Effects of erythrocyte flexibility on microvascular perfusion and oxygenation during acute anemia

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Cabrales P. Effects of erythrocyte flexibility on microvascular perfusion and oxygenation during acute anemia. Am J Physiol Heart Circ Physiol 293: H1206–H1215, 2007. First published April 20, 2007; doi:10.1152/ajpheart.00109.2007.—Responses to exchange transfusion using red blood cells (RBCs) with normal and reduced flexibility were studied in the hamster window chamber model during acute moderate isovolemic hemodilution to determine the role of RBC membrane stiffness in microvascular perfusion and tissue oxygenation. Erythrocyte stiffness was increased by 30-min incubation in 0.02% glutaraldehyde solution, and unreacted glutaraldehyde was completely removed. Filtration pressure through 5-μm pore size filters was used to quantify stiffness of the RBCs. Anemic conditions were induced by two isovolemic hemodilution steps using 6% 70-kDa dextran to a hematocrit (Hct) of 18% (moderate hemodilution). The protocol continued with an exchange transfusion to reduce native RBCs to 75% of baseline (11% Hct) with either fresh RBCs (RBC group) or reduced-flexibility RBCs (GRBC group) suspended in 5% albumin at 18% Hct; a plasma expander (6% 70-kDa dextran; Dex70 group) was used as control. Systemic parameters, microvascular perfusion, capillary perfusion [functional capillary density (FCD)], and oxygen levels across the microvascular network were measured by noninvasive methods. RBC deformability for GRBCs was significantly decreased compared with RBCs and moderate hemodilution conditions. The GRBC group had a greater mean arterial blood pressure (MAP) than the RBC and Dex70 groups. FCD was substantially higher for RBC (0.81 ± 0.07 of baseline) vs. GRBC (0.32 ± 0.10 of baseline) and Dex70 (0.38 ± 0.10 of baseline) groups. Microvascular tissue PO2 was significantly lower for Dex70 and GRBC vs. RBC groups and the moderate hemodilution condition. Results were attributed to decreased oxygen uploading in the lungs and obstruction of tissue capillaries by rigidified RBCs, indicating that the effects impairing RBC flexibility are magnified at the microvascular level, where perfusion and oxygenation may define transfusion outcome.

microcirculation; red blood cell membrane; glutaraldehyde; functional capillary density; extreme hemodilution; plasma expander; transfusion; intravascular oxygen

RED BLOOD CELLS (RBCs) are available for transfusion as early as 3–4 days after collection, and with modern preservation techniques they can be administered up to 42 days after collection (12). Factors limiting their efficacy in blood replacement develop in proportion to their period of storage, which may be due in part to the depletion of 2,3-diphosphoglyceric acid (DPG) and adenosine triphosphate (ATP), reduced RBC deformability (35), and a significant increase in abnormally shaped RBCs (32). Although synthesis of DPG does occur in RBCs after transfusion, it is a slow process, taking up to 24 h to reach normal levels (44). Lowered DPG concentration in RBCs increases the oxygen affinity for hemoglobin (Hb), potentially limiting oxygen supply to the tissues, leading to tissue hypoxia. Studies have shown that transfusion does not augment oxygen supply in critically ill patients on transfusion, suggesting that stored blood is less efficacious than anticipated (12). Mechanistically, these storage lesions may also impair oxygen delivery to tissues by decreasing microvascular perfusion and reducing the amount of oxygen released from Hb (29).

The deformability of RBCs due to their membrane flexibility is a factor in maintaining normal blood flow in the microcirculation, allowing their transit through capillaries whose lumen is narrower than the cell diameter (9). The major determinants of RBC deformability are cell geometry, intracellular fluid viscosity, and the viscoelastic properties of the cell membrane (9, 17). Several methods for studying RBC deformability in vitro have been reported in the literature (9, 28). However, the role of RBC deformability in the maintenance of capillary perfusion is not well established.

The present study was carried out to determine the effect of changes in RBC deformability on in vivo changes in functional capillary density (FCD) during acute anemia. FCD is defined as the number of capillaries with passage of RBCs per unit surface in the field of view of a microscopically observed tissue, a parameter found to be critical in defining tissue survival (24). The experimental hamster window chamber model was subjected to moderate hemodilution via two isovolemic exchanges with 6% 70-kDa dextran to induce an acute anemic state [hematocrit (Hct) 18%]. After anemia, the animals were randomly exchange transfused with fresh RBCs or RBCs whose flexibility was reduced by glutaraldehyde incubation (GRBCs). In each case, Hct was adjusted to 18% to maintain the anemic state. Changes in microvascular function were characterized by determining effects on capillary flow, microhemodynamic changes, and tissue oxygenation. RBC deformability was characterized by passing RBCs through a polycarbonate filter at different flow rates.

