CATEGORY

Acute 40 percent exchange-transfusion with hemoglobin vesicles (HbV) suspended in recombinant human serum albumin solution: degradation of HbV and erythropoiesis in a rat spleen for 2 weeks

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BACKGROUND: Hemoglobin vesicles (HbVs; diameter, 251 ± 81 nm) are artificial O_2 carriers. Their efficacy for acute exchange transfusion has been characterized in animal models. The subsequent profiles of recovery involving the degradation of HbV in the reticuloendothelial system (RES) and hematopoiesis remain unknown, however.

STUDY DESIGN AND METHODS: Isovolemic 40 percent exchange transfusion was performed in 60 male Wistar rats with HbV suspended in 5 g per dL recombinant human serum albumin (HSA; HbV/rHSA, [Hb] = 8.6 g/dL), stored rat RBCs suspended in rHSA (sRBC/rHSA), or rHSA alone. Hematological and plasma biochemical analyses and histopathological examination focusing on the spleen were conducted for the subsequent 14 days.

RESULTS: The reduced hematocrit (Hct) level (26%) for the HbV/rHSA and rHSA groups returned to its original level (43%) in 7 days. Plasma erythropoietin was elevated in all groups: the rHSA group showed the highest value on Day 1 (321 ± 123 mIU/mL) relating to the anemic conditions (HbV/rHSA, 153 ± 22; sRBC/rHSA, 63 ± 7; baseline, 21 ± 3). Simultaneously, splenomegaly occurred in all the groups as HbV/rHSA > rHSA > sRBC/rHSA. Histopathologically, the accumulated HbV in the spleen was undetectable by Day 14, but hemosiderin was deposited in slight quantities for both the HbV/rHSA and sRBC/rHSA groups. Considerable amounts of erythroblasts were apparent in the spleens of both the rHSA and the HbV/rHSA groups.

CONCLUSION: HbVs were phagocytized and degraded in RES, a physiological compartment for the degradation of RBCs, and the elevated erythropoietic activity resulted in the complete recovery of Hct within 7 days in the rat model.

Hemoglobin (Hb)-based O_2 carriers (HBOCs) have been developed progressively for use as a transfusion alternative. Some are now undergoing clinical trials.[1,2] Advantages of HBOCs include the absence of blood-type antigenicity and infectious pathogens and stability for long-term storage when compared with RBC transfusion.[3] Considerably shorter $t_{1/2}$ of the HBOCs in the blood stream (2-3 days) limit their use,[4] but they are applicable for shorter periods of use as: 1) a resuscitative fluid for hemorrhagic shock during an emergency situation temporarily or for bridging until RBCs are available;[5] 2) a fluid for preoperative hemodilution or perioperative O_2 supply fluid for a hemorrhage during elective surgery to avoid or delay allogeneic transfusion;[6] 3) a priming solution for the circuit of an extracor-

ABBREVIATIONS: HBOC(s) = hemoglobin-based O_2 carrier(s); HbV(s) = hemoglobin vesicle(s); MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin count; RES = reticuloendothelial system; sRBC(s) = stored red blood cell(s).

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poreal membrane oxygenator during cardiac surgery;[7] and 4) an alternative for use for other potential indications, for example, so-called O_2 therapeutics to oxygenate ischemic tissues.[8,9]

A phospholipid vesicle or liposome-encapsulating concentrated human Hb (Hb-vesicle, HbV) is an HBOC.[10,11] The cellular structure of the HbV (particle diameter, approx. 250 nm) has characteristics that resemble those of natural RBCs because both have lipid bilayer membranes that prevent the direct contact of Hb with blood components and the endothelial lining, thus shielding all side effects of molecular Hb levels.[12,13] Once in circulation, HbV particles are captured by the phagocytes in the reticuloendothelial system (RES or mononuclear phagocytic system) and are metabolized in the physiologically normal pathway after topload infusions.[14-17]

We tested the efficacy of HbV suspended in plasma-derived and recombinant human serum albumin (rHSA) in extreme normovolemic hemodilution (80-90% blood exchange) and resuscitation from hemorrhagic shock. They have a comparable O_2-transporting capacity with RBCs.[18-21] We reported results for only a few hours of observation after extensive blood exchange, however.

This study undertakes, for the first time, a longer period of observation (2 weeks) after moderate and clinically relevant isovolemic exchange transfusion of a 40 percent estimated blood volume with HbV suspended in a 5 g per dL rHSA solution.[21] We analyzed plasma biochemical, hematological, and histopathological examinations, particularly addressing the degradation of HbV in RES and erythropoietic activity after the reduced Hct. Splenomegaly was more dominant than hepatomegaly after single and repeated infusions of HbV in our previous studies.[14,15,17] Senescent RBCs are known to be captured and degraded in the spleen.[22] For that reason, we conducted infusion of stored homologous RBCs to compare the relative impacts on the spleen.

MATERIALS AND METHODS

Preparation of HbVs suspended in rHSA

HbVs were prepared under sterile conditions, as reported in previous studies.[23,24] The Hb was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dL) contained 14.7 mmol per L pyridoxal 5′-phosphate (Sigma-Aldrich Corp.., St. Louis, MO) as an allosteric effector at a molar ratio of pyridoxal 5′-phosphate/Hb of 2.5. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (NOF Corp., Tokyo, Japan), at a molar composition of 5/5/1/0.033. The lipopolysaccharide content, measured with a modified Limulus amebocyte lysate test, was less than 0.1 EU per mL.[25] The physicochemical parameters are P_{50}, 27 Torr; 251 ± 81-nm particle diameter; and less than 3 percent MetHb content. Before use, the HbV suspension ([Hb] = 10 g/dL, 8.6 mL) was mixed with a solution of rHSA (25 g/dL, 1.4 mL; Nipro Corp. Osaka, Japan) to regulate the rHSA concentration in the suspending medium to 5 g per dL. Consequently, the Hb concentration became 8.6 g/dL.[21] Under these conditions, the colloid osmotic pressure and the viscosity (300/sec, 37°C) of the HbV/rHSA were 20 mmHg and 2.9 cP, respectively.

Preparation of stored homologous RBC suspended in rHSA

Blood was withdrawn from donor Wistar rats via the caudal vena cava during ether anesthesia. This was mixed with an RBC preservation fluid, CPDA-1 (C.A. Karmi, Kawasumi Laboratories Inc., Tokyo, Japan) at the volume ratio of 10 percent. The mixture was stored under sterile conditions at 4°C for 1 week because rat RBCs stored for 1 week are reportedly as fragile as the human RBC stored for 4 weeks.[27] After preservation, the stored blood was centrifuged for 10 min at 4000 ×g, and then the supernatant and the buffy coat were removed. The sedimented RBCs were resuspended in saline and centrifuged. This procedure was repeated twice. Finally, the RBCs were suspended in a 5 g per dL rHSA solution to prepare stored homologous RBCs suspended in rHSA (sRBC/rHSA). The Hb concentration was regulated at 8.6 g per dL, the same Hb concentration of HbV/rHSA.

Exchange transfusion and 2-week observations

Experiments were conducted with 65 male Wistar rats (223 ± 20 g body weight; Saitama Experimental Animals Supply Co., Kawagoe, Japan). During cannulation and exchange transfusion, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (1 mL/kg; Abbott Laboratories, North Chicago, IL). Polyethylene catheters were introduced into the right common carotid artery. Blood withdrawal and sample injection were repeated through one line at 1 mL per 30 seconds. Samples were HbV/rHSA (n = 20), sRBC/rHSA (n = 20), and rHSA only (n = 20). Five rats were used for baseline measurements.

The systemic blood volume was estimated to be 56 mL per kg of the total body weight.[27] Blood was exchanged under the assumption of normovolemia. Therefore, to estimate the necessary amount of HbV, the exchange was assumed to consist of repeating the number of cycles of 1.0-mL withdrawal and sample infusion. The level of exchange, 40 percent, is therefore given as

$$40\% = 100 \times \{1 - [(0.056 \times \text{body weight} - 1.0)/(0.056 \times \text{body weight})]^n\}. \quad (1)$$

The volume exchanged was calculated as $n \times 1.0$ (mL).[28] The sample volume is calculated as 6.0 mL for a rat body weight of 220 g.

After the blood exchange, the catheter was removed, the artery was ligated, and the neck skin was sutured with a stitch. The rats were housed in cages in a barrier room at the animal experimental facility of Keio University. Rats were provided ad libitum access to food and water in a temperature-controlled environment with a 12-hour dark-light cycle.

