# Blood viscosity maintains microvascular conditions during normovolemic anemia independent of blood oxygen-carrying capacity

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Cabrales, Pedro, Judith Martini, Marcos Intaglietta, and Amy G. Tsai. Blood viscosity maintains microvascular conditions during normovolemic anemia independent of blood oxygen-carrying capacity. Am J Physiol Heart Circ Physiol 291: H581-H590, 2006. First published March 3, 2006; doi:10.1152/ajpheart.01279.2005.—Responses to exchange transfusion with red blood cells (RBCs) containing methemoglobin (MetRBC) were studied in an acute isovolemic hemodiluted hamster window chamber model to determine whether oxygen content participates in the regulation of systemic and microvascular conditions during extreme hemodilution. Two isovolemic hemodilution steps were performed with 6% dextran 70 kDa (Dex70) until systemic hematocrit (Hct) was reduced to 18% (Level 2). A third-step hemodilution reduced the functional Hct to 75% of baseline by using either a plasma expander (Dex70) or blood adjusted to 18% Hct with all MetRBCs. In vivo functional capillary density (FCD), microvascular perfusion, and oxygen distribution in microvascular networks were measured by noninvasive methods. Methylene blue was administered intravenously to reduce methemoglobin (rRBC), which increased oxygen content with no change in Hct or viscosity from MetRBC. Final blood viscosities after the entire protocol were 2.1 cP for Dex70 and 2.8 cP for MetRBC (baseline, 4.2 cP). MetRBC had a greater mean arterial pressure (MAP) than did Dex70. FCD was substantially higher for MetRBC [82 (SD 6) of baseline] versus Dex70 [38 (SD 10) of baseline], and reduction of methemoglobin to oxyhemoglobin did not change FCD [84% (SD 5) of baseline]. Po2 levels measured with palladium-meso-tetra(4-carboxyphenyl)porphyrin phosphorescence were significantly changed for Dex70 and MetRBC compared with Level 2 (Hct 18%). Reduction of methemoglobin to oxyhemoglobin partially restored Po<sub>2</sub> to Level 2. Wall shear rate and wall shear stress decreased in arterioles and venules for Dex70 and did not change for MetRBC or rRBC. Increased MAP and shear stress-mediated factors could be the possible mechanisms that improved perfusion flow and FCD after exchange for MetRBC. Thus the fall in systemic and microvascular conditions during extreme hemodilution with low-viscosity plasma expanders seems to be, in part, from the decrease in blood viscosity independent of the reduction in oxygen content.

microcirculation; extreme hemodilution; plasma expander; intravascular oxygen; methemoglobin; methylene blue; functional capillary density

CORRECTION OF BLOOD LOSSES commences with the initial restitution of volume by means of plasma expanders, followed by the reinstatement of oxygen-carrying capacity via blood transfusion on reaching the so-called transfusion trigger. Multiple factors are responsible for reaching this point, including the amount of the blood loss and the dilution due to fluid infused to restore volume. As anemia progresses, oxygenation becomes a concern, when the oxygen-carrying capacity may be insufficient to supply the metabolic demand, and a blood transfusion is performed. The level that triggers the decision is variable, because the transfusion trigger is not a fixed threshold that determines the reintroduction of red blood cells (RBCs) into the circulation. The transfusion decision depends on an analysis of systemic parameters, age, and the nature and cause of the blood loss. Presently, the threshold for blood transfusion is set at  $\sim$ 7 g of hemoglobin (Hb) per 100 ml of blood, being higher for older populations and lower for younger and otherwise healthy individuals.

A new interpretation for the decision to maintain blood volume via the transfusion of blood is that RBCs are required to restore or maintain blood viscosity at a physiological level (40). Current studies (39) have shown that if blood viscosity is severely decreased by hemodilution, microvascular function is impaired, and tissue survival is jeopardized due to the local microscopic maldistribution of blood flow, rather than the deficit in oxygen delivery. Clearly, a limit is reached when extreme hemodilution is no longer able to maintain the metabolic needs of the tissue. However, microvascular studies (3, 6, 39) in extreme hemodilution show that this limit could be significantly lower than current transfusion triggers if blood viscosity is partially maintained during hemodilution by increasing plasma viscosity.

Restoration of blood viscosity during hemodilution and hemorrhage is desirable, because it maintains functional capillary density (FCD). FCD is defined as the number of capillaries with passage of RBC per unit surface of the field of view of a microscopically observed tissue. This microvascular parameter was found to be critical in defining tissue survival by Kerger et al. (19), who showed the correlation between maintenance of FCD above a specific threshold and survival in extended hemorrhagic shock (19). FCD also has been shown to be determined by the maintenance of capillary pressure, which, in extreme hemodilution, can be obtained by using highviscosity plasma expanders (39).

Blood conserved by conventional means for transfusion carries a limited amount of oxygen on introduction into the circulation. Oxygen transport by transfused RBCs begins several (2–5) hours later (36). Consequently, a conventional blood transfusion (using stored blood) may not fully restore oxygen-carrying capacity in acute conditions. However, it restores blood volume and blood viscosity. Furthermore, it provides rapid clinical benefits.

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In this study, our laboratory tested the hypothesis that in a three-step extreme hemodilution exchanging 110% of the blood volume, systemic and microvascular conditions in the hamster window model depend mostly on blood rheological properties unrestrained by the oxygen-carrying capacity. To partially maintain the rheological properties of blood, we exchange transfused RBCs in which Hb was converted to methemoglobin and, therefore, did not have the oxygen-binding capacity (creating an effective oxygen-carrying capacity of 11% Hct). As a control, hemodilution with 6% dextran 70 kDa (Dex70) was implemented to 11% Hct. Additionally, in this study, methemoglobin was rapidly reduced to oxyhemoglobin by using methylene blue to restore oxygen-carrying capacity to 18% Hct and account for stress and extension of exchange protocol.

