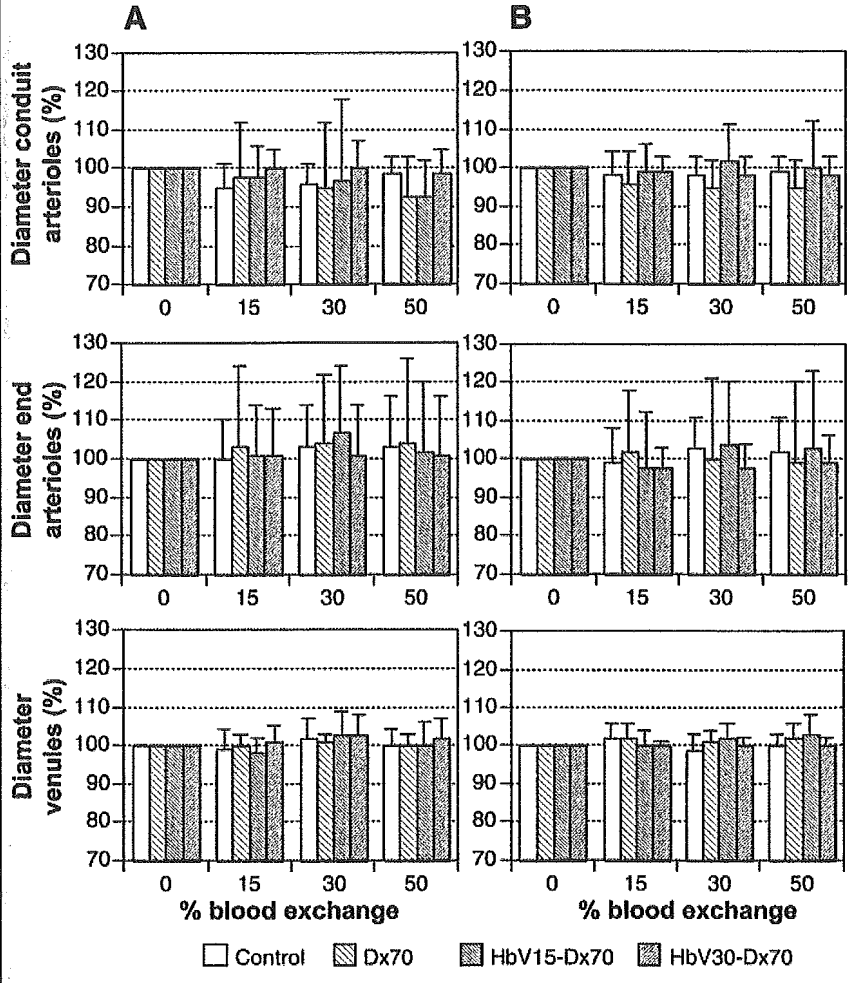

Fig. 1. Microvascular diameters in the anatomically perfused (*A*) and ischemic (*B*) tissues at baseline and after stepwise exchange of 15%, 30%, and 50% total blood volume with 6% Dextran 70 (Dx70) and Hb vesicles (HbVs) with a P_{50} of 15 mmHg (HbV15) and 30 mmHg (HbV30) suspended in Dx70. Data are given as a percentage of baseline and represent means ± SD.

lutions are summarized in Table 1. Oncotic pressure and viscosity were measured with a colloid osmometer (model 4420, Wescor; Logan, UT) and a cone-plate viscometer (PVII+, Brookfield Engineering; Middleboro, MA), respectively (30).

Protocol. The animals were kept under light anesthesia with a continuous infusion of 50 mg/ml pentobarbital given at a rate of ~0.5 mg·min^{-1}·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). A constant temperature in the animal and flap preparation was maintained by means of a heating pad and by keeping the room temperature at 28°C. Normovolemic hemodilution was achieved by simultanous replacement of withdrawn blood over 10 min. Hemodilution was performed in three steps at an interval of 1 h, thus reaching levels of 15%, 30%, and 50% of blood exchange. Measurements were taken at the end of each interval.

Inclusion criteria were a normal vascular anatomy, sufficient optical clarity of the preparation, and a mean arterial pressure above 60 mmHg.

The animals were euthanized 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 utilized for statistical analysis. If the assumption of a normal distribution was appropriate, data were presented as means ± SD; otherwise, they represented median and 25th and 75th percentiles. For normally distributed data, the time-related differences between repeat measurements and the differences between the groups were assessed by the paired and unpaired ANOVA, respectively, whereas the nonparametric Friedman and Kruskal-Wallis tests were used for not normally distributed data analysis. All tests were followed by the Bonferroni post test. A value of $P < 0.05$ was taken to represent statistical significance.

RESULTS

Five animals (1 control, 1 Dx70, 1 HbV15-Dx70, and 2 HbV30-Dx70) did not fulfill the inclusion criteria and were excluded from this study.

The systemic data are summarized in Table 2. Similar hematocrits were obtained in all hemodiluted animals. After the 50% blood exchange, hemodilution with Dx70 resulted in a mean total Hb concentration of 6.5 g/dl, whereas the addition of HbV to the diluents enhanced the total Hb concentration to 8.7 and 8.0 g/dl, respectively ($P < 0.05$). Arterial P_{O_2} was gradually raised after each step of hemodilution, reaching 71 mmHg in the Dx70 group ($P < 0.01$ vs. baseline) and 53 and 52 mmHg for HbV15-Dx70 [not significant (NS)] and HbV30-Dx70 ($P < 0.05$), respectively (both $P < 0.05$ vs. Dx70). Furthermore, the blood exchange was followed by gradual reductions of arterial P_{CO_2} ($P <$

0.01 for Dx70; NS for the HbV groups) and increases in pH (NS).

At baseline, the microhemodynamic data were similar in all groups. The diameters and centerline velocities for conduit arterioles, end arterioles, and venules in each part of the flap are summarized in Table 3. Mean centerline velocities were significantly reduced in the ischemic vessels compared with the anatomically perfused vessels ($P < 0.01$).

The behavior of the microvascular diameters in both parts of the flap are shown in Fig. 1. A slight vasoconstriction was observed in the conduit arterioles after hemodilution with Dx70 (93 ± 10% in the anatomically perfused tissue and 95 ± 7% in the ischemic tissue, both NS) and HbV15-Dx70 (93 ± 9% anatomically perfused, NS), whereas the diameters remained virtually stable in the other microvessels in all groups.

Microvascular blood flow did not show any relevant changes in the anatomically perfused vessels (Fig. 2), whereas it was significantly increased in all vessels in the ischemic tissue due to hemodilution ($P < 0.05$ for Dx70 and HbV15-Dx70; $P < 0.01$ for HbV30-Dx70). The highest values were obtained with the 30% and 50% blood exchanges, reaching 144% (108–160%) for Dx70, 166% (152–192%) for HbV15-Dx70, and 141% (118–165%) for HbV30-Dx70.

Mean P_{O_2} ranged between 23.0 ± 4.3 and 26.7 ± 2.2 mmHg in the anatomically perfused tissue. Tissue P_{O_2} was significantly reduced in the ischemic part, where the values were between 7.7 ± 3.2 and 10.9 ± 3.4 mmHg ($P < 0.01$). In the anatomically perfused tissue, oxygenation was virtually not influenced by hemodilution with or without HbVs (Fig. 3). In the ischemic tissue, a transient improvement was observed during hemodilution with Dx70. The maximum was obtained after the 30% exchange (121 ± 17% of baseline, $P < 0.05$). Each step of blood exchange caused an increase in ischemic tissue oxygenation if the diluents contained HbV. Tissue P_{O_2} was enhanced up to 217 ± 67% and 164 ± 33% for HbV15-Dx70 and HbV30-Dx70, respectively (both $P < 0.01$ vs. baseline and other groups).