Five rats were selected randomly from each group at 1, 3, 7, and 14 days for sequential measurements. At each time point, the rats were anesthetized with a 1.5 percent sevoflurane-mixed air inhalation. After measuring the body weight, approximately 150 µL of blood was withdrawn from the tail vein via an indwelling needle (24-gauge; Nipro Corp.) for Hct measurement with glass capillaries, and blood cell counts with an automatic blood cell counter (Model KX-21, Sysmex Corp., Kobe, Japan). The animals were laparotomized and approximately 6 mL of blood was withdrawn from the caudal vena cava for the plasma biochemical tests. The organs were resected en bloc and fixed in a 10 percent formalin neutral buffer solution (Wako Pure Chemical Industries Ltd., Tokyo, Japan) and then embedded in paraffin. Four-micrometer sections were stained with the hematoxylin-eosin, Berlin blue, and Giemsa methods.

The collected blood (approx. 6 mL) was centrifuged (5,000 × g, 10 min) to separate the plasma, which was then ultracentrifuged (50,000 × g, 20 min) to sediment the HbV particles from the plasma at 1 and 3 days after the exchange transfusion with HbV/rHSA to avoid their interference by HbV particles in the plasma biochemical assays.[29] The obtained transparent serum specimens contained no Hb, indicating that no hemolysis of HbV occurred. They were stored at −80°C until biochemical tests at BML, Inc. (Kawagoe, Japan). Erythropoietin (EPO) was measured with radioimmunoassay. Because the rat EPO shows a high degree of homology with human EPO, the rat EPO cross-reacts in the assay of the antihuman EPO.[30]

The experimental protocol was fully approved by the Laboratory Animal Care and Use Committee of School of Medicine, Keio University. It also complied with the *Guide for the Care and Use of Laboratory Animals*.[31]

Statistical analyses

Data are reported as mean ± standard deviation (SD) for all measurements. Differences between the control (baseline) group and a treatment group were analyzed with a one-way analysis of variance followed by Fisher's protected least significant difference test. The changes were considered significant if the p value was less than 0.01.

RESULTS

Body and spleen weights and hematological tests

Rats of all groups well tolerated the 40 percent blood exchange; they survived until their intentional euthanization. The rats survived this intervention because of the normovolemic exchange transfusion while maintaining the blood colloid osmotic pressure with 5 g per dL rHSA as the suspending medium. All rats gained weight until their euthanization (Fig. 1). No noticeable change occurred in their behavior or appearance such as the pilomotor response.

The spleen:body weight ratio increased significantly for the HbV/rHSA group at 1 and 3 days after the exchange. It returned to a level that was comparable to the baseline at 14 days. The rHSA group also showed significant splenomegaly at 3 days, but no splenomegaly at 1 day. At 14 days, the spleen weight reverted to the baseline level. The sRBC/rHSA group also showed moderate, but significant, splenomegaly on Days 1, 3, and 7.

The Hct before the exchange transfusion was approximately 43 percent. It decreased to about 26 percent for the HbV/rHSA and rHSA groups. Both groups showed a monotonic Hct increase; at 7 days, the Hct showed a complete recovery to the baseline level (about 43%) and an overshooting at 14 days (approx. 46%). In the sRBC/rHSA group, the Hct level at 1 day was much higher than that of the other groups because of the sRBC infusion. The Hct level, however, was slightly lower than for the other groups at 7 and 14 days. The mean corpuscular Hb (MCH), mean corpuscular volume (MCV), and mean corpuscular Hb count (MCHC) values remained within normal ranges (data not shown); however, MCH and MCHC of the HbV/rHSA group at 1 and 3 days were not measured because of the presence of HbV. The sRBC/rHSA group showed slightly lowered MCV and MCH levels at 1 day. In contrast to Hct, platelet and white blood cell counts showed nonsignificant decreases at 1 day and then maintained rather steady values. The plasma Hb concentration derived from HbV after the exchange transfusion was estimated as 4.4 g per dL, which decreased, respectively, to 1.8 ± 0.1, 1.1 ± 0.1, and 0 g per dL on Days 1, 3, and 7.

Plasma biochemical tests

The plasma EPO level, an indicator of an anemic, hypoxic, or stressed condition, increased significantly from 21 ± 3 IU per L in the normal condition to 312 ± 123 IU per L for the rHSA group at 1 day, which was significantly higher than for the HbV/rHSA group (153 ± 22 IU/L) or the sRBC/rHSA group (63 ± 7 IU/L; Fig. 2). After 3 days, they decreased to less than 100 IU/L; at 7 days, they reverted to the baseline level.

Regarding the other routine analytes, apartase aminotransferase showed slight increases on Day 1 for all

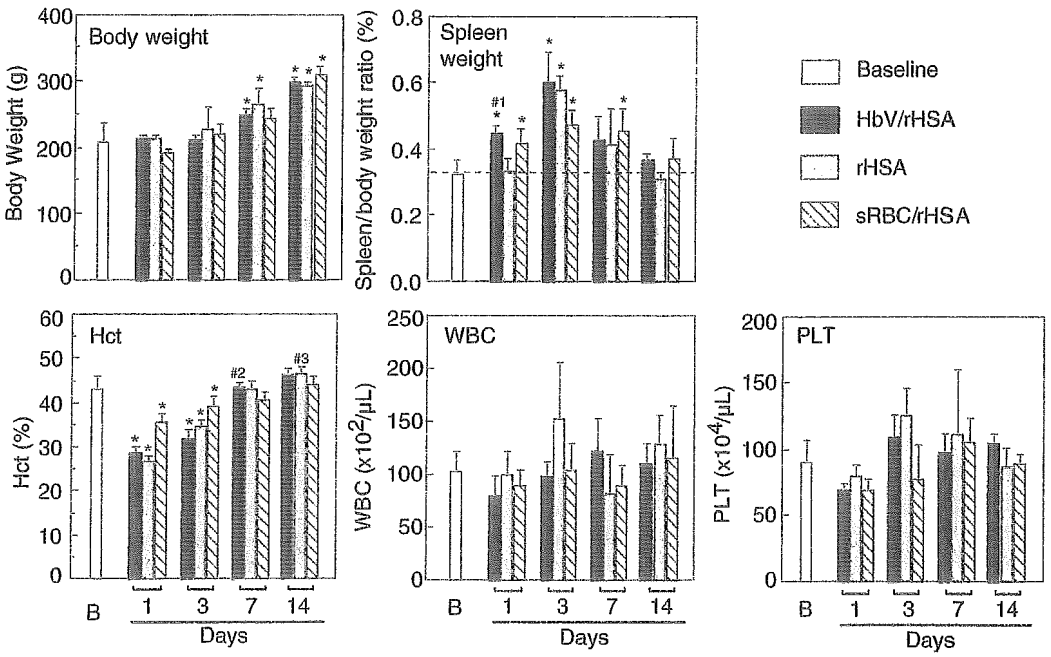

Fig. 1. Changes in body weight, spleen:body weight ratio, and hematological parameters after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. The spleen:body weight ratio (baseline, 0.32 ± 0.04%) increased significantly for the HbV/rHSA group at 1 day (0.45 ± 0.03%) and 3 days (0.60 ± 0.09%). It returned to the baseline at 14 days (0.37 ± 0.02%). The rHSA group also showed significant splenomegaly at 3 days (0.58 ± 0.05%) and returned to 0.31 ± 0.02 percent at 14 days. The sRBC/rHSA group also showed splenomegaly at 1, 3, and 7 days (0.42 ± 0.04, 0.48 ± 0.04, and 0.46 ± 0.06%, respectively). The baseline Hct level was 43 percent; it decreased to about 26 percent for the HbV/rHSA and rHSA groups. At 7 days, they showed complete recovery to approximately 43 percent and then further increased to approximately 46 percent at 14 days. The values are means ± SD. The broken line indicates the baseline value. *Significantly different from the baseline ($p < 0.01$); #1significantly different from the rHSA group ($p < 0.01$); #2$p = 0.0288$ versus sRBC/rHSA; #3$p = 0.0353$ versus sRBC/rHSA. B = baseline.