### METHODS

Animal preparation. Investigations were performed in male Golden Syrian hamsters (55-65 g; Charles River, Boston, MA) fitted with a dorsal skinfold chamber window. Animal handling and care followed the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985). Experimental protocol was approved by the local Animal Care Committee. The hamster chamber window model is widely used for microvascular studies in the unanesthetized state, and the complete surgical technique is described in detail elsewhere (9, 13). Briefly, the animal was prepared for chamber implantation with a 50 mg/kg ip injection of pentobarbital sodium anesthesia. After hair removal, sutures were used to lift the dorsal skin away from the animal, and one frame of the chamber was positioned on the animal's back. The chamber consists of two identical titanium frames with a 15-mm circular window (12-mm diameter circular visible field). With the aid of backlighting and a stereomicroscope, one side of the skinfold was removed following the outline of the window until only a thin layer of retractor muscle and the intact subcutaneous skin of the opposing side remained. The cover glass was placed on the exposed skin held in place by the other frame of the chamber. The intact skin of the other side was exposed to the ambient environment. The animal was allowed at least 2 days for recovery before the preparation was assessed under the microscope for any signs of edema, bleeding, or unusual neovascularization. Barring these complications, the animal was anesthetized again with pentobarbital sodium. Arterial and venous catheters were implanted in the carotid artery (PE-50) and jugular vein (PE-50), respectively, and filled with a heparinized saline solution (30 IU/ml) to ensure patency at the time of the experiment. Catheters were tunneled under the skin and exteriorized at the dorsal side of the neck, where they were attached to the chamber frame with tape. Three to four days after the initial surgery, the microvasculature was examined, and only animals passing established systemic and microcirculatory inclusion criteria, which include having tissue void of low perfusion, inflammation, or edema, were entered into the study (39).

Inclusion criteria. Animals were suitable for the experiments if 1) systemic parameters were within normal range, namely, heart rate >340 beats/min, mean arterial blood pressure (MAP) >80 mmHg, systemic Hct >45%, and arterial Po<sub>2</sub> (Pa<sub>O2</sub>) >50 mmHg; and 2) microscopic examination of the tissue in the chamber observed under a ×650 magnification did not reveal signs of edema or bleeding. Hamsters are a fossorial species with a lower Pa<sub>O2</sub> than that of other rodents because of adaptation to the subterranean environment. However, microvascular Po<sub>2</sub> distribution in the chamber window model is the same as in other rodents, such as mice (4).

Systemic parameters. MAP and heart rate were recorded continuously (MP 150; Biopac System, Santa Barbara, CA). Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes (25  $\mu$ l; ~50% of the heparinized glass capillary tube is filled). Hb content was determined spectrophotometrically from a single drop of blood (B-Hemoglobin; Hemocue, Stockholm, Sweden).

Blood chemistry and biophysical properties. Arterial blood was collected in heparinized glass capillaries (0.05 ml) and immediately analyzed for PaO<sub>2</sub>, arterial PCO<sub>2</sub> (PaCO<sub>2</sub>), base excess, and pH (Blood Chemistry Analyzer 248; Bayer, Norwood, MA). The comparatively low  $Pa_{O_2}$  and high  $Pa_{CO_2}$  of these animals is a consequence of their adaptation to a fossorial environment. Blood samples for viscosity and colloid osmotic pressure measurements were quickly withdrawn from the animal with a heparinized 5-ml syringe at the end of the experiment for immediate analysis or refrigerated for next-day analysis. The small animal size allowed for only 3 ml of blood to be withdrawn from the animal after an experiment, which was not a sufficient volume to measure colloid osmotic pressure, blood viscosity, and plasma viscosity. Viscosity was measured in a DV-II plus (Brookfield Engineering Laboratories, Middleboro, MA) cone/plate viscometer with a CPE-40 cone spindle at a shear rate of  $160 \text{ s}^{-1}$ . Colloid osmotic pressure was measured by using a 4420 Colloid Osmometer (Wescor, Logan, UT) (44).

*FCD.* Functional capillaries, defined as those capillary segments that have RBC transit of at least a single RBC in a 30- to 45-s period, in 10 successive microscopic fields were assessed, totaling a region of 0.46 mm<sup>2</sup>. Each field had between two and five capillary segments with RBC flow. FCD (in cm<sup>-1</sup>), i.e., total length of RBC-perfused capillaries divided by the area of the microscopic field of view, was evaluated by measuring and adding the length of capillaries that had RBC transit in the field of view. The relative change in FCD from baseline levels, after each intervention, is indicative of the extent of capillary perfusion (5, 37).

*Microhemodynamics*. Arteriolar and venular blood flow velocities were measured on-line by using the photodiode cross-correlation method (16) (Photo Diode/Velocity Tracker model 102B; Vista Electronics, San Diego, CA). The measured center line velocity (*V*) was corrected according to vessel size to obtain the mean RBC velocity (26). A video image-shearing method was used to measure vessel diameter (*D*) (17). Blood flow (*Q*) was calculated from the measured values as  $Q = \pi \times V(D/2)^2$ . Changes in arteriolar and venular diameter from baseline were used as indicators of a change in vascular tone. This calculation assumes a parabolic velocity profile and has been found to be applicable to tubes of 15- to 80-µm internal diameter and for Hcts in the range of 6–60% (26). Wall shear stress (WSS) was defined by WSS = WSR ×  $\eta$ , where WSR is the wall shear rate given by 8·*V*/*D* and  $\eta$  is the microvascular blood viscosity or plasma viscosity.

Estimates of microvascular blood viscosity made from systemic samples tend to overestimate the actual changes of viscosity in the microcirculation because microvascular Hct is lower than systemic Hct. At Hct  $\sim 11\%$ , the contribution of RBCs to whole blood viscosity is linear and accounts for  $\sim 0.70$  cP, which is the difference between blood and plasma viscosity. According to Lipowsky and Firrell (25), "... the relationship between systemic arteriolar venular and systemic hematocrit is illustrated by a tendency toward equilibrium during extreme hemodilution." For extreme hemodilution, this relationship converges to an average value of  $\sim 0.7$  of the ratio between microvascular and systemic Hct, at Hct of  $\sim 10\%$ . Therefore, viscosity data were corrected for extreme hemodilution by linearly reducing the RBC viscosity contribution by 70%. The same procedure was used for the normal blood data, where the Hct reduction is 0.58 for arterioles and 0.68 for venules (25). However, because at normal Hct levels, blood viscosity is not linearly proportional to Hct, we used actual viscosity versus Hct (dilution with hamster blood) data to obtain the corrected value for blood viscosity.