DISCUSSION

The principal findings of this study were that *1)* oxygenation in the ischemic, collateralized, and hypoxic flap tissue was raised with each step of hemodilution with both HbV solutions and that higher values

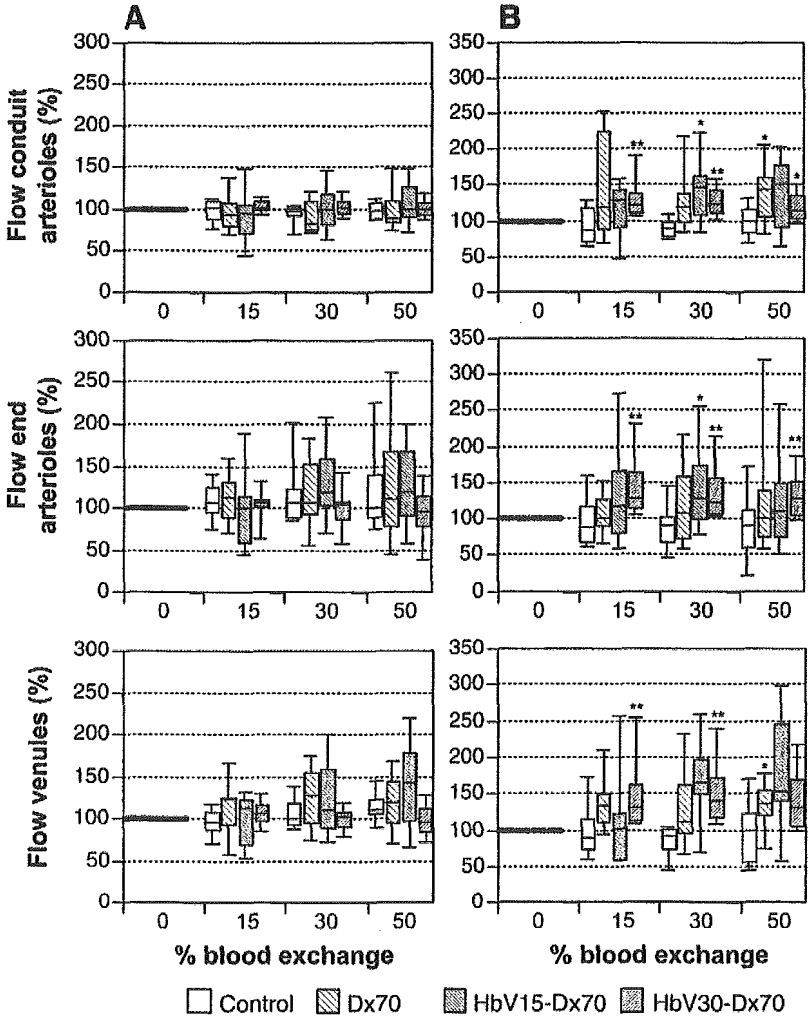

Fig. 2. Microvascular blood flow in the anatomically perfused (*A*) and ischemic (*B*) tissues at baseline and after stepwise exchange of 15%, 30%, and 50% total blood volume with 6% Dx70, HbV15-Dx70, and HbV30-Dx70. Data are given as a percentage of baseline and are presented in box plots reflecting 10th percentile, 25th percentile, median, 75th percentile, and 90th percentile. *$P < 0.05$ and **$P < 0.01$ vs. baseline.

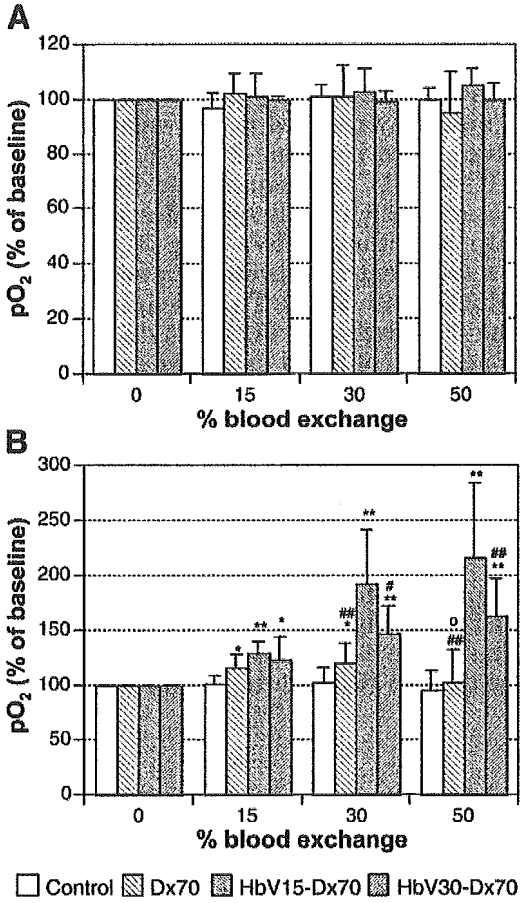

Fig. 3. P_{O_2} in the anatomically perfused (*A*) and ischemic (*B*) tissues at baseline and after stepwise exchange of 15%, 30%, and 50% total blood volume with 6% Dx70, HbV15-Dx70, and HbV30-Dx70. Data are given as a percentage of baseline and represent means ± SD. *$P < 0.05$ and **$P < 0.01$ vs. baseline; #$P < 0.05$ and ## $P < 0.01$ vs. HbV15-Dx70; °$P < 0.01$ vs. HbV30-Dx70.

were obtained *2*) by increasing the oxygen affinity of HbV and *3*) with HbV-containing solutions compared with Dx70 only.

Normovolemic hemodilution with Dx70 transiently improved the oxygenation in the ischemic tissue, reaching a peak of 121% of baseline at the 30% blood exchange. This level of hemodilution is considered to provide the highest RBC flux at the capillary level (15), thus resulting in maximal oxygen transport capacity both systemically (28) and in locally ischemic tissue (25). Furthermore, arterial P_{O_2} was increased at this level of hemodilution due to hyperventilation. As we (5) demonstrated in previous experiments in the same model, increased arterial P_{O_2} values were transferred as far as the collateral arterioles, which is where the blood circulation enters the ischemic tissue. In the present study, however, the improvement of oxygenation in the ischemic tissue was by far higher if HbV was added to the Dx70 solution, although microvascular blood flow, Hb concentration, and arterial P_{O_2} were similar or lower. Moreover, the oxygenation was enhanced despite a simultaneous decrease in total Hb concentration due to hemodilution with the HbV-Dx70 solutions. These results suggest that under the given conditions, the presence of HbVs increases the capacity of blood to deliver oxygen to the ischemic tissue and that the effect is related to the level of blood exchange with HbV solutions.

The effect may be achieved due to the small size of the HbVs, which might allow them to perfuse capillaries that are no longer accessible to RBCs due to intraluminal obstructions or reduced perfusion pressure that have to be assumed in the ischemic, collateralized tissue in the present model. Indeed, circulating HbVs could be observed in capillaries that were no longer considered functional because of the cessation of RBC flux (23). However, our previous experiments (6) showed that the improvement of oxygenation in the ischemic tissue obtained after hemodilution with HbV solutions was dependent on an increased RBC flux in this tissue, which indicates that mechanisms different from the passage of HbVs through vascular obstructions may be present.

On the basis of previous intravascular oxygen tension measurements, it was estimated that 40–50% of the systemic arterial oxygen content exited the upstream vasculature before reaching the collateralized, ischemic flap tissue (5, 24). It has been shown in both experimental (8, 24) and theoretical (31) studies that oxygen delivery may be delayed in favor of the downstream vasculature if oxygen carriers with increased oxygen affinity are infused. It is conceivable that this effect was responsible for the increased ischemic tissue oxygenation obtained with HbV15-Dx70 in our study. The high oxygen affinity of HbV15 did not seem to hamper the unloading of oxygen to this tissue, which is promoted by the high oxygen tension gradient and the increased residence time of circulating blood (11). Furthermore, it may be assumed that oxygen delivery is facilitated due to metabolic acidosis, thus causing a shift of the oxygen dissociation curve to the right.

The results obtained with HbV30-Dx70 suggest that prevention of oxygen loss in the upstream vasculature may have been accomplished without raising the oxygen affinity of HbV. It has been shown that the diffusion of oxygen through the plasma may substantially be influenced by adding oxygen carriers (14, 16, 17). According to the Stokes-Einstein equation, the diffusivity of oxygen is inversely proportional to the size of the plasma-bound oxygen carrier and the viscosity of the suspension. In mathematical models and in vitro experiments, facilitated oxygen diffusion was ascribed to small-sized Hbs (14, 16, 17, 21), whereas, because of their large size, this effect was abolished if HbVs were used (21). Although not measured in our study, there is sufficient evidence to assume a marked increase in viscosity of the plasma suspension obtained during hemodilution with the HbV-Dx70 solutions, because an increase in plasma viscosity from 1.2 to 1.4 cP has been observed in hamsters after severe hemodilution with Dx70 (30), which has a threefold lower viscosity than HbV-Dx70. Taken together, it may be assumed that hemodilution with the HbV-Dx70 solutions caused a retention of oxygen in the upstream vasculature, which

was related to both the size of the HbV and the increasing composite viscosity of the plasma suspension consisting in original hamster blood plasma, Dx70 molecules, and HbV, and which resulted in a shift of oxygen delivery in favor of the collateralized, ischemic and hypoxic flap tissue.

Additional studies will be necessary to outline the influence of both HbV concentration and viscosity of the solutions, and the long-term benefit of the observed improvement in oxygenation would yet have to be confirmed by evaluating the functionality and survival of the jeopardized tissue. Moreover, our data may not be extrapolated to ischemic conditions in other, vital organs, such as the myocardium or cerebral tissue, in which the oxygen demand is substantially higher than in the present flap tissue.

In summary, we conclude that up to a 50% blood exchange, normovolemic hemodilution with HbV-Dx70 solutions led to a dose- and oxygen affinity-dependent improvement of oxygenation in the ischemic, hypoxic flap tissue, which was not related to the oxygen-carrying capacity of the circulating blood. Thus our study strongly emphasizes the potential function of HbVs as a therapeutic that may be used to improve the delivery of oxygen to ischemic and hypoxic tissues, which exceeds its role as a simple RBC substitute.