7 groups (HbV/rHSA, 70 ± 5 U/L; rHSA, 69 ± 12; sRBC/rHSA, 72 ± 9; baseline, 60 ± 7), but it reverted to the original level, whereas alanine aminotransferase was stable. Alkaline phosphatase and γ-glutamyltransferase showed significant or nonsignificant reductions for all groups throughout the experiment. Creatine phosphokinase was stable for 14 days. For all groups, creatinine and uric acid were maintained at low levels for 14 days (data not shown). Amylase showed some significant reduction, but did not change markedly for 14 days (Fig. 3). In contrast, lipase showed significant and marked increases for the HbV/rHSA group for 3 days, but it tended to decrease after 7 days.

Regarding plasma lipid components in the HbV/rHSA group, the total cholesterol and free cholesterol showed significant increases with maximum values at 3 days (Fig. 3). Nevertheless, they returned to their original levels at 7 days. The β-lipoprotein tended to decrease after the exchange transfusion, showing significant reductions at 3 and 7 days for the rHSA group. The high-density lipoprotein cholesterol also tended to decrease with a significant reduction at 3 days for the rHSA group. Triglyceride tended to decrease for all groups with a significant difference in the HbV/rHSA group at 1 and 3 days, partly because of ultracentrifugation of the plasma fractions, and in the rHSA group at 7 days. At 14 days, they generally recovered to the baseline level. The phospholipid tended to decrease with significant differences for all groups. Free fatty acid tended to increase at 14 days. The serum bilirubin (<0.1 mg/dL) remained at a low level throughout the experiment.

In electrolyte concentrations, Fe^{3+} showed significant reductions at 1 and 3 days for the HbV/rHSA group, at 3 and 7 days for the rHSA group, and at 3 days for the sRBC/rHSA group, but they returned to the original level at 14 days (Fig. 3).

Histopathological study

Histopathological examination revealed no significant changes in the lung, heart, and kidney in all groups. At 1 and 3 days after infusion, significant amounts of HbV phagocytized by macrophages in the marrow and Kupffer

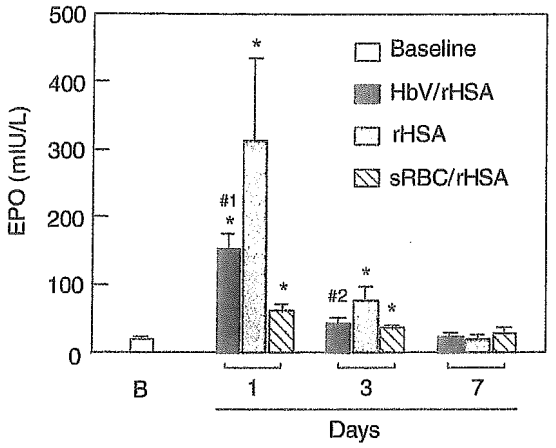

Fig. 2. Plasma EPO activity after 40 percent exchange transfusion with HbV/rHSA or rHSA. All groups showed significant increases at 1 day. However, the HbV/rHSA groups showed a lower level than the rHSA group. The values are means ± SD. *Significantly different from the saline group ($p < 0.01$); #1$p = 0.0222$ versus rHSA; #2$p = 0.0195$ versus rHSA. B = baseline.

cells in the liver were observed. However, HbV decreased significantly at 7 days and was undetectable at 14 days. At 3 days after infusion, the pancreas in the HbV/rHSA group showed no significant morphological changes in spite of the increased lipase activity.

Sections of the spleen of the HbV/rHSA group, which is stained with Giemsa method, revealed the accumulation of HbV particles in the red pulp zone at 1 and 3 days after the exchange transfusion. The amount of the accumulated HbV decreased at 7 days and then became undetectable at 14 days (Fig. 4). Throughout the period examined in this study, nests composed of erythroblasts and proerythroblasts were formed in the splenic cord, especially at 3 and 7 days, indicating extramedullar erythropoiesis. Nest formation was remarkable for the rHSA group at 3 days. Hematopoietic activity was also observed at 3 days in the marrow of the HbV/rHSA group that contained erythroblastic islets.

The Berlin blue method indicated the presence of hemosiderin in macrophages of the spleen in the HbV/rHSA group at 7 days. This hemosiderin deposition increased until 14 days (Fig. 5). A small amount of hemosiderin was confirmed in the Kupffer cells of the liver at 14 days. Hemosiderin deposition, however, was undetected in the marrow. In addition, in the sRBC/rHSA group, hemosiderin deposition was present in the spleen macrophages at 14 days.

DISCUSSION

A main finding of this study is that the reduced Hct level after the 40 percent exchange transfusion with HbV/rHSA returned to the original level after 7 days; furthermore, the accumulated HbVs in RES became undetectable within 14 days. Significant splenomegaly is attributable to the combination of the accumulation of HbV in the red pulp zone and the considerable presence of nests of erythroblasts in the splenic cord in response to the EPO secretion, but these observations subsided within 14 days.

Extensive studies of circulation kinetics and organ distribution of isotope-labeled HbV clarified that HbV accumulates preferentially in the RES.[11,16] One cause of the splenomegaly is the accumulation of HbV particles in the red pulp zone, as shown in Fig. 4. It subsided completely within 14 days, however. Gradual increases in the plasma cholesterol levels by 3 days after infusion and lack of disruption of the HbV in the plasma suggest that the cholesterols are liberated from the RES after the HbVs are captured by the RES and destroyed in the phagosomes of the macrophages.[14,15] In our previous studies of topload HbV infusions, significant increases in the high-density lipoprotein cholesterol, β-lipoprotein, and phospholipids were observed as surplus amounts.[15,17] In contrast, we observed no such significant increases after the 40 percent blood exchange, only decreases. A large demand of nutrients should pertain for hematopoiesis and so on; also, the lipid components from HbV might be utilized efficiently for proliferation.

During the metabolism of Hb, we would expect a release of bilirubin and iron. They did not increase in the plasma within 14 days, however. The released heme from Hb in HbV might be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells of the liver and the spleen macrophages.[15,31] Bilirubin would normally be excreted in the bile as a normal pathway, and no obstruction or stasis of the bile should occur in the biliary tree. Berlin blue staining revealed considerable deposition of hemosiderin in the liver and spleen, even after 14 days. Normally, iron from a heme is stored in the ferritin molecule.[33] Both ferritin and hemosiderin release iron. They are anticipated to induce hydroxyl radical production followed by lipid peroxidation.[34] The iron release rate from hemosiderin, however, is substantially less than that from ferritin.[35] Consequently, the excess amount of iron would then normally be stored in an insoluble and less toxic form as hemosiderin. Hemosiderosis is often observed in patients who have received repeated blood transfusions because of the shorter $t_{1/2}$ of the stored RBCs. Moderate splenomegaly and hemosiderin deposition were also confirmed in the spleen in the sRBS/rHSA groups of this study, partly because of the accumulation and degradation of stored RBCs with the lowered membrane deformability and shortened circulation $t_{1/2}$.[26] These results indicate that the metabolism of heme from HbV and the iron storage is within the physiological capacity that has been well characterized for the metabolism of senescent RBCs.[35]