*Hemoglobin oxygen equilibrium curves.* Oxygen saturation of freshly collected hamster blood was investigated by deoxygenation of oxygen-equilibrated oxyhemoglobin in a Hemox buffer (pH 7.35) at 37.6°C, using a Hemox Analyzer (TCS).

Microvascular Po<sub>2</sub> distribution. High-resolution microvascular Po<sub>2</sub> measurements were made by using phosphorescence quenching microscopy (33). This method for measuring oxygen levels is based on the oxygen-dependent quenching of phosphorescence emitted by albumin-bound metalloporphyrin complex after pulsed light excitation. The phosphorescence decay curves were converted to oxygen tensions with the use of a fluorescence decay curve fitter (model 802; Vista Electronics, Ramona, CA) (18). This technique has been used in this animal preparation and others for both intravascular and extravascular oxygen tension measurements. Tissue Po<sub>2</sub> measurements are possible in this preparation because the albumin-bound dye equilibrates between the plasma and tissue compartments as a consequence of the increased permeability of subcutaneous connective and adipose tissue to albumin. Animals received a slow intravenous injection of 15 mg/kg body wt at a concentration of 10.1 mg/ml of a palladium-mesotetra(4-carboxyphenyl)porphyrin (Porphyrin Products, Logan, UT). The dye was allowed to circulate for 10 min before Po2 was measured (18, 33, 41).

In our system, intravascular measurements are made in an optical rectangular window (5  $\mu$ m × 15  $\mu$ m) over the image of the vessel of interest, with the longest side of the rectangular slit positioned parallel to the vessel wall. Tissue Po<sub>2</sub> is measured in regions void of large vessels within intercapillary spaces (10  $\mu$ m × 10  $\mu$ m) (38). In the present configuration of the oxygen-measuring system, tissue Po<sub>2</sub> values are obtained with a repeatability of 1–3 mmHg, capturing the emission from an area related to the tissue of ~75  $\mu$ m × 100  $\mu$ m (33). Measurements in regions with large tissue gradients in the vicinity of arterioles are made by shaping the slit in a rectangular format, with its longest side placed along the outside of the vessel wall, and varying the length until an acceptable signal-to-noise ratio is obtained (18). Perivascular Po<sub>2</sub> measurements are made by placing the center line of the measuring slit at a distance that is one-tenth of the inner vessel diameter from the blood tissue interface.

*Tissue oxygen delivery and extraction.* Calculations of oxygen delivery and extraction are made with *Eqs. 1* and 2:

$$O_{2 \text{ delivery}} = \left[ (\text{RBC}_{O_2\text{Hb}} \gamma S_A\%) + (1 - \text{Hct})\alpha PO_{2A} \right] \dot{Q} \qquad (1)$$

$$O_{2 \text{ extraction}} = \left[ (\text{RBC}_{\text{Hb}} \gamma S_{\text{A}-\text{V}} \%) + (1 - \text{Hct}) \alpha \text{Po}_{2\text{A}-\text{V}} \right] \dot{Q} \quad (2)$$

where RBC<sub>02,Hb</sub> is the effective Hb in RBCs (g Hb/dl blood),  $\gamma$  is the oxygen-carrying capacity of Hb (1.34 ml O<sub>2</sub>/g Hb), S<sub>A</sub>% is the arteriolar oxygen saturation, (1-Hct) is the plasma fraction of blood,  $\alpha$  is the solubility of oxygen in plasma (3.14  $\times$  10<sup>-3</sup> ml O<sub>2</sub>/dl mmHg), PO<sub>2 A</sub> is the arteriolar partial pressure of oxygen, Q is the microvascular flow relative to baseline, and subscript A-V indicates the difference between arterioles and venules.

Preparation of RBCs containing methemoglobin. Fresh cells were collected from the animal under study during the initial hemodilution steps. The sample was centrifuged, and the supernatant and RBCs were transferred into tubes and resuspended in an equivalent amount of normal saline and mixed gently for 2 min with sodium nitrite (100  $\mu$ l of 1 M sodium nitrite per 5 ml of RBCs). The cells were then centrifuged at 2,100 g, washed three times with 5 ml of heparinized saline, and stored as packed cells at 4°C. Aliquots of these cells were tested, and only those cells with 95–100% methemoglobin were used (14). The RBCs containing methemoglobin (MetRBC) were resuspended in fresh-frozen plasma taken from another animal to produce a Hct of 18% or Hb of 5.8 g Hb/dl. Care was taken to avoid removing samples from the layer between the plasma and RBCs that contained white blood cells. RBC manipulation (pipette, reaction, and transfer) was performed in a laminar flow hood for sterility.

Acute isovolemic exchange transfusion (hemodilution) protocol. Progressive hemodilution to a final systemic Hct level of 11% was accomplished with three isovolemic exchange steps. This protocol is described in detail in our previous reports (2, 6, 39). Briefly, the volume of each exchange-transfusion step was calculated as a percentage of the blood volume, estimated as 7% of body weight. An acute anemic state was induced by lowering systemic Hct to 18% with two steps of progressive isovolemic hemodilution using 6% Dex70 (70 kDa, mean molecular weight; Pharmacia) in saline (exchange *Levels 1* and 2). *Level 1* exchange was 40% of blood volume, and *Level 2* was 35% of blood volume.

After *Level 2* exchange, animals were randomly divided into three experimental groups by assigning each animal to an experimental group according to a sorting scheme based on a list of random numbers (1). One group was used to study oxygen at *Level 2* (*Group 1, Level 2*). In two groups of animals, *Level 2* exchange was followed by *Level 3*, exchanging 35% of the blood volume by using Dex70 (*Group 2, Dex70*) or metRBCs at a Hct of 18% suspended in fresh-frozen plasma (*Group 3, MetRBC*). Figure 1 illustrates the experimental protocol.

