, et al: Haemodynamics and oxygen tension in the microcirculation of ischaemic skin flaps after neural blockade and haemodilution. *Br J Plast Surg* 1999; 52:565–572
- Ungerstedt U: Microdialysis: Principles and applications for studies in animals and man. *J Intern Med* 1991; 230:365–373
- Harris AG, Schropp A, Schütze E, et al: Implementation of the microdialysis method in the hamster dorsal skinfold chamber. *Res Exp Med* 1999; 199:141–152
- Kuo L, Davis MJ, Chilian WM: Endothelial modulation of arteriolar tone. *News Physiol Sci* 1992; 7:5–9
- Sakai H, Hara H, Yuasa M, et al: Molecular dimensions of Hb-based O₂ carriers determine constriction of resistance arteries and hypertension. *Am J Physiol* 2000; 279:H908–H915
- Sakai H, Hara H, Tsai AG, et al: Constriction of resistance arteries determines I-NAME-induced hypertension in a conscious hamster model. *Microvasc Res* 2000; 60:21–27
- Sakai H, Takeoka S, Wettstein R, et al: Systemic and microvascular responses to hemorrhagic shock and resuscitation with Hb vesicles. *Am J Physiol* 2002; 283:H1191–H1199
- Sakai H, Tsai AG, Rohlfis RJ, et al: Microvascular response to hemodilution with Hb vesicles as red blood cell substitutes: Influence of O₂ affinity. *Am J Physiol* 1999; 276: H552–H562
- Nishide H, Chen XS, Tsuchida E: Facilitated oxygen transport with modified and encapsulated hemoglobins across non-flowing solution membrane. *Artif Cells Blood Substitut Immobil Biotechnol* 1997; 25:335–346
- Page TC, Light WR, McKay CB, et al: Oxygen transport by erythrocyte/hemoglobin solution mixtures in an *in vitro* capillary as a model of hemoglobin-based oxygen carrier performance. *Microvasc Res* 1998; 55:54–64
- Sakai H, Suzuki Y, Kinoshita M, et al: O₂ release from Hb-vesicles evaluated using an artificial narrow O₂-permeable tube: Comparison with RBC and acellular Hb. *Am J Physiol* 2003; 285:H2543–H2551
- Vadapalli A, Goldman D, Popel AS: Calculations of oxygen transport by red blood cells and hemoglobin solutions in capillaries. *Artif Cells Blood Substitut Immobil Biotechnol* 2002; 30:157–188



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

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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 [PO_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 HbV₈ and HbV₂₉, respectively) and observed their O_2 -releasing behavior from an occluded arteriole in a hamster skinfold window model. Conscious hamsters received HbV₈ or HbV₂₉ at a dose rate of 7 ml/kg. In the microscopic view, an arteriole (diameter: $53.0 \pm 6.6 \mu\text{m}$) was occluded transcutaneously by a glass pipette on a manipulator, and the reduction of the intra-arteriolar PO_2 100 μm down from the occlusion was measured by the phosphorescence quenching of preinfused Pd-porphyrin. The baseline arteriolar PO_2 (50–52 mmHg) decreased to about 5 mmHg for all the groups. Occlusion after HbV₈ infusion showed a slightly slower rate of PO_2 reduction compared with that after HbV₂₉ infusion. The arteriolar O_2 content was calculated at each reducing PO_2 in combination with the O_2 equilibrium curves of HbVs, and it was clarified that HbV₈ showed a significantly slower rate of O_2 release compared with HbV₂₉ and was a primary source of O_2 (maximum fraction, 0.55) overwhelming red blood cells when the PO_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 reticuloendothe-

rial 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 PO_2 is low and RBCs release most of their O_2 before reaching the capillary circulation. As an example, if tissue PO_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.

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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 (HbV₂₉) 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 (HbV₈) 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 μ m) and deoxygenated with N₂ bubbling for storage. The endotoxin content was measured with a modified Limulus amoebocyte lysate assay, and the level was less than 0.2 EU/ml (27). The O₂ equilibrium curves (OECs) of HbV₂₉ and HbV₈ were obtained by a Hemox Analyzer (TCS-Medical Products; Philadelphia, PA), as shown in Fig. 1. The physicochemical parameters of the HbVs are listed in Table 1.

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

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

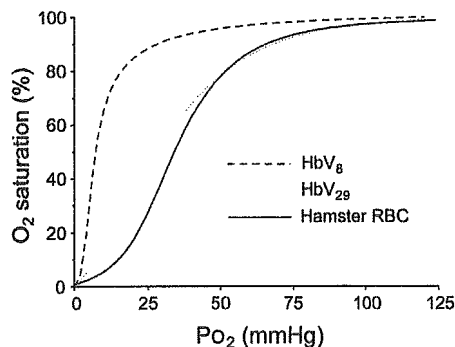


Fig. 1. Oxygen equilibrium curves (OECs) of Hb vesicles (HbVs) at a P_{O_2} where Hb is half-saturated (P_{50}) of 8 mmHg (HbV₈) and 29 mmHg (HbV₂₉) measured with a Hemox Analyzer (TCS Medical Products) at 37°C compared with hamster blood. RBC, red blood cells.

Table 1. Physicochemical properties of HbV₈ and HbV₂₉ compared with hamster blood

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

HbV₈ and HbV₂₉, Hb vesicles (HbVs) at 8- and 29-mmHg P_{O_2} at which Hb is 50% saturated (P_{50}); PLP, pyridoxal 5'-phosphate. *Size of hamster red blood cells (RBCs) (39).