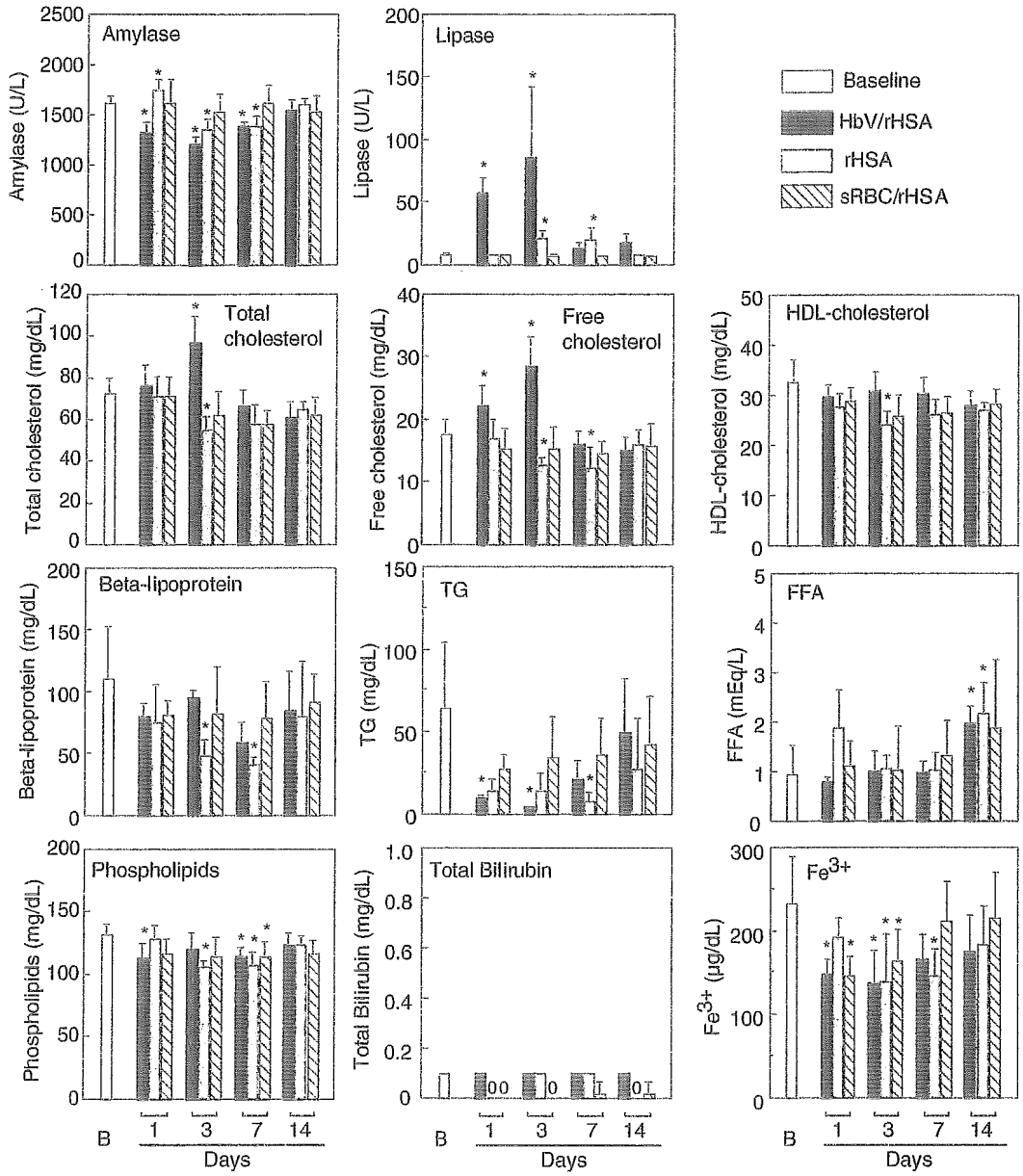

Fig. 3. Plasma biochemical tests representing the metabolism of the components of HbV (lipids and Hb) and pancreatic function after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. The values are means ± SD. *Significantly different from the saline group ($p < 0.01$). TG = triglyceride; FFA = free fatty acid; B = baseline.

Interestingly, not only the HbV/rHSA and sRBC/rHSA groups, but also the rHSA group, showed a significant splenomegaly at 3 days, even though the rHSA group showed no symptoms on Day 1. In rats, extramedullary hematopoiesis induced by hypoxia is localized predominantly in the spleen.[37,38] We observed extensive nests of erythroblasts in the splenic cords, especially at 3 days. It is not plausible that the rHSA as an xenogeneic protein accumulates in the spleen macrophages, according to the fact the ^{125}I-labeled rHSA in a rat showed no specific distribution to the spleen.[39,40] Therefore, the splenomegaly for the rHSA group is attributed to the erythropoiesis stimulated by the significant increase in the plasma EPO level.

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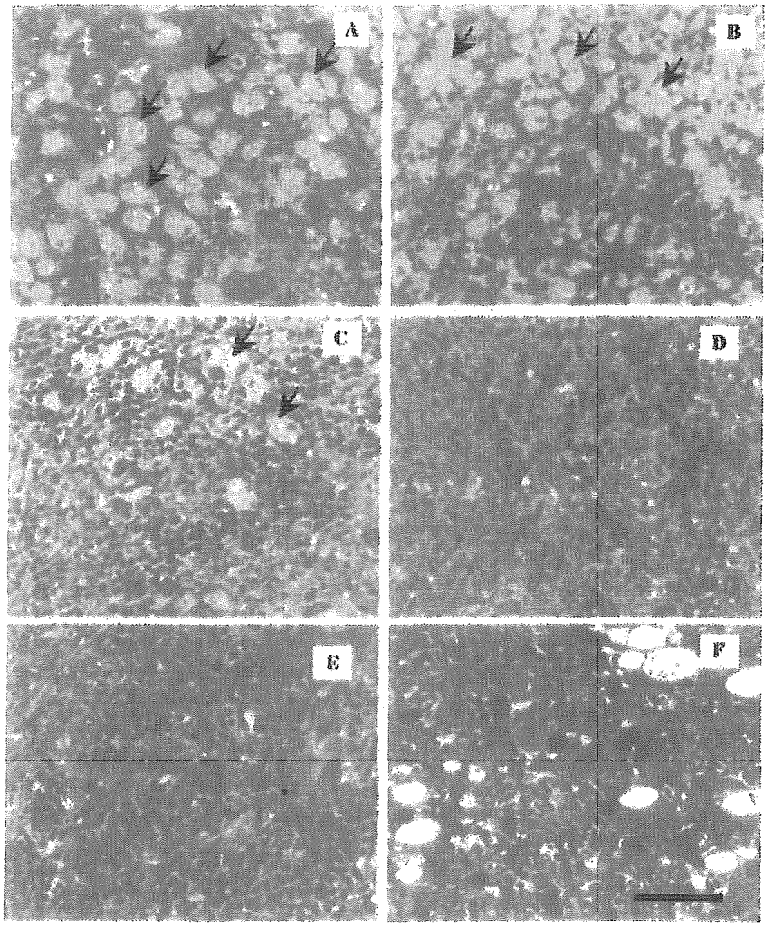

Fig. 4. Histology of rat spleen and marrow after exchange transfusion with HbV/rHSA or rHSA alone. (A-C) Respective images of the spleen of the HbV/rHSA group at 1, 3, and 7 days. Accumulated HbV particles are visible as light-blue areas (black arrows). Nests of erythroblasts are visible as dark blue cells (red arrows). The domain of the HbV particles decreased significantly at 7 days. (D) Spleen of the HbV/rHSA group at 14 days. HbV particles had disappeared, whereas the erythroblast nests remained, as indicated by the red arrows. (E) Spleen of the rHSA group at 3 days. The erythroblast nest formation is remarkable. (F) Marrow of the HbV/rHSA group at 3 days. Hematopoietic activity is visible. Bar = 50 μm (Giemsa method).

Plasma EPO release from the kidney strongly reflects an anemic condition, depending on the O_2-carrying capacity of the circulating blood.[41,42] The highest EPO level was seen in the rHSA group, indicating that its anemic condition was the severest. Because of the short $t_{1/2}$ and MetHb formation,[43] the HbV/rHSA also showed a significant increase in the EPO level. It was considerably lower than that of the rHSA group, however. The sRBC/rHSA group also showed a moderate increase in the EPO level probably caused by the reduced Hct by the exchange transfusion. Accordingly, the splenomegaly for the HbV/rHSA and sRBC/rHSA groups is also partly attributable to the nests of erythroblasts for erythropoiesis that was sufficient for recovery from the reduced Hct. Interestingly, both HbV/rHSA and rHSA groups tended to show higher Hct values than the sRBC/rHSA group at 7 and 14 days, probably because of the enhanced erythropoiesis caused by the higher levels of EPO excretions than for the sRBC/rHSA group. The MCH, MCV, and MCHC levels were normal overall, supporting our inference of normal erythropoiesis.

Routine plasma biochemical tests showed that the hepatic function was maintained despite the large amount of HbV that were captured and degraded by Kupffer cells. Significant reductions were seen in the amylase activity, whereas a transient increase in lipase activity was observed consistently in our previous topload infusion experiments; this should be due to the up regulation of lipase in response to the infusion of phospholipid vesicles.[15,17,44]

In conclusion, all rats tolerated the 40 percent exchange transfusion with HbV/rHSA and showed complete Hct recovery within 7 days. Although transient splenomegaly and the hemosiderin deposition were confirmed, no excess iron was found in the blood. The recycling or excretion of iron as well as lipid components should be on the physiological pathway that is known for the degradation of senescent RBCs. Although some aspects remain unresolved, the present results offer important information on the safety and handling of HbV during preoperational or perioperational infusion in a clinical setting.

