Modulation of Perfusion and Oxygenation by Red Blood Cell Oxygen Affinity during Acute Anemia

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Responses to exchange transfusion using red blood cells (RBCs) with modified hemoglobin (Hb) oxygen (O_2) affinity were studied in the hamster window chamber model during acute anemia to determine its role on microvascular perfusion and tissue oxygenation. Allosteric effectors were introduced in the RBCs by electroporation. Inositol hexaphosphate (IHP) and 5-hydroxymethyl-2-furfural (5HMF) were used to decrease and increase Hb- O_2 affinity. *In vitro* P50s (partial pressure of O_2 at 50% Hb saturation) were modified to 10, 25, 45, and 50 mm Hg (normal P50 is 32 mm Hg). Allosteric effectors also decreased the Hill coefficient. Anemic condition was induced by isovolemic hemodilution exchanges using 6% dextran 70 kD to 18% hematocrit (Hct). Modified RBCs (at 18% Hct in 5% albumin solution) were infused by exchange transfusion of 35% of blood volume. Systemic parameters, microvascular perfusion, capillary perfusion (functional capillary density, FCD), and microvascular PO_2 levels were measured. RBCs with P50 of 45 mm Hg increased tissue PO_2 and decreased O_2 delivery (DO_2) and extraction (VO_2) and RBCs with P50 of 60 mmHg reduced FCD, microvascular flow, tissue PO_2 , DO_2 and VO_2 . Erythrocytes with increased Hb- O_2 affinity maintained hemodynamic conditions, DO_2 and decreased tissue PO_2 . This study shows that in an anemic condition, maximal tissue PO_2 does not correspond to maximal DO_2 and VO_2 .

Keywords: microcirculation; hemodilution; functional capillary density; tissue oxygen; hemoglobin allosteric effectors

Red blood cells (RBCs) contain a high concentration of hemoglobin (Hb), which binds reversibly with O_2 . The amount of O_2 released in the periphery depends not only on the quantity of the circulating RBC, but also on the O_2 affinity of the intracellular Hb determined by allosteric factors. O_2 partial pressure (PO_2) in the lungs is the highest, whereas that of the tissue capillaries the lowest, depending on the organ it ranges from 15 to 30 mm Hg (1). Under physiologic conditions, only about 25% of the oxygenated Hb will be deoxygenated while 75% recirculates, returning to the lungs. Thus, a major fraction of the O_2 present in blood does participate in the processes of O_2 metabolism.

Interactions of Hb with allosteric effectors provide for maximal O_2 release while simultaneously maintaining the highest possible O_2 partial pressure in the microvascular system. Shifting O_2 release to higher O_2 partial pressures (so-called right-shifted) would theoretically facilitate O_2 release in the microcirculation, improving O_2 supply to the tissues. The consequence of a substantial change of the *in vivo* P50 (PO_2 at 50% Hb saturation) is not well documented due to the difficulty in obtaining substantial and long-term low O_2 affinity. Few methods have been reported to raise P50 *in vivo*, where the induced P50 increases were always modest and/or sustained for only a short time. Teisseire and coworkers

CLINICAL RELEVANCE

This study shows that the criterion for an optimal blood Hb-oxygen affinity for exchange transfused blood is not unique and requires differentiating between microvascular function and tissue oxygen tension.

reported a method for increasing P50 in anesthetized piglets using inositol hexaphosphate (InsP6) (2, 3). InsP6 is an effective Hb allosteric effector (4, 5); however, since it cannot diffuse through the erythrocytic membrane, it has to be introduced into erythrocytes using a reverse osmotic lysis process or electroporation (6, 7).

Experimental evidence in isolated canine muscle and humans supports that the rate at which O_2 diffuses from the erythrocyte to the tissue determines maximal O_2 uptake (8, 9). The O_2 diffusion can be divided in the O_2 conductance within the tissue and release from the erythrocyte Hb to the tissue (8). For a given O_2 supply (flow $\times$ arterial O_2 content), the O_2 extracted by tissue according to Fick's law is determined by the O_2 diffusing capacity and the PO_2 gradient between the blood and the tissue. If the O_2 conductance is held constant, Hb- O_2 affinity should regulate the rate at which the O_2 is released. Therefore, O_2 released could be increased when changes in Hb- O_2 affinity create the most advantageous arteriolar-to-tissue PO_2 driving gradient. Complete direct assessment of microvascular hemodynamic and O_2 transport has not been performed in a concurrent manner before. The study of Stein and Ellsworth suggest that an increase in Hb- O_2 affinity does not affect the amount of O_2 delivered across the capillary network. They found that blood O_2 content was higher in the high-affinity group at control and hypoxia. However, their experimental conditions did not account for hemodynamics; therefore, convective O_2 transport was not taken in to consideration (10).

This study addresses whether Hb- O_2 affinity leads to changes in tissue O_2 release by regulating the arterial/interstitial tissue PO_2 gradient. It was carried out to determine systemic and microvascular conditions, after transfusion of RBCs with modified Hb- O_2 affinity in an acute anemic state. Moderated isovolemic hemodilution was used to amplify the effects of altering blood O_2 transport properties. The hamster window chamber model was subjected to moderated hemodilution via two isovolemic exchanges with 6% dextran 70 kD to induce an acute anemic state (18% hematocrit [Hct]). After anemia, animals were further exchange transfused with Hb- O_2 affinity modified RBCs (P50s of 10, 25, 32, 45, 60, mm Hg) in such a fashion that 1/3 of the circulating RBCs had modified P50s. In each case, Hct remained at approximately 18% to maintain the same anemic state. Changes in microvascular function were characterized in terms of effects on capillary flow and tissue oxygenation.

