Lowering of Blood Pressure by Increasing Hematocrit with Non-Nitric Oxide-Scavenging Red Blood Cells

Beatriz Y. Salazar Vázquez^{1,2}, Pedro Cabrales³, Amy G. Tsai^{2,3}, Paul C. Johnson², and Marcos Intaglietta^{2,3}

¹Faculty of Medicine, Universidad Juárez del Estado de Durango, Durango, Mexico; ²Department of Bioengineering, University of California, San Diego, La Jolla, California; and ³La Jolla Bioengineering Institute, La Jolla, California

Isovolemic exchange transfusion of 40% of the blood volume in awake hamsters was used to replace native red blood cells (RBCs) with RBCs whose hemoglobin (Hb) was oxidized to methemoglobin (MetHb), MetRBCs. The exchange maintained constant blood volume and produced different final hematocrits (Hcts), varying from 48 to 62% Hct. Mean arterial pressure (MAP) did not change after exchange transfusion, in which 40% of the native RBCs were replaced with MetRBCs, without increasing Hct. Increasing Hct with MetRBCs lowered MAP by 12 mm Hg when Hct was increased 12% above baseline. Further increases of Hct with MetRBCs progressively returned MAP to baseline, which occurred at 62% Hct, a 30% increase in Hct from baseline. These observations show a parabolic "U" shaped distribution of MAP against the change in Hct. Cardiac index, cardiac output divided by body weight, increased between 2 and 17% above baseline for the range of Hcts tested. Peripheral vascular resistance (VR) was decreased 18% from baseline when Hct was increased 12% from baseline. VR and MAP were above baseline for increases in Hct higher than 30%. However, vascular hindrance, VR normalized by blood viscosity (which reflects the contribution of vascular geometry), was lower than baseline for all the increases in Hct tested with MetRBC, indicating prevalence of vasodilation. These suggest that acute increases in Hct with MetRBCs increase endothelium shear stress and stimulate the production of vasoactive factors (e.g., nitric oxide [NO]). When MetRBCs were compared with functional RBCs, vasodilation was augmented for MetRBCs probably due to the lower NO scavenging of MetHb. Consequently, MetRBCs increased the viscosity related hypotension range compared with functional RBCs as NO shear stress vasodilation mediated responses are greater.

Keywords: blood pressure; shear stress; NO bioavailability; hematocrit; plasma layer

Increased hematocrit (Hct) above baseline is usually associated with elevation of systemic blood pressure due to the increase in blood viscosity. These effects were found in studies in which Hct was increased 40% or more above baseline (1–3). However, a recent study by Martini and coworkers (4) showed that mean arterial pressure (MAP) increases only when Hct is increased 20% above baseline using functional RBCs, and that MAP decreased when the change in Hct were less than 20%. In this range, cardiac output (CO) also increased and peripheral vascular resistance (VR) decreased (4, 5).

According to Martini and colleagues (4), the cause of this paradoxical effect is that acute augmentation in Hct directly increases shear stress on the endothelium. This phenomenon

CLINICAL RELEVANCE

Increasing blood viscosity via an increase in hematocrit reduces peripheral vascular resistance, lowering blood pressure and increasing perfusion via the increase in cardiac index.

may be due, in part, to the increase in viscosity and the decrease in cell-free layer (plasma layer) width, affecting blood properties and the endothelium interface, where shear signals are produced.

Increased endothelial shear stress promotes production of endothelium vasoactive and nonvasoactive factors (6). Knock-out mice deficient in endothelial nitric oxide (NO) synthases, and hamsters treated with N (G)-nitro-L-arginine methyl ester (L-NAME), did not lower MAP at Hct, whereas wild-type mice and untreated hamsters showed maximal reduction in MAP. These findings led to the conclusion that MAP decreased as the product of vasodilation mediated by endothelial NO. This explains that the decrease in VR is proportional to the initial increase in Hct; however, this is eventually counteracted by the increase in VR due to the increase in blood viscosity. There is a point at which the vasodilator effect due to the increase in shear stress no longer compensates for the raise in viscosity, and VR and MAP are increased above baseline.

Evidence for a direct link between blood viscosity, shear stress, the production of NO, and vasodilation was reviewed by Smieško and Johnson (7), who showed that increasing flow locally in arterioles (and therefore shear stress) caused "flow-dependent vasodilation." de Wit and coworkers (8) showed that elevating plasma viscosity (and presumably shear stress) causes NO-mediated dilation in hamster skeletal muscle. Tsai and colleagues (9) showed that increasing plasma viscosity in extreme hemodilution in the hamster window chamber model increased shear stress, flow, and perivascular NO (measured with NO-specific microelectrodes). Shear stress also elicits the production of prostacyclin; however, this mediator appears to provide residual vasodilatory effects compared with those that are NO mediated (10).

Changes in Hct may also affect NO bioavailability due to changes in NO scavenging by blood hemoglobin (Hb). The width of the plasma layer should decrease when Hct increases, bringing red blood cells (RBCs) closer to the endothelium, enhancing NO scavenging and counteracting effects of increased NO production (11, 12). Increasing Hct with non-oxygen-carrying (and therefore non-NO-scavenging) RBCs should extend the positive balance of vasodilation and the range over which the increase in Hct lowers MAP compared with oxygen functional RBCs.

