TABLE 1. EFFECTS OF BLOCKADE OF CSE BY PPG ON BASAL BILE OUTPUT AND BILIARY HCO_3^- EXCRETION

Groups	*Basal bile output (μl/min/g of liver)*	*Biliary HCO_3^- concentration (mmol/L)*
Vehicle ($n = 6$)	1.73 ± 0.09	27.9 ± 1.2
PPG ($n = 6$)	2.11 ± 0.05*	33.0 ± 0.7*

*$p < 0.05$ as compared with the vehicle-treated control group.

We determined the effects of systemic administration of PPG on bile output and biliary constituents *in vivo* according to the identical protocol used in Fig. 1. As shown in Table 1, the PPG administration significantly stimulated basal bile output by 15%. The biliary concentration of HCO_3^- was also significantly elevated in the PPG-treated group. As PPG inhibits CSE and could not only reduce endogenous H_2S, but also modify cysteine metabolism, it is necessary to examine the direct effects of exogenous H_2S administration on hepatobiliary function. However, such experiments were difficult, because the administration of NaHS, the H_2S-donating reagent, is known to change systemic blood pressure *in vivo* through its vasorelaxing action (22). We thus used livers perfused *ex vivo* with the taurocholate-free Krebs solution to prove roles of CSE-derived H_2S in the basal bile excretion.

As illustrated in Fig. 3, the hepatic vascular resistance was comparable among four groups tested (*e.g.*, vehicle, PPG, PPG + NaHS, and PPG + NAC). Under these circumstances, the basal bile output was significantly elevated by 20% in perfused livers of the PPG-treated rats as compared with those treated with vehicle. This response was slightly greater than that observed in the experiments *in vivo* (Table 1), presumably because the perfusion of the organ was carried out under cholate-free conditions, as discussed later in Results. The choleretic response elicited by the PPG treatment was repressed by coperfusion of NaHS at 30 μmol/L, the concentration being comparable to the PPG-sensitive fraction of the gas generation. On the other hand, coperfusion of the same concentration of NAC, a reagent entering cells to yield cysteine, did not alter the CSE-elicited choleretic response. Like the aforementioned observations *in vivo* (Table 1), the PPG treatment significantly enhanced biliary HCO_3^- concentrations, and coperfusion of 30 μmol/L NaHS completely attenuated the changes in the perfused rat livers. On the other hand, the NAC coperfusion did not repress the PPG-induced elevation of the HCO_3^- concentration (Fig. 3B).

As HCO_3^- serves as a putative constituent yielding the driving force for bile formation, we determined if the bile acid-independent bile formation is elevated in livers of the PPG-treated groups. As seen in Fig. 4, where the output was plotted as a function of biliary fluxes of bile salts, the *y*-intercept of the line for the PPG-pretreated groups became markedly decreased and dissociated from that for the control groups. The difference between the two groups became smaller with increasing fluxes of bile salts, but the difference was still evident when the flux of bile salts reached the physiologic levels (70 nmol/min/g of liver). Such a dependency of the PPG effect on bile salts was consistent with the current data indicating differences in the choleretic responses between *in vivo*

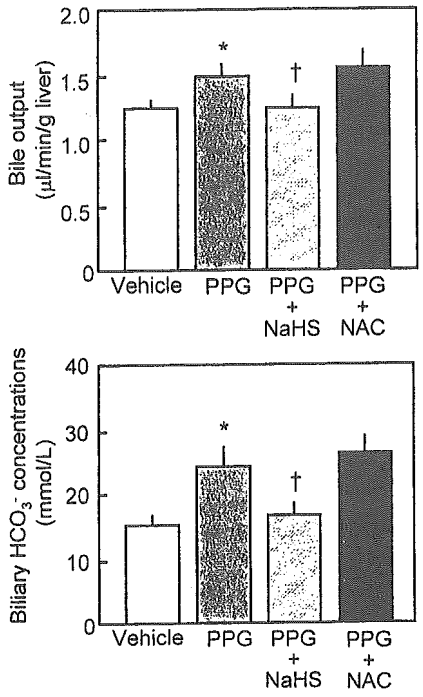

FIG. 3. Effects of the CSE blockade by PPG and supplementation of NaHS on the basal bile output and biliary HCO_3^- concentration in *ex vivo* perfused rat livers. PPG at 1.5 mmol/kg was administered *in vivo* intraperitoneally at 4 h prior to the isolation of the perfused liver. Either NaHS or NAC was perfused *ex vivo* into the liver at a concentration of 30 μmol/L, when necessary. Data indicate means ± SE of seven to nine separate experiments in each group. *$p < 0.05$ as compared with the vehicle-treated group; †$p < 0.05$ versus the PPG-treated group.

(Table 1) and *ex vivo* (Fig. 3) perfused livers. We further investigated whether biliary output of glutathione, another major constituent for bile acid-independent bile formation, could also be elevated under the blockade of CSE. As seen in Fig. 5, total amounts of glutathione excreted into bile was comparable irrespective of the PPG treatment, suggesting that this constituent plays little role in generation of the osmotic driving force, if any. Interestingly, the ratio between reduced and oxidized forms of glutathione (GSH/GSSG) was significantly elevated by the CSE blockade with PPG. Moreover, the PPG-induced elevation of GSH/GSSG in bile was further elevated with coperfusion with 30 μmol/L NaHS. As one might expect, the PPG pretreatment significantly caused a reduction of total glutathione presumably through inhibition of the transsulfuration pathway. The PPG-elicited decrease in hepatic glutathione contents was unchanged upon administration of NaHS, suggesting that the event is not mediated by endogenous H_2S. Among the three groups, >90% of glutathione was present as the reduced form (data not shown). These results suggest that suppression of CSE-derived H_2S accelerates biliary excretion of GSH, whereas its hepatic contents are reduced. Moreover, exogenous supplementation of the gas under the CSE blockade further increases its excretion into bile. Physiologic implications of this phenomenon will be mentioned later in the Discussion. Collectively, the present results suggest that H_2S endogenously generated by

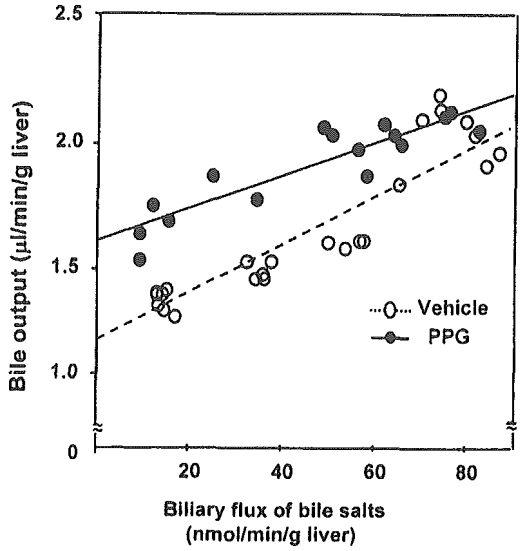

FIG. 4. Alterations in bile salt-independent fraction of bile output by the blockade of CSE by PPG. PPG at 1.5 mmol/kg was administered *in vivo* intraperitoneally at 4 h prior to the isolation of the perfused liver. Note the significant elevation ($p < 0.05$) of the y-intercept by the PPG treatment, and the difference in the basal bile output between the two groups becomes smaller with increased excretion of bile salts in bile.

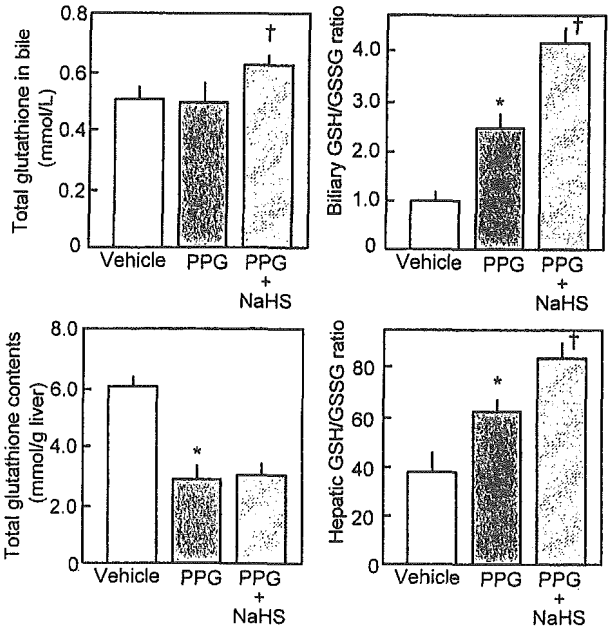

FIG. 5. Effects of the CSE blockade by PPG and supplementation of 30 μmol/L NaHS on biliary excretion and hepatic contents of glutathione in perfused rat livers. PPG at 1.5 mmol/kg was administered *in vivo* intraperitoneally at 4 h prior to the isolation of the perfused liver. Data indicate means ± SE of seven to nine separate experiments in each group. $^*p < 0.05$ as compared with the vehicle-treated group; $^\dagger p < 0.05$ versus the PPG-treated group.