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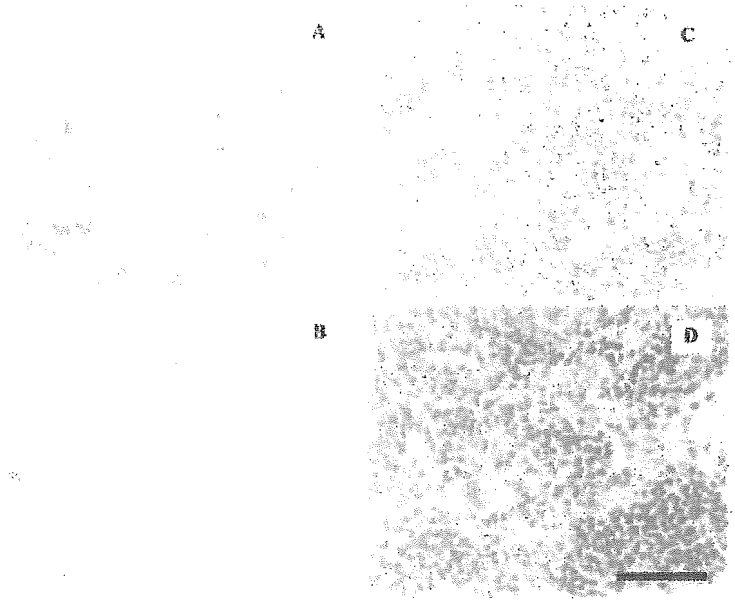

Fig. 5. Histology of rat spleen, liver, and marrow 14 days after exchange transfusion with HbV/rHSA or sRBC/rHSA. Spleen (A), liver (B), and marrow (C) of the HbV/rHSA group. The spleen and liver contained slight hemosiderin deposition, but not the marrow. The spleen of the sRBC/rHSA group (D) also contained slight hemosiderin deposition. Bar = 50 μm (Berlin blue method).

hematopoietic activities. The rHSA was obtained from Nipro Corp.

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

Animal and flap preparation. A hamster skin flap model was used as previously described in detail (7, 8, 10–12). Anesthesia was induced by pentobarbital sodium (Nembutal) injected intraperitoneally (100 mg/kg body wt; Abbott Laboratories, Chicago, IL). The carotid artery and external jugular vein were cannulated for administration of anesthesia, blood exchange, laboratory analysis, and monitoring arterial blood pressure (Type514; Spacelabs, Hillsboro, OR). Catheterization and flap dissection were performed with the aid of an operating microscope at ×10 magnification (Wild; Heerbrugg, Switzerland). An island flap measuring 3 × 2 cm was dissected from the shaved and epilated back skin of the animal. The flap consisted of skin and a thin layer of panniculus carnosus muscle, and it was perfused by one vascular axis, which bifurcates into two equal-sized branches within the flap, each of them supplying a separate vascular territory. One of the branches was transected after being secured with microsurgical ligatures, thus rendering the corresponding vascular territory ischemic. This tissue was merely perfused by a collateral vasculature connecting the two vascular networks. During surgery, the flap was irrigated with 0.9% NaCl solution to prevent the flap from drying out. The animal was placed on a specially designed Plexiglas stage including a platform for fixation of the flap. During surgery, 4 mg papaverine hydrochloride (Sigma Chemical, St. Louis, MO) dissolved in 1 ml physiological saline solution were applied to the pedicle by a soaked cotton tip to prevent vascular spasm.

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

Laboratory analysis. Blood samples were collected in 40-μl heparin-washed microtubes for measurement of total Hb concentration and arterial blood gases with the use of the Radiometer ABL 625 system (Radiometer; Copenhagen, Denmark). By validating this system, we have found that the vesicle-bound hemoglobin concentration may be overestimated by maximally 10%, whereas the results were not affected by the lipid concentrations present in our study. Hematocrit was determined by centrifugation. The colloid osmotic pressure of the diluents was measured with a colloid osmometer (model 4420; Wescor, Logan, UT) with a 30-kDa cutoff membrane. The viscosity was measured with a cone-plate viscometer (PVII+; Brookfield Engineering, Middleboro, MA) or a capillary rheometer (Anton Parr DCS 300; Parr Physica, Graz, Austria) at 37°C. Viscosities of blood and plasma were measured 4 h after hemodilution with a Höppler-type viscosimeter (HAAKE Messtechnik, Karlsruhe, Germany). The physicochemical characteristics of the solutions are summarized in Table 1. Oxygen content (ml/dl) in the carotid artery was calculated according to the equation

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

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

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

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

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

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

Tissue oxygen tension. Partial tissue oxygen tension was assessed with combined bare fiber probes with a tip diameter of 450 μm (Oxylite probes; Oxford Optronix, Oxford, UK). The sensitive tip of the oxygen probe (100-μm diameter) consists of ruthenium-III-(Tris)-chloride, which measures P_{O_2} by fluorescence quenching of the dye. A T-type thermocouple was attached to the probe, which was coated with a biocompatible sleeve of polyurethane. According to the manufacturer, the bare fiber probe provides resolutions of <1 mmHg and 0.1°C for partial oxygen tension and temperature, respectively, and the sampling area of the oxygen sensors is 0.25–0.35 mm^3. The probes were inserted into the subcutaneous tissue in the middle of each vascular territory under visual microscopic control. Care was taken to place the probes in such a way that no arterioles or large venules lay within the sampling area.

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

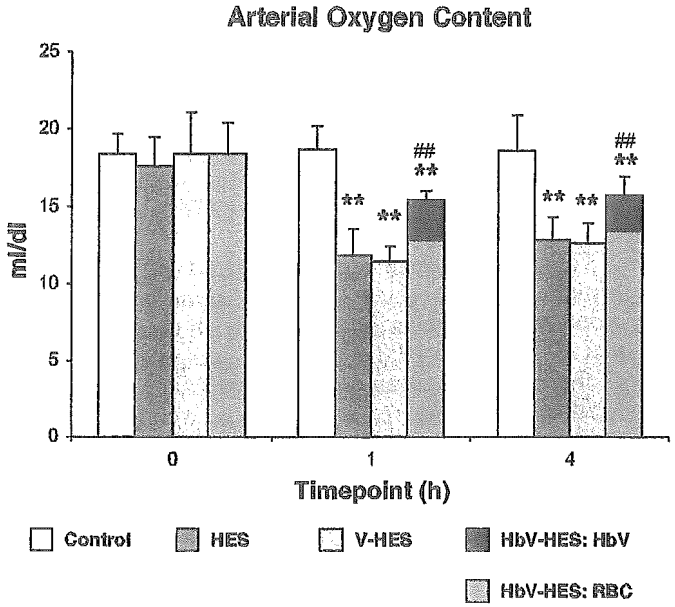

Fig. 1. Oxygen content in carotid artery at baseline and 1 and 4 h after hemodilution with 6% hydroxyethyl starch (HES) and vesicles with (HbV-HES) and without (V-HES) Hb suspended in HES, including relative contribution of red blood cells (RBCs) and HbV. Data are given as percentages of baseline and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