DISCLOSURES

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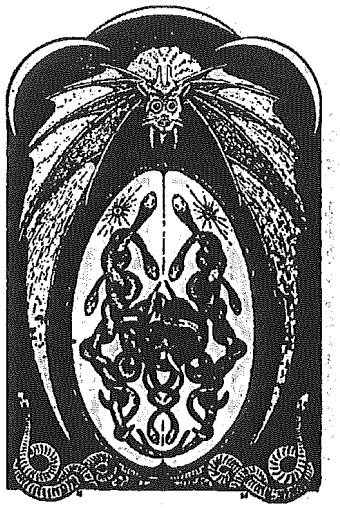

CHAPTER 160

Blood Substitutes' Efficacy Microvascular and Rheological Determinants

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The development of a blood substitute, also called "artificial" blood, or more exactly an oxygen-carrying plasma expander (OCPE), is still a major goal of transfusion medicine, driven by blood shortages, problems associated with the transmission of disease by available blood, and the complex logistics of acquiring, analyzing, storing, distributing, and delivering the needed blood. To date blood is unequaled (or appears to be) in its capacity to restore circulatory volume; however, it is often remarked that if blood were proposed today as an oxygen-carrying volume restoration fluid, it would not be approved by regulatory agencies.

Blood is needed in the presence of blood losses; however, the initial anemia is usually inconsequential to the organism's survival, whereas the associated hypovolemia is tolerated only within a narrow margin. As a result, a blood substitute should ideally target both events, possibly sequentially. Therefore, the development of an effective blood substitute is also in part related to availability and detailed understanding of an effective plasma expander, which is the term used to describe a volume restoration fluid used prior to reaching the transfusion trigger, or the point in volume restitution at which the introduction of an oxygen carrier, blood, is determined to be essential.

Blood exerts its principal functions in the microcirculation, the locale of exchange of the materials that it transports. Thus any attempt to introduce a blood surrogate must ensure efficacy at this level. In contrast, most efforts aimed at developing artificial blood were made in the absence of detailed information and analysis of how oxygen is managed at the level of the microcirculation. The cornerstones in the development of artificial blood up to the present are that this fluid should restore most of the oxygen-carrying capacity of the shed blood, that it is beneficial for the resulting mixture of remaining blood and resuscitation fluid (e.g., OCPE) to have a viscosity lower than that of natural blood, and that the material should have very low oxygen affinity so that oxygen would be readily released when blood arrives to the microcirculation.

These principles have guided the development of products that are now undergoing clinical trials. It is apparent that the initial impetus for the development of artificial blood was based on the restoration of systemic functions after acute blood losses with little or no emphasis on understanding the behavior of the resuscitation fluids in the microcirculation, which is the organ system where blood performs its functions. This was in part due to the imperfect understanding of how oxygen is managed in the microcirculation in both normal and pathophysiological conditions, the role and regulation of shear stress–dependant mediators produced by the endothelium, and the lack of techniques for measuring the key transport parameters that determine efficacy in maintaining microvascular function upon the introduction of a blood surrogate.

Oxygen-carrying capacity and oncotic pressure were prescribed to be similar to that of blood, while the experience with hemodilution suggested that improvements in transport would be obtained by lowering blood viscosity to values significantly below those of whole blood. An additional

presumed beneficial modification was the use of oxygen carriers based on modified hemoglobins with high p50s, presumed to facilitate oxygen unloading and tissue oxygenation. As acellular modified molecular hemoglobin became the oxygen carrier of choice, it was found this material was vasoactive, causing hypertension, which is deleterious in resuscitation. Vasoactivity was attributed to hemoglobin scavenging NO, leading to vasoconstriction, which gave rise to a significant effort aimed at modifying the hemoglobin molecule so that its affinity for NO was reduced.

At present an optimal OCPE is still perceived to have the following properties: oxygen-carrying capacity equivalent to 10 to 14 g/dL hemoglobin, p50 greater than 30 mmHg, viscosity 1 cP, oncotic pressure approximately 25 mmHg, and low NO binding. However, studies in the microcirculation show that a fluid configured according these concepts yields problematic outcomes in terms of resuscitation from anemic hypovolemia. Furthermore, since the most favored source of hemoglobin is human, even if it were possible to obtain a one to one conversion from blood to blood substitute, the problem of blood shortages is not solved. In an effort to circumvent the human hemoglobin source there have been various attempts to obtain hemoglobin by recombinant technology. Biopure Inc. (Boston, MA) has progressed to Phase III clinical trials with a molecular hemoglobin-based fluid derived from bovine blood. However, recombinant technology has not progressed to the development of an efficacious product to date, and the bovine-based product was not developed on the basis of microcirculatory data.

The Design of an Efficacious Oxygen-Carrying Plasma Expander

To date there are virtually no rivals to hemoglobin as a transporter of oxygen from the lung to the tissue because of its ability to bind a large amount of oxygen through a chemical reaction. The discovery that fluorocarbons could dissolve a comparatively large amount of oxygen, albeit at high oxygen partial pressures, suggested using this vehicle as the oxygen transporter. However, the use of fluorocarbon-based blood replacement fluids has not materialized, in part because of the lack of definitive experimental studies on the physiology related to altered blood physical properties and changes in the distribution of oxygen partial pressure in the circulation.

Various modifications of hemoglobin have optimized its performance and mostly eliminated the vasoactivity of this molecular species. Human hemoglobin remains the most favored source because of the well-defined methodology for obtaining blood from donors, which in advanced medical systems is virtually free of parasitic, bacterial, or viral contamination. The present formulations of bovine hemoglobin appear to be vasoactive and in the long term could present unknown risks of introducing diseases that may have extraordinarily long incubation times.

If the perceived and now frequently reported blood shortage is the driving force behind the development of hemoglobin-based oxygen-carrying blood substitutes, then the use of human hemoglobin is problematic since the processing technology and formulation would require that a unit of original blood yield at least an equivalent unit of "artificial blood," a zero-sum result that does not relieve shortages. A realistic process should produce several units of equivalent hemoglobin-based oxygen-carrying blood substitute from a unit of natural blood.

The present goal in devising a human hemoglobin-based blood substitute is to circumvent the inherent toxicity of the hemoglobin molecule and to be as efficacious as an equivalent unit of natural blood but at a lower hemoglobin concentration than blood, thus introducing a multiplying factor between the original source of human hemoglobin and the final product. Advances in microvascular technology allow us to critically analyze each of the "cornerstone" precepts that have guided the development of blood substitutes to date, namely the viscosity of the material, the affinity for oxygen, the effective concentration, and the resulting colloidal osmotic pressure when the material is present in the circulation. In the following these parameters will be analyzed from a microcirculatory perspective.

The Role of Viscosity in Oxygen Transport

Blood viscosity depends on red blood cell concentration (hematocrit) and on plasma viscosity. The manipulation of these two viscosities is the basis of the clinical practice of hemodilution. Accordingly, the restitution of blood losses with conventional plasma expanders can be effectively and safely accomplished up to a 50 percent loss of the red blood cell mass, using fluids with plasma-like viscosity. The decrease in viscosity due to hemodilution causes a compensatory increase in cardiac output due to the lowered flow resistance, thus maintaining oxygen delivery.

A specific decrease in oxygen-carrying capacity is one of the parameters that defines the so-called transfusion trigger. Microcirculatory experimental studies do not support the contention that lack of oxygen-carrying capacity is the actual determining factor in the decision of transfusing blood. In the awake hamster window chamber model, neither oxygen-carrying capacity nor tissue oxygenation is in jeopardy with red blood cell losses of two-thirds of the original mass (Figure 1).

A factor that is significantly changed upon reaching the transfusion trigger is blood viscosity, which is approximately half of normal, because of the loss of red blood cells. Thus, additional losses of red blood cells will further reduce blood viscosity, which is strongly dependent on hematocrit. The reduction of blood viscosity is initially compensated by increased cardiac output. However, cardiac output seldom doubles, and blood viscosity has the potential of decreasing

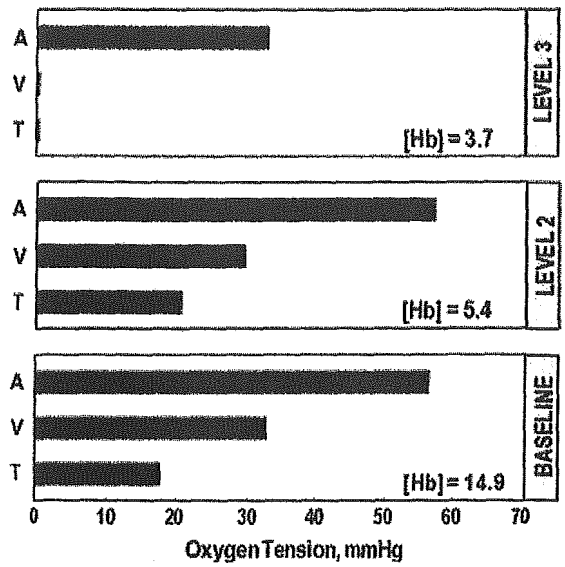

Figure 1 Distribution of oxygen in arterioles (A) and venules (V) in the tissue (T) in the awake hamster window model as a function of total hemoglobin (Hb, grams per deciliter). Nonovolemic hemodilution was achieved using dextran 70 kDa. It is apparent that a loss of the order of two thirds of red blood cells has no influence on microcirculatory pO_2 of this model. (see color insert)

to 25 percent of normal upon further reductions of hematocrit, leading to the significant lowering of functional capillary density (FCD, capillaries with red blood cell transit), a condition detrimental to survival in hemorrhagic shock.