CSE modulates the basal excretion of HCO_3^- in bile, playing a role in the regulation of the basal bile output through mechanisms dependent on bile acid-independent choleresis.

DISCUSSION

The present study first provided evidence for the presence of considerable amounts of H_2S in the liver. Furthermore, the gas appeared to serve as an endogenous modulator of the basal bile formation in the liver. Mechanisms for regulation of the basal bile formation involve the bile acid-independent process rather than bile acid-dependent one. Several lines of the current data support this concept: First, the effect of blockade of CSE, the enzyme producing ~50% of the basal H_2S generation, causes an increase in the bile acid-independent bile output *ex vivo* and *in vivo*. Second, as judged by data from *ex vivo* perfused livers, the difference in the excretion between PPG-treated and -untreated groups becomes increased as the biliary excretion of bile salts is reduced (Fig. 4), suggesting that the bile acid-independent fraction plays a major role. Thirdly and most importantly, between the two major biliary constituents for this fraction, HCO_3^-, but not glutathione, is elevated upon the CSE blockade and repressed by supplementation with H_2S, indicating that the former is attributed to generating the driving force for the bile formation. These results collectively suggest that stimulation of HCO_3^- plays an important role in the bile acid-independent choleresis elicited by suppression of CSE-derived H_2S generation.

As seen in alterations in hepatic contents of glutathione, PPG not only suppressed CSE-derived H_2S, but also reduced the glutathione contents. As the decrease in the hepatic glutathione contents was not restored by supplementation of NaHS, this event is not mediated by the gas, but occurs as a consequence of CSE-dependent transsulfuration processes. Of interest is that biliary excretion of total glutathione [reduced (GSH) and oxidized (GSSG) forms of glutathione] was unchanged despite the reduction in their hepatic contents. Furthermore, the relative amounts of GSH in bile were increased with supplementation of NaHS. Considering biochemical properties of the gas as a potent reductant with small molecular weight, this result raised a possibility that exogenously administered H_2S is utilized to increase reducing equivalents for GSH in bile. Several possibilities should be taken into account for mechanisms by which H_2S increases the ratio of GSH/GSSG in bile: First, the blockade of CSE by PPG could inhibit the conversion of cysteine into H_2S and thereby save this amino acid for the glutathione synthesis even when the supply of the substrate from the transsulfuration pathway is inhibited. Secondly, H_2S could be used directly as a reducing equivalent to increase GSH in bile. Thus, the role of CSE-mediated conversion of cysteine into H_2S for a fail-safe mechanism to maintain the reducing equivalent deserves further studies to provide evidence that the gas serves as a novel endogenous reductant.

Among gaseous substances detected in mammalian tissues, H_2S has recently been suggested to account for a novel neurovascular transmitter, although receptor mechanisms for the gas signal transduction remain largely unknown. The current results first suggest that the liver could have the ability to ex-

ecute remodeling of HCO_3^- excretion and increase the basal bile formation when exposed to disease conditions causing a decrease in the enzyme activity; such circumstances involve cirrhosis and surgical insults as previously reported both experimentally and clinically (8, 20). When considering effects of other gaseous mediators on the quality control of bile excretion, which were previously reported from our laboratory and other, it is not unreasonable to hypothesize that the liver could utilize multiple gases to regulate biliary function under physiologic and pathologic conditions. In the rat model of endotoxemia, nitric oxide (NO) suppresses oxidative phosphorylation via blockade of mitochondrial cytochrome *c* oxidase, and thereby down-regulates bile acid-dependent bile formation (17, 19). Although mechanisms for transcriptional regulation of the CSE expression remain largely unknown, previous studies revealed that exposure to excess NO caused up-regulation of the CSE expression in aortic tissues and increased endogenous generation of H_2S to modulate the vascular tone. As shown in the current study, the excess dose of exogenous NaHS supplementation reduced the basal bile output, suggesting that H_2S causes cholestasis with its excess amounts. In this context, quantitative determination of these two gases in the endotoxemic liver deserves further studies provided that the functional link of their overproduction to biliary function can be demonstrated.

On the other hand, the current results together with our previous data collectively suggest that a reduction of H_2S and an increase in carbon monoxide (CO) share common roles in the regulation of bile formation in that both events stimulate excretion of bile constituents besides bile salts. CO at micromolar levels not only modulates sinusoidal tone (18), but also has the ability to induce choleresis and to stimulate biliary excretion of major organic anions such as glutathione and bilirubin-IXα through mechanisms involving multidrug resistance protein 2 (13). Such effects of CO on biliary excretion occur in a concentration-specific manner, and excess concentrations of the gas repress the choleretic response and lead to cholestasis through the increase in paracellular junctional permeability and suppression of bile canalicular contractility (11, 16). In contrast to CSE, heme oxygenase-1 is up-regulated by surgical insults or by liver cirrhosis, and the parenchyma is exposed to high concentrations of CO (9, 12, 21). Thus, under disease conditions, overproduced CO and reduced H_2S could cooperatively increase the bile acid-independent fraction of bile output through increased excretion of organic anions and HCO_3^-, respectively. Although physiologic implication of the current observations remains to be fully understood, such remodeling of a quality of bile could benefit the increasing solubility of organic anions or protect against cholestasis possibly occurring under the aforementioned disease conditions. Further investigation is necessary to examine if alterations of these gases could regulate a quality of bile cooperatively with modulation of H_2S generation under a variety of hepatobiliary disease conditions.

ACKNOWLEDGMENTS

This study was supported by the 21st Century Center-of-Excellence Program and the Leading Project for Biosimulation, and partly supported by Grant-in-Aid for Creative Science Research 13GS0015 from the Ministry of Education, Sciences and Technology of Japan, as well as by Advanced Medical Technology in Health Sciences Research Grants from Ministry of Health and Welfare in Japan.

ABBREVIATIONS

CBS, cystathionine β-synthase; CO, carbon monoxide; CSE, cystathionine γ-lyase; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; HCO_3^-, bicarbonate; H_2S, hydrogen sulfide; NAC, *N*-acetylcysteine; NO, nitric oxide; PPG, propargylglycine.

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Received for publication December 3, 2004; accepted December 10, 2004.

Am J Physiol Heart Circ Physiol 288: H1885–H1892, 2005.
First published November 24, 2004; doi:10.1152/ajpheart.01004.2004.

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

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Submitted 1 October 2004; accepted in final form 18 November 2004

Cabrales, Pedro, Hiromi Sakai, Amy G. Tsai, Shinji Takeoka, Eishun Tsuchida, and Marcos Intaglietta. Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution. *Am J Physiol Heart Circ Physiol* 288: H1885–H1892, 2005. First published November 24, 2004; doi:10.1152/ajpheart.01004.2004.—The oxygen transport capacity of phospholipid vesicles encapsulating purified Hb (HbV) produced with a P_{O_2} at which Hb is 50% saturated (P_{50}) of 8 (HbV_8) and 29 mmHg (HbV_{29}) was investigated in the hamster chamber window model by using microvascular measurements to determine oxygen delivery during extreme hemodilution. Two isovolemic hemodilution steps were performed with 5% recombinant albumin (rHSA) until Hct was 35% of baseline. Isovolemic exchange was continued using HbV suspended in rHSA solution to a total [Hb] of 5.7 g/dl in blood. P_{50} was modified by coencapsulating pyridoxal 5′-phosphate. Final Hct was 11% for the HbV groups, with a plasma [Hb] of 2.1 ± 0.1 g/dl after exchange with HbV_8 or HbV_{29}. A reference group was hemodiluted to Hct 11% with only rHSA. All groups showed stable blood pressure and heart rate. Arterial oxygen tensions were significantly higher than baseline for the HbV groups and the rHSA group and significantly lower for the HbV groups compared with the rHSA group. Blood pressure was significantly higher for the HbV_8 group compared with the HbV_{29} group. Arteriolar and venular blood flows were significantly higher than baseline for the HbV groups. Microvascular oxygen delivery and extraction were similar for the HbV groups but lower for the rHSA group ($P < 0.05$). Venular and tissue P_{O_2} were statistically higher for the HbV_8 vs. the HbV_{29} and rHSA groups ($P < 0.05$). Improved tissue P_{O_2} is obtained when red blood cells deliver oxygen in combination with a high- rather than low-affinity oxygen carrier.