METHODS

Animal preparation. Investigations were performed in 55- to 65-g male golden Syrian hamsters (Charles River Laboratories, Boston, MA) fitted with a dorsal skinfold window chamber. The hamster window chamber model is used widely for microvascular studies in the unanesthetized state, and the complete surgical technique is described in detail elsewhere (10, 15). Arterial and venous catheters filled with a heparinized saline solution (30 IU/ml) were implanted. Catheters were tunneled under the skin, exteriorized at the dorsal side of the neck, and securely attached to the window frame. The microvascularity was examined 3–4 days after the window implantation.
surgery, and animals were entered into the study after meeting established systemic and microcirculatory inclusion criteria, which exclude preparations whose tissue has low perfusion, inflammation, and edema (15). Animal handling and care followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the local animal care committee.

Inclusion criteria. Animals were suitable for the experiments if 1) systemic parameters were within normal range, namely, heart rate (HR) >340 beats/min, mean arterial blood pressure (MAP) >80 mmHg, systemic Hct >45%, and arterial oxygen partial pressure (Pao2) >50 mmHg, and 2) microscopic examination of the tissue in the chamber under high magnification did not reveal signs of edema or bleeding. Hamsters are a fossorial species with a lower Pao2 than other rodents due to their adaptation to the subterranean environment. However, microvascular Pao2 distribution in the window chamber model is similar to that of other rodents (7).

Erythrocyte preparation. Male retired breeder golden Syrian hamsters (donors) weighing 90–120 g were anesthetized with pentobarbital sodium and exsanguinated via a carotid catheter over a period of 5 min into a Vacutainer tube (purple stoppered) containing 100 μl of 15% EDTA. RBCs were washed three times by centrifugation in phosphate-buffered saline (PBS, 0.01 M, pH 7.4: 0.109 g/dl Na2HPO4, 0.032 g/dl NaH2PO4, 0.9 g/dl NaCl), and the buffy coat was removed each time.

RBCs were hardened by incubation for 30 min at room temperature in 0.02% glutaraldehyde in 0.01 M PBS (pH 7.4). Glutaraldehyde-hardened RBCs (GRBCs) were washed three times in PBS and then resuspended in PBS with 5% human serum albumin to Hct of 18%. The cell suspension was checked under a high-magnification microscope to ensure that the cells maintained their normal biconcave discoid shape.

RBC deformability. A filtration method was used to assess the deformability of fresh RBCs and hardened RBCs. In brief, polycarbonate filters (Nuclepore, Pleasanton, CA) with a mean pore size of 5 μm, a 13-mm diameter, and a mean pore density of 4 x 10^5/cm^2 were perfused with diluted cell suspensions by means of an infusion pump (model 944, Harvard Apparatus, South Natick, MA). Cell suspensions or suspending medium (buffer) were delivered through the filter at a rate of 0.5, 0.8, 1.0, 1.2, and 1.5 ml/min. The pressure drop across the filter was measured with a differential pressure transducer (MP 150, BIOPAC Systems, Santa Barbara, CA).

RBC deformability was assessed by calculating the ratio β of the resistance to flow through a pore with RBCs present (Rca) to resistance of the pore with suspending medium only (Rc). β was calculated according to the method of Skalak et al. (37, 38), using the ratio of the initial pressure drop across the filter (P0) with cells present (obtained by extrapolation of the pressure-time curve at constant flow to the point of zero RBC trapping at time 0) to the pressure drop (P0) with buffer alone

\[ \beta = \frac{P_0 - 1}{h} \]

where v is the ratio of mean cell volume to pore volume and h is the packed RBC fraction of the perfusate. All filtration measurements were performed at Hct of 18% in triplicate (different filters) and averaged. Figure 1A presents the filtration pressure (Fig. 1A) and β (resistance to flow through a pore: Fig. 1B) for GRBCs and fresh RBCs at different flow rates.