Although capillaries do not appear to be the determinant structure for the supply of oxygen in some tissues, the maintenance of FCD in shock is a critical parameter in determining outcome independently of tissue oxygen tension (pO_2), suggesting that extraction of metabolic by-products may be as critical to a capillary function as oxygenation. The relationship between FCD and survival has been demonstrated in experimental analysis of conditions during prolonged hemorrhagic shock, where the principal microvascular functional difference between survivors and nonsurvivors was that survivors maintained FCD above a threshold of about 40 percent of that present in the normal organism; there were no other significant differences between groups [1].

The blood viscosity threshold that causes the decrease in FCD appears to coincide with the decision to transfuse blood. In other words, the transfusion trigger may also be a viscosity trigger; therefore results obtained with blood transfusions may also be achieved by increasing plasma viscosity. Thus use of red blood cells solely for the purpose of increasing blood viscosity may be avoided by introducing a material that increases plasma viscosity in the circulation. In this scenario blood viscosity resulting from the balance between the diminished red blood cell concentration and the increased plasma viscosity leads to the maintenance of vascular resistance. Tsai et al. [2] explored this phenomenon by inducing extreme hemodilution with low-viscosity dextran 70 kDa. At 11 percent hematocrit, FCD and microvascular flow were significantly reduced from control. However, when plasma viscosity was maintained above 2 cP by the introduction of high-viscosity dextran 500 kDa, FCD was maintained near to control values, and microvascular flow increased significantly above control, though hematocrit was 11 percent. This effect was not found if extreme hemodilution was performed with the Biopure product Oxyglobin, even at a total blood hemoglobin content of 6.7 g Hb/dL. High-viscosity plasma also caused blood flow to increase significantly above nonhemodiluted values because of the release of shear-dependent generated endothelial relaxing factors.

Counterintuitive Rheological Findings

High-viscosity plasma restores mean arterial blood pressure (MAP) in hypotension without vasoconstriction. Systemic blood viscosity depends on hematocrit squared; thus viscous losses in major vessels of the circulation can be minimized even when plasma viscosity is increased, shifting pressure and pressure gradients from the systemic to the peripheral circulation. Reduced blood viscosity decreases shear stress and the release of vasodilators, causing vasoconstriction, which negates the benefit of reducing the rheological component of vascular resistance. Vascular resistance depends on the first power of blood viscosity and the 4th power of vessel radius. Therefore reducing blood viscosity with low viscosity plasma might decrease flow and oxygen delivery to the tissue if there is an associated vasoconstrictor stimulus. However, increased flow and/or increased viscosity augments shear stress on the endothelium since the elevation of plasma viscosity causes sustained NO-mediated dilatation in the hamster muscle microcirculation, supporting this interpretation. Enhancement of shear stress on the vessel wall results in the release of prostacyclin and NO from the vascular endothelium. Endogenous NO release reduces total peripheral resistance during moderate hemodilution. Increased wall shear stress following increased blood flow induces vasodilation [3] showing that the link between shear stress and vasodilatation is well established.

Experimental verification of the beneficial effects of high-viscosity plasma during hemodilution in the microcirculation is evidenced by effects on FCD, perfusion and vasodilatation. More recently it was demonstrated by Cabrales et al. [4] that an increase in capillary pressure is the principal mechanical event that governs the effects due to perfusion with high-viscosity plasma.

Hemodilution, Blood Viscosity, and Vasoactivity

It is now apparent that low viscosity molecular hemoglobin solutions lower FCD independently of the intrinsic

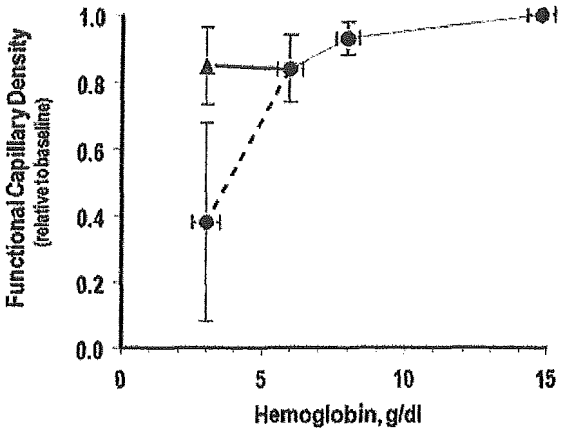

Figure 2 Changes of FCD following isovolemic hemodilution with low- and high-viscosity plasma expanders. ● Hemodilution with dextran 70 kDa maintains FCD up to a hematocrit (hemoglobin) that is 40 percent of normal. Further hemodilution with the same low-viscosity diluent causes the fall of FCD to pathologically low levels. ▲ Continuation of hemodilution with dextran 500 kDa after reaching 40 percent of normal hemoglobin with hemodilution with dextran 70. FCD is maintained to normal levels by the increased plasma viscosity. Redrawn from Tsai et al. [2].

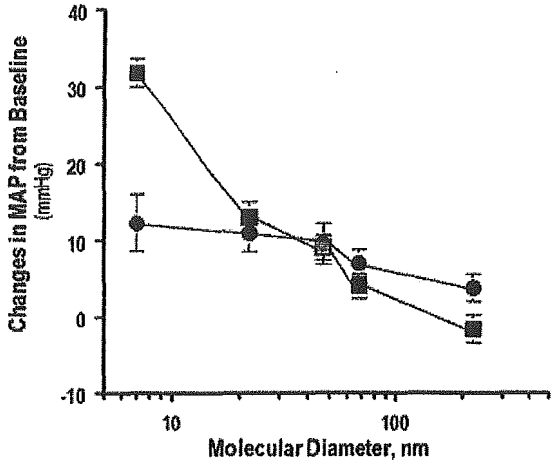

Figure 3 Changes in mean arterial blood pressure after a 5 percent by volume (5 Hb g/dL) topload infusion of free hemoglobin solutions of different molecular diameters and vesicle encapsulated hemoglobin. ■ Pressor effect after infusion. ● Pressor effect 3 hours after infusion. Redrawn from Sakai et al. [5]. (see color insert)

vasoactive properties of the hemoglobin molecule because they cause a significant decrease in blood viscosity after reaching the transfusion trigger. An additional factor attendant to the restoration of blood volume upon reaching the transfusion trigger with a plasma-like viscosity fluid is that this process brings the organism to near extreme hemodilution conditions, characterized by decreased shear stress on the endothelium, lowering the production of endothelial-derived vasodilators. Increasing plasma viscosity to about 2.0 to 2.5 cP increases shear stress and the production of vasodilators, which breaks up the vicious circle caused by extreme hemodilution, compensatory vasoconstriction and low viscosity.

Experimental results shown in Figure 2 show that the maintenance of FCD is not directly linked to oxygen delivery, but to mechanical factors related to the viscosity of the perfusion fluid and the production of vasodilators by mechanotransduction in the endothelium. Therefore an acellular oxygen carrier should maintain plasma viscosity above a specific threshold, while ensuring that overall blood viscosity does not exceed normal values.

Low blood viscosity can be compensated for by hemoglobin solutions with high viscosity. This can be achieved by mixing the hemoglobin molecule with a viscogenic material such as hydroxyethyl starch (HES) at suitable concentrations, or by modifying the hemoglobin molecule to produce an intrinsically viscous solution by increasing its molecular dimension. This latter process can be implemented by polymerization or conjugation with various molecules such as starch and polyethylene glycol, as described by Sakai et al. [5], who showed that the pressor effect is inversely related to molecular size (Figure 3). Hemoglobin molecules with these and several other beneficial features are polyethylene glycol conjugated hemoglobin molecules [6].

The Vasoconstrictive Effect of Hemoglobin

Natural hemoglobin molecules are presumed to be vasoconstrictive because of their ability to scavenge NO. However, recent experimental evidence shows that whereas NO binding is virtually identical for most hemoglobin molecules [7], the vasoconstrictive effect is not, being essentially absent in polyethylene glycol modified hemoglobins and in some very large hemoglobin polymers.

NO is produced by the endothelium as a result of shear stress and other processes. The affinity of the hemoglobin molecule for NO is due to the physical similarity between NO and O_2. Thus in general, hemoglobins with high affinity for O_2 generally also have a high affinity for NO, and vice versa. The production of genetically modified hemoglobins that appear to have little affinity for NO, while maintaining a normal affinity for O_2, may challenge this generalization; however, the fact remains that interfering with NO production with administration of L-arginine methyl ester hydrochloride (L-NAME) or scavenging NO with cell free unmodified hemoglobin causes the constriction of aortic rings, and an increase in blood pressure in experimental subjects.