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

PHOSPHOLIPID VESICLES encapsulating concentrated hemoglobin (Hb) solution [Hb vesicles (HbV) or liposome-encapsulated Hb] provide oxygen-carrying capacity to plasma expanders, reproducing several of the characteristics of red blood cells (RBC) suspended in plasma. HbV contain Hb at a high concentration within a cell membrane-like structure. Their oxygen dissociation curve can be adjusted by varying the concentration of pyridoxal 5′-phosphate (PLP). A widely accepted premise for designing a blood substitute is that its Hb should have an oxygen dissociation curve like that of RBC or one that is right shifted, i.e., having a high P_{50} to facilitate the unloading of oxygen (P_{50} is the partial pressure of oxygen at which the Hb molecule is 50% saturated). In a previous study by Sakai et al. (16), vesicles were formulated with P_{50} values set at 9, 16, and 30 mmHg. The study showed that optimal tissue oxygen conditions were obtained when 80% of the circulating blood was substituted with HbV whose P_{50} was 16 mmHg, a value considerably lower than the usual value of 28 mmHg for normal blood (16). Oxygen-carrying capacity was found to be well above the oxygen supply limitation.

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

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

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

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

METHODS

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

Preparation of HbV with different P_{50}. HbV were prepared under sterile conditions as previously reported (12, 15). Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Japanese Red Cross Society (Tokyo, Japan). The encapsulated purified Hb (38 g/dl) contained 0 or 14.7 mM PLP (Sigma Chemical, St. Louis, MO) as an allosteric effector at a molar ratio of [PLP]/[Hb] = 0 or 2.5, respectively. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate at a molar ratio of 5:5:1 (Nippon Fine Chemical, Osaka, Japan) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (0.3 mol% of the total lipid; NOF, Tokyo, Japan) (17). HbV with a 250-nm diameter were suspended in a physiological saline solution in which [Hb] = 10 g/dl, sterilized with filters (Dismic, pore size 0.45 μm; Toyo Roshi, Tokyo, Japan), and deoxygenated with N_2 bubbling for storage (14). The content of lipopolysaccharide was <0.1 EU/ml.

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

In a previous study (16), HbV were suspended in 8 g/dl HSA. However, we changed to 5 g/dl rHSA because it showed better microvascular perfusion in the hamster window model (i.e., increased red cell velocity and functional capillary density) than 8 g/dl HSA. The suspension was filtered through sterile filters (pore size 0.45 μm; Millipore, Billerica, MA). The characteristics of HbV are listed in Table 1, with all parameters being almost identical except oxygen affinity (HbV_8, P_{50} = 8 mmHg; HbV_{29}, P_{50} = 29 mmHg).

Table 1. *Physical characteristics of solutions*

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

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

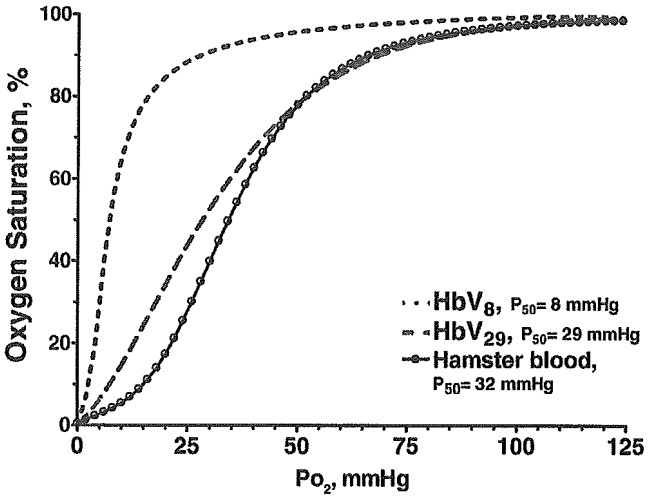

Fig. 1. Oxygen dissociation curves for phospholipid vesicles encapsulating purified Hb (HbV) produced with a Po_2 at which Hb is 50% saturated (P_{50}) of 8 (HbV_8) and 29 mmHg (HbV_{29}) vs. the dissociation curve for hamster blood (P_{50} = 32 mmHg).

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

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

After *level 2*, the animals were randomly divided into three experimental groups by being assigned to an experimental group according to a sorting scheme based on a list of random numbers (1). *Level 2* exchange was followed by *level 3* exchange. Hemodilution with 5% rHSA solution was continued with one group of the *level 2* hemodiluted animals, the experimental group rHSA, until Hct was decreased to 11% of baseline (Fig. 2). The test materials were studied by assigning the remainder of the *level 2* animals to groups labeled HbV_8 (P_{50} = 8 mmHg) and HbV_{29} (P_{50} = 29 mmHg) and were hemodiluted using these materials, reducing Hct to 11%. Plasma Hb concentrations derived for HbV_8 and HbV_{29} after exchange of 35% blood volume are estimated around 2.0–2.3 g/dl for both groups (35% of estimated total Hb content) (21).

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

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

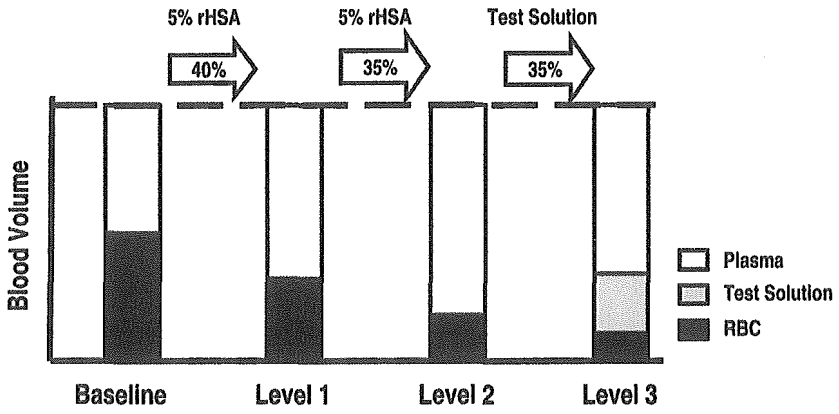

Fig. 2. Hemodilution was attained by means of a progressive, stepwise, isovolemic blood exchange-transfusion protocol. Volume of each exchange-transfusion step was calculated as a percentage of the blood volume, estimated as 7% of body weight. An acute anemic state was induced by lowering systemic Hct, using a 5% recombinant human serum albumin (rHSA) solution, in 2 progressive steps of isovolemic hemodilution labeled *level 1* and *level 2* exchanges. *Level 3* exchange was achieved by a third hemodilution that continued using rHSA or the vesicle solutions HbV_8 or HbV_{20} suspended in 5% rHSA (test solutions). RBC, red blood cells.

analyzed for arterial P_{O_2} (Pa_{O_2}), arterial P_{CO_2} (Pa_{CO_2}), base excess (BE), and pH (Blood Chemistry Analyzer 248; Bayer, Norwood, MA). The comparatively low Pa_{O_2} and high Pa_{CO_2} values of these animals is a consequence of their adaptation to a fossorial environment. Blood samples for viscosity and colloid osmotic pressure measurements were quickly withdrawn from the animal with a heparinized 5-ml syringe at the end of the experiment for immediate analysis.

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

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

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

Microvascular P_{O_2} distribution. High-resolution microvascular P_{O_2} measurements were made using phosphorescence-quenching microscopy (18), a method based on the oxygen-dependent quenching of phosphorescence emitted by albumin-bound metalloporphyrin complex after pulsed light excitation. Phosphorescence microscopy is not dependent on the level of dye within the tissue, and the decay time is inversely proportional to the P_{O_2} level. The phosphorescence decay curves were converted to oxygen tensions by using a fluorescence decay curve fitter (model 802; Vista Electronics) (9). This technique has been used in this animal preparation and others for both intravascular and extravascular oxygen tension measurements, because albumin exchange between plasma and tissue allows for sufficient concentrations of albumin-bound dye within the interstitium to achieve an adequate signal-to-noise ratio. Animals received a slow intravenous injection of 15 mg/kg body wt at a concentration of 10.1 mg/ml of a palladium-*meso*-tetra(4-carboxyphenyl)porphyrin (Porphyrin Products, Logan, UT). P_{O_2} measurements were made 20 min after porphyrin injection, allowing it to be distributed to all the tissues.

In our system, intravascular measurements are made by placing an optical rectangular window (5 × 40 μm) within the vessel of interest, with the longest side of the rectangular slit positioned parallel to the vessel wall. Tissue P_{O_2} is measured in regions void of large vessels within intercapillary spaces with an optical window size of ~10 × 10 μm, which allows us to precisely establish the localization of the P_{O_2} measurements in arterioles, venules, and the interstitium (20). The phosphorescence decay due to quenching at a specific P_{O_2} yields a single decay constant, and in vitro calibration has been demonstrated to be valid for in vivo measurements. Intravascular and perivascular P_{O_2} measurements were made in the arterioles studied, and intravascular P_{O_2} measurements were made in venules. Interstitial tissue P_{O_2} was measured in regions distant from visible underlying and adjacent vessels.