MATERIALS AND METHODS

Animal Preparation

Investigations were performed in 55- to 65-g male Golden Syrian Hamsters (Charles River Laboratories, Boston, MA) fitted with a dorsal

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skinfold window chamber. The hamster window chamber model is widely used for microvascular studies in the unanesthetized state, and the complete surgical technique is described in detail elsewhere (11, 12). Arterial and venous catheters filled with a heparinized saline solution (30 IU/ml) were implanted in to the carotid and jugular vessels. Catheters were tunneled under the skin, exteriorized at the dorsal side of the neck, and securely attached to the window frame. Animal handling and care followed the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the local animal care committee.

Inclusion Criteria

The microvasculature was examined 3 to 4 days after the window implantation surgery, and only animals passing an established systemic and microcirculatory inclusion criteria were included in the study. Animals were suitable for the experiments if: (1) systemic parameters were within normal range, namely, heart rate (HR) greater than 340 beats/minute, mean arterial blood pressure (MAP) greater than 80 mm Hg, systemic Hct greater than > 45%, and arterial O₂ partial pressure (PaO₂) greater than 50 mm Hg; and (2) microscopic examination of the tissue in the chamber observed under a $\times 650$ magnification did not reveal signs of low perfusion, inflammation, edema, or bleeding. Hamsters are a fossorial species with a lower arterial Po₂; however, their microvascular Po₂ distribution in the chamber window model is similar to that of other rodents (13).

Erythrocyte Preparation

Male retired breeder Golden Syrian Hamsters (donor), weighing 120 to 150 g, were anesthetized with sodium pentobarbital and exsanguinated via a carotid catheter over a period of 5 minutes into a vacutainer tube (purple-stoppered) containing 100 μ l of 15% EDTA. Red cells were washed three times by centrifugation in phosphate-buffered saline (PBS, 0.01 M, pH 7.4, Na₂HPO₄ 0.109 g/dl; NaH₂PO₄ 0.032 g/dl; NaCl 0.9 g/dl) and the buffy coat was removed each time.

Electroporation was used for encapsulation of allosteric effectors into RBCs. The pulse generator used for RBC electroporation was a BTX T100 (Biotechnologies and Experimental Research, San Diego, CA). This device delivers an exponentially decaying pulse, whose voltage and duration can be monitored by a pulse checker (BTX). The electroporation chamber used for the encapsulation of the allosteric effectors was a cuvette with parallel stainless steel electrodes with a 1.9-mm gap. Erythrocytes were suspended at 50% Hct in a normotonic saline solution of allosteric effectors, inositol hexaphosphate (IHP, Na salt) or 5-hydroxymethyl-2-furfural (5HMF) (Sigma, St. Louis, MO). Concentrations of IHP were 20 mM and 5 mM, and for 5HMF they were 20 mM and 5 mM. Allosteric effectors solutions were neutralized to pH 7.4 using hydrochloric acid, before mixing with RBCs. The RBC suspension in the electroporation cuvette was subjected to pulses of 3.5 kV/cm and 3 minutes at 4°C. After 5 minutes of incubation at 4°C, resettling of the cells was performed by incubation at 37°C for 1 hour (6, 7). The erythrocytes were then washed twice with PBS with 0.5% human serum albumin at 37°C for 30 minutes.

Hemoglobin Oxygen Saturation

O₂ equilibrium curves for RBCs were measured by deoxygenation of O₂-equilibrated samples in the Hemox buffer at 37.6°C, using a Hemox Analyzer (TCS Scientific Corporation, New Hope, PA) (13).

Acute Isovolemic Exchange Transfusion (Hemodilution) Protocol

Acute anemia was induced by two isovolemic hemodilution steps. This protocol was described in detail in our previous reports (14–16). Briefly, the volume of each exchange-transfusion step was calculated as a percentage of the blood volume (BV), estimated as 7% of body weight (BW). The acute anemic state was induced by lowering systemic Hct to 18% by two steps of progressive isovolemic hemodilution using 6% dextran 70 (70 kD, mean molecular weight; Pharmacia, Uppsala, Sweden). First exchange was 40% of BV and second exchange was 35% of BV, respectively, producing a moderate hemodilution state.

Moderate hemodilution animals were randomly divided into five experimental groups by assigning each animal to an experimental group

according to a sorting scheme based on a list of random numbers (17). Exchange transfusion protocol was continued by exchanging 35% of the BV using RBCs with modified Hb-O₂ affinity (10, 25, 32, 45, and 60 mm Hg). RBCs were exchanged using the respective RBCs suspended in 5% albumin at Hb of 6 g/dl ($\sim$ 18% Hct). The experimental groups were labeled E10, E25, E32, E45, and E60.

Blood was simultaneously withdrawn at the same rate from the carotid artery catheter according to a previously established protocol (15, 18). Blood samples were withdrawn at the end of the experiment for subsequent analysis of viscosity, colloid osmotic pressure (COP), and Hb-O₂ affinity. The duration of the experiments was 4 hours. Each exchange and the respective observation time point after exchange were completed in 1 hour. Systemic and microcirculation data was taken after a stabilization period of 10 minutes.