Because mixed blood and dilution material was withdrawn during the exchanges, a 110% blood volume exchange was needed to reduce the functional Hct to 11% (2, 6, 34, 39, 42). Dilution material was infused and filtered (in-line, 0.22- $\mu$ m filter) into the jugular vein catheter at a rate of 100  $\mu$ l/min. Blood was simultaneously withdrawn at the same rate from the carotid artery catheter according to a previously established protocol (2, 6, 34, 39, 42). Blood samples were withdrawn at the end of the experiment for subsequent analysis of viscosity and colloid osmotic pressure. The duration of the experiments was 4 h. Each exchange and the respective observation time point postexchange were fully completed in 1 h. Systemic and microcirculation data were taken after a 5-min stabilization period.

After the assessment of systemic and microvascular parameters (30 min) in animals transfused with MetRBC, an intravenous infusion of methylene blue was given in a dose of 1 mg/kg. methemoglobin levels were decreased significantly to <6% in 15 min, and all the systemic and microvascular parameters were measured again, producing a new experimental group (*Group 4*, rRBC).

*Effects of methylene blue.* Nonhemodiluted hamsters were studied after intravenous infusion of 1 mg/kg methylene blue to establish the effects of methylene blue on microvascular and systemic regulation.

## Exchange Protocol



Fig. 1. Hemodilution was attained by means of a progressive, stepwise, isovolemic, blood exchange-transfusion protocol in which the red blood cell (RBC) volume (shaded bar) is continuously decreased and the plasma volume is increased (open bar) while maintaining total blood volume constant (represented by dotted line). Volume of each exchange-transfusion step was calculated as a percentage of blood volume, estimated as 7% of body weight. An acute anemic state was induced by lowering systemic Hct two progressive steps of isovolemic hemodilution (40 and 35% of blood volume) using 6% dextran 70 (Dex70), labeled Level 1 and Level 2. Level 3 was achieved by a third hemodilution step (35% of blood volume) using either 6% Dex70 or RBCs containing methemoglobin (MetRBC) at an Hct of 18% suspended in fresh-frozen plasma. Hemodilution levels achieved are slightly different from volumetric fluid exchanges because exchanged volume cannot be withdrawn before diluents are introduced. After assessment of systemic and microvascular parameters in MetRBC group, an intravenous infusion of methylene blue was given to decrease methemoglobin levels, producing a new experimental group (rRBC).

Experimental setup. The unanesthetized animal was placed in a restraining tube with a longitudinal slit from which the window chamber protruded and then fixed to the microscopic stage of a transillumination intravital microscope (BX51WI; Olympus, New Hyde Park, NY). The animals were given 20 min to adjust to the change in the tube environment before measurements were made. The tissue image was projected onto a charge-coupled device camera (Cohu 4815) connected to a videocassette recorder and viewed on a monitor. Measurements were carried out using a ×40 (Lumpfl-Wir, numerical aperture 0.8, Olympus) water immersion objective. The same sites of study were followed throughout the experiment so that comparisons could be made directly to baseline levels. The animals were randomly started with low or high oxygen, followed by the opposite (high or low) to reduce the bias based on the order of gas exposure. Systemic parameters, FCD, vessel diameter, velocity, and intravascular oxygen tension were measured after 10-min exposure.

Data analysis. Results are presented as means (SD). Figures are presented as box-whisker plots separating the data into quartiles, with the top of the box defining the 75th percentile, the line within the box giving the median, and the bottom of the box showing the 25th percentile. The top "whisker" defines the 95th percentile, and the bottom whisker defines the fifth percentile. Data within each group were analyzed by ANOVA for repeated measurements (Kruskal-Wallis test). When appropriate, post hoc analyses were performed with the Dunn's multiple comparison test. Microhemodynamic measurements were compared with baseline levels obtained before the experimental procedure. Microhemodynamic data are presented as absolute values and ratios relative to baseline values. A ratio of 1.0 signifies no change from baseline, whereas lower and higher ratios are indicative of changes proportionally lower and higher than baseline (i.e., 1.5 would mean a 50% increase from the baseline level). The same vessels and functional capillary fields were followed so that direct comparisons to their baseline levels could be performed, allowing for more robust statistics for small sample populations. All statistics were calculated with the use of GraphPad Prism 4.01 (GraphPad Software, San Diego, CA). Changes were considered statistically significant if P < 0.05.

## RESULTS

Eighteen animals were entered into the hemodilution microcirculation study. Four animals were used as plasma donors for the study. All animals tolerated the entire hemodilution protocol without visible signs of discomfort. The animals were randomly assigned to the following experimental groups: *Level* 2 (n = 6); Dex70 (n = 6); or MetRBC and rRBC (n = 6). All groups used were statistically similar (P > 0.20) in systemic and microcirculation parameters at baseline, *Level 1*, and *Level 2*.

Systemic parameters. The exchange protocol significantly reduced Hct to 28.4% (SD 1.8) for Level 1 and to 18.4% (SD 0.9) for Level 2. Hct and Hb for the Level 3 exchange are given in Table 1. The MetRBC group did not show a decrease in Hct or Hb content from Level 2, because of the additional Hb contributed by metRBCs. Dex70 decreased Hct and Hb significantly from Level 2. Intravenous methylene blue was administered to accelerate the reduction of methemoglobin to oxyhemoglobin in the MetRBC group, restoring 96% of the total Hb to oxyhemoglobin (rRBC). Hct after methylene blue (rRBC) was not significantly different from MetRBC. The oxygen content for the rRBC group increased markedly after methylene blue administration from a condition with high methemoglobin (MetRBC).

MAP was not changed from baseline [104 mmHg (SD 6)] after *Level 1* exchange [95 mmHg (SD 11)], and on further hemodilution with Dex70, MAP decreased to 90 mmHg (SD 9) at *Level 2. Level 3* exchanges lowered MAP, as is shown in Table 2. Systemic arterial blood gas analysis showed a statistically significant rise in Pao<sub>2</sub> and Pa<sub>CO<sub>2</sub></sub> from baseline after *Level 3* for all groups (P < 0.05). Arterial pH was not statistically changed from baseline. Blood base excess was statistically significantly decreased after *Level 3* hemodilution compared with baseline in Dex70 (Table 2).