Tissue oxygen delivery and extraction. The microvascular methodology used in our studies allows a detailed analysis of oxygen supply in the tissue. Calculations of O_2 delivery, defined as the amount of oxygen delivered by the arterioles to the microcirculation per unit time normalized relative to baseline, and O_2 extraction, defined as the amount of oxygen released by blood to the tissue by the microcirculation per unit time normalized relative to baseline, were made using *Eqs. 1* and *2*:

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

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

where RBC_{Hb} is the [Hb] in RBC (expressed in g/dl of blood), HbV_{Hb} is the [Hb] in HbV (expressed in g/dl of blood), γ is the oxygen-carrying capacity of Hb at 100% saturation (or 1.34 ml O_2/g Hb), Sa% indicates the arteriolar oxygen saturation of RBC or HbV, S(a-v)% indicates the arteriovenous difference in oxygen saturation of RBC or HbV, (1 − Hct) is the fractional plasma volume (and converts the equation from units per dl of plasma to per dl of blood), α is the solubility of oxygen in plasma and is equal to 3.14×10^{-3} ml O_2/dl plasma mmHg, Pa_{O_2} is the arteriolar partial pressure of oxygen, $P(a\text{-}v)_{O_2}$ is the arteriovenous difference in P_{O_2}, and Q is the microvascular flow for each microvessel as a percentage of baseline. The oxygen dissociation curves were determined as described before. In this analysis, microvascular Hct was corrected according to the findings of Lipowsky and Firrell (10).

Experimental procedure. Baseline systemic, microvascular, and hemodynamic characterizations were performed before the start of the exchange. After each exchange and a stabilization period of 10 min, systemic and/or microvascular measurements were performed. Exchanges began every hour. After the *level 3* exchange transfusion, the same measurements were repeated, and then the P_{O_2} distribution was determined using phosphorescence-quenching microscopy (9). The duration of the experiment was 3–4 h.

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

RESULTS

Exchange transfusion. Twenty-four animals (55–65 g body wt) entered into the exchange-transfusion (hemodilution) protocol, and all tolerated the experiment without any visible discomfort. Microvascular studies were completed in six preparations for each test material, namely, the *level 2* rHSA, HbV_8, and HbV_{29}. The data were analyzed using a model for computing oxygen delivery to the tissue at the microscopic level.

Hematological changes. The exchange-transfusion protocol resulted in a final Hct ranging from 11.0 ± 0.5 to 11.4 ± 0.6%. The HbV_8 and HbV_{29} groups had a final plasma Hb concentration of 2.1 ± 0.1 g/dl, which increased the total Hb concentration in blood (RBC + Hb in plasma) to 5.7 ± 0.2–0.3 g/dl after completion of the *level 3* exchange transfusion. Thus oxygen-carrying capacities at this level were similar to those found at *level 2*, where total blood Hb concentration was 5.7 ± 0.3 g/dl (Hct 18.1 ± 0.7) (Table 2).

Systemic and blood gas parameters. Changes in the systemic parameters are presented in Fig. 3. Mean arterial pressure was statistically lower for the extreme hemodilution tests with rHSA and the HbV_{29} group and attained the highest value with HbV_8 viscosity. Heart rate after hemodilution followed by exchange transfusion with the HbV solutions was ~10% higher than baseline at the *level 3* exchange. The slight increase in heart rate was not statistically different.

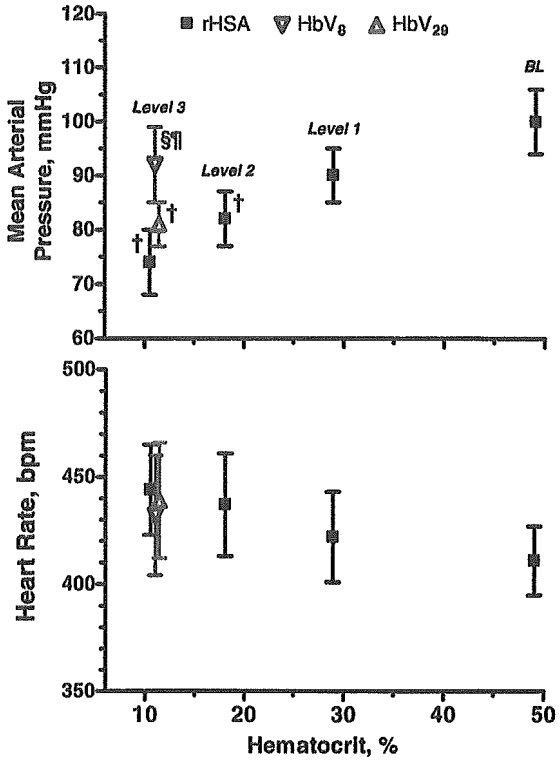

Fig. 3. Mean arterial blood pressure and heart rate [in beats/min (bpm)] at baseline (BL) (Hct 49%) and *level 1* (Hct 29%), *level 2* (Hct 18%), and *level 3* (Hct, 11%) hemodilutions. *Level 1* and *level 2* exchanges were performed with 5% rHSA as diluent. *Level 3* exchange was used to evaluate the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

Analysis of arterial blood gases (Table 2) showed a statistical increase in P_{O_2} after hemodilution and exchange transfusion. Pa_{CO_2} was unchanged from baseline after hemodilution. Blood pH was not statistically changed. At *level 3* exchange, BE was positive and not statistically different between HbV groups, but it was negative and statistically different from baseline for the rHSA group ($P < 0.05$).

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

Table 2. *Laboratory parameters during exchange protocol*

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

Values are means ± SD. Baseline values include all animals in the study. No significant differences were detected between the baseline values of each group or between the values after *level 1* and *level 2* exchange before the exchange with test solutions. Hct, systemic hematocrit; Hb, hemoglobin content of blood; Pa_{O_2}, arterial partial O_2 pressure; Pa_{CO_2}, arterial partial pressure of CO_2; BE, base excess. *$P < 0.05$ compared with baseline; †$P < 0.05$ compared with *level 3* rHSA; ‡$P < 0.05$ compared with *level 3* HbV_8 to *level 3* HbV_{29}.

Table 3. *Rheological properties and COP*

Fluid	Blood Viscosity, cp	Plasma Viscosity, cp	COP, mmHg	*n*
Blood	4.2±0.7	1.2±0.1	17.6±0.7	6
Level 2 rHSA	2.0±0.2*	0.9±0.1	17.2±0.8	4
Level 3 rHSA	1.6±0.2*	0.9±0.1	17.4±1.1	5
Level 3 HbV_8	1.9±0.3*	1.0±0.1	17.3±0.8	6
Level 3 HbV_{29}	2.0±0.4*	1.0±0.1	17.8±1.0	5

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

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

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

Microhemodynamics. After *level 3* exchange, arteriolar and venular diameters were not statistically different from baseline for any of the groups. Arteriolar flow velocities attained the highest value for the HbV_8 group, being 1.90 relative to baseline, which was statistically significant. The same effect was found in the venular microcirculation, where blood flow velocity was 2.20 relative to baseline. HbV_{29} exchange transfusion lowered both arteriolar and venular velocities relative to the values attained at the *level 2* exchange. However, venular velocity in this group was statistically significantly higher than in baseline. Notably, the *level 2* hemodilution with rHSA caused significantly higher blood flow velocities in the arteriolar and venular microcirculation (Fig. 4).

Combining data for the RBC flow velocity and diameter allowed calculation of the arteriolar and venular blood flows (Fig. 5). The results of this calculation showed that all exchanges caused blood flow to increase. Arteriolar and venular blood flows at *level 2* exchange with the use of rHSA were significantly higher than those at baseline. However, continuing hemodilution with this material to *level 3* exchange did not sustain the increase, and arteriolar and venular blood flow, although showing a tendency to remain elevated, were not statistically different from baseline values.

Level 3 exchange transfusion with HbV_8 and HbV_{29} caused blood flow to be significantly higher than baseline. Furthermore, the HbV_8 group showed consistently higher blood flows than the HbV_{29} group; however, the trend was not statistically significant.

Functional capillary density. The number of capillaries with RBC passage upon *level 3* hemodilution in the rHSA, HbV_8, and HbV_{29} groups was 62 ± 9, 76 ± 12, and 72 ± 13% of baseline, respectively. These values were statistically different from baseline but not statistically different with respect to each other (Fig. 6).