In this study, we test the hypothesis that increasing the Hct using RBCs with decreased NO scavenging will extend the range in which increases in Hct cause hypotension when compared with similar increases in Hct using functional RBCs. NO scavenging by the native RBCs was reduced by isovolemic exchange transfusion of 40% of the animals' blood volume (BV) in the hamster

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Correspondence and requests for reprints should be addressed to Marcos Intaglietta, Ph.D., Department of Bioengineering, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92037-0412. E-mail: mintagli@ucsd.edu

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window chamber model with MetRBCs, RBCs with Hb previously oxidized to methemoglobin (MetHb). MetHb has a 5,000-fold decreased reaction rate with NO relative to either oxy or deoxyHb (13–15). The exchange transfusion did not affect BV, and reduced native oxygen carrying capacity by 40%, and above the level at which the systemic oxygen supply falls below the metabolic requirements of hamsters.

MATERIALS AND METHODS

Animal Preparation

Investigations were performed in 55- to 75-g male Golden Syrian Hamsters (Charles River Laboratories, Boston, MA). Animal handling and care were provided following the procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The study was approved by the local Animal Subjects Committee. The window chamber model is widely used for microvascular studies in the unanesthetized state, and the complete surgical technique is described in detail elsewhere (16, 17). Briefly, hamsters were prepared for chamber implantation with a 50 mg/kg intraperitoneal injection of sodium pentobarbital 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. A chamber consists of two identical titanium frames with a 15-mm circular window. 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. Saline and then a cover glass were placed on the exposed skin held in place by the other part 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 catheter implantation. The animal was anesthetized again with sodium pentobarbital. Arterial and venous catheters (PE-50) were implanted in the carotid artery and jugular vein. The catheters were filled with a heparinized saline solution (30 IU/ml) to ensure their 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. The window chamber preparation was used in these experiments because it provides a well-established animal model to compare results with those of a previous study by Martini and colleagues (4). The window chamber keeps the catheters out of reach of the animal during the recovery period. Experiments were performed three days after the initial surgery, and only with animals that passed established systemic inclusion criteria.

Inclusion Criteria

Animals were suitable for the experiments if systemic parameters were within normal limits, namely, heart rate (HR) greater than 340 beats/minute, MAP greater than 80 mm Hg and less than 125 mm Hg, systemic Hct greater than 45%, and PaO_2 greater than 50 mm Hg.

Preparation of Functional RBCs and RBCs Containing MetHb

Functional RBCs (oxygen functional RBCs). RBCs were obtained as described by Martini and coworkers (4). Briefly, blood was collected from a donor in a heparinized syringe, centrifuged 5 minutes at 2,000 rpm, and buffy coat was discarded and packed RBCs were stored. Packed cells Hct was adjusted to the desired Hct by dilution using plasma from the donor.

MetRBCs (accelerated oxidation via nitrite). Oxidized RBCs were prepared using the RBCs collected from donor. RBCs were resuspended in an equivalent amount of normal saline and mixed gently for 2 minutes with sodium nitrite (100 μl of 1 M sodium nitrite per 5 ml of resuspended RBCs). Cells were then washed three times using heparinized saline and centrifuged at 2,100 rpm. MetHb-loaded RBCs (MetRBC) were stored at 4°C. Aliquots of these cells were tested, and only those cells with 95 to 100% MetHb were used. To measure MetHb, we collected approximately 50 μl of blood in microhematocrit tubes. MetHb was determined using a Co-oximeter (IL 682; Instrumentation Laboratory, Lexington, MA). Calibration was ensured using standard levels at 5.2, 2.6, and 1.2% MetHb (RNA Medical, Bayer Diagnostics,

Medfield, MA). In normal conditions, the concentration of cell-free Hb in the hamster is lower than 0.08 g/dl, and cell-free MetHb cannot be detected. MetRBCs were re-suspended in fresh frozen plasma (FFP) taken from hamster donors to produce the desired Hct.

MetRBC (autoxidation). A second method was used to prepare MetRBCs, without adding nitrite. Briefly, previously washed RBCs were incubated fully oxygenated at 37°C for 36 to 48 hours. MetRBCs produced this way were tested for MetHb levels, as described before, and only those with 85 to 100% MetHb were used. RBC manipulation (pipette, reaction, and transfer) was performed in a laminar flow hood for sterility.

Isovolemic Exchange Transfusion with Functional RBCs and MetRBCs

Exchange transfusion was 40% of the estimated animals' BV, 7% of the animals' body weight (BW). Since animal weights ranged between 55 and 75 g, their BVs ranged between 3.9 and 5.3 ml, and their actual exchange transfusion volumes ranged between 1.4 and 2.1 ml, and varied between the animals. The RBCs were infused using a dual syringe pump ("33" syringe pump; Harvard Apparatus Inc., Holliston, MA) into the jugular vein catheter at a rate of 100 $\mu\text{l}/\text{minute}$. Blood was simultaneously withdrawn from the carotid artery catheter at the same rate.