Hemoglobin oxygen saturation. The oxygen equilibrium curve for hamster RBCs was measured by deoxygenation of oxygen-equilibrated samples in Hemox buffer at 37°C with a Hemox Analyzer (TCS Scientific, New Hope, PA). The analyzer measures the O2 pressure with a Clark-type O2 electrode (Yellow Springs Instruments, Yellow Springs, OH) and simultaneously calculates the Hb saturation via a dual-wavelength spectrophotometer (4).

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 was described in detail in our previous reports (6, 8, 42). 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 (mean molecular mass 70 kDa; Pharmacia). The first exchange step was 40% of BV and the second was 35% of BV.

At Hct of 18%, corresponding to a condition of moderate hemodilution, animals were randomly assigned to four experimental groups with a sorting scheme based on a list of random numbers (1). One group was used to study microvascular oxygen distribution at moderate hemodilution (MH group). The remaining three groups were exchanges of 35% of BV using Dextran 70 (Dex70 group), fresh
RBCs (RBC group), or GRBCs (GRBC group). RBC and GRBC groups were exchanged with the respective RBCs suspended in 5% albumin at Hct of 18%. This procedure caused two-thirds of the circulating RBCs to be the normal original RBCs and the remaining one-third to be normal reinforced RBCs or GRBCs. Figure 2 illustrates the experimental protocol.

Since both mixed blood and dilution material were withdrawn during the exchanges, a 110% blood volume exchange was needed to reduce the functional Hct to 11% (8, 41). Blood was simultaneously withdrawn at the same rate from the carotid artery catheter according to a previously established protocol (8, 41). Blood samples were withdrawn at the end of the experiment for subsequent analysis of viscosity and colloid osmotic pressure (COP). The duration of the experiments was 4 h. Each exchange and the respective observation time point after exchange were fully completed in 1 h. Systemic and microcirculation data were taken after a stabilization period of 10 min.

**Systemic parameters.** MAP and HR were recorded continuously (MP 150, BIOPAC Systems). Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes (25 μl, filling ~50% of the heparinized glass capillary tube). 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 (50 μl) and immediately analyzed for PaO2, arterial PaCO2 (PaCO2), base excess (BE), and pH (Blood Chemistry Analyzer 248, Bayer, Norwood, MA). The comparatively low PaO2 and high PaCO2 of these animals is a consequence of their adaptation to a fossorial environment. Blood samples for viscosity and COP measurements were quickly withdrawn from the animal into a heparinized 5-ml syringe at the end of the experiment. Blood chemistry and biophysical properties were analyzed immediately or refrigerated for next-day analysis. Viscosity of the experimental protocol.

**Microhemodynamics.** Arteriolar and venular blood flow velocities were measured online by the photodiode cross-correlation method (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. A video image-shearing method was used to measure vessel diameter (D) (22). Blood flow (Q) was calculated from the measured values as Q = π × 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 diameters and for Hct in the range of 6–60% (26). Wall shear stress (WSS) for each microvessel was defined by WSS = WSR × ν, where WSR is the wall shear rate given by 8VD⁻¹ and ν is the microvascular blood viscosity at this WSR.

**Microvascular PO2 distribution.** High-resolution noninvasive microvascular PO2 measurements were made by phosphorescence quenching microscopy (PQM) (23, 42). PQM is based on the oxygen-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 PO2 level, causing the method to be more precise at low PO2 levels. This technique is used to measure both intravascular and extravascular PO2 since the albumin-dye complex continuously extravasates the circulation into the interstitial tissue (23, 42). Tissue PO2 was measured in tissue regions between functional capillaries. PQM allows for precise localization of the PO2 measurements without subjecting the tissue to injury. These measurements provide a detailed understanding of microvascular oxygen distribution and indicate whether oxygen is delivered to the interstitial areas.

**Oxygen delivery and extraction.** The microvascular methodology used in our studies allows a detailed analysis of oxygen supply in the tissue. Calculations are made with Eqs. 2 and 3 (8):

\[
O_2\text{ delivery} = [(RBC_{Hb} \times \gamma \times S_A\% ) + (1 - Hct)] \\
\times \alpha \times P_{O2}\text{a} \times Q \tag{2}
\]

\[
O_2\text{ extraction} = [(RBC_{Hb} \times \gamma \times S_{AV}\% ) + (1 - Hct)] \\
\times \alpha \times P_{O2}\text{a-V} \times Q \tag{3}
\]

where RBC_{Hb} is the Hb in RBCs (g Hb/dl blood), γ is the oxygen carrying capacity of saturated Hb (1.34 ml O2/g Hb), S_A% is the arteriolar oxygen saturation, (1 – Hct) is the fractional plasma volume (dl plasma/dl blood), α is the solubility of oxygen in plasma (3.14 × 10⁻³ ml O2/dl blood at 37°C), and Q is blood flow (dl/min).