Microvascular oxygen distribution. Oxygen tension measured using phosphorescence microscopy after *level 3* exchange transfusion in the rHSA, HbV_8, and HbV_{29} groups showed that these materials produced virtually identical distributions of arteriolar microvascular P_{O_2} (arterioles averaged 49.5 mmHg), although HbV_8 tended to be higher (Fig. 7). The decrease of RBC from *level 2* to *level 3* did not decrease the arteriolar P_{O_2}. Venular P_{O_2} after *level 3* was significantly lower than at *level 2* exchange in all cases (rHSA, 7.2 ± 3.2 mmHg; HbV_8, 15.1 ± 3.7 mmHg; HbV_{29}, 9.6 ± 4.2 mmHg).

Tissue P_{O_2} values at *level 3* exchange were consistently lower than those at *level 2* exchange (20.1 ± 2.2 mmHg), with the difference being statistically significant. The highest was attained by the HbV_8 group, being 14.0 ± 2.2 mmHg. By comparison, tissue P_{O_2} for the HbV_{29} group was 9.2 ± 2.7 mmHg and for the rHSA group, 2.6 ± 1.4 mmHg, which was significantly lower compared with the HbV_8 and HbV_{29} groups (Fig. 7).

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

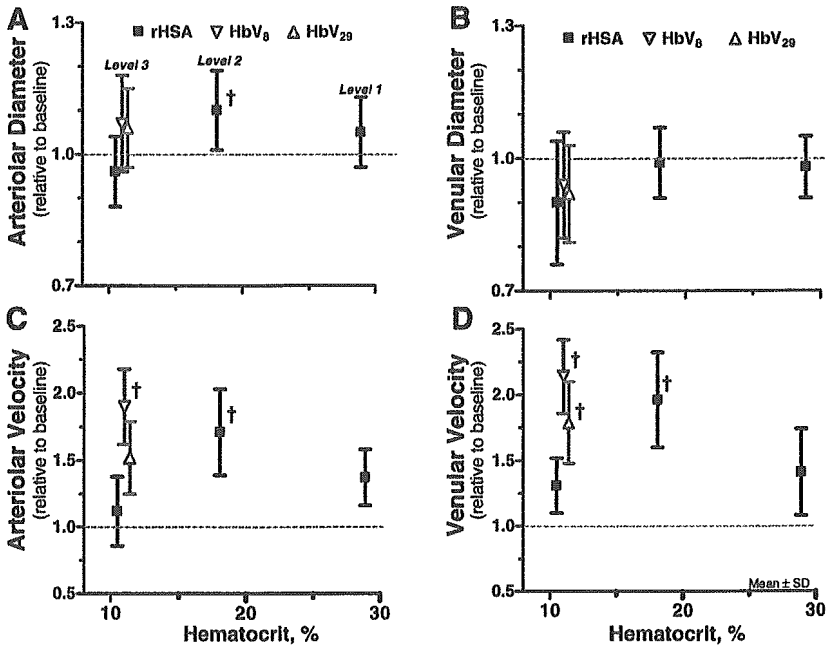

Fig. 4. Changes relative to baseline in arteriolar and venular hemodynamics at the *level 1*, *level 2*, and *level 3* exchanges. Dashed lines represent baseline level. †$P <$ 0.05 relative to baseline. Arteriolar (*A*) and venular (*B*) diameters (μm, means ± SD, *n* = no. of vessels studied) in each animal group were as follows. Baseline: arterioles (A), 61.2 ± 10.2, *n* = 80; venules (V), 62.0 ± 12.3, *n* = 80. *Level 1* with rHSA: A, 65.6 ± 12.4; V, 61.1 ± 12.4. *Level 2* with rHSA: A, 66.1 ± 14.6; V, 64.3 ± 15.2. *Level 3* with rHSA: A, 63.7 ± 14.4, *n* = 20; V, 61.0 ± 16.7, *n* = 20. *Level 3* with HbV_8: A, 64.9 ± 15.7, *n* = 20; V, 64.6 ± 18.2, *n* = 20. *Level 3* with HbV_{29}: A, 66.1 ± 16.2, *n* = 20; V, 63.1 ± 18.0, *n* = 20. Arteriolar (*C*) and venular (*D*) RBC velocities (mm/s, means ± SD) in each animal group were as follows. Baseline: A, 4.9 ± 1.3; V, 1.7 ± 0.5. *Level 1* with rHSA: A, 6.3 ± 1.4; V, 2.0 ± 0.7. *Level 2* with rHSA: A, 7.9 ± 1.5; V, 2.6 ± 0.9. *Level 3* rHSA: A, 5.2 ± 2.0, *n* = 20; V, 1.9 ± 1.1, *n* = 20. *Level 3* with HbV_8: A, 7.2 ± 1.8, *n* = 20; V, 3.3 ± 1.0, *n* = 20. *Level 3* with HbV_{29}: A, 7.0 ± 1.7, *n* = 20; V, 3.0 ± 0.9, *n* = 20.

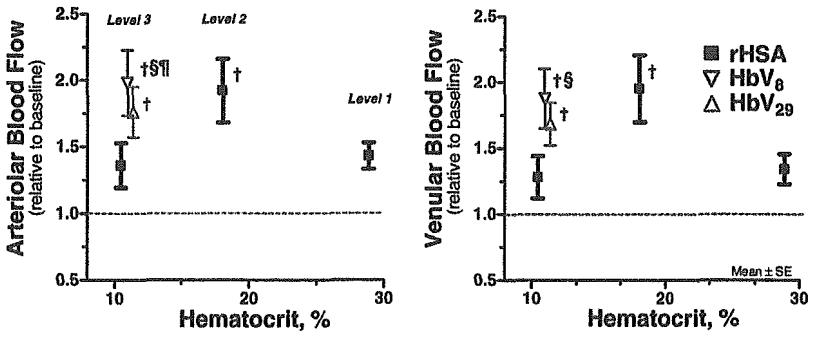

Fig. 5. Arteriolar and venular flow (nl/s, means ± SD, n = no. of vessels studied) in each animal group were as follows. Baseline: arterioles (A), 14.8 ± 7.1, n = 76; venules (V), 5.0 ± 2.9, n = 76. *Level 1* with rHSA: A, 21.9 ± 9.7; V, 5.8 ± 3.6. *Level 2* with rHSA: A, 27.2 ± 16.1; V, 8.3 ± 4.2. *Level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

microcirculation. It is apparent that exchanging RBC for HbV_8 maintains oxygen delivery to the tissue, whereas HbV_{29} reduces this by ~20%, and continued hemodilution with a non-oxygen-carrying material significantly depresses oxygen delivery to the tissue, reducing this to half of that attained at the *level 2* hemodilution.

DISCUSSION

The principal finding of this study is that under identical extreme hemodilution conditions, with the use of vesicles encapsulating Hb with normal P_{50} (HbV_{29} = 29 mmHg) and low P_{50} (HbV_8 = 8 mmHg), tissue P_{O_2} is statistically significantly higher when the high oxygen affinity material is used, namely, 14.0 ± 2.2 vs. 9.2 ± 2.7 mmHg. The significantly increased tissue P_{O_2} attained with HbV_8 appears to be due to a series of incremental improvements in microvascular and macrovascular hemodynamics comprising the increase of arteriolar blood flow and mean arterial blood pressure, which was significantly higher ($P < 0.05$) for HbV_8 than for HbV_{29}.

In the hemodilution procedures of this study, blood was exchanged with a rHSA solution as a colloidal plasma expander, which was the same suspending medium used for the Hb vesicles. Therefore, in these experiments, we can make a direct comparison between an oxygen-carrying and non-oxygen-carrying blood substitute, uncomplicated by the presence of additional materials. Our results show that the *level 2* hemodilution with rHSA leads to maintained functional capillary density and significantly improved arteriolar and venular blood flow, although somewhat lowered central blood pressure. The latter finding is not necessarily negative and may reflect a lowered overall peripheral vascular resistance due to the decrease of blood viscosity after hemodilution. The fact that microvascular flow is significantly increased indicates that the *level 2* hemodilution with rHSA provides the tissue with adequate microvascular perfusion and that this colloid is an adequate plasma expander.

Average oxygen delivery and extraction were somewhat greater for HbV_8 than for HbV_{29}. These are calculated values and are not statistically significantly different; however, the same difference was found in all micro and macro parameters measured in this study.

The *level 2* hemodilution and the succeeding *level 3* hemodilution with either HbV_8 and HbV_{29} resulted in the same total Hb concentration in the circulation (5.7 and 5.8 g Hb/dl); however, oxygen delivery was lower with HbV_{29} and lowest with rHSA, as might be expected due to the low Hb content (3.7 g Hb/dl) in the absence of plasma Hb for the rHSA group. Therefore, because all groups had the same Hct at the *level 3* hemodilution, the sustained oxygen consumption and tissue P_{O_2} relative to the rHSA group clearly demonstrate that Hb vesicles release oxygen. However, the vesicles with the lowest P_{50} provide an oxygen delivery capacity identical to that of blood at *level 2* hemodilution, whereas vesicles with a high P_{50} lower oxygen delivery at the microcirculatory level, an effect probably caused by the decreased blood flow associated with HbV_{29}.