The concept that hemoglobin extravasation and its location between the endothelium and smooth muscle is the principal factor causing hypertension and vasoconstriction is also questionable because the extravasated molecule will eventually saturate. In fact the presence of a NO-avid mole-

cule in plasma is sufficient to distort the diffusion field of NO from the endothelium, whereby hemoglobin does not need to extravasate to be vasoconstrictive.

NO scavenging does not provide a consistent explanation for the pressor effect of free hemoglobin in the circulation that is applicable to the different hemoglobin modifications. The lack of correlation between pressor responses and NO scavenging characteristic of hemoglobin molecules led McCarthy et al. [8] to propose that hypertension following the introduction of molecular hemoglobin in the circulation is caused by a mechanism related to the process of facilitated diffusion of oxyhemoglobin. According to this hypothesis the presence of molecular hemoglobin causes an additional flux of oxygen in the plasma layer due to the diffusion of oxyhemoglobin. Although the diffusion constant of hemoglobin is low, the amount of oxygen carried is large because hemoglobin binds a large amount of oxygen. The net result of this process is that a comparatively small concentration of molecular hemoglobin augments oxygen transfer to the vessel wall, leading to a hyperoxia signal, and consequently a vasoconstrictive response.

In vivo, peripheral vascular resistance is autoregulated at the level of the arterioles by a mechanism that senses oxygen tension, producing vasodilatory signals when blood and tissue po_2 is low, and vice versa. This conceptualization is supported by the finding that large hemoglobin molecules are not vasoactive, although they carry oxygen. As an example, poly (ethylene glycol) (PEG) surface decorated hemoglobins (PEG-Hb) have consistently been shown to be vasoinactive. These molecules have a large volume because of the water bound by PEG. Since the diffusion constant is inversely proportional to molecular radius, it can be shown that PEG-Hb has a diffusion constant that is about one fifth that of the native hemoglobin.

Experimentation with different levels of hemoglobin surface decoration show that vasoinactivity may be partially related to the degree to which the surface of the hemoglobin molecule is shielded by the water–PEG combination [6]. This phenomenon suggests that free hemoglobin may also cause a pharmacological effect mediated at the surface of the endothelium, and that conjugation of hemoglobin with PEG may produce a shield that prevents this process.

The vasoconstrictive effects of molecular hemoglobin may have several components that sometimes reinforce each other. When blood viscosity becomes too low, there is a reflex vasoconstriction that attempts to maintain perfusion pressure, a phenomenon independent of blood oxygen-carrying capacity. Oxygen regulation plays a crucial role since the arteriolar walls and the tissue sense both the rate of oxygen delivery from the red blood cell column and local po_2. When molecular hemoglobin is present in plasma, there is a significant additional flux of oxygen to the arteriolar wall by facilitated diffusion, a process enhanced with right-shifted oxygen dissociation hemoglobin molecules. NO scavenging can also be a factor that may be balanced by increased NO (and/or prostacyclin) production resulting from elevated shear stress caused by high-viscosity hemoglobin molecules. Furthermore, considering that modest amounts of small molecular hemoglobin can elicit a pressor response, a pharmacological effect due to "naked" small hemoglobin molecules in the circulation may also be present.

Vasoconstriction limits perfusion and decreases FCD. Although healthy organisms could probably compensate for moderate hypertensive episodes leading to corresponding decreases in FCD, these same episodes may place the organism in jeopardy if they are superposed to other vasoconstrictive stimuli, such as those inherent to hemorrhagic shock. Conversely, high plasma viscosity is critical in resuscitation, as an OCPE is administered in conditions of extreme hemodilution because there is no need for using these products prior to reaching the transfusion trigger.

Optimal Oxygen Disassociation Properties

The development of oxygen carriers has implicitly assumed that the oxygen dissociation curve should be right shifted, thus facilitating the release of oxygen. This approach does not consider the longitudinal gradient of oxygen tension in the circulation, whereby a right-shifted dissociation curve favors oxygen unloading from small arteries and arterioles. Hemodilution with hemoglobin-filled vesicles of different p50 in the hamster window chamber model has shown that improved tissue oxygenation is obtained when this parameter is 16 mmHg, instead of 34 mmHg (Department of Polymer Chemistry, Waseda University, Tokyo, Japan). PEG-conjugated hemoglobin (Hemospan, 4% Mal-PEG hemoglobin) produced by Sangart (San Diego, CA), with a p50 of 5 mmHg, used at low concentration in hemodilution maintains FCD and positive acid–base balance.

This apparent paradox may be understood by analyzing the distribution of oxygen in the microcirculation as shown in Figure 4, where oxygen tension in the microcirculation in normal conditions has a baseline tissue po_2 level of 22 to 24 mmHg (which appears to be common for most tissues). It is notable that although oxygen is regulated to achieve this partial pressure in the tissue, anaerobic metabolism occurs when tissue po_2 is below 2.4 to 2.9 mmHg.

A possible rationale for the high po_2 tissue regulation is that the organism has excess oxygen-carrying capacity, not only as a requirement for extreme efforts, but also for compensation of oxygen delivery inhomogeneity in the microcirculation. The effect of this inhomogeneity becomes apparent in considering the variability of oxygen partial pressure distribution in the hamster window chamber, which is of the order of $\pm$ 4 mmHg. This variability is a consequence of the quasirandom distribution of the transport properties of the microcirculation, and therefore intrinsic to any level of tissue oxygenation. In conditions of extreme hemodilution tissue po_2 decreases to 3 to 5 mmHg; thus, if

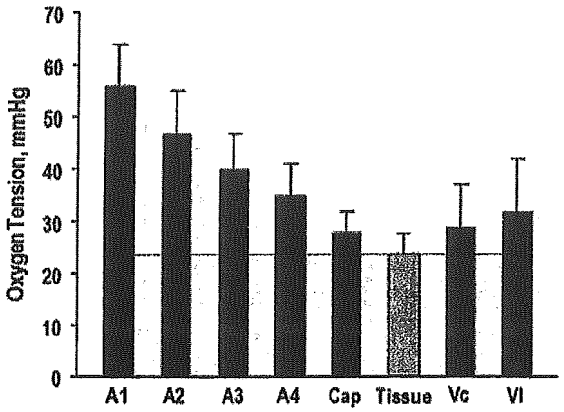

Figure 4 Distribution of pO_2 in the microvessel of the microcirculation of the hamster window preparation as a function of the arteriolar order of branching (As) and the venular order of branching (Vs). It is apparent that tissue pO_2 is narrowly regulated to a value in the range of 22 to 24 mmHg, which is significantly higher than the level associated with anaerobic metabolism.

the same variability is present, there is a significant amount of tissue that is anoxic. In the presence of a fraction of the oxygen-carrying capacity that can only be released at very low pO_2s, a form of targeted oxygen delivery, the effects of this variability will be nullified, ensuring that all the tissue, even at low pO_2 is oxygenated above the anaerobic threshold.

Tissue pO_2 levels that may be considered harmful could, in fact, be quite safe if it were possible to eliminate the inherent variability of oxygen delivery shown by the variability of tissue pO_2. A small quantity of a low-p50 hemoglobin oxygen carrier in the circulation accomplishes this because it delivers oxygen only to portions of the tissue where the anoxic threshold is passed, while the presence of even significant amounts of right-shifted hemoglobin would have no effect since most of the bound oxygen would be unloaded in oxygenated regions.

Cross-linked or polymerized hemoglobins developed so far have a high p50, presumed to be beneficial since it facilitates oxygen unloading. However, pO_2 in the microcirculation is regulated so that there is a significant decrease in oxygen tension from the systemic circulation to the capillaries, which typically have a pO_2 of about 30 mmHg. At this p50 half of the blood oxygen is delivered by arterioles in normal conditions; however, if the p50 of the OCPEs is above this value, as in the case of Oxyglobin (p50 = 54 mmHg), most of the oxygen in the blood should be delivered by the arterioles if this material were to replace blood. These vessels extract a significant amount of oxygen from the circulation while consuming a major portion of this oxygen flux, thus increasing their oxygen supply increases tissue oxygen inhomogeneity, which is further aggravated by the vasoconstrictor autoregulatory response already discussed.

Oxygen-Carrying Capacity

Measurements of pO_2 in the microcirculation utilizing the technique of phosphorescence oxygen quenching show that when hemodilution carried out to a total hemoglobin content in red blood cells of 5.6 g/dL, then tissue oxygen is somewhat higher than normal but not statistically significant. The required oxygen-carrying capacity can also be obtained by a simple calculation that relates the whole-body oxygen consumption and cardiac output, which yields a nearly identical number for the organism at rest. Therefore, in principle, the oxygen-carrying capacity of an OCPE does not need to reproduce the value for normal blood and can be significantly lower.