Blood biophysical properties after exchange. Blood viscosity, plasma viscosity, and plasma colloid osmotic pressure after hemodilution for all the groups are presented in Table 2. Blood viscosities for *Level 2*, Dex70, MetRBC, and rRBC were significantly lower than baseline; Dex70 was also lower than the other exchanged groups. Plasma viscosity was raised from baseline only for Dex70. However, this value was not significantly different from the other hemodiluted groups. All test materials did not affect plasma colloid osmotic pressure. The introduction of solutions into the circulation causes autotrans-

Table 1.	Systemic	parameters	before	and	after	exchange	protocol
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		Hemodilution					
				Level 3			
	Baseline	Level 2	Dex70	MetRBC	rRBC		
n Hct, % Hb, g/dl MetHb, %	12 49.4±1.2 14.8±0.6 <1%	12 18.4±0.9* 6.0±0.7* <1%	$6 \\ 11.1 \pm 0.9^{*\dagger} \\ 3.7 \pm 0.5^{*\dagger} \\ < 1\%$	$ \begin{array}{c} 6 \\ 18.6 \pm 0.7 * \\ 6.2 \pm 0.4 * \ddagger \\ 32 \pm 5 * \dagger \ddagger P \end{array} $	$\begin{array}{r} & & & & & & \\ & & & & & & \\ & & & & & $		
MAP, mmHg HR, beats/min	104±6 414±33	90±9†* 443±25	$64\pm8^{*}$ † 418±41	88±8*‡ 432±21	87±8*‡ 419±26		
$Pa_{O_2}$ , mmHg $Pa_{CO_2}$ , mmHg $pH_a$ $BE_a$ , mmol	$55.2\pm6.5$ $54.3\pm6.2$ $7.35\pm0.03$ $3.0\pm2.0$	$82.0\pm8.3*$ 49.9±5.2* 7.35±0.05 1.6±1.2	$105.3 \pm 16.7 * \dagger \\ 39.1 \pm 7.8 * \dagger \\ 7.32 \pm 0.04 \\ -4.6 \pm 2.6 * \dagger$	$87.8 \pm 10.1^{*}$ $46.4 \pm 7.2^{*}$ $7.36 \pm 0.03$ $0.8 \pm 1.1^{\ddagger}$	$79.6 \pm 6.2^{*} \ddagger 47.6 \pm 5.4^{*} \\ 7.35 \pm 0.02 \\ 1.4 \pm 1.5 \ddagger$		

Values are means  $\pm$  SD; *n*, no. of animals studied. Baseline included all the animals in the study. No significant differences were detected between baseline values of each group or between values after *Level 1* and *Level 2* exchange before exchange with test solutions. Hct, systemic hematocrit; Hb, hemoglobin content of blood; MAP, mean arterial blood pressure; BE<sub>a</sub>, arterial base excess Dex70, 6% dextran 70 kDa; MetRBC, red blood cells (RBCs) containing methemoglobin; rRBC, RBCs with reduced methemoglobin; HR, heart rate; pH<sub>a</sub>, arterial pH. \**P* < 0.05 compared with baseline; †P < 0.05 compared with *Level 2*;  $\ddagger P < 0.05$  compared with Dex70.

		Hemodilution				
				Level 3		
	Baseline	Level 2	Dex70	MetRBC/rRBC		
n	6	6	6	6		
Blood viscosity, cP	$4.2 \pm 0.3$	$2.9 \pm 0.5^{*}$	$2.1\pm0.2*$ †	$2.8 \pm 0.4 * \ddagger$		
Plasma viscosity, cP	$1.2 \pm 0.1$	$1.3 \pm 0.2$	$1.4 \pm 0.2^*$	$1.3 \pm 0.2$		
Plasma COP, mmHg	$17.6 \pm 0.7$	$16.9 \pm 0.6$	$16.6 \pm 0.8$	$16.4 \pm 0.5$		
Arteriolar						
WSR, $s^{-1}$	$736 \pm 108$	$1,040\pm213$	774±302†	845±270/1,038±311‡		
WSS, dyn/cm	$31 \pm 5$	30±6	16±6*†	$25\pm 8/30\pm 9$ ‡		
WSS <sub>micro</sub> , dyn/cm	$21 \pm 4$	$21 \pm 4$	13±5*†	$17 \pm 4/21 \pm 7$ ‡		
Venular						
WSR, $s^{-1}$	$505 \pm 126$	$647 \pm 186$	382±163*†	552±181/530±141‡		
WSS, dyn/cm	$21 \pm 5$	19±6	8±4*†	$16\pm 6/15\pm 5$ ‡		
WSS <sub>micro</sub> , dyn/cm	12±3	$11 \pm 4$	5±3*†	9±4/9±3‡		

Table 2.	Blood	rheolog	vical	properties.	WSR.	and	WSS
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Values are means  $\pm$  SD; *n*, no. of animals studied. Shear rate of 160 s<sup>-1</sup> at 37° C. Hematocrits are presented in Table 1. COP, colloid osmotic pressure at 27°C; WSR, wall shear rate; WSS, wall shear stress; WSS<sub>micro</sub>, microcirculation WSS. \**P* < 0.05 compared with baseline (nonhemodiluted animals); †*P* < 0.05 compared with *Level 2*; ‡*P* < 0.05 compared with Dex70.

fusion, which dilutes the introduced materials. leading to virtually identical colligative properties at this level of exchange (Hct 18%), regardless of the relative values of colloid osmotic pressure in the bulk solutions. This effect is also, in part, due to test materials being diluted on introduction into the circulation.

Microhemodynamics. The changes in diameter, RBC velocity, and blood flow of large feeding and small arcading arterioles (range 52–71  $\mu$ m) and small collecting venules and large venular vessels (range 50–74  $\mu$ m) were measured after each hemodilution step. Arteriolar diameter was unchanged after Level 1 exchange. On further blood exchange to Level 2, arterioles dilated to 1.09 (SD 0.12) of baseline [Level 2, number of vessels (N) = 64]. This trend reversed after Level 3 exchange with Dex70, resulting in a slight arteriolar vasoconstriction to 0.90 (SD 0.21) of baseline (Dex70; N = 20, P <0.05 to Level 2). After the Level 3 exchange with MetRBC, arteriolar diameter remained dilated to 1.15 (SD 0.19) of baseline (MetRBC; N = 24; P < 0.05 to Dex70). After methylene blue administration, arteriolar diameters were 1.09 (SD 0.18) of baseline (rRBC; N = 24; P < 0.05 to Dex70). Arteriolar microvascular tone changes are presented in Fig. 2A.