The differences in tissue P_{O_2}, mean arterial blood pressure, and arteriolar blood flow between HbV_8 and HbV_{29} show that in designing a blood substitute, it is not sufficient to provide adequate oxygen-carrying capacity. Once a suitable oxygen carrier is available, it also must be able to maintain or enhance other circulatory transport parameters, particularly flow. The Hb vesicles used in this study are vasoinactive, and the difference in P_{50} appears to be a factor in improving flow condition that is not related to vasoactivity. An explanation for this may be related to the inherent variability of tissue P_{O_2} shown in this and other studies (4, 22), which may be enhanced in extreme hemodilution. This variability determines that if average tissue P_{O_2} is low, portions of the tissue may become anoxic. Introducing a small quantity of a low-P_{50} Hb oxygen carrier into the circulation will deliver oxygen only to those parts of the tissue

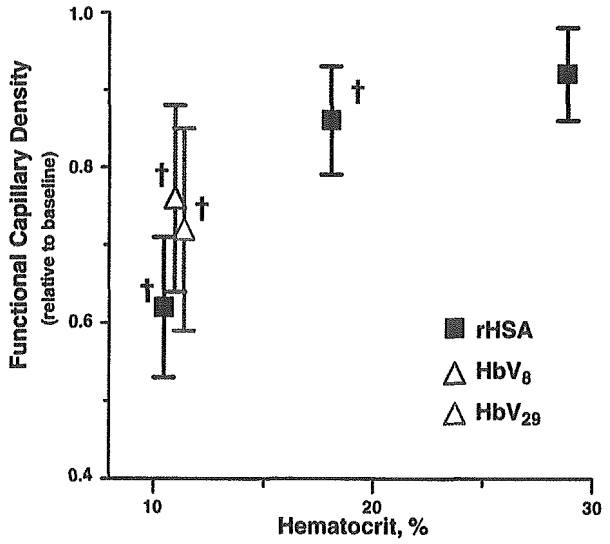

Fig. 6. Functional capillary density after the *level 1*, *level 2*, and *level 3* exchanges for the different test fluids. All values are relative to baseline levels. †$P < 0.05$ relative to baseline.

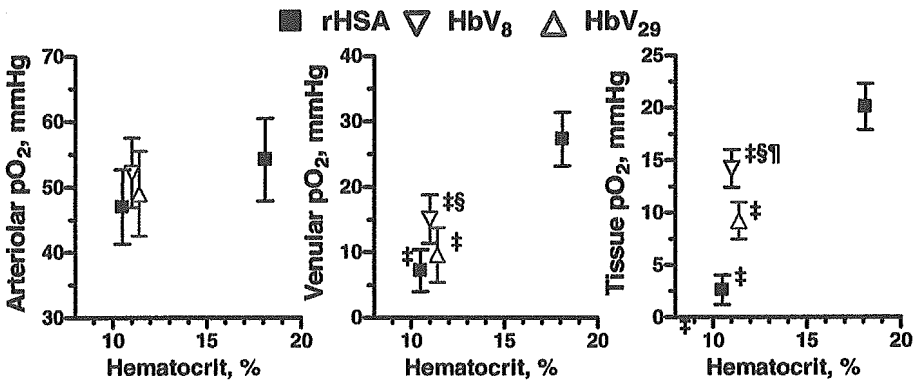

Fig. 7. Intravascular P_{O_2} after the *level 2* and *level 3* hemodilutions. Values are presented as means ± SD. ‡$P < 0.05$ compared with *level 2* with rHSA; §$P < 0.05$ compared with *level 3* with rHSA; ¶$P < 0.05$, *level 3* with HbV_8 vs. *level 3* with HbV_{29}.

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

Considering the significantly improved blood pressure and the trend toward higher flow for HbV_8 (in the absence of vasoconstriction and changes in the rheological properties of blood), it is possible that in conditions of extreme hemodilution the cardiac function should be improved because of the proposed more homogenous heart tissue oxygenation using HbV_8 vs. HbV_{29}.

In summary, the present results show that either HbV_8 or HbV_{29} are efficient oxygen carriers that do not cause vasoactivity. The experiments were carried out using rHSA as a hemodiluent, and this material was adequate as a plasma volume substitute. Oxygen extraction was similar for both oxygen carriers; however, HbV_8 appeared to be beneficial at the systemic level, because base excess remained at baseline levels, whereas it was decreased for HbV_{29}. This finding suggests that improved tissue P_{O_2} and microcirculatory oxygen delivery may be efficient in other tissues. The improvement obtained may be specific to the conditions of these experiments in which the vesicles were tested for their capacity to restore tissue P_{O_2}, FCD, and oxygen extraction in the microcirculation during extreme hemodilution. The significant differences in the tissue oxygen parameters produced by the presence of low-P_{50} Hbs vs. an identical oxygen carrier with normal P_{50} suggests that small amounts of Hbs with high oxygen affinity may have therapeutic effects in the treatment of ischemic conditions (6).

ACKNOWLEDGMENTS

We greatly acknowledge A. Barra and C. Walter (Univ. of California-San Diego) for technical assistance and Drs. K. Sou and Y. Teramura (Waseda University) for preparation of the HbV suspension.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute (NHLBI) Bioengineering Research Partnership Grant R24-HL-64395, NHLBI Grants R01-HL-62354 and R01-HL-62318 (to M. Intaglietta), and NHLBI Program Project Grant P01-HL-71064-01 (to Dr. J. Friedman) and by U.S. Army Grant PR023085, Health Sciences Research Grants (Research on Regulatory Science), the Ministry of Health, Labour, and Welfare, Japan, and grants in aid for Scientific Research from the Japan Society for the Promotion of Science (B16300162).

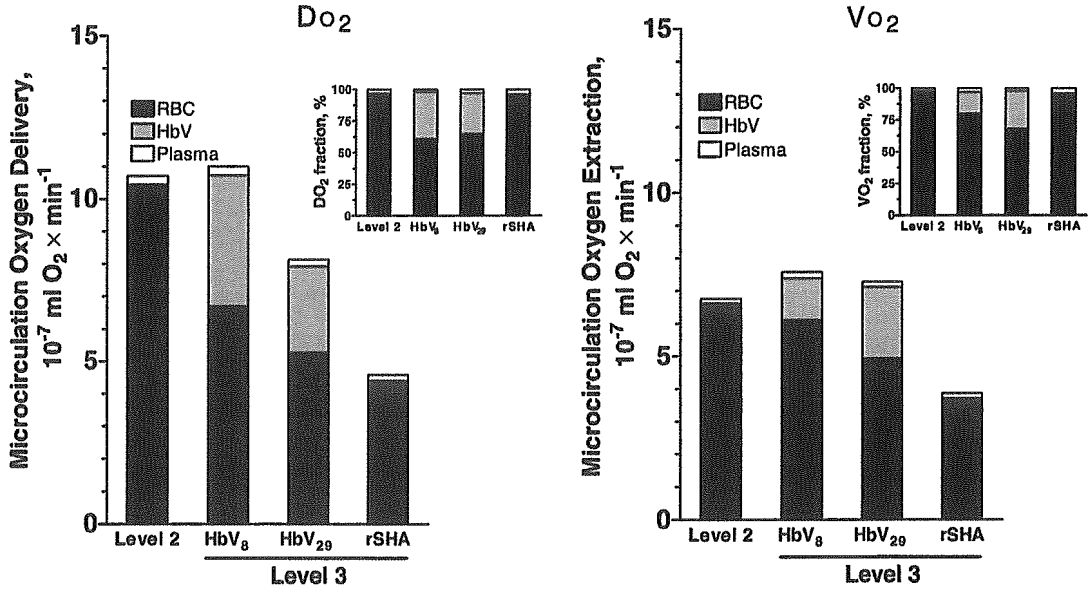

Fig. 8. Arterial oxygen delivery (D_{O_2}) and extraction (V_{O_2}) before and after the *level 3* hemodilution. Calculations of global oxygen transport are not directly measurable in our model; however, the changes relative to baseline can be calculated using the measured parameters. These calculations can be identified as those presented without standard deviations to focus on their tendencies rather than on the variability of the measurement.

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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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Supported, in part, by the Swiss National Foundation for Scientific Research (grants 32-054092.98 and 32-065149.01 to D. Erni and grant 32-050771.97 to M. Leunig); the Department of Clinical Research, University of Berne, Switzerland; a grant for Health Sciences Research (Research on Pharmaceutical and Medical Safety, Artificial Blood Project) from the Ministry of Health, Labour, and Welfare, Japan; and by Grants in Aid for Scientific Research from the Japanese Society for the Promotion of Science (B16300162).