Colloid Osmotic Pressure

It is generally assumed that a blood substitute should have a colloid osmotic pressure similar to that of blood and in the range of 20 to 25 mmHg; however, several plasma expanders have zero colloid osmotic pressure (saline, Ringer's lactate) and small-volume resuscitation utilizes fluids with very high osmotic properties. To date there is no definitive answer on what is the osmotic and/or oncotic property that is most appropriate, and in all probability this is a variable that depends on the type of blood loss to be corrected. Resuscitation with noncolloidal fluids leads to tissue edema. Conversely fluids with high colloidal and osmotic pressures cause tissue fluid to come into the vascular compartment, thus decreasing the amount of fluid to be administered. Most conditions of hemorrhage are associated with endothelial edema, which has been demonstrated to be rapidly reversed upon the introduction of hyperosmotic and hyperoncotic fluids. Volume expansion fluids such as hydroxyethyl starch have relative high colloid osmotic pressures, typically in the range of 30 to 50 mmHg depending on formulation. Small molecule hemoglobin–based OCPEs have their oncotic pressure adjusted to be that of plasma, but PEG-hemoglobin modified OCPEs tend to have higher oncotic pressures.

Synthesis of an Effective Oxygen-Carrying Plasma Expander

An OCPE based on the preceding concepts is a fluid with properties fundamentally different from those of blood, since it has low oxygen-carrying capacity, p50 is low and in the neighborhood of 5 mmHg, viscogenic properties are such that when introduced into the circulation plasma viscosity should be of the order of 2.0 to 2.5 cP, and colloidal osmotic pressure can be high. A fluid with these properties can be obtained by conjugating hemoglobin with PEG, and various formulations have been tested in both animal experiments and human trials with excellent results. Notably this

formulation is vasoinactive, and its NO-scavenging characteristics do not appear to be relevant since these fluids have the same NO binding constant as other vasoactive formulations that are vasoactive [7].

These fluids are in some cases more effective than blood because they are designed to maintain FCD, which is as necessary as restoring tissue oxygenation for the recovery from blood losses. Because in the foreseeable future OCPEs will use human hemoglobin, these fluids are practical: Their hemoglobin content is low, and more than two units of blood equivalent unit of resuscitation fluid can be obtained from one unit of blood. Finally, this low oxygen-carrying capacity is practical and safe because it yields a significant improvement of microvascular function.

Experimental Evidence

The effectiveness of different resuscitation modalities was tested experimentally in studies of extreme hemodilution and hemorrhagic shock in the microcirculation of the hamster chamber window model, which allows microcirculatory monitoring in the awake condition for a period up to 1 week, after the effects of the surgical intervention have subsided. Extreme hemodilution was chosen because in most instances, lowering systemic hematocrit to 50 percent of baseline with a suitable plasma expander does not alter microvascular hemodynamics and transport in our experimental model. Animals were hemodiluted to 60 percent of normal with dextran 70 kDa, and further hemodiluted to a final hematocrit of 11 percent using the different products simulating blood losses initially remedied with conventional plasma expanders, which upon passing the transfusion trigger are corrected with an oxygen-carrying blood substitute.

A compendium of findings in extreme hemodilution to 50 percent of normal with dextran 70 kDa and further hemodilution to a final hematocrit of 11 percent with the different products is shown in Figure 5, including results obtained with PEG-Hb vesicles developed at Waseda University, Tokyo, using a somewhat different protocol where extreme hemodilution was achieved with a continuous exchange of a hemoglobin vesicle suspension. FCD is shown as a function of blood base excess, which represents systemic conditions and suggests the definition of *critical functional capillary density* as the value for this parameter at which base excess is no longer sustained and drops following modest reductions of total blood hemoglobin, that is, in the neighborhood of a 50 percent FCD reduction. The most important result is that normal base excess is obtained with total blood hemoglobin of 5 percent, if 1 percent of this is Mal-PEG-Hb—a result not found with other OCPEs.

Extreme hemodilution is not a clinically relevant procedure and serves only to study basic mechanisms. A clinically relevant test is to rescue a subject in hemorrhagic shock. Studies were therefore conducted to determine the effects of resuscitation with blood, starch, and Mal-PEG-Hb in a conventional 50 percent bleed shock protocol. The animals were resuscitated after 1 hour without any additional volume manipulation using shed blood, HES, and Mal-PEG-Hb with 25 percent of the blood volume. The results, shown in Figure 6, indicate that Mal-PEG-Hb is superior to both HES and blood in reestablishing microvascular function. Concurrently it was found that base excess was higher in the Mal-PEG hemoglobin-resuscitated animals than in the blood-resuscitated animals. An explanation for these findings is that low p50 hemoglobin targets oxygen delivery of oxygen to only the anoxic tissue.

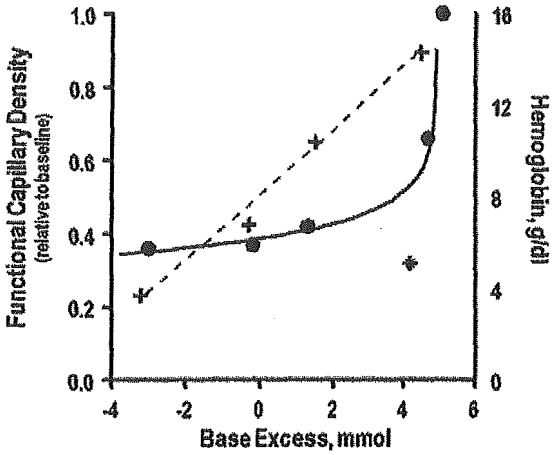

Figure 5 Relationship between total circulating hemoglobin and base excess, and FCD and base excess, for different hemoglobin modifications and concentrations, including hemoglobin vesicles, in normovolemic hemodilution experiments. The data marked ● shows the relationship between FCD and base excess, showing that MAL-Peg-Hb (▼) yields high FCD and base excess at low hemoglobin concentrations. It is apparent that base excess is a direct function of hemoglobin concentration (+) with the exception of MAL-Peg-Hb (✱), which presents normal base excess at a very low total hemoglobin content. (see color insert)

An extreme hemorrhage study was performed with Mal-PEG-Hb in which rats were 50 percent exchange transfused before hemorrhage with either αα-cross-linked hemoglobin, or 4 percent Mal-PEG hemoglobin (Figure 7). These animals were then subjected to a continuous exponential bleed (1 hour, 60 percent of blood volume) whereby at the end of the second hour after the start of bleeding 50 percent of the control animals succumbed. In these experiments it was found that at the end of one hour all animals that received Mal-PEG hemoglobin before hemorrhage survived, while all of those receiving αα-cross-linked hemoglobin did not survive.

Summary and Conclusions

The revision of microvascular physiology related to modifying basic transport properties of blood such as plasma viscosity, p50, and hemoglobin concentration shows

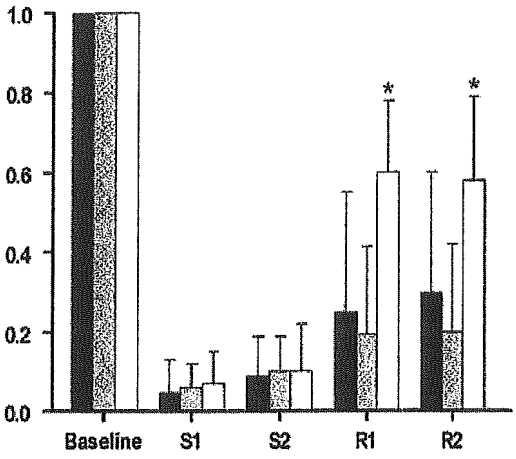

Figure 6 Recovery of FCD during resuscitation from 1 hour hemorrhagic shock with identical volumes of shed blood (*black bars*), 5 percent HES (*shaded bars*), and 4 percent MAL-Peg-Hb (*white bars*). S1 and S2 initial and final conditions during the shock period. R1, Recovery immediately after resuscitation; R2, 1 hour after resuscitation. $p < 0.05$ relative to shed blood and HES.

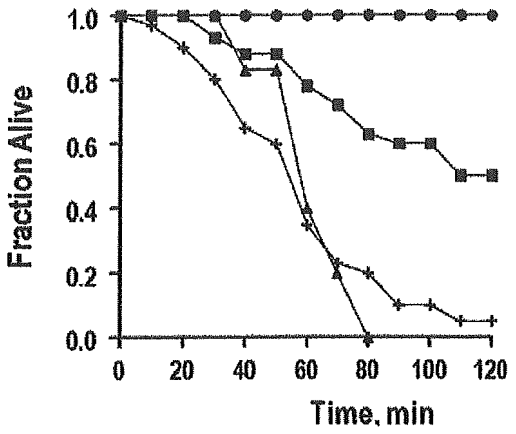

Figure 7 Controlled bleeding in rats that are 50 percent exchange transfused with MAL-Peg-Hb (●), αα-cross-linked hemoglobin (▲), and a polymerized hemoglobin (+), versus controls (■) with no treatment. The study was designed so that 50 percent of the untreated (not transfused controls) would survive 120 minutes.

that blood or a bloodlike fluid may not be the optimal oxygen-carrying volume-restoring fluid. A critical parameter for either oxygen-carrying or noncarrying blood replacements is their viscosity, which is a factor in maintaining capillary flow.