Systemic Parameters

MAP and heart rate (HR) were recorded continuously (MP 150; Biopac System, Santa Barbara, CA). Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes. Hb content was determined spectrophotometrically (B-Hemoglobin; Hemocue, Stockholm, Sweden).

Blood Chemistry and Biophysical Properties

Arterial blood was collected in heparinized glass capillaries (50 μ l) and immediately analyzed for PaO₂, PaCO₂, base excess (BEa), and pHa (Blood Chemistry Analyzer 248; Bayer, Norwood, MA). Blood samples for viscosity and COP measurements were quickly withdrawn into heparinized 5-ml syringes at the end of the experiment. Viscosity was measured in a DV-II plus (Brookfield, Middleboro, MA). COP was measured using a 4420 Colloid Osmometer (Wescor, Logan, UT).

Microvascular Experimental Setup

The unanesthetized animal was placed in a restraining tube with a longitudinal slit from which the window chamber protruded, then fixed to the microscopic stage for transillumination with the intravital microscope (BX51WI; Olympus, New Hyde Park, NY). Animals were given 20 minutes to adjust to the tube environment before any measurement. 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 $\times 40$ (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.

Functional Capillary Density

Functional capillaries, defined as those capillary segments that have RBC transit of at least a single RBC in a 45-second period in 10 successive microscopic fields, were assessed, totaling a region of 0.46 mm². The relative change in functional capillary density (FCD) from baseline levels after each intervention is indicative of the extent of capillary perfusion (15).

Microhemodynamics

Arteriolar and venular blood flow velocities were measured online by using the photodiode cross-correlation method (Photo Diode/Velocity Tracker Model 102B; Vista Electronics, San Diego, CA). The measured centerline velocity (V) was corrected according to vessel size to obtain the mean RBC velocity. A video image-shearing method was used to measure vessel diameter (D) (19). Blood flow (Q) was calculated from the measured values as $Q = \pi \times V (D/2)^2$. This calculation assumes a parabolic velocity profile and has been found to be applicable to tubes of 15 to 80 μ m internal diameters and for Hcts in the range of 6 to 60% (20).

Microvascular Po₂ Distribution

High-resolution noninvasive microvascular Po₂ measurements were made using phosphorescence-quenching microscopy (PQM) (16). PQM is based on the O₂-dependent quenching of phosphorescence emitted by albumin-bound metalloporphyrin complex after pulsed light excitation. PQM is independent of the dye concentration within the tissue and is well suited for detecting hypoxia because its decay time is

inversely proportional to the Po_2 level, causing the method to be more precise at low Po_2 s. This technique is used to measure both intravascular and extravascular Po_2 since the albumin-dye complex continuously extravasates from the circulation into the interstitial tissue (16). Tissue Po_2 was measured in tissue regions in between functional capillaries. PQM allows for precise localization of the Po_2 measurements, without subjecting the tissue to injury. These measurements provide a detailed understanding of microvascular O_2 distribution and indicate whether O_2 is delivered to the interstitial areas.

Oxygen Delivery and Extraction

The microvascular methodology used in our studies allows a detailed analysis of O_2 supply in the tissue. Calculations are made using Equation 1, O_2 delivery (DO_2), and Equation 2, O_2 extraction (VO_2) (15).

$$\text{DO}_2 = [(\text{RBC}_{\text{Hb}} \times \gamma \times \text{S}_A \%) + (1 - \text{Hct}) \times \alpha \times \text{Po}_{2A}] \times Q \quad (1)$$

$$\text{VO}_2 = [(\text{RBC}_{\text{Hb}} \times \gamma \times \text{S}_{A-V} \%) + (1 - \text{Hct}) \times \alpha \times \text{Po}_{2A-V}] \times Q, \quad (2)$$

where RBC_{Hb} is the hemoglobin in RBCs [$\text{g}_{\text{Hb}}/\text{dl}_{\text{blood}}$], γ is the O_2 carrying capacity of saturated hemoglobin [$1.34 \text{ ml O}_2/\text{g}_{\text{Hb}}$], $\text{S}_A \%$ is the arteriolar O_2 saturation, $(1 - \text{Hct})$ is the fractional plasma volume [$\text{dl}_{\text{plasma}}/\text{dl}_{\text{blood}}$], α is the solubility of O_2 in plasma [$3.14 \times 10^{-3} \text{ ml O}_2/\text{dl}_{\text{plasma}} \text{ mm Hg}$], Po_{2A} is the arteriolar Po_2 , $A-V$ indicates the arteriolar/venular differences, and Q is the microvascular flow. O_2 saturations were measured as described above from samples obtained at the end of the experiment.

Data Analysis

Tabular results are presented as mean $\pm$ standard deviation. The *box-whisker plot* separates 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 *upper whisker* defines the 95th percentile, the *lower whisker* the 5th percentile. Data within each group were analyzed using analysis of variance for repeated measurements (ANOVA, Kruskal-Wallis test). When appropriate, *post hoc* analyses were performed with the Dunn's multiple comparison test. Microhemodynamic data are presented as absolute values and ratios relative to baseline values. A ratio of 1.0 signifies no change from baseline, while 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 using GraphPad Prism 4.01 (GraphPad Software, Inc., San Diego, CA). Changes were considered statistically significant if $P < 0.05$.