Systemic Parameters and Blood Chemistry

MAP and HR were recorded continuously (MP 150; Biopac System, Santa Barbara, CA) except during the actual blood exchange. Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes (Readacrit Centrifuge; Clay Adams, Division of Becton-Dickinson, Parsippany, NJ). Hb content was determined spectrophotometrically from a single drop of blood (B-Hemoglobin; Hemocue, Stockholm, Sweden). Arterial blood was collected in heparinized glass capillaries (0.05 ml) from the carotid catheter and immediately analyzed for PaO_2 , PaCO_2 , and pH (Blood Chemistry Analyzer 248; Bayer, Norwood, MA). The comparatively low PaO_2 and high PaCO_2 of these animals is a consequence of their adaptation to a fossorial environment.

Measurements of Blood Viscosity

Blood was collected in heparinized syringes and analyzed for viscosity in a cone/plate viscometer (DV-II plus; Brookfield Engineering Laboratories, Middleboro, MA). Viscosities are reported at 150 s^{-1} shear rate.

Blood Rheological Changes Due to Nitrite-Accelerated Oxidation

Blood samples collected from donors were prepared as described before for exchange transfusion (functional RBCs and MetRBCs), then mixed with heparinized fresh blood. Final Hct of the samples was fixed to 52%. These samples consisted of either 50% fresh heparinized blood mixed with either 50% RBCs or 50% MetRBCs, and viscosity-shear rate relations were measured using a cone and plate viscometer (DV-II plus; Brookfield). Studied shear rates were in the range of 10 to 750 s^{-1} .

Cardiac Output Measurements

CO was measured with the modified thermodilution technique described by Cabrales and coworkers (18). Due to volume addition with each CO measurement (150 μl of saline), measurements were made twice in the same animal. Cardiac index (CI) is defined as the measured CO divided by the BW, and used because it eliminates CO animal size dependence.

Measurement of Plasma Layer Width

The cell-free layer between the erythrocyte column and the vessel was continuously determined from video images of microcirculatory vessels (19). In this technique, the light intensity of a linear array of pixels perpendicular to the vessel axis is continuously recorded from a video image of a microcirculatory vessel (FASTCAM ultima SE, framing rate up to 4,500/second; Photron, San Diego, CA). An optical threshold level is used to establish the interface between the cell-free layer and the erythrocyte column on the line intensity data along a perpendicular cross-section of a blood vessel in the video image (19). The distance

between this optical threshold and the interface between the endothelial surface and the plasma is measured continuously using image analysis software (MATLAB; Mathworks, Natick, MA).

Plasma Nitrite Concentration

Nitrite plasma concentration was measured at baseline, at 30 minutes, and at 1 hour after the exchange, to ensure the absence of increased nitrite levels as a result of the method used to produce the MetRBCs. The Griess reactant was added to convert nitrites into a colorimetric compound (20). Optical absorbance was determined at 540 nm (Lambda 20 UV/VIS spectrometer; Perkin Elmer, Foster City, CA).

Experimental Protocol

Unanesthetized animals were placed in a restraining tube. They were given 30 minutes to adjust to the tube environment before baseline measurements (MAP, HR, Hct, Hb, and blood gases). After analysis of baseline parameters, baseline CO was measured. Animals were exchange transfused and followed for 90 minutes after the exchange. MAP and HR measurements were taken every 30 minutes; each measurement represents the average of MAP over 4 minutes. One hour after the end of the exchange transfusion protocol CO was measured again. Assessment of the effects of changes in MAP as a function of Hct were made 1 hour after the end of the exchange, to avoid artefacts created by the exchange transfusion protocol (which, although carefully implemented, resulted in cardiovascular in minor perturbations that subsided after 30 min). Hct and Hb were measured again 1 hour after the end of exchange protocol. Blood oxygen-carrying capacity was measured spectrophotometrically as the difference between total Hb and MetHb, from blood samples obtained 1 hour after the exchange protocol. Blood for viscosity determinations was collected at the end of the observation period.

Experimental Groups

Identical exchange transfusion protocol was used in all groups. MetRBC: animals exchange transfused with MetRBCs; OxyRBC: animals exchange transfused with functional RBCs (data previously published [4]). Additional experiments to complete similar range of Hct variation for both groups were added to previous results (4). Close to 60% of the native RBCs remained in the animals after the exchange protocol. The MetRBC group had approximately 60% of the native RBC (~ 10 g/dl), which maintained oxygen carrying capacity (Figure 1).

Comparison after 30% Increase in Hct and L-NAME Treatment

Changes in MAP after a target 30% increase in baseline Hct (effective Hct $\sim 62\%$) were compared between animals transfused with functional RBCs and MetRBCs. These two groups were subsequently treated with L-NAME (30 mg/Kg, 20 μ l/min continuous infusion intravenously), and 10 minutes after stabilization MAP was recorded (21).