Microvascular 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 for transillumination with the intravital microscope (BX51WI, Olympus, New Hyde Park, NY). Animals were given 20 min to adjust to the tube environment before any measurement. 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 with a ×40 (LUMPF-LIR, 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.

**Microhemodynamics and Erythrocyte Membrane Flexibility.**
10^{-3} \text{ ml} \text{ O}_2/\text{dl} \text{ plasma mmHg}, P_{O_2, A} \text{ is the arteriolar partial pressure of oxygen, } P_{O_2, A, V} \text{ indicates the arteriolar/venular differences, and } Q \text{ is microvascular flow. Oxygen saturations were measured as described above.}

Data analysis. Tabular results are presented as means ± SD. 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 and the lower whisker the 5th percentile. Data within each group were analyzed with analysis of variance for repeated measurements (ANOVA, Kruskal-Wallis test). When appropriate, post hoc analyses were performed with 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). 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 GraphPad Prism 4.01 (GraphPad Software, San Diego, CA). Changes were considered statistically significant if \( P < 0.05 \).

RESULTS

Twenty-four animals were entered into the hemodilution microcirculation study. Six 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: MH (\( n = 6 \)), Dex70 (\( n = 6 \)), GRBC (\( n = 6 \)), and RBC (\( n = 6 \)). All groups used were statistically similar (\( P > 0.20 \)) in systemic and microcirculation parameters at baseline and under MH. Systemic and microhemodynamic data for baseline and MH were obtained by combining data from all experimental groups. Blood typing and crossmatching tests are not necessary with hamsters, based on previous experience with this species. No changes in body temperature were detected during the protocol, indicating no immune responses due to inappropriate transfusion.

Systemic parameters. The first exchange step significantly reduced Hct to 29.4 ± 2.2% and the second step to 18.4 ± 0.7%. Hct and Hb during the exchange protocol are given in Table 1. GRBC and RBC groups did not show an initial decrease in Hct or Hb content from MH, because of the additional Hb contributed by fresh RBCs or GRBCs. The added concentration of Hb was 2.3 g/dl in the GRBC group and 2.5 g/dl in the RBC group. The Dex70 group had significantly decreased Hct and Hb compared with RBC, GRBC, and MH groups. Hct decreased after exchange transfusion with GRBCs from 18.2 ± 0.6% to 16.1 ± 0.8% after 1 h postexchange. Hct for RBC and Dex70 groups did not change over the observation period. Plasma Hb was not detected (<0.1 g/dl) after transfusion for both groups.

MAP decreased from baseline (108 ± 6 mmHg) to 97 ± 7 mmHg at MH. After three exchanges, MAP was lowered for RBC and Dex70 groups, as shown in Table 2. Systemic arterial blood gas analysis showed a statistically significant rise in PaO_2 and decrease in PaCO_2 from baseline after extreme hemodilution and exchange transfusion with GRBCs and fresh RBCs (\( P < 0.05 \)). Arterial pH was statistically changed from baseline in the Dex70 group, while other groups did not show statistically significant changes. Blood BE was statistically significantly decreased after the third hemodilution exchange compared with baseline in all groups; the Dex70 and GRBC groups were significantly different from the MH group (Table 2).

Blood biophysical properties after exchange. Rheological properties of the fresh RBCs and the GRBC preparation before infusion are presented in Fig. 3A. Blood viscosity, plasma viscosity, and plasma COP after hemodilution for all groups are presented in Table 2. Blood viscosity for MH, Dex70, GRBC, and RBC groups was significantly lower than baseline and in the Dex70 group was also lower than in the other exchanged groups. Figure 3B shows changes of blood viscosity at the end of the experiment at different shear rates. It should be noted that Hct decreased from the time of infusion to the time of sample collection for viscosity measurements in animals infused with GRBCs. Hct at the time of blood viscosity measurement was 16.1 ± 0.8% for the GRBC group. Only the Dex70 group had an increase in plasma viscosity from baseline. However, it was not significantly different from the other hemodiluted groups. In all groups, plasma COP did not change.