RESULTS

Twenty-five animals were entered into the hemodilution microcirculation study. Eight animals were used as blood donors. All animals tolerated the entire hemodilution protocol without visible signs of discomfort. The animals were assigned randomly to the experimental groups: E10 ($n = 5$), E25 ($n = 5$), E32 ($n = 5$), E45 ($n = 5$), and E60 ($n = 5$). All groups used were statistically similar ($P > 0.20$) in systemic and microcirculation parameters at baseline and moderate hemodilution with dextran 70. Systemic and microhemodynamic datasets for baseline and moderate hemodilution (MH) were obtained by combining data from all experimental groups. Blood typing and cross-matching tests are not necessary with hamsters, based on previous experience.

RBC Oxygen Affinity

The O_2 dissociation properties of the modified erythrocytes used and the blood after exchange are presented in Table 2 and Figure 1. Introduction of allosteric effectors changed P50 of RBCs to 10 and 25 mm Hg for 5HMF and to 45 and 60 mm Hg for InsP6. Cells exposed only to electroporation maintained a similar P50 than the native RBCs (32 mm Hg). The amount of modified Hb- O_2 affinity RBCs transfused was estimated by comparing the actual P50 of the circulating blood to the P50 of each of the cell populations, measured independently. The result of this calculation is given in Table 2, showing that approximately 1/3 of modified RBCs were in circulation.

Systemic Parameters

The first two steps of the exchange transfusion protocol leading to MH with dextran 70 reduced Hct to $29.6 \pm 2.0\%$ and $18.6 \pm 0.6\%$, respectively. Hct and Hb after the exchange transfusion protocol are given in Table 1. Hct after exchange transfusion remained stable until the end of the experiment. Plasma Hb was not detected in transfused groups at any time (0.1 g/dl detection limit of the instrument). These observations indicate that the modified cells appeared to circulate unhindered, and independently of the electroporation procedure.

TABLE 1. SYSTEMIC PARAMETERS DURING EXCHANGE PROTOCOL

	Exchange Transfuse						
	Baseline	MH	E10	E25	E32	E45	E60
n	25	25	5	5	5	5	5
Hct, %	48.8 ± 0.9	18.6 ± 0.6*	18.2 ± 0.9	18.7 ± 0.6	18.5 ± 0.7	18.6 ± 0.6	18.1 ± 0.5
Hb, g/dl	14.7 ± 0.5	5.9 ± 0.5*	5.5 ± 0.7	5.7 ± 0.5	5.7 ± 0.4	5.5 ± 0.5	5.3 ± 0.4
MAP, mm Hg	108 ± 6	94 ± 6*	88 ± 7*	85 ± 7*	86 ± 8*	87 ± 6*	92 ± 5*
HR, bpm	447 ± 27	432 ± 28	438 ± 31	442 ± 22	431 ± 28	452 ± 18	401 ± 24
PaO ₂ , mm Hg	57.9 ± 5.2	78.0 ± 6.4*	84.3 ± 6.9*	83.3 ± 7.2*	85.8 ± 7.5*	88.1 ± 6.6*	92.7 ± 6.8*†
PaCO ₂ , mm Hg	54.1 ± 5.6	48.9 ± 5.2	43.3 ± 5.8	44.0 ± 6.2	42.9 ± 6.6	41.3 ± 7.4*	39.7 ± 5.5*†
pH _a	7.348 ± 0.016	7.356 ± 0.024	7.364 ± 0.018	7.352 ± 0.021	7.361 ± 0.026	7.374 ± 0.027	7.337 ± 0.029
BE _a , mmol	3.3 ± 1.4	1.4 ± 1.1	0.9 ± 1.5	1.1 ± 1.6	0.7 ± 1.4	0.5 ± 1.6*	-0.6 ± 1.6*
Blood V., cp	4.2 ± 0.2		2.8 ± 0.2	2.9 ± 0.2	2.9 ± 0.2	2.8 ± 0.2	2.8 ± 0.2
Plasm V., cp	1.2 ± 0.1		1.3 ± 0.2	1.3 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	1.3 ± 0.2
COP, mm Hg	17.6 ± 0.5		18 ± 1	17 ± 1	18 ± 1	17 ± 2	17 ± 1

Definition of abbreviations: BE_a, arterial base excess; COP, colloid osmotic pressure; Hb, hemoglobin content of blood; Hct, systemic hematocrit; HR, heart rate; MAP, mean arterial blood pressure; MH, moderate hemodilution; PaCO_2 , arterial partial pressure of CO_2 ; PaO_2 , arterial partial O_2 pressure.

* $P < 0.05$ compared with baseline

† $P < 0.05$ compared with MH.

Values are means $\pm$ SD. Baseline included all the animals in the study. No significant differences were detected between the baseline, first exchanged with dextran 70 and MH values of each group.

TABLE 2. BLOOD OXYGEN AFFINITY

Group	Exchange Blood		Blood after Exchange		Exchange %
	P50 (mm Hg)	n	P50 (mm Hg)	n	
E10	11.5 ± 0.5	1.38 ± 0.08	24.5 ± 0.8	2.08 ± 0.11	37.0%
E25	26.6 ± 0.4	1.59 ± 0.09	30.1 ± 0.6	2.16 ± 0.07	36.4%
E32	32.2 ± 0.3	2.88 ± 0.05	32.2 ± 0.2	2.88 ± 0.04	—
E45	45.2 ± 0.6	2.70 ± 0.06	36.8 ± 0.3	2.72 ± 0.05	35.5%
E60	60.1 ± 0.5	2.32 ± 0.05	41.0 ± 0.4	2.49 ± 0.05	31.4%

Definition of abbreviations: n, Hill coefficient; P50, half the binding sites saturated with oxygen.