Statistical Analysis

Results are presented as mean $\pm$ SD unless otherwise noted. MAP changes are reported in absolute values. Changes in Hct are presented as percent changes from baseline Hct. CI is also reported as a change from baseline. Data on VR and vascular hindrance (VR divided by blood viscosity) are presented as relative to levels at baseline. A ratio of 1.0 signifies no change from baseline, while lower or higher ratios are indicative of changes proportionally higher or lower than baseline. Comparison of baseline values from the three different groups of animals was performed using one-way ANOVA, and *post hoc* analyses were performed with the Bonferroni's multiple comparison test. Data within each group were analyzed using ANOVA for repeated measures and followed by the Bonferroni's multiple comparison test. Changes were considered statistically significant if $P < 0.05$. The data were fitted to second-order polynomials and the resulting curves were compared by means of the F-test, and considered to be different if the F-test indicated a significantly smaller sum of squares for the deviations in each individual fit compared with the deviation in the fit to the pooled data (22).

Figure 1. Concentration of reduced hemoglobin (Oxy and DeoxyHb) in blood after increasing hematocrit (Hct) by exchange transfusion with MetRBCs. The concentration of total Hb and MetHb was measured spectrophotometrically; reduced Hb was calculated as the difference between total Hb and MetHb.

RESULTS

Experiments were performed in 40 animals; 16 were used for the measurement of MAP as a function of changes in Hct, 9 were used to measure CO and calculated VR, and 4 were used to determine the concentration of nitrite in plasma. Three animals were used to obtain a 30% increase in Hct with MetRBCs, and were compared with four animals whose Hct was increased by 30% using functional RBCs (data added to previously published study [4], OxyRBC group). Four additional animals were used to determine the effect of Hb oxidation; they were exchange transfused with MetRBC (produced by autoxidation) to increase Hct 12% above baseline. The average weight of animals was 63 ± 15 g.

Systemic parameters were measured at baseline and at 1 hour after exchange transfusion. Hct, Hb, MAP, HR, blood gas parameter, and blood viscosity after exchange with MetRBC are given in Table 1. Comparisons between systemic parameters for both study groups (MetRBC and OxyRBC) after 30% increase in Hct are given in Table 2. As in other studies,

TABLE 1. LABORATORY PARAMETERS

	Baseline	1 h after Exchange
Hct, %	47.9 ± 2.0	$54.7 \pm 6.2^*$
Hb, g/dl	14.8 ± 0.6	$16.8 \pm 1.9^*$
MetHb, g/dl		$7.5 \pm 1.9^*$
MAP, mm Hg	102.4 ± 6.5	$96.4 \pm 7.5^*$
Heart rate, bpm	432 ± 35	446 ± 32
PaO ₂ , mm Hg	56.4 ± 5.6	$62.4 \pm 5.6^*$
PaCO ₂ , mm Hg	54.2 ± 4.2	51.6 ± 6.0
Arterial pH	7.344 ± 0.007	7.346 ± 0.008
BE, mmol/l	3.0 ± 1.4	2.2 ± 1.6
Viscosity, cp		
Blood [†]	4.2 ± 0.4	$4.7 \pm 0.5^*$
Plasma [†]	1.2 ± 0.1	1.2 ± 0.1
COP, mm Hg	17.8 ± 1.6	17.6 ± 1.8

Definition of abbreviations: BE, base excess; COP, colloid osmotic pressure at 27°C; Hb, hemoglobin content of blood; Hct, systemic hematocrit; MAP, mean arterial blood pressure; MetHb, methemoglobin.

Values are means $\pm$ SD. Baseline included all the animals in the study.

* $P < 0.05$ compared with baseline.

[†] Shear rate of 160 s⁻¹ at 37°C.

TABLE 2. LABORATORY PARAMETERS AFTER 30% INCREASE IN HCT

	MetRBC		OxyRBC	
	Baseline	1 h	Baseline	1 h
Hct, %	48.3 ± 0.6	62.1 ± 1.4	47.8 ± 1.3	61.2 ± 1.6
Hb, g/dl	14.7 ± 0.4	18.7 ± 0.9	14.5 ± 0.5	18.4 ± 0.8
MetHb, g/dl		8.4 ± 1.0		
MAP, mm Hg	102.4 ± 6.5	96.4 ± 7.5	105.0 ± 2.4	115.2 ± 2.4
Heart rate, bpm	441 ± 22	426 ± 21	447 ± 26	430 ± 24
PaO ₂ , mm Hg	66.5 ± 8.2	72.0 ± 2.9	52.5 ± 6.4	69.1 ± 4.4
PaCO ₂ , mm Hg	55.7 ± 7.3	52.1 ± 2.3	48.2 ± 5.6	51.7 ± 5.6
Arterial pH	7.371 ± 0.014	7.335 ± 0.017	7.355 ± 0.051	7.343 ± 0.037
BE, mmol/l	6.0 ± 4.9	1.3 ± 0.6	2.7 ± 2.6	2.3 ± 0.8
Viscosity, cP				
Blood*		6.6 ± 0.5		6.5 ± 0.4

Definition of abbreviations: BE, base excess; Hb, hemoglobin content of blood; Hct, systemic hematocrit; MAP, mean arterial blood pressure; MetHb, methemoglobin.