RBC oxygen affinity. The oxygen dissociation properties of fresh RBCs and GRBCs before infusion are presented in Fig. 4A. Incubation in glutaraldehyde reduced P_{50} (P_{02} required to achieve 50% Hb saturation) to 22.2 mmHg compared with 31.8 mmHg for fresh RBCs. Changes in oxygen dissociation for the hamster blood after exchange transfusion in the RBC and GRBC groups are presented in Fig. 4B. Blood P_{50} at the end of the experiment was 29.3 mmHg for the GRBC group and 32.0 mmHg for the RBC group.

Microhemodynamics. The changes in diameter, RBC velocity, and blood flow of large feeding and small arcading arte-

| Table 1. Systemic parameters during exchange protocol |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| n               | Baseline        | MH              | Dex70           | GRBC            | RBC             |
| Hct, %          | 48.7 ± 1.0      | 18.4 ± 0.7†     | 11.1 ± 0.9††    | 18.2 ± 0.6††    | 18.5 ± 0.7††    |
| [Hb], g/dl      | 14.6 ± 0.6      | 6.0 ± 0.5†      | 3.7 ± 0.5††     | 6.0 ± 0.5†      | 6.2 ± 0.4†      |
| MAP, mmHg       | 108 ± 26        | 92 ± 7†        | 64 ± 8††       | 93 ± 7††       | 86 ± 8†        |
| HR, beats/min   | 433 ± 24        | 443 ± 25       | 418 ± 41††     | 392 ± 24††     | 429 ± 26†      |
| PaO_2, mmHg     | 561 ± 6.1       | 80.4 ± 8.2††   | 105.3 ± 16.7†† | 94.3 ± 10.2††  | 82.8 ± 6.8††   |
| pH_8            | 7.346 ± 0.018   | 7.345 ± 0.025  | 7.323 ± 0.022††| 7.332 ± 0.026  | 7.352 ± 0.018  |
| BE_8, mmol      | 3.6 ± 1.5       | 1.7 ± 1.3†     | 4.6 ± 2.6††    | 2.8 ± 1.4†     | 2.12 ± 1.1††   |

Values are means ± SD. Baseline included all the animals in the study. No significant differences were detected between the baseline and moderate hemodilution (MH) values of each group; Dex70, Dextran 70; RBC, red blood cell; GRBC, glutaraldehyde-hardened RBC; Hct, systemic hematocrit; [Hb], hemoglobin content of blood; MAP, mean arterial blood pressure; HR, heart rate; PaO_2, arterial partial pressure of O_2; PaCO_2, arterial partial pressure of CO_2; pH_8, arterial pH; BE_8, arterial base excess. *\( P < 0.05 \) vs. MH; †\( P < 0.05 \) vs. baseline; ‡\( P < 0.05 \) vs. Dex70; §\( P < 0.05 \) vs. GRBC.
riles (range 49–76 μm) and small collecting venules and large venular vessels (range 52–78 μm) were measured after each hemodilution step. Arteriolar diameter was unchanged after the first exchange (Fig. 5A). On further blood exchange to Hct of 18%, arterioles dilated to 1.07 ± 0.11 of baseline [MH group; n = 6]. After the level 3 exchange with Dextran 70, resulting in a slight arteriolar vasoconstriction to 0.93 ± 0.06 of baseline (Dex70 group; N = 32), arteriolar diameter constricted to 0.92 ± 0.09 of baseline (GRBC group; N = 33; P < 0.05 vs. MH and RBC). On the other hand, exchange with fresh RBCs sustained arteriolar dilation at 1.10 ± 0.15 of baseline (RBC group; N = 31; P < 0.05 vs. Dex70 and GRBC groups). Arteriolar microvascular tone changes are presented in Fig. 5A.

Venular changes due to the hemodilution protocol are shown in Fig. 5B. After the second exchange, venules dilated to 1.02 ± 0.11 of baseline (MH group; N = 140). When the exchange protocol was continued with Dextran 70, venules constricted to 0.90 ± 0.12 of baseline (Dex70 group; N = 36, P < 0.05 vs. baseline and MH group). Exchange with GRBCs did not change venular diameters from baseline levels, which were 0.98 ± 0.14 of baseline (GRBC group; N = 32). Exchange with fresh RBCs did not change venular diameter, which was 1.06 ± 0.17 of baseline (RBC group; N = 36) and not statistically different from baseline.