The amount of modified Hb-O₂ affinity red blood cells transfused was estimated by comparing the actual P50 of the circulating blood to the P50 of each of the cell populations, measured independently.

MAP was not changed from baseline (108 ± 6 mm Hg) after first hemodilution exchange (98 ± 12 mm Hg). MH decreased MAP to 94 ± 6 mm Hg. Exchange transfusion lowered MAP for all groups when compared to baseline, but not different from MH, as shown in Table 1. Systemic arterial blood gas analysis showed a statistically significant rise in arterial Po₂ from baseline for all groups compared to baseline. Arterial PCO₂ decreased significantly from baseline for E45 and E60. Among hemodiluted groups, arterial Po₂ and PCO₂ were significantly different for E60 compared to MH, other groups did not present any difference compared to MH. Arterial pH was not statistically changed from baseline or MH for any of the exchange transfused groups. Blood base excess (BE) was statistically significantly decreased from baseline for E45 and E60, E60 was also different from MH (Table 1).

Blood Biophysical Properties after Exchange

Blood viscosity, plasma viscosity and plasma COP after hemodilution for all the groups are presented in Table 1. Blood viscosities were lower than baseline for all exchange transfused groups and no differences among groups were measured, further supporting the contention that all transfused cells had similar circulatory behavior, independently of the change in P50 induced. Plasma viscosity did not change from baseline. Plasma COP did not change after exchange transfusion.

Microhemodynamics

The changes in diameter, RBC velocity, and blood flow were measured after each hemodilution step for (I) large feeding and

small arcading arterioles (range 47–78 μm) and (2) small collecting and large venular vessels (range 45–81 μm). Upon further blood exchange, arterioles dilated to 1.06 ± 0.10 of baseline (MH, n = 122; n, number of vessels). Exchange transfusion with low Hb-O₂ affinity RBCs (E60), resulted in a slight arteriolar vasoconstriction to 0.91 ± 0.06 of baseline (n = 25, P < 0.05 to baseline and to all the other groups). Exchange with left-shifted RBCs (E10 and E25) produced significant arteriolar vasodilation compared with baseline. Arteriolar diameter remained unchanged, compared with baseline for E32 and E45. Arteriolar microvascular tone changes are presented in Figure 2A; absolute diameters and relative changes to baseline are given in the figure legend.

Venular changes due to the hemodilution protocol are shown in Figure 2B and relative changes to baseline are given in the Figure 2 legend. MH dilated venules to 1.06 ± 0.08 of baseline (n = 138). Exchange transfusion with high-affinity RBCs (E10) produced venular vasodilation, compared with baseline. Venular diameter in the other transfused groups (E25, E32, E45, and E60) did not change from baseline.

The arteriolar and venular blood flows, after exchange transfusions, are presented in Figures 2C and 2D and absolute values are given in Figure 2 legend. Arteriolar and venular blood flows, before exchange transfusion (MH) were statistically increased from baseline. Arteriolar and venular blood flows, after exchange transfusions E10, E25, E32, and E45, were higher than baseline and no different from MH. The group transfused with low-affinity RBCs (E60) showed lower arteriolar and venular blood flows compared to baseline.

Figure 2. Relative changes to baseline in arteriolar and venular hemodynamics for E10, E25, E32, E45 and E60. The broken line represents baseline level. †P < 0.05 compared with E10; ‡P < 0.05 compared with E25; §P < 0.05 compared with E32; and ¶P < 0.05 compared with E45. Diameters (μm, mean ± SD) in A (arteriolar) and B (venular) for each animal group were as follows: Baseline (arterioles [A], 63.2 ± 6.4, n = 122; venules [V], 66.3 ± 7.2, n = 138); MH (A, 67.4 ± 7.7; V, 71.1 ± 7.8); E10 (A, 68.2 ± 7.2, n = 23; V, 73.5 ± 7.5, n = 25); E25 (A, 69.7 ± 8.4, n = 26; V, 70.8 ± 8.1, n = 28); E32 (A, 68.3 ± 9.1, n = 24; V, 71.2 ± 8.0, n = 31); E45 (A, 64.3 ± 7.7, n = 24; V, 68.1 ± 9.3, n = 26); E60 (A, 58.2 ± 8.4, n = 25; V, 65.2 ± 7.7, n = 28) (n = number of vessels studied). Calculated flows (nl/s, mean ± SD) in C (arteriolar) and D (venular) for each animal group: Baseline (A, 14.0 ± 4.9; V, 7.9 ± 2.8); MH (A, 18.9 ± 6.3; V, 8.3 ± 3.2); E10 (A, 18.2 ± 5.8; V, 9.8 ± 3.3); E25 (A, 19.4 ± 6.4; V, 9.7 ± 3.8); E32 (A, 17.8 ± 6.2; V, 9.6 ± 3.8); E45 (A, 15.6 ± 5.2; V, 7.8 ± 3.4); E60 (A, 12.1 ± 4.3; V, 7.6 ± 2.8).

Figure 1. Red blood cell (RBC) Hb O₂ affinity of modified RBCs and after exchange transfusion protocol. (A) O₂ dissociation curves after introduction of allosteric effectors. (B) Blood O₂ dissociation curves after exchange transfusion. Introduction of allosteric effector into the RBCs changed P50 and cooperativity. The fraction of RBCs exchanged affected the colligative properties of the blood.