DOI: 10.1097/01.CCM.0000159195.12111.CE

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.

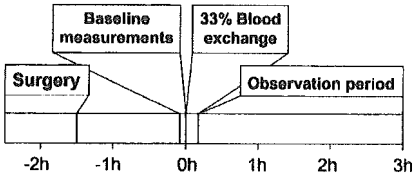

Figure 1. Diagram illustrating sequence of interventions.

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 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.

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.

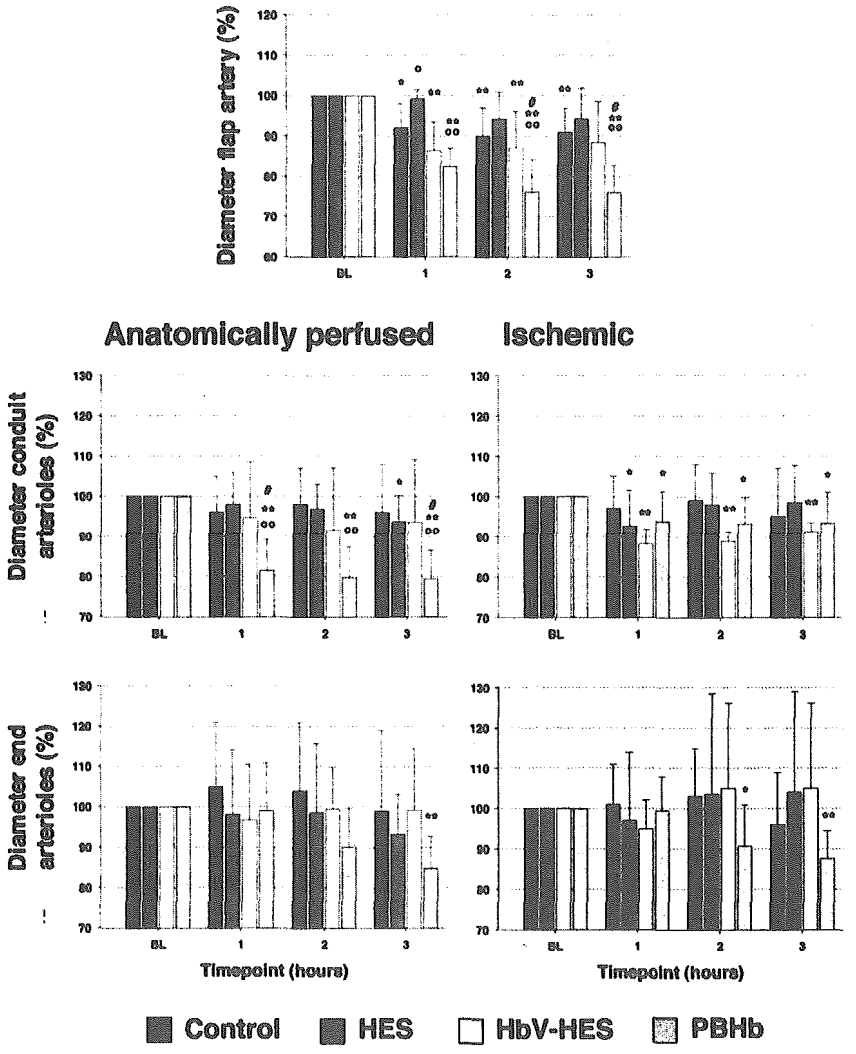

Figure 2. Microvascular diameters in flap artery, conduit arterioles, and end arterioles in the anatomically perfused and ischemic tissues at baseline (*BL*) and after hemodilution with 6% hydroxyethyl starch (*HES*), hemoglobin vesicles suspended in HES (*HbV-HES*), and polymerized bovine hemoglobin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

operating microscope at ×10 magnification (Wild, Heerbrugg, Switzerland). An island flap measuring 3 × 2 cm was dissected from the shaved and epilated back skin of the animal. The flap consisted of skin and a thin layer of panniculus carnosus muscle and 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 Po_2 and Pco_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 (Axioplan 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 ×40 objective resulting in a total optical magnification of ×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-μm fibers. The sensitive tip of the oxygen probe (100 μm in diameter) consisted of Ruthenium-III-(tris)-chloride, which measured Po_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

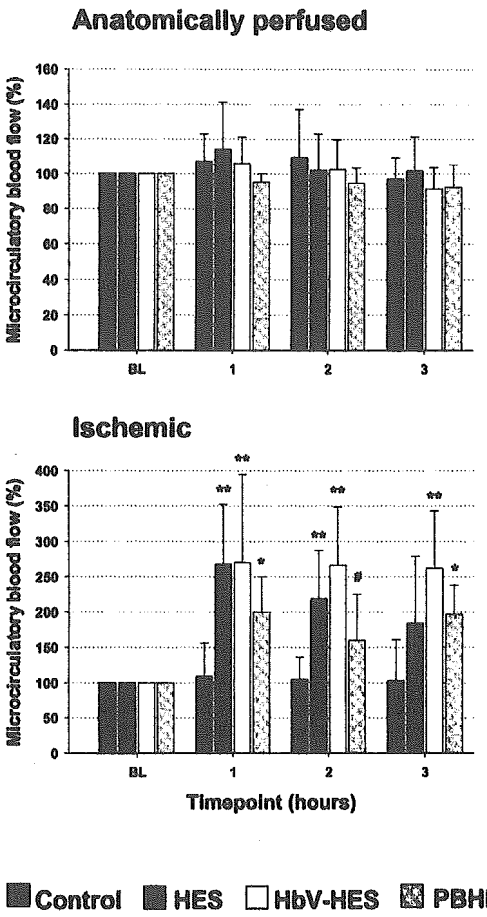

Figure 3. Microcirculatory blood flow in the anatomically perfused and ischemic tissues at baseline (*BL*) and after hemodilution with 6% hydroxyethyl starch (*HES*), hemoglobin vesicles suspended in HES (*HbV-HES*), and polymerized bovine hemoglobin (*PBHb*). Data are given as a percentage of baseline 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 μL/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 mg/mL pentobarbital given at a rate of approximately 0.5 $mg \cdot min^{-1} \cdot kg$ 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, Po_2, and Pco_2 outside the normal ranges at baseline (7.19–7.29, 35–55 torr, and 45–65 torr, respectively). The animals were killed with an overdose of pentobarbital at the end of the experiment.

Statistical Analysis. The InStat version 3 program (Graph Pad Software, San Diego, CA) was used for statistical analysis. The data were presented as mean ± 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 ± 0.9 g/dL, whereas the addition of HbV to the diluent enhanced the total Hb concentration to 11.2 ± 1.3 g/dL ($p < .05$ vs. HES), and hemodilution with PBHb resulted in a total Hb concentration of 13.6 ± 0.9 g/dL ($p < .05$ vs. HbV-HES). However, this difference became less pronounced over time. Hemodilution increased mean Po_2 from 43 torr to mean values of >60 torr and mean pH from 7.23–7.27 to mean values of 7.31–7.34, whereas mean Pco_2 decreased from 53–57 torr to 41–47 torr (all $p < .01$ vs. baseline).