Arteriolar and venular blood flows after hemodilution are presented in Fig. 5, C and D. The results are given as a box-whisker plot to show the trend of this parameter calculated from vessel diameter and RBC velocity (absolute values are given in the legend of Fig. 5). Arteriolar blood flows were statistically increased from baseline for MH and RBC groups; the GRBC group showed lower arteriolar blood flows compared with MH and RBC groups. Arteriolar and venular flow increased from baseline after moderate hemodilution with Dextran 70. Further exchanges with GRBCs and Dextran 70 reduced both arteriolar and venular blood flows to being statistically lower than baseline and MH and RBC groups.

**DISCUSSION**

The principal finding of this study is that at moderate hemodilution (18% Hct), if 30% of RBCs have a reduced flexibility (GRBCs), functional oxygen delivery is reduced to the same degree as in hemodilution to Hct 11% with normal RBCs. This difference was not apparent by comparing MAP, which was the same for both exchanges while capillary and microvascular flows were severely compromised in the GRBC exchange. Tissue oxygenation was affected even though Hb concentration was 6 g/dl, since arteriolar oxygen content was significantly lower for GRBC than for RBC perfusion. Hemodilution using 6% 70-kDa dextran to Hct 11% (3.7 g/dl of Hb) produced a significant decrease in MAP, microvascular
flow, and FCD, in addition to a negative acid-base balance and reduced tissue oxygen. MAP and microvascular flow were maintained after hemodilution with fresh RBCs (RBC group).

Systemic hemodynamic parameters with normal RBC flexibility at 18% Hct (MH and RBC groups) were different compared with the group that received GRBCs, independent of the maintenance in oxygen carrying capacity. At Hct of 18%, if 30% of the RBCs are replaced by GRBCs, microvascular hemodynamics significantly decrease oxygen delivery to the microcirculation. Oxygen tensions in the microcirculation and systemic and microvascular conditions were similar for the group with only two hemodilution steps (MH group) compared with the group exchange transfused with fresh RBCs (RBC group). Therefore, RBC deformability has a significant effect on microvascular function, although blood viscosity was not different between RBC and GRBC groups at high shear rates (>150 s⁻¹). Reduced RBC deformability significantly impairs microvascular flow to an extent comparable to conditions of extreme anemia (Dex70 group); thus these and other findings show that RBC properties play a significant role in modulating tissue perfusion.

Exchange transfusion with GRBCs significantly lowered arteriolar PO₂ by comparison with MH, fresh RBCs, and extreme hemodilution with Dextran 70. Previous studies show that the rate of oxygen exiting from arterioles before blood arriving to the capillaries is significant (25); therefore low flow rates lead to the depletion of arteriolar oxygen and lowering of arteriolar blood PO₂. This effect was found after GRBC transfusion, which decreased RBC flow velocity and produced a lower arteriolar PO₂. Venules exhibited a similar effect as a consequence of their lowered flow velocity after exchange with GRBCs leading to low PO₂, which was the same as for Hct of 11% (Dex70 group) but significantly decreased compared with MH and RBC groups. Thus the residence time of the blood within the vessel segments critically influences the amount of oxygen that diffuses into the surrounding tissue, affecting oxygen delivery to the capillary network.

In our experiments, blood containing a fraction of RBCs with reduced flexibility arrives at the arterioles with significantly less oxygen than blood with 100% normal RBCs at the same Hb concentration. This effect suggests that rigidified RBCs acquire less oxygen in their passage through the lung.

**Fig. 3.** Viscosity before and after the exchange protocol. A: rheological properties of GRBCs and fresh RBCs at 18% Hct. B: blood rheological properties after the exchange transfusion. Viscosities in the range of shear rates found in the microcirculation and circulation (50 – 450 s⁻¹) were the similar for the animals that received GRBCs (GRBC group) and fresh RBCs (RBC group). The fraction of RBCs added to the animals is approximately 1/3 of the RBCs in circulation. Rheological effects of GRBCs appear to be mitigated by the decrease in Hct from the time of infusion to the time of blood sample collected for viscosity measurement.