Venular changes due to the hemodilution protocol are shown in Fig. 2*B* as a function of the material infused during the *Level 3* exchange. Venules were unchanged after *Level 1* exchange. *Level 2* exchange dilated venules to 1.05 (SD 0.13) of baseline (*Level 2*, N = 68). When the exchange protocol was continued to *Level 3* by using Dex70, venules constricted to 0.89 (SD 0.15) of baseline (Dex70, N = 22, P < 0.05 to baseline and *Level 2*). *Level 3* exchange with MetRBC did not change venular diameters from baseline levels, which were 1.04 (SD 0.14) of baseline (MetRBC; N = 24; P < 0.05 to Dex70). Methylene blue did not change venular diameters, which were 1.06 (SD 0.17) of baseline (rRBC; N = 24; P < 0.05 to Dex70).

Figure 2, C and D, shows the change in RBC velocity in arterioles and venules after exchange for the different groups. The arteriolar and venular blood flows after hemodilution are presented in Fig. 2, E and F. The results are given as means (SD) to show the trend of this parameter calculated from vessel diameter and RBC velocity (absolute values are presented in

Fig. 2 legend). Arteriolar blood flows were statistically increased from baseline for *Level 2*, MetRBC, and rRBC. For the Dex70 group, both arteriolar and venular blood flows were not sustained, being statistically lower than those of baseline and other groups with higher Hct (*Level 2*, MetRBC, and rRBC). Venular flow for *Level 2*, MetRBC, and rRBC was not different from that for baseline.

*FCD.* After *Level 1* exchange, all animals showed a reduction in FCD [93% (SD 6) of baseline, P < 0.05 to baseline]. *Level 2* reduced FCD to 87% (SD 8) of baseline (P < 0.05 to baseline). FCD was further reduced for Dex70 [38% (SD 10) of baseline; P < 0.05 to baseline, *Level 2*, MetRBC, and rRBC]. FCD for MetRBC [82% (SD 6) of baseline; P < 0.05 to baseline] and rRBC [84% (SD 5) of baseline; P < 0.05 to baseline) was not different from *Level 2*. Intravenous infusion of 1 mg/kg of methylene blue did not change FCD for hemodiluted animals.

*Microvascular oxygen distribution.* Oxygen tension measured by using phosphorescence quenching microscopy after the exchange-transfusion for *Level 2*, Dex70, MetRBC, and rRBC is shown in Fig. 3. Dex70 and MetRBC yielded a lower arteriolar Po<sub>2</sub>, statistically significantly different from *Level 2*. Arteriolar Po<sub>2</sub> for Dex70 was also different from rRBC. Tissue Po<sub>2</sub> for Dex70 and MetRBC was statistically lower than for *Level 2* and rRBC but not statistically different from each other. According to previous studies in this species, normal tissue Po<sub>2</sub> is 21.7 mmHg (SD 3.5). Methylene blue treatment partially restored microvascular oxygen (arteriolar, venular, and tissue) tension to *Level 2*.

*Microvascular oxygen delivery and extraction.* Figure 4 shows the result of the analysis of oxygen delivery and release in the microcirculation. It is evident that exchanging RBCs with MetRBC decreased oxygen-carrying capacity to levels similar to Dex70. Oxygen delivery and extraction was increased for MetRBC compared with Dex70, because of the better perfusion. The contribution of reduced MetRBC by methylene blue in the rRBC group restored oxygen delivery to a similar degree as *Level 2*. However, *Level 2*, MetRBC, and rRBC apparently had similar levels of oxygen extraction in the microcirculation. Conversely, Dex70 reduced oxygen delivery



Fig. 2. Relative changes to baseline in arteriolar and venular hemodynamics for *Level 2*, Dex70, MetRBC, and rRBC. Dotted horizontal line represents baseline level.  $\dagger P < 0.05$  relative to baseline;  $\star P < 0.05$  compared with *Level 2*. Diameters [in  $\mu$ m, and means (SD)] in *A* (arteriolar) and *B* (venular) for each animal group were as follows: baseline [arterioles, 59.1 (SD 6.2), n = 64; venules, 61.6 (SD 9.5), n = 68]; *Level 2* [arterioles, 63.5 (SD 10.5); venules, 62.8 (SD 9.4); Dex70 [arterioles, 55.3 (SD 13.7), n = 20; venules, 53.2 (SD 11.0), n = 22]; MetRBC [arterioles, 68.3 (SD 12.1), n = 24; venules, 64.7 (SD 11.5), n = 24; rRBC [arterioles, 65.3 (SD 11.1), n = 24; venules, 64.1 (SD 10.6), n = 22]; *n*, number of vessels studied. RBC velocities [in mm/s, means (SD)] in *C* (arteriolar) and *D* (venular) for each animal group were as follows: baseline [arterioles, 4.8 (SD 1.0); venules, 2.4 (SD 0.6); *Level 2* [arterioles, 6.7.1 (SD 1.7); venules, 3.5 (SD 1.0); Dex70 (arterioles, 4.1 (SD 1.5); venules, 1.7 (SD 0.8)]; MetRBC [arterioles, 6.0 (SD 1.9); venules, 2.7 (SD 1.0)]; rRBC [arterioles, 6.8 (SD 2.0); venules, 2.8 (SD 1.1)].

by about one-fifth and extraction by the tissue by about one-third of *Level 2*, respectively.

*Wall shear rate.* The calculated WSR and WSS for arterioles and venules using measured viscosity and microcirculation apparent viscosity are shown in Table 2. Tabulated results from the present study show what appears to be a threshold of WSR as well as the corresponding WSS that is required to sustain microvascular perfusion, FCD, and oxygen extraction.

*Systemic and microvascular effects of methylene blue.* Four animals were used to study the effects of methylene blue. Methylene blue infused intravenously (1 mg/kg) did not change any hemodynamic or microvascular parameters in non-hemodiluted animals. Table 3 summarizes the parameters before infusion and 5 and 30 min after infusion of methylene blue.