Analysis of the microvascular consequences of changing blood rheological conditions and particularly plasma shows that low plasma viscosity is not of universal benefit. Patients following trauma, peripheral arterial occlusive disease, and acute myocardial infarction have elevated plasma viscosity, a condition presumed to be pathological. However, there are situations where increased viscosity may be a protective or beneficial mechanism.

Plasma expanders are not used after reaching the transfusion trigger because the reduction of blood oxygen-carrying capacity beyond this point is assumed to jeopardize tissue oxygenation, according to the systemic evaluation of the organism portrayed by blood gases. Conditions in the microcirculation and local microscopic tissue environment when the reduction of red blood cells is extended beyond the transfusion trigger have not been consistently explored and presently show that oxygen-carrying capacity is not the major factor in determining tissue survival.

Studies show that the transfusion trigger is also the limit for the organism to adapt to low blood viscosity in acute conditions; thus *the conventional transfusion trigger is also a viscosity trigger*. Since the administration of a molecular oxygen carrier is physically similar to continuing fluid therapy after reaching the transfusion trigger, the maintenance of FCD requires the increase of plasma viscosity which through shear stress–dependent mechanisms operating in the endothelium ensures the maintenance of optimal microvascular function. Oxygen-carrying capacity is exhausted upon red blood cell (or hemoglobin) losses that are significantly greater than those represented by the transfusion trigger. However, these losses of oxygen-carrying capacity do not need to be compensated on a one-to-one basis, if microvascular function (i.e., FCD) is maintained and an oxygen carrier is introduced only to deliver oxygen to anoxic tissue regions. This approach ensures a uniform maintenance of the whole organism above the anaerobic threshold, while limiting the amount of oxygen carrier needed to maintain metabolism. Thus the combination of maintenance of microvascular function and targeted oxygen delivery is the primary determinant of an efficacious human hemoglobin-based blood substitute that is more effective than blood in acute conditions and that also expands the available blood supply, since a unit of blood yields more than two units of surrogate blood.

Glossary

Functional capillary density: Number of capillaries in a unit volume of tissue that presents the passage of red blood cells. This parameter is experimentally determined by measuring the length of red blood cell–perfused capillaries in a microscopic field of view.

Microvascular function: A combination of parameter including flow, number of open capillaries, intact vascular permeability, and level of vessels tone that allows for the proper interaction between blood and tissue at the microscopic level.

Oxygen-carrying capacity: The amount of oxygen in milliliters at standard atmospheric conditions and temperature contained in a fluid.

p50: Partial pressure of oxygen at which hemoglobin is 50 percent saturated with oxygen.

Plasma expander: A fluid used to restore circulatory volume when oxygen-carrying capacity is adequate.

Transfusion trigger: Level of blood hemoglobin at which the decision is made to introduce red blood cells into the circulation in order to restore oxygen-carrying capacity.

Vasoactivity: Inherent property of compounds that cause vasoconstriction and the elevation of systemic blood pressure.

Acknowledgments

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Biographies

Amy G. Tsai obtained her Ph.D. in bioengineering at the University of California, San Diego, where she is currently a Senior Research Scientist. She is widely recognized for her findings on oxygen consumption by the microvasculature and the development of high-viscosity plasma expanders. She is an expert in mathematical modeling, measuring methods for the in vivo study of the microcirculation and small animal experimentation.

Dr. Pedro Cabrales received his Ph.D. from the Universidad de los Andes, Bogotá, Colombia, studying the microvascular effects of extreme hemodilution with perfluorocarbons. He specializes in hemodynamic transport phenomena, having developed techniques for the analysis of tissue oxygenation at the microscopic level. He is presently at the Laboratory of Microhemodynamics of the University if California, San Diego.

Dr. Hiromi Sakai received his Ph.D. in polymer chemistry from Waseda University, Tokyo, Japan, where he is now Associate Professor. He specialized in the synthesis and characterization of oxygen carriers from the viewpoint of molecular assembly. For several years he was a visiting scholar at the University of California, San Diego, where he developed expertise in microhemodynamics. He is currently working on the optimization of oxygen carriers using in vivo methods in order to determine their safety and efficacy.

Prof. Marcos Intaglietta received his Ph.D. in applied mechanics from the California Institute of Technology in Pasadena and developed his academic career at the University of California, San Diego, where he is one of the founders of the bioengineering program and department. His specialty is the study of transport phenomena in the microcirculation and the development of blood substitutes. He has developed and implemented most of the methods presently used for the study of the microcirculation.

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Performances of PEG-modified hemoglobin-vesicles as artificial oxygen carriers in microcirculation

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Abstract. Hemoglobin-Vesicles (HbV; diameter, 250 nm) are artificial O_2 carriers encapsulating purified and concentrated human Hb solution in phospholipid vesicles, and their safety and efficacy, as a transfusion alternative, have been studied. In this paper, we summarized the characteristics of HbV that have been clarified by the microcirculatory observations.

Keywords: Blood substitutes, liposome, microcirculation, EDRF, oxygenation

1. Introduction

Hemoglobin (Hb)-based O_2 carriers (HBOCs) have been developed for use as a transfusion alternative and some of them are now in the process of clinical trials [1]. The advantages of the HBOCs are the absence of blood-type antigenicity and infectious pathogens, and stability for long-term storage when compared with the RBC transfusion [2]. A phospholipid vesicle or liposome encapsulating concentrated human Hb (Hb-vesicle, HbV) has been developed as an O_2 carrier [2–7]. The cellular structure of the HbV (particle diameter, ca. 250 nm) has characteristics similar to those of natural RBCs, since both have lipid bilayer membranes that prevent the direct contact of Hb with the components of blood and the endothelial lining [8]. The reasons for the Hb encapsulation in red blood cells (RBCs) should be: (1) a decrease in the high viscosity of Hb and a high colloidal osmotic pressure; (2) prevention of the removal of hemoglobin from the blood circulation; and (3) preservation of the chemical environment in the cells such as the concentration of phosphates (2,3-DPG, ATP, etc.) and other electrolytes. Moreover, during the long history of the development of Hb-based O_2 carriers (HBOCs), many side effects of molecular Hb have become apparent. These side effects of molecular Hb would imply the importance of the cellular structure.

Our *in vivo* studies of HbV have revealed the sufficient O_2 transporting efficiency comparable to RBCs [9–12], the safety in terms of blood compatibility [13], and prompt degradation in the reticuloendothelial system [14–17], all of which make us confident about advancing to the further development of HbV.

In this paper, we focus on the performances of our polyethylene-glycol (PEG)-modified HbV from the viewpoint of hemorheology and microcirculation.

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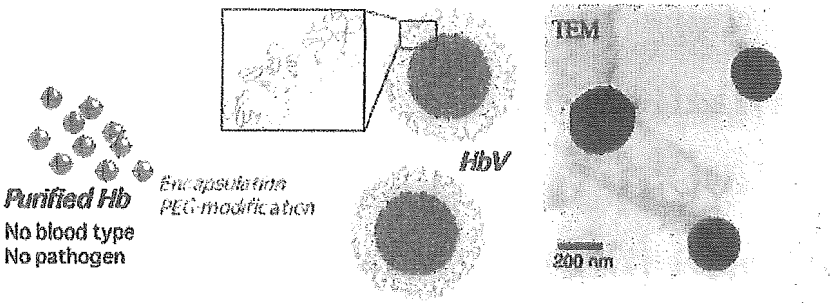

Fig. 1. Hemoglobin-vesicles (HbV) encapsulate the ultrapurified and concentrated human Hb solution (35 g/dl) with phospholipid bilayer membrane, and the surface is modified with polyethylene glycol chains. The well-regulated particle size (about 250 nm) was confirmed by TEM. One particle contains about 30,000 Hb molecules and about 6000 PEG chains were fixed on the surface.

2. Impact of PEG-modification of HbV

The rheological property of an artificial oxygen carrier is important because the infusion amount should be significantly large and that may affect the blood viscosity and hemodynamics. One HbV contains about 30,000 Hb molecules so that the suspension of HbV does not have colloid osmotic pressure (COP) (Fig. 1). The HbV suspended in 5 g/dl HSA at [Hb] = 10 g/dl shows comparable COP and viscosity to the blood.