**Fig. 4.** Oxygen-hemoglobin (Hb) RBC affinity before and after the exchange protocol. A: oxygen-Hb dissociation curve for GRBCs and fresh RBCs. B: oxygen-Hb dissociation curve after the exchange transfusion. RBC incubation in glutaraldehyde reduced PO₂ required for 50% Hb saturation (P₅₀) and cooperativity. The fraction of GRBCs exchanged changed the colligative properties of the blood, decreasing overall P₅₀.
This contention is supported by the results from the study of Betticher et al. (3), who measured pulmonary oxygen diffusion capacity as a function of RBC deformability and found that it increases with increased deformability and vice versa. Mechanistically, this effect should be related to the rate of oxygen uptake depending roughly on the second power of the surface area-to-volume ratio of RBCs, whereas the rate of release is much less dependent on the size and shape of the RBC (45).

The increased flexibility of RBCs is associated in part with increased surface area-to-volume ratio, supporting the concept of decreased oxygen uploading by rigid RBCs in the lung. This effect, in combination with the associated reduced arteriolar flow and FCD, should explain why tissue and venular PO$_2$ values are significantly reduced relative to perfusion after normal RBC transfusion.

Arteriolar and venular diameters in this experimental model decreased significantly after extreme hemodilution with 70-kDa dextran as shown here and in several studies (8, 41, 43). These effects are probably due to a reflex response that tends to centralize perfusion once oxygen supply becomes limited (5). Exchange transfusion with GRBCs at Hct of 18% led to tissue oxygen delivery and extraction similar to that attained with Dextran 70 (Fig. 8). Oxygen distribution in arterioles (Fig. 7) for all the experimental groups does not present anoxic hypoxia for the section of the cardiovascular network included in the hamster window model, curtailing any hypoxic vasodilatory responses. PaO$_2$ values were lower for the GRBC group, although not sufficiently low enough to be sensed by chemoreceptors and to induce vasodilation in an attempt to compensate for the hypoxia. The hypoxic conditions in the study were anemic, in which blood oxygen content is decreased, but the PaO$_2$ values are near normal.

Lowered capillary pressure is the principal cause for lowering FCD (8). Capillary pressure can be reduced by either a reduction in cardiac performance or an increase in peripheral vascular resistance. In these experiments, infusion of GRBCs maintained blood pressure at the level of MH and significantly greater than in the RBC and Dex70 groups, suggesting that
cardiac performance was not impaired because it was able to sustain MAP. Therefore, an increase in vascular resistance via vasoconstriction (evidenced by a lowering of microvascular diameter) was responsible for the increase in flow resistance in the arterioles, which is most likely to cause a reduction in FCD downstream.

While functionally GRBC and Dextran 70 perfusion led to the same hemodynamic and oxygenation outcome, they are mechanistically different. GRBC perfusion involves a significantly higher concentration of functional Hb, and therefore a significantly greater oxygen carrying capacity than available with Dextran 70. Basically, in the case of Dextran 70, the blood lacks sufficient RBCs, and in the GRBC exchange transfusion group a fraction of the RBCs modifies blood properties, disturbing perfusion. A factor that may differentiate perfusion between the GRBC and RBC groups is the release of ATP from RBCs. Hypoxia and mechanical deformation promote ATP release from RBCs via activation of G protein-dependent signaling, as shown by Sprague and coworkers (30) and reports indicating that ATP release is proportional to the degree of mechanical deformation and the lowering of ATP release from chemically stiffened RBCs (18). Since ATP stimulates nitric oxide (NO) production in endothelial cells, leading to relaxation of the smooth muscle, the lack of RBC ATP release may lead to decreased NO production and increased vascular resistance.

MH followed by exchange transfusion with fresh or rigidified RBCs caused microhemodynamic WSR and WSS conditions that were significantly different, although whole blood rheological properties were moderately unaltered. RBC exchange transfusion maintained WSR and WSS close to baseline and MH, while these parameters were 70% and 50% lower than baseline for the GRBC group, respectively. The nature and magnitude of shear stress play an important role in maintenance of the function of the blood vessel, affecting vessel diameter via modulation of the release of autacoids (prostacyclin, NO, etc). A previous study by Tsai et al. (40) in the same model and protocol showed that increased WSS was associated with an increase in the concentration of perivascular NO and a vasodilator effect. Thus the significantly lowered WSS is related to the observed vasoconstriction, although it may be a consequence rather than the cause.