## DISCUSSION

The principal finding of this study is that extending hemodilution from 18% Hct by exchange transfusion of methemoglobin-inactivated RBCs, reducing functional oxygen-carrying capacity to 11% Hct, sustained systemic and microvascular conditions better than hemodilution with 6% Dex70 to 11% Hct. Blood consisting of a mixture of normal and inactivated RBCs with a concentration of Hb capable of binding oxygen of 3.1 g/dl sustained MAP, FCD, and microvascular perfusion but did not maintain tissue oxygenation as a consequence of the limited carrying capacity. Conversely, hemodilution using only 6% Dex70 to Hct 11% had Hb of 3.7 g/dl, with significantly lower MAP, microvascular flow, and FCD, in addition to a negative base excess and compromised tissue oxygenation. Microvascular flow and MAP from hemodilution to 18% were maintained by an additional exchange transfusion with methemoglobin-inactivated RBCs suspended in fresh-frozen plasma, which did not carry oxygen. When methemoglobin in RBCs was reduced to oxyhemoglobin, oxygenation conditions were partially restored to hemodilution to 18% Hct.

Systemic hemodynamic parameters were the same for all conditions with an 18% Hct, independent of the significant difference in oxygen-carrying capacity. An Hct of 18% with 32% of the Hb inactivated reduced oxygen delivery and extraction, decreasing tissue and venular oxygen tension. Oxygen tensions in the microcirculation, systemic, and microvascular conditions were similar for the group with only two steps of dilution (*Level 2*) compared with the group exchange transfused with methemoglobin RBCs after reduction to oxyhemoglobin by methylene blue (rRBC).



Fig. 3. Intravascular partial oxygen pressure after *Level 3* exchange hemodilution with test materials. Po<sub>2</sub> (mmHg, means (SD) in each animal group was as follows: *Level 2* [arterioles, 49.7 (SD 6.8), n = 20; venules, 28.2 (SD 4.8), n = 22; tissue, 17.9 (SD 2.7), n = 20]; Dex70 [arterioles, 32.9 (SD 3.9), n = 20]; venules, 3.8 (SD 1.2), n = 22; tissue, 1.8 (SD 1.1), n = 20]; MetRBC [arterioles, 37.9 (SD 4.1), n = 24; venules, 5.4 (SD 2.9), n = 24; tissue, 3.9 (SD 5.1), n = 24; venules, 14.9 (SD 3.9), n = 24; tissue, 14.7 (SD 2.9), n = 20]; n, number of vessels or locations studied.  $\star P < 0.05$  compared with *Level 2*.

Maintenance of both systemic and microvascular function is assured up to Hb levels that are significantly lower than those proposed by accepted transfusion triggers, if blood viscosity is maintained above specific levels. This suggests that the current transfusion triggers may indicate the need for restoring blood viscosity earlier than needed for restoring oxygen-carrying capacity during hemodilution or anemic conditions. In this context, we propose that the present transfusion triggers could be, in part, due to blood viscosity triggers. The significant subjective improvement usually experienced in anemia after a blood transfusion may be partially due to the increase in blood viscosity and the production of shear stress-mediated endothelial factors (35). The results of the study support the hypothesis that a reduction in oxygen-carrying capacity may not be the only determining factor in setting the threshold for the transfusion trigger.

Lowering blood viscosity has been considered to be beneficial and presently embodied in the practice of hemodilution. The present results showed that the concept of a beneficial effect due to lowered blood viscosity in transfusion is limited to a certain extent. A minimum level of blood viscosity appears to be necessary for generating shear stress and stimulating the release of vasoregulatory factors such as nitric oxide and prostacyclin (35). These effects have been demonstrated in acute models of extreme hemodilution (4, 6, 35, 39) and shock resuscitation (3). This assertion is supported by our findings that essentially the same level of oxygen-carrying capacity during extreme hemodilution with low blood viscosity (Dex70) leads to a significantly depressed systemic and microvascular outcome when compared with a sustained viscosity condition attained with inactivated RBCs (at the same overall level of oxygen-carrying capacity).

The lack of morbidity due to the maintenance of moderately elevated blood viscosity has been addressed in several studies. Chen et al. (7) maintained plasma viscosity fourfold (4 cP) and found the reduction of vascular hindrance (vascular resistance independent of viscosity) in vital organs. Our laboratory (6, 39) showed that the reduction of FCD found after decreasing systemic Hct to 18% of baseline with low-viscosity plasma expanders was not decreased further by continuing hemodilution with high-viscosity plasma expanders. Waschke et al. (43) found that cerebral perfusion was not changed when blood was replaced with fluids with the same oxygen-carrying capacity and viscosities varying from 1.4 to 7.7 cP. Krieter et al. (21) increased plasma viscosity using dextran 500 kDa and found that medians in tissue  $Po_2$  in skeletal muscle were maximal at 3 cP of plasma viscosity and that there were no changes in tissue oxygenation and organ perfusion when blood was he-



Fig. 4. Arterial oxygen delivery and extraction after hemodilution.  $\star P < 0.05$  compared with *Level 2*. Calculations of global oxygen transport are not directly measurable in our model. However, changes relative to baseline can be calculated using the measured parameters. Extraction was calculated as the difference of averaged arterioles and venules for each animal. Difference in oxygen delivery and extraction between *Level 2* and rRBC is not statistically significant.

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Table	3.	Systemic	and	microvascular	effects
of met	thyl	ene blue			

		After I	nfusion
	Baseline	5 min	30 min
Systemic			
MAP, mmHg	$106 \pm 6$	$108 \pm 8$	$104 \pm 7$
HR, beats/min	436±31	$452 \pm 38$	$442 \pm 29$
Microvascular			
Arteriolar diameter, µm	$57 \pm 12$	$59 \pm 13$	$59 \pm 11$
(relative to baseline)		$(1.04 \pm 0.08)$	$(1.02 \pm 0.07)$
Venular diameter, µm	$64 \pm 10$	63±12	$65 \pm 14$
(relative to baseline)		$(0.97 \pm 0.06)$	$(0.99 \pm 0.07)$

Values are means  $\pm$  SD; Four animals were studied to measure systemic parameters; 22 arterioles and 24 venules were used to measure microvascular parameters. Infusion of 1 mg/kg of methylene blue did not induce any significant differences from baseline values.

modiluted in these conditions. Doss et al. (12) found that endogenous nitric oxide release reduced total peripheral resistance during moderate hemodilution. de Wit et al. (10) found that an elevation in plasma viscosity caused sustained nitric oxide-mediated dilatation in the hamster muscle microcirculation. Our study amply supports these findings (35).