Functional Capillary Density

After first exchange with dextran 70, all animals showed a reduction in FCD (0.92 ± 0.05 of baseline, $P < 0.05$ to baseline). MH reduced FCD to 0.88 ± 0.07 of baseline ($P < 0.05$ to baseline). FCD was reduced from MH for E60 and E45, 0.65 ± 0.08 and 0.73 ± 0.05 of baseline, respectively ($P < 0.05$ to baseline). FCD for E10, E25, and E32 mm Hg were 0.78 ± 0.06 , 0.82 ± 0.05 , and 0.80 ± 0.06 of baseline, respectively (Figure 3).

Microvascular Oxygen Distribution

Microvascular and tissue O_2 tensions are shown in Figure 4. E25 yielded the highest arteriolar PO_2 , and E60 the lowest when compared with E32 (normal P50 of the hamster). Venular PO_2 were elevated for E45 compared with all the groups. Interstitial tissue PO_2 for E45 was statistically higher than all the groups, but not statistically different from normal tissue PO_2 at full Hct 21.7 ± 3.5 mm Hg (21).

Microvascular Oxygen Delivery and Extraction

Figure 5 shows the result of the analysis of microvascular DO_2 and VO_2 . VO_2 was statistically lower for E45 compared with normal hamster P50 (E32). Exchange transfusion with low-affinity RBCs (E60) decreased DO_2 when compared with normal (E32) and DO_2 and VO_2 compared against high-affinity RBCs (E10 and E25).

DISCUSSION

The principal finding of this study is that under identical anemic conditions (18% Hct), exchange transfusion of 35% of the blood with RBCs having P50 ranging from 10 to 60 mm Hg produced a trend whereby maximal vasodilation, microvascular flow, intravascular arteriolar O_2 tension, microvascular DO_2 , and VO_2 corresponded to exchange transfused RBCs with P50 of 25 mm Hg. Mean arterial blood pressure and arterial PO_2 were also less affected after exchange transfusion with 25 mm Hg P50, although the changes were not statistically different. Exchange transfusion with RBC with lower P50 than normal yields normal/optimal microvascular and systemic conditions, while introducing RBCs with elevated P50 negatively impacted microvascular and systemic conditions. Moderated increase in P50 produced substantial changes in venular and tissue PO_2 s, which tend to track each other, and did not follow the trend of the other parameters. Significant change in O_2 distribution in the microcirculation was caused by the introduction of a comparatively small proportion of modified P50 RBCs. This suggests that native and modified RBCs are independent effectors

of O_2 delivery regulation, and that the O_2 sensor mechanisms is particularly sensitive to the presence of low-affinity RBCs rather than being sensitive to the average affinity of the circulating blood.

Systemic Versus Microvascular Response

Infusion of RBCs with different O_2 affinities maintained blood pressure and blood gas parameters, including acid-base balance in the acute anemic state (Hb 5.3 g/dl). However, microcirculatory conditions were significantly different with the infusion of low-affinity RBCs, which induced vasoconstriction and compromised blood flow. FCD was decreased in all groups, compared with baseline, as a consequence of hemodilution. FCD further decreased with the infusion of low Hb- O_2 affinity RBCs (P50 of 45 and 60 mm Hg).

Hb allosteric effectors that increase DO_2 to the tissue led to arteriolar constriction and reduced blood flow in the microcirculation and a tendency to maintain (increase) systemic pressure. This result is in agreement with previous observations (2, 3) following the introduction of RBCs with right-shifted Hb- O_2 affinity, where the benefit from enhanced oxygenation due to the facilitated release of O_2 is opposed by vasoconstriction and reduced blood flow. MAP in general was significantly reduced by hemodilution, but to a lesser degree in animals exchange transfused with RBCs of lower Hb- O_2 affinity (Table 1). Exchange transfusion of 35% of BV with RBCs with low Hb- O_2 affinity (P50 of 60 mm Hg) decreased blood P50 by 28% (P50 of 41 mm Hg) and resulted in the highest blood pressure in the group exchanged with treated RBCs. RBCs with P50 of 10 mm Hg decreased blood P50 by 25% (P50 of 24 mm Hg). Microvascular resistance was estimated by assuming that systemic pressure is directly transmitted to the microcirculation, and calculated by dividing MAP by microvascular flow. This calculation shows that microvascular resistances were reduced to 55 to 60% of baseline for all exchange groups except for the E60 group (P50 of 60 mm Hg), where resistance was restored to baseline levels. This effect may be due to vasoconstriction since the exchange lowered resistance by 36% of baseline, while the decrease of blood viscosity was 65% of baseline.

As in previous studies, hemodilution in combination with a vasoconstrictor effect caused a significant decrease in FCD (13). The lowest FCD coincided with the most pronounced vasoconstriction (Figures 2). The decrease in vascular tone (arterioles and venules) after introduction of right-shifted RBCs, with the consequent reduction in perfusion, can be attributed to the response to an autoregulatory process that attempts to limit the oversupply of O_2 to the vascular smooth muscle (22).