RBC storage may affect tissue oxygen availability by impairing microcirculatory hemorheology. RBC deformability is an energy-dependent process, which shows a time-dependent decline during ex vivo storage (20). The actual results showed that a significant decrease in flexibility of part of the population of RBCs did not affect blood viscosity at high shear rates (>150 s⁻¹) but impaired microvascular perfusion. Cells with diminished flexibility did not maintain oxygenation, which may be a secondary consequence of the decrease in capillary perfusion (FCD) without occlusion of the microcirculation by...
these less deformable cells (41). Storage-related loss of deformability or increased aggregation may account for impaired microvascular oxygenation following transfusion, an effect reported in several preclinical studies (11, 29, 33).

The RBC deformability is decreased in a number of clinical states, including diabetes, sickle cell disease, and sepsis. Numerous studies in both animal and human sepsis using a variety of techniques have documented decreases in RBC deformability (16, 34). The decreased deformability in septic RBCs has been implicated in alteration of microvascular hemodynamics with a concomitant decrease in oxygen utilization and tissue ischemia (31). Multiple mechanisms for reduced RBC deformability in sepsis have been postulated, including membrane changes induced by lipid peroxidation, oxidative stress, hemoglobin cross-linking, decreased intracellular ATP, loss of membrane surface sialic acid, and NO (21, 31, 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 (41). Consequently, a conventional blood transfusion (using stored blood) may not fully restore oxygen carrying capacity in acute conditions. However, it does restore blood volume and blood viscosity. Blood transfusions have immediate subjective as well as physiological and clinical beneficial effects that are not fully explained by the restoration of oxygen carrying capacity, since this occurs as much as several hours later, depending on the storage period of the transfused blood. Recent studies showed that an increase in Hct in a normal organism led to a rapid increase in NO production via increased shear stress (27). Similar effects were obtained when Hct was decreased via hemodilution and plasma viscosity was increased, raising shear stress and consequently augmenting the levels of peripheral NO, producing a stable and homogeneously perfused microcirculation (40). Therefore, the beneficial effects of blood transfusions may be in part linked to the increase or restoration of shear stress and mechanotransduction by blood viscosity.

The viscosities of rigidified and normal RBC suspensions (with similar Hct) were different from each other before they were exchange transfused into the animals (Fig. 3A). However, as shown in Fig. 3B, viscosity was not different between the two groups (GRBC and RBC), while Hct decreased significantly in the GRBC exchange from 18.2% to 16.1% after 1 h. Given the strong dependence of viscosity on Hct, this result suggests that GRBCs were selectively separated from the pool of circulating RBCs. A mechanism behind this effect is the important role of reduced RBC deformability in the removal of senescent RBCs from the circulation by the spleen (13). This process, although prominent in the spleen, may also occur in other microvascular networks that become obstructed by the rigidified RBCs (2, 36).

Oxygen affinity of GRBCs was increased as found in previous studies (14, 19). The overall change in oxygen affinity of blood was minor, since only ~30% of the circulating RBCs had a lower P50 (Fig. 2). In principle, higher oxygen affinity (lower P50) should hinder oxygen delivery to the tissue. However, this effect was not present in this study because of the very low tissue and venular PO2 resulting from GRBC perfusion. Arguably, the low tissue PO2 values could be attributed to the increased oxygen affinity; however, the effect is more likely due to the inherently low oxygen delivery by the arteriolar supply, which was about one-third of that attained by RBC perfusion at the same Hb concentration. The lack of an effect on oxygen extraction by the lowered P50 is also shown by extraction being 75% of arteriolar oxygen delivery by RBC perfusion vs. 100% by GRBC perfusion.

In summary, this study shows that maintenance of vascular homeostasis during acute anemic states requires sustenance of microvascular function. Compromised vital perfusion and consequently maldistributed oxygenation may affect survival. Previous studies showed that transfusions of stored RBCs, which do not necessarily raise the effective capacity of blood to transport oxygen on transfusion, still provided beneficial effects because of the effectiveness in restoring perfusion, which is a crucial factor for oxygen delivery and the flushing out of metabolites. However, these cells maintained their flexibility; thus RBC flexibility appears to be a critical factor in ensuring microvascular function as well as ensuring efficient oxygen uploading in the lungs and offloading in the tissue.

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REFERENCES