We tested he function of PEG-modified and unmodified HbV as a blood replacement in the subcutaneous microvasculature of awake hamsters during severe hemodilution in which 80% of the red blood cell mass (70 ml/kg) was substituted with suspensions of the vesicles in 5% HSA solution [18,19]. Both materials yielded normal mean arterial pressure, heart rate, and blood gas parameters, which could not be achieved with albumin alone. Subcutaneous microvascular studies showed that PEG-modified HbV/HSA significantly improved microhemodynamic conditions (flow rate, functional capillary density, vessel diameter, and oxygen tension) relative to unmodified HbV/HSA. PEG-modified HbV was homogeneously dispersed in the plasma phase while the unmodified HbV showed aggregation in venules and capillaries. Even though it was confirmed *in vitro* that the aggregates dissociated reversibly at higher shear rates, it is unlikely that they would dissociate in vessels where the flow rate or shear stress was low. Aggregation and decreased flow rate may constitute a vicious circle that reinforces negative effects on blood flow. PEG reduced vesicular aggregation and viscosity, improving microvascular perfusion relative to the unmodified type. From this result, PEG modification is important for HbV in microvascular blood flow.

3. Interaction with NO and CO

As clinical trials of the chemically modified Hbs are extended to include larger numbers of individuals, it becomes apparent that the principal side effect consistently reported in the administration of acellular Hb solutions is hypertension presumably because of vasoconstriction. Hypertension, a well-defined reaction of the acellular intramolecularly cross-linked Hb (XLHb), was proposed to be beneficial in the treatment of hypotension concomitant to hemorrhagic shock [20]. However, vasoconstriction reduces blood flow, lowering functional capillary density, and therefore affecting tissue perfusion and oxygenation [21,22]. Nitric oxide (NO) scavenging by Hb due to intrinsic high affinity of NO to Hb is the mechanism presumed to cause vasoconstriction and hypertension [23,24].

We analyzed the relationship between the constriction of resistance vessel and hypertension after administration of acellular Hb and the extent to which the effect is dependent on the size of acellular Hb molecules modified by polymerization, polymer conjugation, and cellular liposome encapsulation [6,25]. Conscious Syrian golden hamsters with dorsal skinfold preparation were used. After the top load infusion of Hb products (7 ml/kg) into arterial catheter into jugular vein, mean arterial pressure, and heart rate were monitored through jugular arterial catheter, and microvascular responses were monitored by an intravital microscopy. The Hb products included intra-molecularly crosslinked Hb (XLHb), PEG-conjugated pyridoxalated Hb (PEG-PLP-Hb), hydroxyethylstarch-conjugated XLHb (HES-XLHb), glutaraldehyde-polymerized XLHb (Poly-XLHb) and HbV. Their molecular diameters were 7, 22, 68 and 224 nm, respectively. The top load infusion of 7 ml/kg of XLHb (5 g/dl) caused the immediate increase of MAP, which was 34 ± 13 mmHg higher 3 hrs after infusion. There was a simultaneous decrease in diameter of A_0 vessels (79 ± 8% of basal value), which caused blood flow to decrease throughout the microvascular network. The diameter of smaller arterioles did not change significantly. Infusion of O_2 carriers of greater molecular size resulted in lesser vasoconstriction and hypertension with HbV showing the smallest changes. Infusion of human serum albumin was used as control and produced no microvascular or systemic effects. Constriction of resistance arteries was found to be correlated to the level of hypertension, and the responses proportional to the molecular dimensions of Hb-based O_2 carriers. Since the results correlate with molecular size it is likely that the effects are related to the diffusion properties of the different hemoglobin molecules.

The liver is a major organ that detoxifies excess amount of heme by the action of heme oxygenase (HO). HO decomposes protoheme IX to generate biliberdin-IXa and CO. Under normal conditions, liver contains at least two OH isozymes for physiologic degradation of the heme: HO-1 and HO-2. One of the important roles of the HO reaction is to generate CO that serves as an endogenous regulator that is necessary for maintaining microvascular blood flow [26]. Since Hb strongly binds with CO (about 200 times stronger than O_2), it is necessary to confirm the effects of HbV in hepatic microcirculation in comparison with stroma free Hb solution. Suematsu et al. studied the perfusion of a rat liver with an acellular Hb solution and HbV, and found out that the Hb solution increased vascular resistance by 30% [27]. The smaller acellular Hb molecules (7 nm) extravasate across the fenestrated endothelium with a pore size of about 100 nm, and reach to the space of Disse. Heme is excessively metabolized by hemeoxygenase-2 to produce CO and bilirubin. Even though CO acts as a vasorelaxation factor in the liver, the excess amount of Hb in the space of Disse rapidly binds CO, resulting in the vasoconstriction and the increase in vascular resistance. On the other hand, Hb-vesicle (250 nm) is large enough to maintain in the sinusoid, and the vascular resistance is maintained.

These results indicate the importance of the size of the oxygen carriers, and the size of HbV is appropriate for the maintenance of microvascular blood flow.

4. Oxygen releasing behavior of HbV and oxygen therapeutics

We measured the O_2 release from HbV perfused through an O_2 permeable fluorinated ethylenepropylene copolymer tube (inner diameter, 28 μm), that was exposed to a deoxygenated environment [28] (Fig. 2). The addition of HbV to RBC did not influence on the O_2-releasing rate. On the other hand, the addition of 50-vol% acellular Hb solution to RBC significantly enhanced the rate of deoxygenation. This outstanding difference in the rate of the O_2 release between the HbV suspension and the acellular Hb solution should mainly be due to the difference in the particle size (250 vs. 8 nm) that affects their

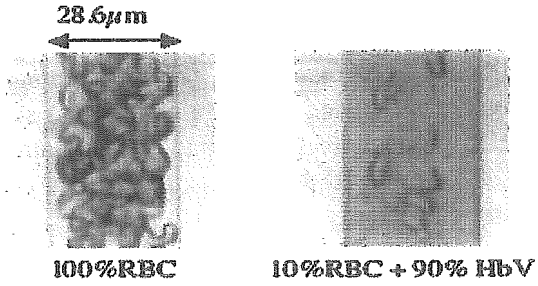

Fig. 2. Flow patterns of RBCs mixed with HbVs suspended in human serum albumin in a narrow tube (diameter, 28 μm) [28]. RBCs tended to flow in the centerline, while the HbV particles were homogeneously dispersed in a suspension medium. The individual particles could not be seen at this magnification. However, semitransparent elements were seen in the suspension medium, indicating the presence of HbV. This experimental model, developed by Maeda et al., was used to analyze the O_2 releasing behavior of HbV and RBC. [Hb] = 10 g/dl; centerline flow velocity, 1 mm/s.

diffusion for the facilitated O_2 transport. It has been suggested that the faster O_2 unloading from the HBOCs is advantageous for tissue oxygenation [29]. However, this concept is controversial regarding the recent findings since an excess O_2 supply would cause autoregulatory vasoconstriction and microcirculatory disorders [22,30]. We confirmed that HbV does not induce vasoconstriction and hypertension, due to not only the reduced inactivation of NO as an endothelium-derived vasorelaxation factor, but also possibly the moderate O_2 releasing rate similar to RBC as confirmed in this study.

One characteristic of HbV is that the O_2 affinity (P_{50}) of Hb can be easily regulated by the amount of coencapsulated allosteric effector, pyridoxal 5′-phosphate [19]. It has been clarified by Erni et al. that oxygenation of an ischemic skin flap, where one branch of feeding arteriole was ligated, was improved by infusion of HbV with a high O_2 affinity (low P_{50}) [31,32]. To clarify the underlying mechanism of ischemic tissue oxygenation, 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 [33]. Conscious hamsters received HbV_8 or HbV_{29} at the dose rate of 7 ml/kg bw. 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 O_2 tension (pO_2) 100 μm down from the occlusion was measured by the phosphorescence quenching of pre-infused Pd-porphyrin. The baseline arteriolar pO_2 (50–52 mmHg) decreased to about 5 mmHg for all the groups. Occlusion after HbV_8 infusion showed slightly slower rate of pO_2 reduction in comparison with that after HbV_{29} infusion. The arteriolar O_2 content was calculated at each reducing pO_2 in combination with the O_2 equilibrium curves of HbVs, and it was clarified that HbV_8 showed significantly slower rate of O_2 release in comparison with HbV_{29} and was a primary source of O_2 (maximum fraction, 0.55) overwhelming red blood cells when the pO_2 was reduced (e.g., <10 mmHg) in spite of a small dosage of HbV.

Accordingly, the result of improved oxygenation of the ischemic skin flap, observed by Erni et al., could be explained by low P_{50} HbVs retaining O_2 in the upstream vessels and delivering it to the ischemic tissue via collateral arterioles, even when these may have significantly slower blood flow. Moreover, an advantage of small HBOCs including HbV is that they are homogeneously dispersed in the plasma phase and therefore can deliver O_2 more homogeneously to the periphery than RBCs because microvascular Hct is heterogeneous particularly in pathological states. In such conditions HbV with a higher O_2 affinity (lower P_{50}) should show a slower O_2 unloading which would be effective for oxygenating ischemic tissues. This result supports the possible utilization of Hb-based O_2 carriers with lower P_{50} for oxygenation of ischemic tissues.

In summary, observation of microcirculation is important for the development of artificial oxygen carriers because it is the site where oxygen is unloaded to the target tissues. From the international collaborative evaluation studies of HbV, we have clarified the rheological property, advantages of the cellular structure, and the performances of HbV not only as a transfusion alternative but also for oxygen therapeutics.

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