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

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

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

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

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

DISCUSSION

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

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

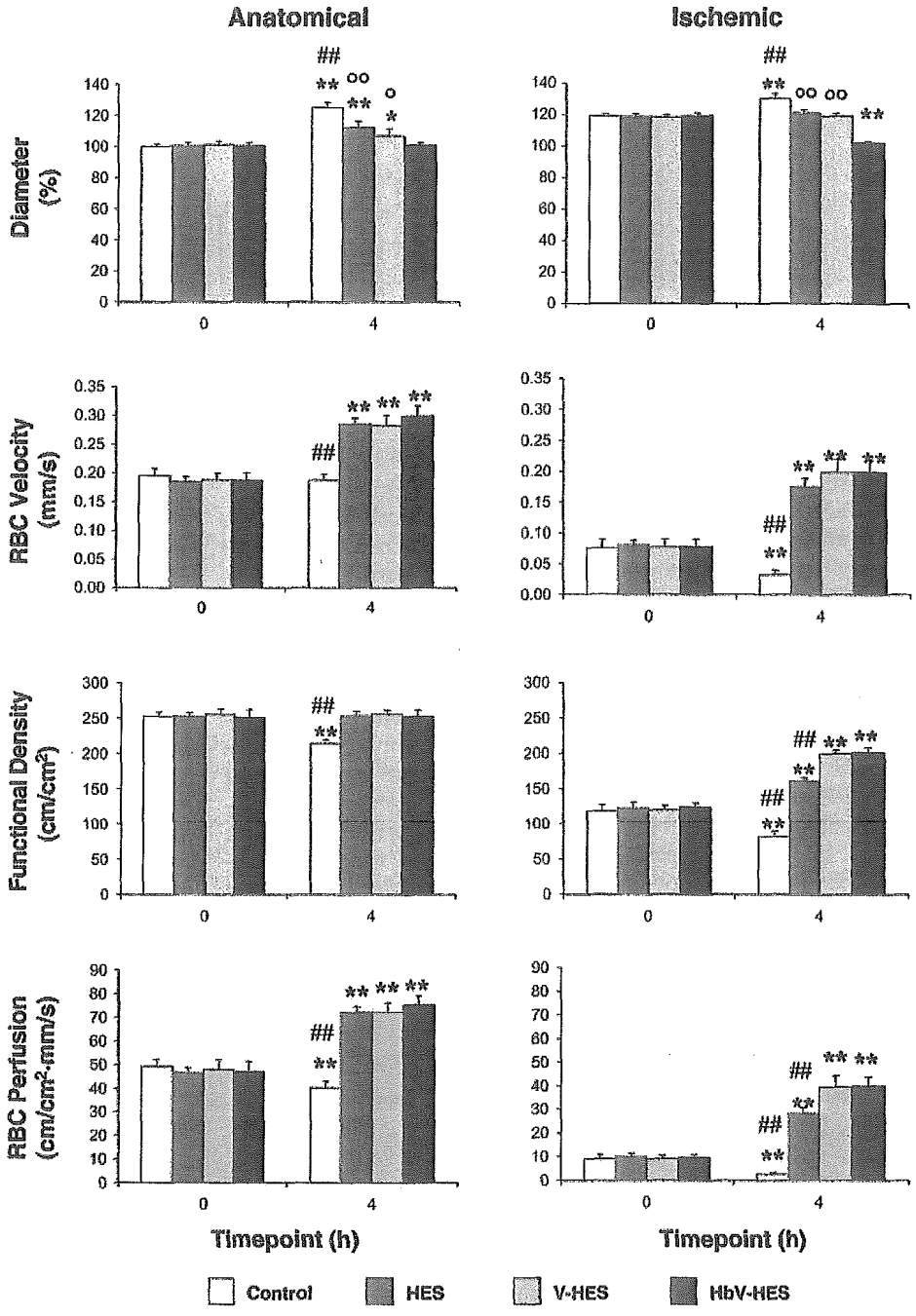

Fig. 2. Capillary hemodynamics in anatomically perfused and ischemic tissues at baseline and 4 h after hemodilution with 6% HES, HbV-HES, and V-HES. Data represent means ± SD. Values for capillary diameter were expressed in percentages of mean in anatomically perfused tissue of control animals at baseline. $*P < 0.05$, $**P < 0.01$ vs. baseline; $\#\#P < 0.01$ vs. other groups; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ vs. HbV-HES.

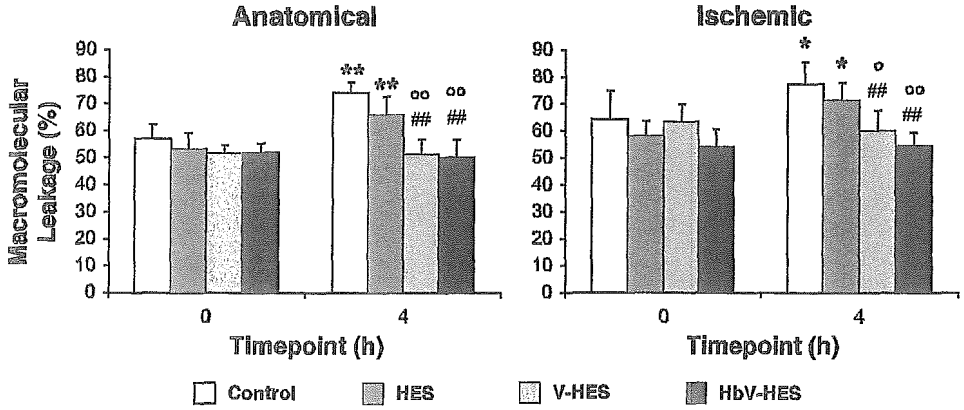

Fig. 3. Macromolecular leakage in anatomically perfused and ischemic tissues at baseline and 4 h after hemodilution with 6% HES, HbV-HES, and V-HES. Data represent means ± SD. $*P < 0.05$, $**P < 0.01$ vs. baseline; $\#\#P < 0.01$ vs. control; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ vs. HES.

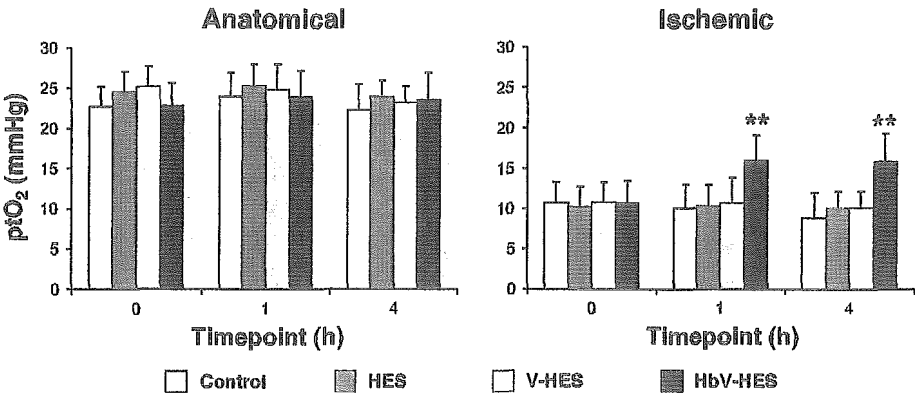

Fig. 4. Partial tissue oxygen tension (P_tO_2) in anatomically perfused and ischemic tissues at baseline and 1 and 4 h after hemodilution with 6% hydroxyethyl starch (HES), HbV-HES, and V-HES. Data represent means ± SD. $^{**}P < 0.01$ vs. baseline and other groups.

viscosity. A dependency of FCD on plasma viscosity has been described for conditions of severe hemodilution (2, 3, 33), which has been ascribed to shear stress-induced, nitric oxide-mediated arteriolar vasodilation being required to maintain capillary pressurization (2, 3, 9). However, during the moderate hemodilution applied in the present study, no such arteriolar vasodilation could be observed, which calls for alternative explanations not only for the behavior of FCD but also of capillary RBC velocity and perfusion.

One interpretation may be found in the changes in macromolecular leakage. This parameter allows for a quantitative assessment of capillary leakage, which is an early sign of inflammation appearing in the course of compromised microcirculation such as that due to trauma (31), hemorrhagic shock (5), or ischemia-reperfusion injury (18), and which is paralleled by the activation of the leukocyte-endothelium interaction particularly in the postcapillary venules. Leukocyte adherence, being an early step in this cascade of events, may augment resistance in this vascular segment considerably and thus impair capillary hemodynamics in critically perfused tissues (19). Compared with both the control group and the HES group, macromolecular leakage was significantly reduced in the animals receiving vesicles. Therefore, it may be postulated that the beneficial effect of the vesicles on the capillary hemodynamics was related to a reduction of postcapillary resistance in terms of blunting leukocyte adherence. The capability of leukocytes to adhere to the endothelial wall may be diminished by increasing shear stress (21), which is proportional to linear flow velocity and viscosity of the plasma and inversely proportional to vascular diameter. Provided that our data on plasma viscosity and capillary hemodynamics may be extrapolated to the conditions in the ischemic postcapillary