Values are means ± SD.

* Shear rate of 160 s⁻¹ at 37°C.

hamsters present a natural Hct variability, which in the present study ranged from 42 to 48%. To account for the variability of results and reduce the number of animals required to detect effects in the range found in previous studies, results are presented as percent changes from baseline Hct. This may be somewhat confusing for Hct changes, since Hct is itself a fraction (commonly reported as a percentage). In this context, Hct should be considered as a concentration of RBCs in the volume of blood, the difference being that the amount of "solute" (i.e., RBCs) is expressed as a volume rather than mass weight.

Blood Oxygen-Carrying Capacity after Exchange Transfusion

In the MetRBC group, blood maintained approximately 60% of baseline oxygen-carrying capacity (9.4 ± 0.5 g/dl) for all the experiments. We strived to maintain similar oxygen-carrying capacity; the final result had some variability, and in two instances Hb was reduced slightly below 60% of baseline, as shown in Figure 1.

Nitrite Levels

Nitrite levels were not different between baseline and 1 hour after the exchange with MetRBCs (baseline 3.6 ± 1.2 μM, 1 hour after exchange 3.9 ± 1.6 μM).

MAP Changes after Increasing Hct with MetRBCs

MAP did not change from baseline when exchange transfusion did not modify baseline Hct, although, 40% of native RBCs were replaced with MetRBCs. Increasing the overall Hct above baseline using higher Hct in the infused MetRBCs during the exchange transfusion caused decreases in MAP proportional to the increases in Hct. MAP reached a minimum and then increased as Hct was progressively increased. Increasing Hct between 0 and 30% with MetRBCs ($45 \pm 3\%$ at baseline versus $59 \pm 3\%$ maximum attained after exchange transfusion) while keeping total BV constant decreased MAP between 1 and 17 mm Hg of baseline, with the maximum interpolated pressure drop of 12 mm Hg based on a second-order polynomial occurring when Hct was increased to 12% of baseline (Figure 2). MAP increased above baseline when Hct was increased 30% above baseline (59% Hct). MAP changes occurred 30 to 60 minutes after exchange transfusion and remained stable for the whole observation period. MAP and MetHb concentration were determined at 1 hour after the end of the exchange transfusion. From previous studies in the same preparation, it was found that MetHb is reduced at the rate of 10%/hour (23). Changes in MAP versus percentage of Hct increase were fitted by a second-order polynomial ($R^2 = 0.87$). The maximal decrease in MAP was not different between MetRBCs produced via nitrite-accelerated oxidation or autoxidation, suggesting no detectable effect of the remaining nitrite contamination in the infused MetRBCs (Figure 2). MAP responses to small increases in Hct studied by Martini and colleagues using functional RBCs (OxyRBC group) are shown in Figure 3, where they are compared with the results of the present study (4).

Cardiac Index

CI increased throughout the range of Hct changes, being approximately 12% higher than baseline. These data are fitted by a second-order polynomial ($R^2 = 0.37$) (Figures 2 and 3). CI

Figure 2. Changes in mean arterial blood pressure (MAP) and cardiac index (CI) as a function change in Hct using MetRBCs. In each instance at least 40% of the native RBCs were exchanged with MetRBCs. Increased Hct above baseline values was attained using additional MetRBCs. Solid circles: in the range between 10 and 15% increases in Hct from baseline were obtained with MetRBC produced by autoxidation of the Hb. No statistical difference was detected after increasing Hct by 10 to 15% using MetRBC, produced by accelerated oxidation via nitrite or autoxidation (incubation), $P = 0.28$. Normovolemia was maintained in all instances. The solid line shows the relation between MAP and Hct if blood viscosity is the only determinant of MAP.

Figure 3. Changes in MAP and CI as a function of the increase in Hct using MetRBCs. The data shown are compared with the data from the study of Martini and colleagues (4, 5) using functional RBCs. Rectangular shadow area in the range between 27 and 30% increases in Hct from baseline shows the data used for direct comparison of the OxyRBC and MetRBC groups. Solid black thick line presents theoretical line in which MAP is only a function of the variation in blood viscosity due to changes in Hct, assumed to be linear in this range as shown by Kameneva and colleagues (31). The maximal difference in blood pressure obtained using MetRBCs and functional RBCs is not statically significant ($P < 0.10$). The difference in CI is significant ($P < 0.01$). The shaded area shows the data obtained from exchange transfusing MetRBCs that were oxidized by exposure to ambient air, and the data used in Figure 7.

and MAP returned to baseline values at approximately the same increase in Hct ($\sim 30\%$).

Blood Rheological Changes

Viscosity of blood samples with Hct approximately 52% of either mix of 50/50 functional RBCs and fresh blood or mix of 50/50 MetRBCs and fresh blood were not different between shear rates of 10 and 450 s^{-1} . At 750 s^{-1} the viscosities were 5.65 (50/50 functional RBCs and fresh blood) and 5.42 cp (50/50 MetRBCs and fresh blood), respectively. This difference cannot be considered statistically significant, because it is within the 0.3-cp resolution limit of the viscometer.