There was a trend indicating that microvascular, and some systemic parameters, tend to be maximized or highest with the E25 group. All microvascular parameters were depressed after exchange transfusion with RBCs with P50 of 60 mm Hg. Moderate overall change in blood P50 induced by these cells significantly compromised O_2 extraction, but maximized tissue PO_2 . Statistically, FCD was similar for all groups; however, it presented the same trend as microvascular arteriolar diameter and flow, and intravascular blood PO_2 , all parameters that were significantly lower with the exchange transfusion of RBCs with P50 of 60 mm Hg. Lower Hb- O_2 affinity did not produce a systemic hypertensive response compared to normal Hb- O_2 affinity, although vasoconstriction was evident for the lowest affinity RBCs. The significant discontinuity in tissue PO_2 found in changing the P50 of the transfused RBCs from 45 to 60 mm Hg suggests the presence of nonlinear effects, whereby the facilitated O_2 release from microvessels consequent to low affinity is counteracted by the combined effects of vasoconstriction and decreased flow and FCD. It is possible that this significant difference in tissue PO_2 represents a threshold for the potential benefit of lowering blood

Figure 3. Effects of plasma viscosity on capillary perfusion during hemodilution. Functional capillary density (FCD) was lower than baseline for all hemodiluted groups ($P < 0.05$). FCD (cm⁻¹) at baseline was as follows: E10 (95 ± 7), E25 (101 ± 9), E32 (96 ± 9), E45 (104 ± 7), and E60 (103 ± 9). $^{\dagger}P < 0.05$ compared with E10; $^{\ddagger}P < 0.05$ compared with E25; $^{\S}P < 0.05$ compared with E32; and $^{\ast}P < 0.05$ compared with E45.

Figure 4. Intravascular and tissue Po₂ after exchange transfusion with modified RBCs Hb-O₂ affinity. [†]*P* < 0.05 compared with E10; [‡]*P* < 0.05 compared with E25; [§]*P* < 0.05 compared with E32; and [¶]*P* < 0.05 compared to E45.

Hb-O₂ affinity, and facilitating tissue O₂ supply, leading to the increase in tissue Po₂.

Our results show that tissue Po₂ tracks venular blood Po₂. Therefore, the well-defined discontinuity in both tissue and venular blood Po₂ occurring upon changing the P50 of the exchange transfused RBCs from 45 to 60 mm Hg suggests the diversion of O₂ to an O₂-consuming compartment not effected at the lower P50s. This additional compartment could be the arteriolar vessels wall, which several studies show to significantly increase O₂ consumption during vasoconstriction (16, 23). Notably, the most significant changes in perfusion were observed between P50s of 45 and 60 mm Hg, although vasoconstriction was significant only for P50 of 60 mm Hg.

Effects of p50 on Tissue Oxygen Supply

Introduction of allosteric factors changed P50 and cooperativity. IHP was used to decrease Hb-O₂ affinity and its effects on Hb cooperativity were lesser than 5HMF, the effector used to increase Hb-O₂ affinity. The presence of two populations of RBCs, the native and the electrophoresis-modified Hb-O₂ affinity RBCs, maintained the sigmoidal O₂ saturation shape (Figure 1), although cooperativity was significantly affected after the infusion of high-affinity RBCs. Infusion of low-affinity RBCs reduced the O₂ saturation of blood arriving to the microcirculation, causing arteriolar O₂ saturation to decrease 14% after exchange with RBCs with P50 of 45 mm Hg and 40% after exchange with RBCs with P50 of 60 mm Hg. Infusion of high Hb-O₂ affinity RBCs maintained arteriolar O₂ saturation, being 75% saturated with O₂ upon arrival to the microcirculation. The saturation of blood in venules was approximately 20% in all groups except for the exchange with RBCs with P50 of 60 mm Hg, which fell to 10%.

Figure 5. Microvascular O₂ delivery and extraction after exchange transfusion. Calculations of global O₂ transport are not directly measurable in our model. However, the 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. Bias in observation for a single vessel and a single animal were eliminated by integrating overall values in a group. [†]*P* < 0.05 compared with E10; [‡]*P* < 0.05 compared with E25; [§]*P* < 0.05 compared with E32; and [¶]*P* < 0.05 compared with E45.

Schumacker and coworkers (24) and Shirasawa and colleagues (25) investigated blood flow regulation during exercise in the presence of red blood cells with high oxygen affinity. Both studies report conclusions similar to ours—namely, that high affinity increases oxygen delivery and lowers tissue Po₂. Similarly, studies in extreme hypoxia show that low P50 RBC tends to maintain tissue Po₂ by increasing arterial O₂ content (26–28). Gutierrez and Andry (26) compared low and high oxygen affinity perfusion during hypoxemia in an isolated limb and found that total O₂ transport was greater with high-affinity red blood cells while the extraction ratio was lower, concluding that increased hemoglobin affinity does not appreciably improve tissue oxygenation in hypoxemia, because of the related diffusion limitation. Notably, in these experiments blood flow was maintained constant; therefore, this result is not directly translatable or comparable to our findings where the tissue and the circulation autoregulates oxygen delivery.

Previous studies suggest that elevated levels of O₂ due to right-shifted Hb in RBCs cause an excessive tissue O₂ supply, resulting in a vasoconstrictor response (22). This has been experimentally documented in several studies using acellular right-shifted modified Hbs (13, 29, 30). When the P50 of the RBCs was increased to 37 mm Hg, there was a sufficient increase in microvascular DO₂, maintaining normal tissue Po₂s with only 35% of the O₂ capacity and a minimal decrease in perfusion. Significant decrease in Hb-O₂ affinity (P50 of 60 mm Hg) caused a strong autoregulatory response, limiting O₂ supply and causing vasoconstriction. These results demonstrate that DO₂ to the tissue cannot be increased beyond the requirements determined by the normal metabolic demand, likely since over-oxygenation leads to detrimental free radical formation and injury. This result supports the role of O₂ availability *per se* as a determinant of local blood flow in the peripheral circulation.