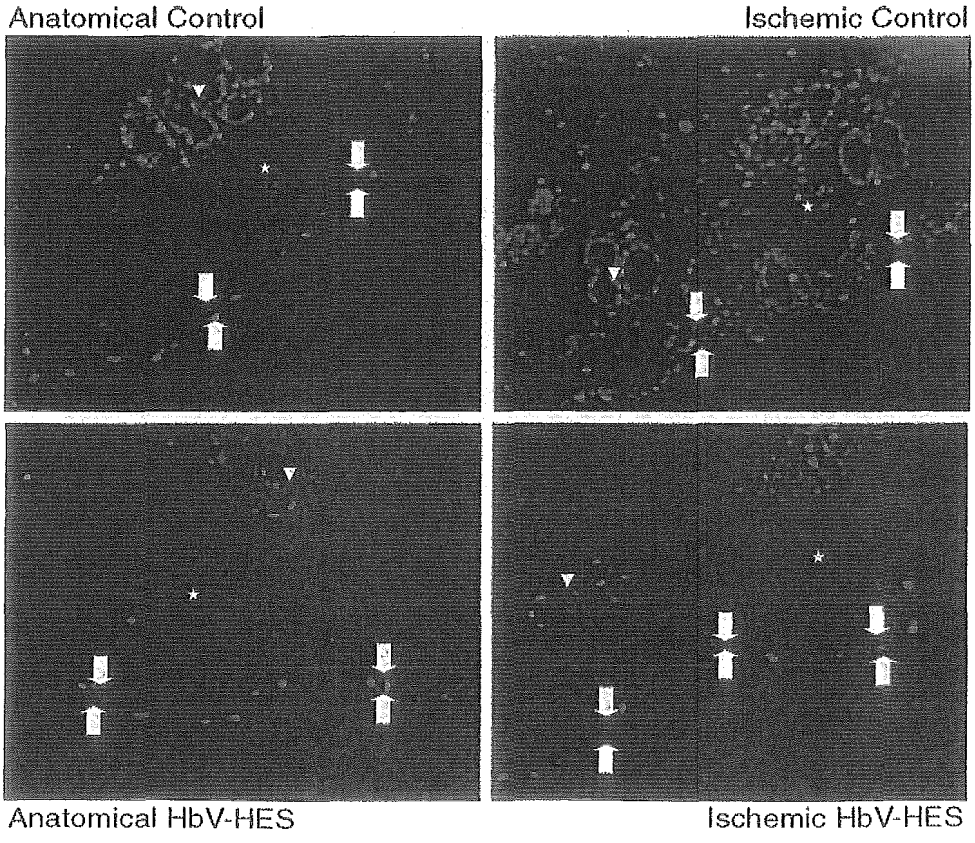

Fig. 5. Transferase-mediated dUTP nick-end labeling (TUNEL) assay of apoptotic cells in anatomically perfused and ischemic tissues 5 h after completion of surgery and 4 h after hemodilution with HbV-HES. Note massive accumulation of red-labeled apoptotic cells in both dermis (☆) and epidermis (arrows) of ischemic tissue and how apoptosis was reduced after hemodilution with HbV-HES. Hair follicles and sebaceous glands are shown (arrowheads).

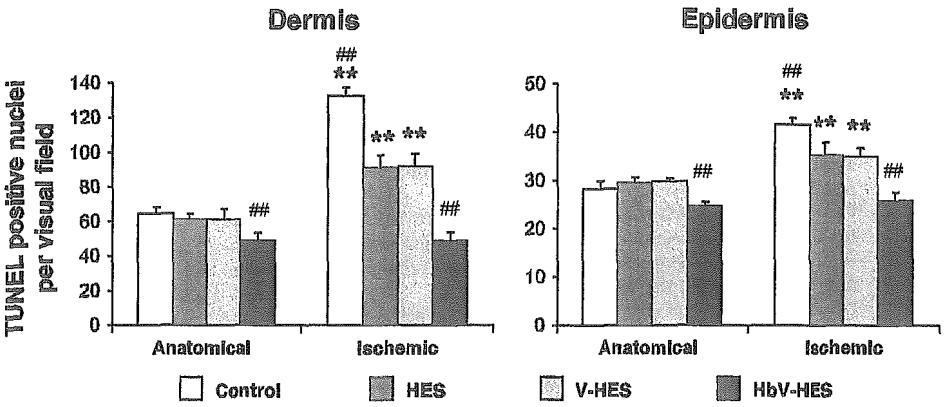

Fig. 6. Density of apoptotic cells in dermis and epidermis of anatomically perfused and ischemic tissues 5 h after completion of surgery and 4 h after hemodilution with 6% HES, HbV-HES, and V-HES. Data represent means ± SD. $^{**}P < 0.01$ vs. anatomically perfused tissue; $^{\#\#}P < 0.01$ vs. other groups.

venules, hemodilution with the vesicle solutions would result in a significant shear stress increase in these vessels compared with baseline and HES, respectively. This mechanism may be of a particular importance in case of ischemia-reperfusion injury after reoxygenation of critically ischemic tissue (16), which may, at least partly, have taken place in the animals receiving HbVs, as evidenced by the improved partial tissue oxygen tension.

In the present preparation, macromolecular leakage appeared to be primarily related to the traumatization of the tissue as a consequence of its surgical manipulation (7), because similar values were obtained in both parts of the flap. However, it is conceivable that the ischemic tissue is more susceptible to changes in postcapillary resistance because of the diminished driving pressure in the collateralized arterioles that are nourished by connecting arterioles, in which perfusion pressures below 30 mmHg were measured, compared with ~45 mmHg in the arterioles feeding the anatomically perfused vasculature (3, 10). With regard to the postulated effect of the vesicles on the postcapillary resistance, this would explain why the vesicle-related improvement of capillary hemodynamics was restricted to the ischemic tissue. Moreover, the vesicle-related increase in capillary perfusion coincided with a decrease in capillary diameters. Given the assumption that the perfusion increase was caused by a reduction of upstream vascular resistance, this would have led to capillary dilation as a result of increased intraluminal capillary pressure (3), whereas intraluminal capillary pressure decreases if vascular resistance is diminished on the postcapillary level. Therefore, the inversely proportional behavior of capillary diameter and perfusion further supports our assumption that the microhemodynamic benefit obtained with the vesicle solutions was predominantly due to its reduction of postcapillary resistance.

Although all capillary hemodynamic parameters in the ischemic tissue were restored to values close to baseline in the anatomically perfused tissue in the V-HES group, this was not sufficient to attenuate hypoxia or hypoxia-induced apoptosis. This suggests that in this group, oxygen delivery to the ischemic tissue is reduced because of a lack of oxygen content in its collateralized, arteriolar inflow, a condition that was presumably circumvented by the presence of Hb in the vesicles because of various reasons. First, the HbVs contribute to a total Hb increase, thus resulting in an enhanced oxygen-carrying capacity not only in terms of arterial oxygen content but also in terms of additional capillary, HbV-related oxygen flow that is not included in the index used to express capillary perfusion in the present study. Second, the high oxygen affinity of the HbVs may have attenuated the unloading of oxygen in the upstream vasculature before reaching the collateralized arterioles, which has been estimated to be as much as 40–50% of the systemic arterial oxygen content (11, 30). This hypothesis is supported by both experimental (15, 25, 30) and theoretical (34) studies, which showed that oxygen delivery may be shifted to the downstream direction if oxygen carriers with high oxygen affinity were infused. Third, because of their small size, HbVs may perfuse capillaries in the compromised microcirculation that are no longer accessible by RBCs. Indeed, HbVs were observed in capillaries showing a cessation of RBC flux (29), which would virtually enhance the density of functional capillaries. Moreover, the occurrence of apoptosis leads to a reduction of oxygen consumption, thus raising partial tissue oxygen tension, provided oxygen delivery remains unchanged. Therefore, the partial tissue oxygen tension increase observed after HbV-HES may underestimate the improvement in oxygen delivery in comparison with the other groups.

In summary, on the basis of the unique constellation in which a HbV solution was compared with a nonoxygen-carrying vesicle solution with identical physicochemical properties, we conclude that the presence of Hb in the vesicles is necessary to obtain an essential improvement of oxygenation and survival in the critically ischemic flap tissue. However, the benefit may, to a certain extent, be ascribed to the rheological changes provided by the vesicles, presumably by reducing postcapillary vascular resistance.