Peripheral Vascular Resistance

VR obtained from the ratio MAP divided by CI was significantly decreased throughout the range of increased Hct. It reached a minimum at the Hct increase of 12% (Figure 4). Figure 5 shows the result of calculated vascular hindrance (VH), for both the MetRBC and OxyRBC groups. VH changes are mostly due to changes in vascular diameter and independent of changes in blood viscosity.

Changes in MAP after 30% Increase in Hct with Functional RBCs and MetRBCs, and NO Synthase Effects (L-NAME)

Independent experiments were made with either functional RBCs or MetRBCs to increase baseline Hct by 30% ($\sim 62\%$ Hct). As shown in Figure 6, there was a significant difference in MAP between the two groups in support of the hypothesis that VR at the same Hct increase was significantly less for the MetRBC group. Treatment of each group with L-NAME increased MAP to identical levels in both groups. Changes in MAP elicited by L-NAME suggest that differences observed between groups were NO related. L-NAME eliminated the NO-vasodilatory responses observed in the OxyRBC group, although the change was more significant in the MetRBC group. This result can be interpreted to be indicative that there was less NO scavenging in the MetRBC group and therefore an increased vasodilatory reserve.

Measurement of Plasma Layer Width

The plasma layer width was measured as a function of arteriolar diameter at baseline Hct and when baseline Hct was increased by 20%. As show in Figure 7, there is a statistical difference in the measurement of the plasma layer width between control and Hct augmented to 20% of baseline. The dependence of plasma layer width on blood vessel diameter was statistically significant for the two Hcts tested. The slopes of the two lines are not statistically significantly different; however, the difference in y-axis intercept is. Assuming that both lines have the same slope, we estimate that increasing Hct by 20% lowers the plasma layer width by about $0.3 \mu\text{m}$ in microvessels ranging from 15 to $35 \mu\text{m}$ in diameter.

Figure 4. Changes in vascular resistance (VR) after exchange transfusion with MetRBCs as a function of the increase in Hct. Vascular resistance was computed from the data in Figure 2. Data obtained by increasing Hct with RBCs (OxyRBC) is reproduced from the study of Martini and coworkers (4, 5). The difference in VR between the two studies is statistically significant ($P < 0.01$). The solid line shows the relation between VR and Hct if blood viscosity is the only determinant of MAP.

Figure 5. VR independent of blood viscosity (Vascular hindrance, VH). This graph shows the overall conditions of vascular tone of the circulation indicating globally whether the circulation is constricted or dilated. It provides an overall average, and may not represent the perfusion of specific tissues. If blood viscosity were the only factor affecting VR, the relationship between VH and Hct should be a horizontal line.

DISCUSSION

The principal finding of this study is that reducing oxygen-carrying capacity (without changing BV) by substituting 40% of native RBCs with non-oxygen-carrying RBCs, whose Hb was converted to MetHb (MetRBCs), did not change MAP or VR. Increasing Hct above baseline without changing BV with MetRBCs, thereby increasing the proportion of MetRBCs beyond the initial 40%, initially reduced MAP to the same extent as in previous experiments using functional RBCs up to an increase in Hct of $\sim 12\%$ (total Hct = 54%).

Augmenting Hct beyond 12% of baseline by increasing the Hct of the exchange transfused MetRBCs (while maintaining BV) reversed the decreased in MAP, which returned to baseline when the increase in Hct was around 30% above baseline ($\sim 62\%$ Hct), leading to a “U” shaped relationship between MAP and

Figure 6. Comparison of the effects on MAP of increasing Hct by 30% from baseline (final Hct $\sim 62\%$) using MetRBC according to the experimental protocol of this study, and using functional RBCs (OxyRBC). L-NAME was administered after cardiovascular conditions stabilized, and MAP was recorded as it reached stable conditions.

Figure 7. Plasma layer width as a function of microvessel size (arterioles and venules) at baseline Hct (48% Hct), and when baseline Hct was increased by 20% (58% Hct) using RBCs. There is no statistical difference between the slopes of the linear regressions; however, the correlation between plasma layer width versus vessel size is statistically significant for both Hcts. The average difference between plasma layer width between baseline and 20% increase Hct is $0.3 \mu\text{m}$, which is statistically significant ($P < 0.05$).

Hct. These effects were paralleled by a consistent elevation of CI; throughout the range of Hcts tested, CI returned to baseline values after an increase in Hct of 30% from baseline.

These results are qualitatively similar to those obtained by Martini and coworkers (4, 5) using functional RBCs, but differ in some important quantitative features, since the range over which MAP is decreased versus the increase in Hct is significantly extended with the use of MetRBCs. In the studies of Martini and colleagues (4, 5) MAP decreased with an Hct increase up to 54% (an increase of 20% above baseline), while in the present study the range is extended to a 30% increase above baseline in Hct. The difference between results for MAP is not statistically significant ($P < 0.10$), while that for CI and VR is significant ($P < 0.01$), as shown in Figures 3 and 4.