The present study demonstrates the beneficial effects resulting from maintaining blood viscosity during anemia. However, this effect may be present at normal Hct levels, as shown by Martini et al. (28), who elevated blood viscosity by the acute transfusion of packed RBCs. In these experiments, the increase of Hct in the range of 8-13% lowered blood pressure by  $\sim 10$ mmHg. This phenomenon was shown to be dependent on changes in shear stress-mediated NO production. Consequently, maintenance of blood viscosity should also promote the beneficial effects associated with the maintenance of nitric oxide and prostaglandin levels in the circulation.

In this study, we used methylene blue to cause RBC reduction of methemoglobin, a process first reported by Smith and Beutler (32) and lately reviewed by Clifton and Leikin (8). Methylene blue infusion is the first-line therapy for methemoglobinemia. Its action depends on the availability of reduced NADPH within the RBCs. After an acute exposure to an oxidizing agent, fresh RBCs respond very efficiently to the methylene blue-accelerated reduction. Only methemoglobin produced by hemoglobin M, abnormal Hb that facilitates oxidation, does not respond to ascorbic acid or methylene blue. Direct evidence that this reaction takes place in vivo is apparent from the increase in oxygen delivery and tissue Po<sub>2</sub> occurring 15 to 30 min on introduction of methylene blue.

The increase in tissue oxygen, venular Po<sub>2</sub>, and microvascular oxygen delivery after the reduction of methemoglobin to oxyhemoglobin with methylene blue was due to an increase in the oxygen-carrying capacity of the blood. However, the possibility of methylene blue altering microvascular tone primarily through inhibition of nitric oxide needs to be addressed. The issue is whether in our experiments, administration of methylene blue to hamsters with 32% methemoglobin could affect microvascular resistance (arteriolar diameter was reduced 30 min after infusion, but this was not statistically significant; P =0.14) through decreases in nitric oxide-mediated vasodilation rather than through a physiological response to the concomitant increase in oxygen-carrying capacity. Whereas intravenous

methylene blue is highly effective in converting methemoglobin to oxyhemoglobin and is used for this purpose clinically, methylene blue could partially inhibit guanylate cyclase in the in vitro vascular segments preparations and in the in vivo models (24). Inhibition of guanylate cyclase prevents nitric oxide signaling (23, 31).

A direct effect on nitric oxide availability sufficiency to increase systemic or microcirculation vascular resistance substantively appears to be unlikely in the actual experiments. First, to specifically address this issue, four hamsters nonhemodiluted and without methemoglobin in circulation were treated with intravenous infusion of methylene blue (1 mg/kg). There was not consistent increase in systemic vascular resistance or microvascular tone in these hamsters when given the same dose of methylene blue and observed over the same time interval as the hamsters with 32% methemoglobin (30-45 min). Therefore, it would seem that the small rise in systemic vascular resistance could be related to the reversal of methemoglobin to oxyhemoglobin and not to a direct effect on endothelial nitric oxide through inhibition of guanylate cyclase. Second, methylene blue is rapidly metabolized and has a short half-life in blood. In one report, the plasma half-time of a 2 mg/kg iv dose of methylene blue was <8 min (11). In clinical settings, methylene blue is administered for shock treatment, and it is given as a prolonged intravenous infusion over at least 30 min, not as a bolus infusion (45). In the actual study, a dose of 1 mg/kg of methylene blue was intravenously given over 1-2 min, followed by a wait of 30-45 min before assessment of parameters, which very likely avoided any substantive vascular effect.

Finally, even with a sustained dose, the effects of methylene blue on shear-mediated factors (nitric oxide, prostacyclin, and endothelin-1) have been inconsistent. It has been described to inhibit NO release (29), to increase the release of endothelin-1 (22), and to enhance the production of flow-induced prostacyclin (30). However, methylene blue does not enter into the cells unless membranes are permeabilized (20). The effects due to prolonged intravenous infusions of methylene blue on systemic vascular resistance have been contradictory. For example, Loeb and Longnecker (27) reported that an intravenous administration of methylene blue in intact, anesthetized rats decreased systemic vascular resistance, whereas, in contrast, administration of N-monomethyl-L-arginine increased systemic vascular resistance. Evgenov et al. (15) observed no effects of infusions of methylene blue alone on hemodynamics in lambs, although infusions of methylene blue did dampen endotoxininduced vasodilation. Zhang et al. (45) studied anesthetized mongrel dogs during endotoxic shock, in which a 2.5 mg/kg dose of methylene blue resulted in inconsequential increases in systemic vascular resistance and decreases in cardiac output of <5%. Thus we believe that the minor hemodynamic changes noted postreduction of the methemoglobin to oxyhemoglobin are not likely to be due to effects of methylene blue and are most likely due to the increase in the oxygen carrying capacity.

In conclusion, microvascular perfusion and systemic conditions can be extended from 18% Hct (6 g Hb/dl) to a reduced oxygen-carrying capacity situation postexchange with methemoglobin-loaded RBCs, which decreases oxygen capacity by 32% to an equivalent functional Hct of 11% by means of maintaining blood rheological properties. This effect was not attained at 11% Hct (Hb of 3.7 g/dl), when the hemodilution from Hct of 18% was continued with 6% Dex70. Blood viscosity is the basic difference between the two conditions, without large differences in the oxygen-carrying capacity due to methemoglobin-inactivated RBCs in one case. These findings lead to the conclusion that there is a range of hemodilution where viscosity maintenance is a primary factor for ensuring compensatory adjustments of physiological conditions. The primary concept of sustaining or maintain blood viscosity before restoration of oxygen-carrying capacity during normovolemic circumstances could be important to lower the presently used blood transfusion trigger, if blood viscosity could be moderately preserved by other means.

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#### DISCLOSURES

M. Intaglietta is a member of the Board of the La Jolla Bioengineering Institute.

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