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

Infusion of HbV₈ and HbV₂₉ and occlusion of an arteriole. The unanesthetized animal was placed in a perforated plastic tube and stabilized under the microscope. Animals were suitable for the experiments if systemic variables were within normal range, namely, heart rate >340 beats/min, mean arterial pressure >80 mmHg, systemic hematocrit >45%, and arterial P_{O_2} >50 mmHg, and microscopic examination of the tissue in the chamber did not reveal signs of edema or bleeding. Baseline measurements of microvascular parameters and P_{O_2} (see below) were performed before the infusion of HbV₈ or HbV₂₉ suspended in physiological saline solution into the venous line at 7 ml/kg. Systemic blood volume was estimated as 70 ml/kg. In our previous reports of resuscitation from hemorrhagic shock or hemodilution, HbVs were suspended in an albumin solution to regulate colloid osmotic pressure (30, 33). However, in the present study, we did not use albumin to minimize the hypervolemic effect. For the same reason, the infusion amount was minimized to equal 10% blood volume (7 ml/kg).

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

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

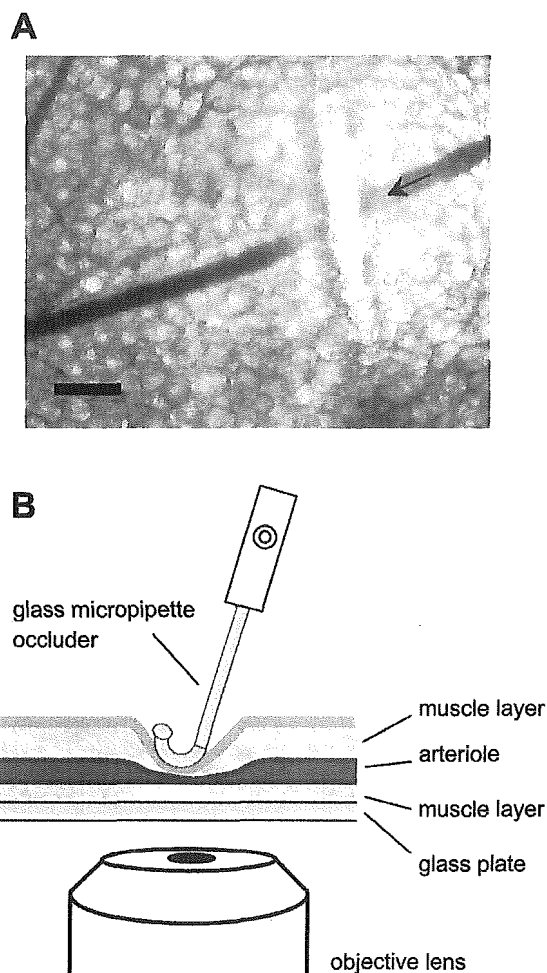


Fig. 2. A: microscopic image of an occluded arteriole in the hamster window chamber. The glass fiber lies across the arteriole. Scale bar = 100 μm . B: schematic representation of occlusion of A showing the different tissue layers of the skin (not to scale).

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 Po_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 Po_2 is given by the following Stern-Volmer equation:

$$\tau_0/\tau = 1 + k_q \times \tau_0 \times \text{Po}_2 \quad (2)$$

where τ_0 and τ are the phosphorescence lifetimes in the absence of molecular O_2 and at a given Po_2 , respectively, and k_q is the quenching constant, with both factors being pH and temperature dependent.

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

The Po_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 Po_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:

$$\text{O}_2 \text{ content} = 23.6 \times \frac{[\text{Sa}_{\text{O}_2}(\text{RBC}) + 0.0667 \times \text{Sa}_{\text{O}_2}(\text{HbV})]}{100} + 2.42 \times \frac{\text{Po}_2}{713} \quad (3)$$

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-Charles's gas law, $PV = nRT$, where P (in atm) is atmospheric pressure, V (in liters) is gas volume, n is mole number, R is the gas constant ($0.082 \text{ atm} \cdot \text{l} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$), and T is absolute temperature [$23.6 \text{ (ml)} = 9.3 \times 10^{-4} \text{ (mol)} \times 0.082 \times (273 + 37) \times 1,000$]. The physically dissolved O_2 content at 1 atm O_2 (713 mmHg after subtracting the vapor pressure of water = 47 mmHg) at 37°C was calculated to be 2.42 ml in 100 ml water. $\text{Sa}_{\text{O}_2}(\text{RBC})$ and $\text{Sa}_{\text{O}_2}(\text{HbV})$ are Sa_{O_2} s of RBCs and HbVs, respectively, at each arteriolar Po_2 during the experiments.

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

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

RESULTS

Hemodynamic properties of arterioles. The profiles of the selected arterioles, diameters, centerline RBC velocities, blood flow rates, and intra-arteriolar Po_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 Po_2 was estimated as 18.61 ± 1.23 ml O_2 /dl blood according to Eq. 3. After the infusion of HbV₈ and HbV₂₉, 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 HbV₈ and HbV₂₉ to whole O_2 content were 1.51 ± 0.01 and 1.25 ± 0.07 ml O_2 /dl blood, respectively. The HbV₈ group showed higher O_2 content than the HbV₂₉ group due to the higher $\text{Sa}_{\text{O}_2}(\text{HbV}_8)$, which was $95.9 \pm 0.6\%$ compared with the $\text{Sa}_{\text{O}_2}(\text{HbV}_{29})$ of $79.6 \pm 4.7\%$.

Changes in Po_2 in arterioles after occlusion in the presence of HbVs. Arteriolar Po_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, Po_2 fell to about 10 and 5 mmHg after 10- and