Our aim was to increase Hct, and therefore blood viscosity, without increasing NO scavenging. We contemplated several approaches to hinder the intrinsic NO-scavenging capacity of Hb, and chose Hb oxidation as the least likely to introduce artefacts, and suitable for short-duration experiments. We considered carboxyhemoglobin (COHb); however, carbon monoxide is released from Hb and the presence of CO in the circulation could be potent vasodilator in the hamster model, as shown by Hangai-Hoger and colleagues (24). Another alternative was inactivation of Hb gas transport carrying capacity using cyanide to form cyanomethemoglobin; however, this reaction affects RBCs membrane (increases fragility) and the cyanide cannot be removed 100% from the treated blood.

When Hct was increased 12% above baseline (i.e., from 48% to 52% Hct) with additional MetRBCs (MetRBCs being now 53% of the total RBCs in circulation), the resulting decrease in MAP obtained with MetRBCs is identical to that obtained by increasing Hct with functional RBCs (OxyRBC group); however, CI does not change as much, causing a lesser change in VR. Since Hct and therefore blood viscosity should be the same in both conditions, it appears that MetRBCs do not produce an extra vasodilator effect beyond that induced by functional RBCs. In fact, in the range of Hcts leading to maximal MAP effects, the vasodilatory effect is reduced compared with that attained by functional RBCs.

Blood viscosity had a tendency to be somewhat lower ($\sim 4\%$) for the blood samples that were 50/50 MetRBCs/fresh blood when compared with samples of functional RBCs/fresh blood at the same Hct ($\sim 52\%$), although the difference cannot be considered significant due to resolution of the technique. Therefore, in principle, part of the difference in response between the two groups could be due to the lower viscosity due to the presence of MetRBCs. However, at the maximal Hct investigated when Hct was increased by 30% and L-NAME was administered, there was no difference in the final MAP. In this condition, NO-based vasodilatory mechanisms are not operational, and a difference in blood viscosity should be evidenced by a change in MAP; however, this was not obtained (Figure 6).

It could be argued that the fall in MAP over the extended range of Hct is due to the lowering of heart function resulting from decreased oxygen delivery to the heart, as a consequence of the lowered intrinsic oxygen carrying RBCs in blood (9.4 ± 0.5 g/dl). This effect is observed when blood oxygen-carrying capacity is lowered in hemodilution below the level that oxygen delivery is compensated by increased CO (3, 25). However, this may not be the full explanation, since increasing Hct by 30% with MetRBCs caused MAP and CI to be above baseline values, indicating that the heart was able to maintain MAP in the face of significantly increased VR due to the increased blood viscosity.

Increasing Hct more than 30% from baseline may present as much as a 40 to 50% increase in blood viscosity, since in the high Hct range the relationship between blood viscosity and Hct is nonlinear (26). This increased viscosity should lead to a proportional increase in VR and MAP; however, this was not observed in the MetRBC group. The absence of a major pressor response indicates that the increase in Hct leads to vasodilation that compensates for the related increase in viscosity.

The presence of significant vasodilation becomes evident when the increased blood viscosity is factored out from VR as shown in Figure 5. This rendition of the data evidences the significant vasodilation caused by increasing Hct with functional RBCs and MetRBCs.

Lowering Hb concentration should lower NO scavenging according to modeling analysis by Buerk (27). This effect would be important in the plasma free layer, which should reduce as Hct increases, thus bringing RBCs closer to the endothelial surface. Lowering the concentration of RBCs in the blood column would decrease NO scavenging, as shown by the analysis of Chen and colleagues (28). We verified the changes of plasma free layer width due to the increase in Hct. Measurements were performed at baseline and after an increase in Hct of 20% using functional RBCs. This value was chosen because this increase in Hct corresponded to levels at which MAP, CI, and VR return to baseline values (4, 5). Therefore, this could be defined as a situation in which the presumed NO bioavailability increases due to augmented shear stress and is balanced by NO scavenging and the increased blood viscosity. Changes in the plasma layer width are due to crowding of RBCs and should occur with the addition of either functional RBCs or MetRBCs. We propose that decreasing the plasma layer width with RBCs increases NO scavenging. Conversely, if the plasma layer width is lowered by an increase in Hct by which approximately 50% of RBCs do not scavenge NO, the resulting vasoactivity is diminished.

There was no effect on MAP or VR when native RBCs were decreased by 40% without changing Hct, a condition in which, presumably, NO scavenging capacity should have been altered by the presence of MetRBCs. However, the effect became apparent when Hct increased significantly. This result could be explained if NO scavenging by RBCs is nonlinearly related to the size of the plasma layer, becoming prominent after a significant decrease of its width.

Results showing the decrease in VR and MAP as Hct increases should be directly related to the increase of shear stress in the blood/endothelium interface due to increased blood viscosity (29). This effect is directly related to flow-dependent vasodilation (7), where increased shear stress is due to increased viscosity rather than blood flow velocity, with vasodilation overcoming the increased viscosity. The subsequent increase of MAP and VR with the increase in Hct is due to viscosity eventually overcoming the effects of vasodilation, and possibly the increased NO scavenging due to the decreased plasma layer.