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

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

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

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

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

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

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

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

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MATERIALS AND METHODS

Preparation of HbVs. HbVs with different P_{50}s were prepared under sterile conditions as previously reported (32, 34, 37). Hb was purified from outdated donated human blood provided by the Japanese Red Cross Society (Tokyo, Japan). HbVs with a P_{50} = 29 mmHg (HbV_{29}) was prepared by adding the allosteric effector pyridoxal 5′-phosphate (PLP; 14.7 mM, Sigma Chemical; St. Louis, MO) to Hb (38 g/dl) at a molar ratio of PLP to Hb = 2.5. HbVs with a P_{50} = 8 mmHg (HbV_8) were prepared by adding no allosteric effector to the Hb solution. The Hb solution was encapsulated within vesicles composed of Presome PPG-I [a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-di-*O*-octadecyl-*N*-succinyl-L-glutamate at a molar ratio of 5:5:1 (Nippon Fine Chemicals; Osaka, Japan)], and the particle size of HbVs was regulated by an extrusion method. The surface of the HbVs was modified with polyethylene glycol (molecular mass: 5 kDa, 0.3 mol% of the lipids in the outer surface of vesicles) using 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-polyethylene glycol (Sunbright DSPE-50H, H-form, NOF; Tokyo, Japan). HbVs were suspended in a physiological salt solution and sterilized with filters (Dismic, Toyo Roshi; Tokyo, Japan; pore size: 0.45 μm) and deoxygenated with N_2 bubbling for storage. The endotoxin content was measured with a modified Limulus amebocyte lysate assay, and the level was less than 0.2 EU/ml (27). The O_2 equilibrium curves (OECs) of HbV_{29} and HbV_8 were obtained by a Hemox Analyzer (TCS-Medical Products; Philadelphia, PA), as shown in Fig. 1. The physicochemical parameters of the HbVs are listed in Table 1.

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

Polyethylene (PE) tubes (PE-10, Becton-Dickinson; Parsippany, NJ; ~1 cm) were connected to PE-50 tubing (~25 cm) via silicone elastomer medical tubes (~4 cm, Technical Products; Decatur, GA) and were implanted in the jugular vein and the carotid artery. They were passed from the ventral to the dorsal side of the neck and exteriorized through the skin at the base of the chamber. Patency of the catheters was ensured by filling them with heparinized saline (40 IU/ml). Microvascular observations of the awake and unanesthetized hamsters were performed 5 days after chamber implantation to mitigate the effects of surgery. The hamster was placed in a perforated plastic tube from which the window chamber protruded to minimize animal movement without impeding respiration. All animal studies were approved by the Animal Care and Use Committee of University of California-San Diego and performed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Washington, DC: National Academy Press, 1996).

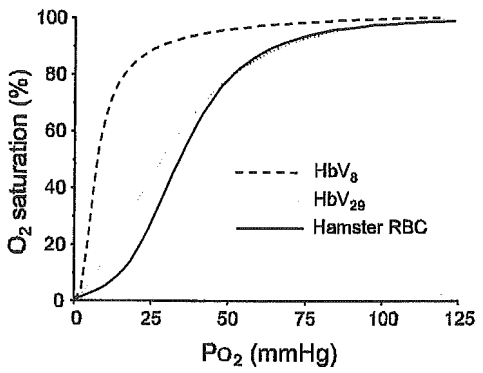

Fig. 1. Oxygen equilibrium curves (OECs) of Hb vesicles (HbVs) at a Po_2 where Hb is half-saturated (P_{50}) of 8 mmHg (HbV_8) and 29 mmHg (HbV_{29}) measured with a Hemox Analyzer (TCS Medical Products) at 37°C compared with hamster blood. RBC, red blood cells.

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

Parameters	HbV_8	HbV_{29}	Hamster Blood
Hb concentration, g/dl	10	10	14.8 ± 0.5
Particle diameter, nm	250 ± 64	247 ± 44	5,000–7,000*
P_{50}, mmHg	8	29	28
Molar ratio of PLP to Hb	0	2.5	
MetHb, %	<3	<1	
HbCO, %	<2	<2	

HbV_8 and HbV_{29}, Hb vesicles (HbVs) at 8- and 29-mmHg Po_2 at which Hb is 50% saturated (P_{50}); PLP, pyridoxal 5′-phosphate. *Size of hamster red blood cells (RBCs) (39).

Infusion of HbV_8 and HbV_{29} and occlusion of an arteriole. The unanesthetized animal was placed in a perforated plastic tube and stabilized under the microscope. Animals were suitable for the experiments if systemic variables were within normal range, namely, heart rate >340 beats/min, mean arterial pressure >80 mmHg, systemic hematocrit >45%, and arterial Po_2 >50 mmHg, and microscopic examination of the tissue in the chamber did not reveal signs of edema or bleeding. Baseline measurements of microvascular parameters and Po_2 (see below) were performed before the infusion of HbV_8 or HbV_{29} suspended in physiological saline solution into the venous line at 7 ml/kg. Systemic blood volume was estimated as 70 ml/kg. In our previous reports of resuscitation from hemorrhagic shock or hemodilution, HbVs were suspended in an albumin solution to regulate colloid osmotic pressure (30, 33). However, in the present study, we did not use albumin to minimize the hypervolemic effect. For the same reason, the infusion amount was minimized to equal 10% blood volume (7 ml/kg).

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

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

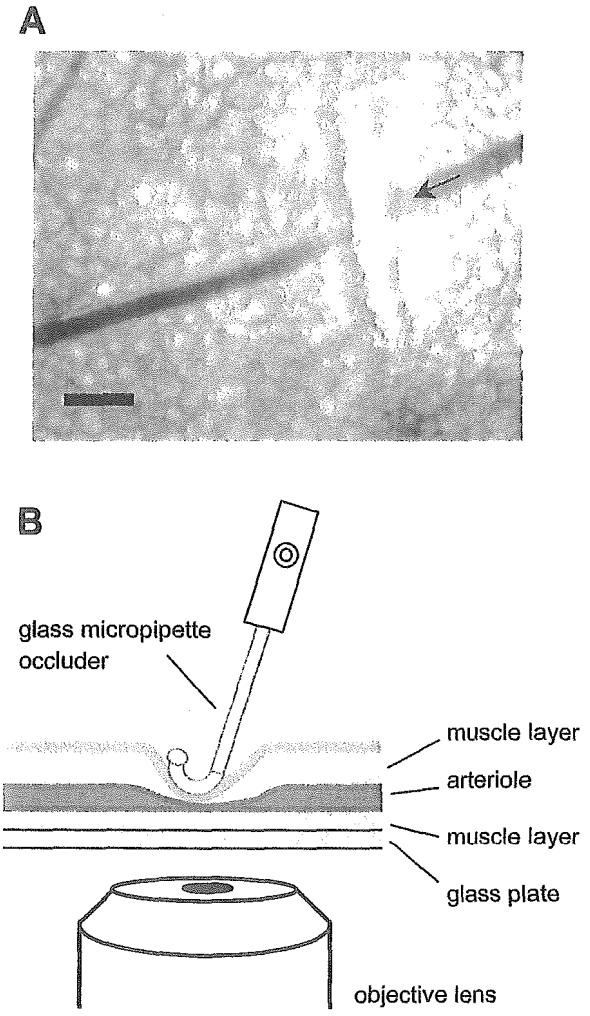

Fig. 2. *A*: microscopic image of an occluded arteriole in the hamster window chamber. The glass fiber lies across the arteriole. Scale bar = 100 μm. *B*: schematic representation of occlusion of *A* showing the different tissue layers of the skin (not to scale).

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

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

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

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

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

where τ_o and τ are the phosphorescence lifetimes in the absence of molecular O_2 and at a given P_{O_2}, respectively, and k_q is the quenching constant, with both factors being pH and temperature dependent. Light was gathered from an optical window of 20 × 5 μm placed longitudinally along the blood vessels. Measurements in the blood compartment were made every second using a single flash.

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

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

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

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

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

RESULTS

Hemodynamic properties of arterioles. The profiles of the selected arterioles, diameters, centerline RBC velocities, blood flow rates, and intra-arteriolar P_{O_2} values before and after infusion of HbVs are listed in Table 2. There was no significant difference between the groups. The O_2 content in blood attributed to hamster RBCs and physically dissolved O_2 at the observed arteriolar P_{O_2} was estimated as 18.61 ± 1.23 ml O_2/dl blood according to *Eq. 3*. After the infusion of HbV_8 and HbV_{29}, the O_2 content increased to 20.30 ± 1.18 and 20.17 ± 1.54 ml O_2/dl blood, respectively, due to the O_2 bound to HbVs. The contributions of HbV_8 and HbV_{29} to whole O_2 content were 1.51 ± 0.01 and 1.25 ± 0.07 ml O_2/dl blood, respectively. The HbV_8 group showed higher O_2 content than the HbV_{29} group due to the higher $Sa_{O_2}(HbV_8)$, which was 95.9 ± 0.6% compared with the $Sa_{O_2}(HbV_{29})$ of 79.6 ± 4.7%.

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