This study shows that high levels of tissue Po₂ do not correspond to the largest DO₂. RBCs with right-shifted Hb raised mean extravascular tissue Po₂ and thus decreased the available O₂ tension gradient that drives diffusion of O₂ from blood into the tissue. RBCs with the native hamster P50 changed microvascular arterio-venous (A-V) O₂ tension difference from 47.2 to 18.7 mm Hg, producing a 54% change in Hb O₂ saturation. Increasing blood P50 by 5 mm Hg (15%) did not change arteriolar Po₂ and significantly increased venular Po₂ to 24.1 mm Hg, decreasing microvascular A-V Po₂ difference to 22.8 mm Hg and a change in O₂ saturation of 42%. As previously noted, tissue Po₂ is in equilibrium with venular Po₂ (31). The absence of a tissue-venular O₂ gradient implies *a priori* that O₂ is diffusing down a concentration gradient from the surrounding extravascular space into the vessel. The interstitial tissue Po₂ results from the diffusion from neighboring tissue regions (arterioles) that have

an elevated P_{O_2} and/or a direct diffusional shunting of O_2 from arterioles. Thus, nearby arterioles are likely the major contributors to the high tissue P_{O_2} generating the inward O_2 gradient. Since arteriolar P_{O_2} sets the upper end of the O_2 gradient available for diffusion, shifts in the dissociation curve have been interpreted as aiding (increased P50) or impeding (decreased P50) tissue oxygenation. This view is supported by the existence of mechanisms such as the Bohr and temperature effects, which change Hb O_2 affinity. However, our results in this study show that a similar tissue O_2 level is achieved with the maximum changes to Hb- O_2 affinity. Moreover, decreasing affinity reduces blood flow and tissue P_{O_2} , while increasing Hb- O_2 affinity requires low tissue P_{O_2} in order to offload O_2 from the blood column.

Our results suggest that allosteric effectors may be used to increase O_2 availability to the tissue under conditions in which O_2 supply is compromised. Low P50s in a fraction of the circulating RBCs causes O_2 to be delivered preferentially in regions with low tissue P_{O_2} . Figure 5 shows that the maximal DO_2 is attained at the lowest P50, a consequence of the large intravascular/extravascular O_2 gradient. The resulting reduction in tissue P_{O_2} can be tolerated if the O_2 gradient between tissue and cell mitochondria is sufficient to maintain cell aerobic metabolism (32).

Effects Due to Electroporation

We assume that circulating electroporetically modified RBCs did not lose Hb through hemolysis and that there was no RBC sequestration, since there was no free plasma Hb after the exchange and Hct did not change after the exchange in any of the transfused groups. These observations also suggest that RBC deformability was not significantly affected by the electroporation process. It is well established that RBCs with increased fragility lose Hb, resulting in plasma-free Hb, increased MAP, and rapid reduction of Hct. However, other studies using RBC electroporation show RBCs remain in circulation for up to 7 days after the introduction of allosteric factors (2). It has been reported that IHP serve as inhibitors for soluble guanylate cyclase-dependent signaling events (33). However, IHP does not diffuse through the erythrocytic membrane, and the excess IHP in the suspending media was removed before infusion; consequently IHP can only appear in plasma if the modified RBCs hemolyze, an effect that was not observed in our experiments. No differences in blood viscosities were found among groups, supporting the contention that all transfused electroporetically modified RBCs had similar unhindered circulatory function.

Our results should be of relevance to the emerging field of blood substitute development, which presents products in clinical trials at the opposite ends of the oxygen affinity spectrum. Notably, a vasoconstrictive molecular bovine hemoglobin-based product with a P50 of 54 mm Hg (Oxyglobin; Biopure Inc., Cambridge, MA) has been in clinical trials for several years with no clear definition of its suitability as a blood substitute due to its vasoactivity, manifested as hypertension upon introduction into the organism (13). A different product based on polyethylene glycol-conjugated hemoglobin has been developed by Sangart Inc. (San Diego, CA) and is now in Phase 3 clinical trials (34). This product has a P50 of approximately 5 mm Hg, is not vasoactive, and may be endowed with positive vasoactivity having vasodilator properties (13). Both products have been extensively analyzed relative to their negative and positive vasoactivity (13), leading to the conclusion that an explanation for the vasoconstrictive effects of bovine hemoglobin is the over supply of oxygen to the arteriolar network and vice versa (13).

In conclusion, this study shows that the criterion for an optimal blood Hb- O_2 affinity for exchange transfused blood is not unique, and requires differentiating between microvascular function and

tissue P_{O_2} . In this study, microvascular function showed a tendency to be optimal after transfusion of left-shifted RBCs. However, if tissue P_{O_2} is the criterion for a most advantageous condition, this can be obtained by transfusing slight right-shifted RBCs. There was no oxygen delivery and extraction limitation in the anemic state tested in the presence of high-affinity RBCs; however, these functions were severely limited with the lowest-affinity RBCs. A notable finding was that high oxygen delivery and extraction found with high-affinity RBCs corresponded to low tissue P_{O_2} s. These results suggest that the tissue at rest presents a variable O_2 consumption related to O_2 availability. It should be noted that these experiments were performed at a specific anemic state, and it is likely that the relationship between DO_2 , VO_2 , and interstitial tissue P_{O_2} will change for different anemic conditions. Finally, it is apparent that interstitial tissue P_{O_2} is not an optimal indicator of tissue O_2 metabolism.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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