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

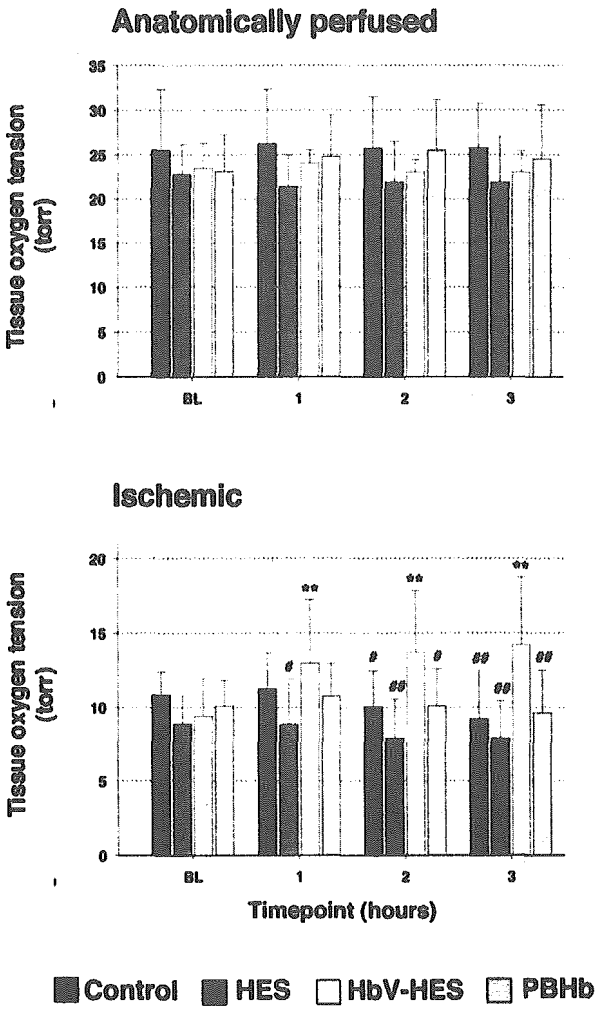

Figure 4. Po_2 in the anatomically perfused and ischemic tissues at baseline (*BL*) and after hemodilution with 6% hydroxyethyl starch (*HES*), hemoglobin vesicles suspended in HES (*HbV-HES*), and polymerized bovine hemoglobin (*PBHb*). Data represent mean values and SD. $**p < .01$ vs. baseline and 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% ± 7% of baseline ($p < .01$ vs. control, $p < .05$ vs. HbV-HES). In the anatomically perfused tissue, diameters were decreased to 79% ± 7% in the conduit arterioles ($p < .01$ vs. baseline and control, $p < .05$ vs. HbV-HES) and 85% ± 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 Po_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 (19–22). No relevant changes occurred in 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 the this study were that the elevation in lactate concentration and lactate/pyruvate ratio in the critically ischemic tissue could be attenuated by isovolemic hemodilution with the oxygen-carrying solutions. This suggests that oxidative energy metabolism was improved in the hypoxic cells, which is crucial for their survival and functional outcome. The effect was superior for HbV-HES, which also yielded markedly higher oxygen tension in the ischemic flap tissue. Both tissue oxygen tension and oxidative energy metabolism are dependent on the oxygen supply to this tissue, which is determined by the oxygen content of the blood entering the collateralized tissue and the perfusion of the latter.

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

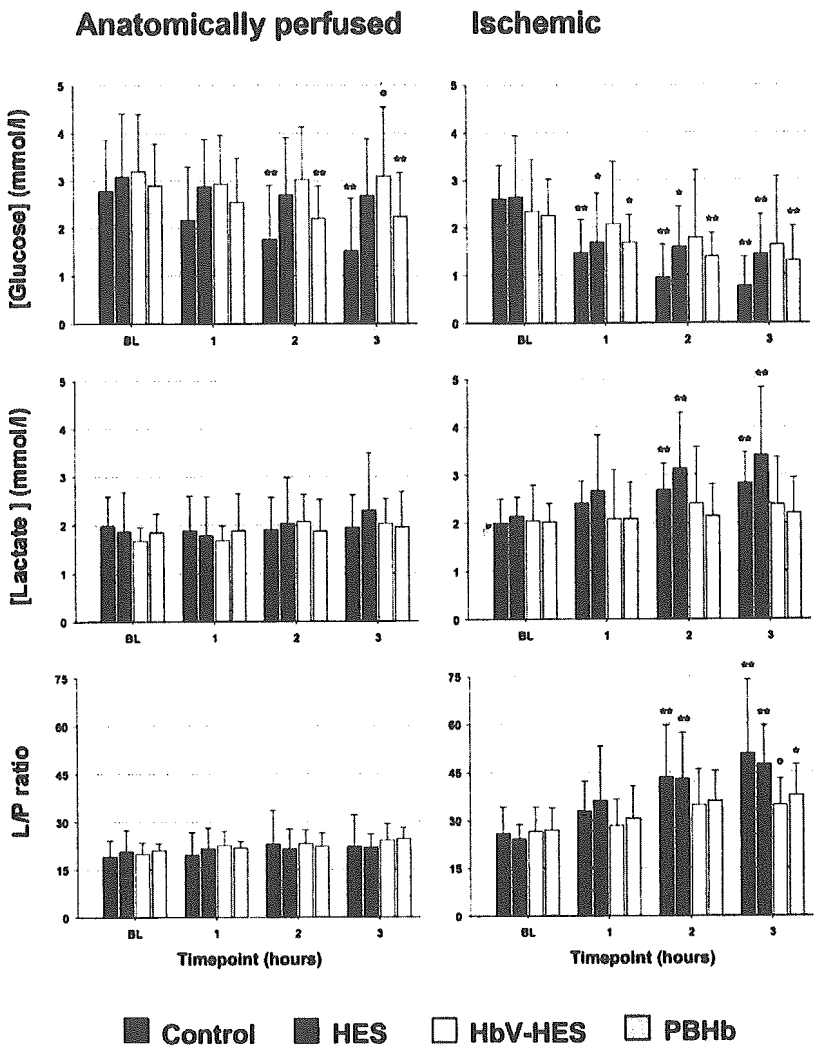

Figure 5. Carbohydrate metabolite concentrations in the anatomically perfused and ischemic tissues at baseline (*BL*) and after hemodilution with 6% hydroxyethyl starch (*HES*), hemoglobin vesicles suspended in HES (*HbV-HES*), and polymerized bovine hemoglobin (*PBHb*). Data represent mean values and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

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

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

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.

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 artifical oxygen carriers may be further enhanced by adding antioxidant enzymes to attenuate any possible ischemia-reperfusion injury (1).

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Am J Physiol Heart Circ Physiol 288: H2897–H2903, 2005.
First published January 28, 2005; doi:10.1152/ajpheart.01184.2004.

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

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[1]*Advanced Research Institute for Science and Engineering, Waseda University, Tokyo, Japan; and* [2]*Department of Bioengineering, University of California-San Diego, and* [3]*La Jolla Bioengineering Institute, La Jolla, California*

Submitted 27 November 2004; accepted in final form 24 January 2005

Sakai, Hiromi, Pedro Cabrales, Amy G. Tsai, Eishun Tsuchida, and Marcos Intaglietta. Oxygen release from low and normal P_{50} Hb vesicles in transiently occluded arterioles of the hamster window model. *Am J Physiol Heart Circ Physiol* 288: H2897–H2903, 2005. First published January 28, 2005; doi:10.1152/ajpheart.01184.2004.—A phospholipid vesicle encapsulating Hb [Hb vesicle (HbV)] has been developed as a transfusion alternative. One characteristic of HbV is that the O_2 affinity [P_{O_2} at which Hb is 50% saturated (P_{50})] of Hb can be easily regulated by the amount of the coencapsulated allosteric effector pyridoxal 5′-phosphate. In this study, we prepared two HbVs with different P_{50}s (8 and 29 mmHg, termed HbV_8 and HbV_{29}, respectively) and observed their O_2-releasing behavior from an occluded arteriole in a hamster skinfold window model. Conscious hamsters received HbV_8 or HbV_{29} at a dose rate of 7 ml/kg. In the microscopic view, an arteriole (diameter: 53.0 ± 6.6 μm) was occluded transcutaneously by a glass pipette on a manipulator, and the reduction of the intra-arteriolar P_{O_2} 100 μm down from the occlusion was measured by the phosphorescence quenching of preinfused Pd-porphyrin. The baseline arteriolar P_{O_2} (50–52 mmHg) decreased to about 5 mmHg for all the groups. Occlusion after HbV_8 infusion showed a slightly slower rate of P_{O_2} reduction compared with that after HbV_{29} infusion. The arteriolar O_2 content was calculated at each reducing P_{O_2} in combination with the O_2 equilibrium curves of HbVs, and it was clarified that HbV_8 showed a significantly slower rate of O_2 release compared with HbV_{29} and was a primary source of O_2 (maximum fraction, 0.55) overwhelming red blood cells when the P_{O_2} was reduced (e.g., <10 mmHg) despite a small dosage of HbV. This result supports the possible utilization of Hb-based O_2 carriers with lower P_{50} for oxygenation of ischemic tissues.

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

PHOSPHOLIPID VESICLES encapsulating concentrated human Hb [Hb vesicles (HbV)] or liposome-encapsulated Hb can serve as a transfusion alternative whose O_2 carrying capacity can be formulated to be comparable to that of blood (1, 5, 8, 16, 24, 30). The capsular structure of HbV (particle diameter ~250 nm) has characteristics similar to those of natural red blood cells (RBCs), because both have membranes that prevent direct contact of Hb with the components of blood and the endothelial lining, mitigating cellular injury due to Hb-mediated prooxidative species (4, 38). Furthermore, Hb encapsulation in vesicles prevents a hypertensive response induced by free Hbs that scavenge the endogenous vasorelaxation factors nitric oxide (NO) and carbon monoxide (12, 18, 26). The safety of HbV has been confirmed in rodent models in terms of the prompt metabolism of the components of HbV in the reticuloendothelial system, which was demonstrated by histopathological analysis and plasma biochemical analysis (28, 29).

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

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

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

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