Comparison of Results with Functional RBCs and MetRBCs (OxyRBC and MetRBC Groups)

The difference in results between the present experiments and those previously made using RBCs (OxyRBC group) (4) would appear to be due to two effects: (1) the limitation on cardiac function due to increase Hct without an associated increase in oxygen-carrying capacity, and (2) reduction in NO scavenging due to lower concentration of reduced Hb (oxy and deoxy) as the plasma layer is reduced. These effects are observed as an increase in the extension of the Hct range which vasodilation compensates for the increase in viscosity.

MAP and CI returned to baseline values at the Hct increase of 30% with MetRBCs, corresponding to a blood viscosity increase of 30% (at Hct of 62% viscosity is ~ 6.58 cp). Therefore it can be assumed that the heart performance is not significantly limited by the reduction in oxygen-carrying capacity. Effects due to the increase in blood viscosity (product of the increase in Hct) should be common to both groups (MetRBC and the OxyRBC), since endothelial function should be similarly affected in both experiments, unless there are other factors involved.

The increased intrinsic oxygen-carrying capacity via increased Hct has been shown to lead to an autoregulatory response, which is attributed to an oxygen-sensing mechanism that maintains oxygen supply at a constant level (30). Therefore, an explanation is that the increase in MAP and VH observed in OxyRBC group was due to oxygen autoregulation, present to a lesser degree when Hct increases with functional RBCs that do not carry oxygen. However, this mechanism does not appear to be operational for the initial small increases in Hct in the OxyRBC group, since oxygen transport capacity of the circulation ($CI \times \Delta Hct$) increases to a maximum of approximately 45% before decreasing presumably due to increased viscosity and NO scavenging. Conversely, autoregulatory effects may not be operational in the MetRBC experiments, since oxygen transport capacity was constant.

Our study provides indirect evidence for NO scavenging by RBCs *in vivo* in terms of changes in VR, a surrogate effect. An additional finding in support of the influence of NO scavenging is that the OxyRBC group has a more pronounced increase in MAP than MetRBC, as evidenced by the steeper slope in the MAP and VR versus Hct plots (Figures 3 and 4). Both slopes are steeper than a theoretical line in which VR is only a function of the variation in blood viscosity due to changes in Hct, assumed to be linear in this range as shown by Kameneva and coworkers (31). An interpretation of this result is that with RBCs there is an extra vasoconstrictor effect that increases VR beyond the effect due to blood viscosity. Furthermore, a reduced effect, mostly due to the increase in viscosity and remaining NO scavenging from the native RBCs, was observed with MetRBCs.

These considerations are further supported by the analysis of the trend of VH, which factors out the effect of blood viscosity on VR. Figure 5 shows that increasing Hct beyond that corresponding to the minimum of MAP for OxyRBC sharply increases VH, an effect due to vasoconstriction, while minimally impacting VH

when the native RBC were replaced by MetRBCs. The significant difference in the behavior of VR for both groups (OxyRBC and MetRBC) suggests the existence of an extra vasoconstrictive effect due to increasing NO-scavenging capacity (blood Hb concentration) as plasma layer is decreased.

Nitrite concentration was measured to ensure the absence of increased nitrite concentration, a consequence of the method used to produce MetRBCs. There was no statistical difference in this parameter between baseline and the time of measurement of MAP. Use of L-NAME showed no difference in MAP between OxyRBC and MetRBC groups, after an Hct increase of 30% from baseline. An Hct increase of 10 to 15% from baseline using RBCs with Hb autoxidated to MetHb, produced similar decrease in MAP than the RBC, whose Hb was rapidly oxidized via nitrite. Thus, we conclude that there was no detectable effect that resulted from the method of preparation of MetRBCs.

In summary, our findings show that increasing Hct reduces VR, lowering MAP and increasing perfusion via the increase in CI. This effect takes place when Hct is increased with functional RBCs and using MetRBCs up to an Hct increase of approximately 12%, where it is maximal. MAP returns to baseline values when Hct is increased 20% for the OxyRBC group and 30% for the MetRBC group. Thus the lowering of MAP persists over a greater increase in Hct with MetRBCs than when RBCs were used. The effects up to these thresholds are due to increasing blood viscosity "enough" to cause shear stress-induced stimulation of vasodilation. Above these thresholds, viscosity "swamps out" the vasodilator effects. Our findings on the occurrence of hypotension and reduction in VR at higher Hct with MetRBCs than with functional RBCs hemoconcentration are compatible with results predicted by mathematical modeling, where the effects are attributed to reduced NO scavenging in the plasma layer (11, 28). Furthermore, eliminating the effects of blood viscosity from the calculation of VR shows that at the Hct when MAP returns to baseline there is still significant vasodilation, showing that at the higher Hcts blood viscosity becomes the determinant factor in regulating VR. In conclusion, the relation between MAP, NO scavenging properties of Hb, blood viscosity, Hct, and blood flow appears to be mediated within the narrow blood tissue interface, the "plasma layer," suggesting that further analysis of the regulation of VR be directed at quantifying effects in this interface.

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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