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Title: Acute reductions in hematocrit increase flow-mediated dilation independent of resting nitric oxide bioavailability in humans

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Acute reductions in hematocrit increase flow-mediated dilation independent of resting nitric oxide bioavailability in humans

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ABSTRACT

Hemoglobin (Hb) may impact the transduction of endothelium-dependent and nitric oxide (NO) mediated vasodilator activity, given its contribution to shear stress stimuli and diverse biochemical reactions with NO. We hypothesized that an acute reduction in [Hb] and hematocrit (Hct) would increase brachial artery flow-mediated dilation (FMD). In eleven healthy males (28±7 years; 23±2 Kg · m^{-2}), FMD (Duplex ultrasound), arterial blood gases, Hct and [Hb], blood viscosity, and NO metabolites (ozone-based chemiluminescence) were measured before and after isovolumic hemodilution, where ~20% of whole blood was removed and replaced with 5% human serum albumin. Hemodilution reduced Hct by 18±2% ($P<0.01$) and whole blood viscosity by 22±5% ($P<0.01$). Plasma nitrite, *S*-nitrosothiols, and total red blood cell NO were collectively reduced by ~15-40% ($P<0.05$ for all). Brachial artery FMD increased by ~160% from 3.8±2.1 to 9.7±4.5% ($P<0.01$). Statistical covariation for the shear stress stimulus did not alter FMD, indicating that the increase in FMD was not directly related to alterations in whole blood viscosity and the shear stimulus. Collectively, these findings indicate that hemoglobin scavenging of NO appears to be an important factor in the regulation of FMD under normal conditions through constraint of endothelium-dependent NO-mediated vasodilation in healthy humans.

KEY POINTS SUMMARY

- Changes in hematocrit influence nitric oxide signaling through alterations in shear stress stimuli and hemoglobin scavenging of nitric oxide; these two regulatory factors have not been assessed simultaneously
- Isovolumic hemodilution led to a marked increase in brachial artery flow-mediated dilation in humans
- The increase in flow-mediated dilation occurred in the face of an unaltered shear stress stimulus for vasodilation and reduced resting steady-state nitric oxide levels in the blood
- Collectively, our data point towards hemoglobin scavenging of nitric oxide as a key regulatory factor of brachial flow-mediated dilation and highlight the importance of simultaneous consideration of nitric oxide production and inactivation when investigating vascular function in humans

INTRODUCTION

The endothelium is a single cell layer that lines the adluminal surface of the vasculature. The production of autocoids within the endothelium and paracrine signaling contribute to vascular homeostasis with endothelial function related to cardiovascular risk (Green *et al.*, 2011). The prototypical pathway by which the endothelium regulates blood flow is through nitric oxide (NO) bioactivation (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). Also implicated in the transduction of NO signaling is hemoglobin (Hb), through its independent influence on NO transport/release, NO scavenging, and rheological properties of the blood (i.e. whole blood viscosity) that impact mechanotransduction of shear stress. Therefore, alterations in hematocrit (Hct) and the concomitant changes in [Hb] and whole blood viscosity would be expected to greatly impact endothelium dependent NO-mediated vasodilation.

Shear stress is the tangential force exerted on the endothelium by blood flow. Acute changes in shear stress lead to mechanotransduction of signaling processes (Lansman *et al.*, 1987; Tessier-Lavigne *et al.*, 1988) that govern NO formation and vasorelaxation (Pohl *et al.*, 1986; Rubanyi *et al.*, 1986; Green *et al.*, 2014), a process termed flow-mediated dilation (FMD). These signaling pathways have been well characterized (Green *et al.*, 2017); however, what often evades experimental consideration is the persistent use of shear rate as a surrogate for shear stress (Leo *et al.*, 2019). While *shear rate* is related to the diameter of a vessel and the velocity of the blood flowing through it, *shear stress* further takes into account the important role of blood viscosity. The viscosity of blood impacts the force exerted on the endothelium, mechanotransduction (Ando *et al.*, 1993), and hence should directly alter FMD (Melkumyants *et al.*, 1989; Melkumyants & Balashov, 1990).

In this regard, Hct would be expected to influence FMD given its relationship with whole blood viscosity (Tremblay *et al.*, 2019*b*). In addition to the role of Hct in determining shear stress, Hb is

intimately intertwined in NO biochemistry. *In vitro* work has demonstrated that a decrease in Hct from 50% to 15% triples the half-life of NO (Azarov *et al.*, 2005) due to reduced NO scavenging by Hb. However, Hb can also protect and convey NO bioactivity through the formation of *S*-nitrosothiols (Doctor & Stamler, 2011) and/or deoxyhemoglobin mediated reduction of NO_2^- to form NO (Kim-shapiro *et al.*, 2006). Therefore, changes in Hct (and [Hb]) may influence NO bioactivation independently from its influence on the shear stress stimulus of an FMD test.

Previous studies have demonstrated inconsistent results related to the influence of Hct/[Hb] on FMD. An inverse correlation between [Hb] and FMD has been reported in both healthy individuals (Madsen *et al.*, 2006) and chronic kidney disease patients with (Sonmez *et al.*, 2010) and without type II diabetes (Yilmaz *et al.*, 2009). However, *within* participants that have undergone an experimental manipulation of [Hb], a proportional relationship between [Hb] and FMD has been described whereby decreases in [Hb] reduced FMD in hemochromatosis patients (Giannattasio *et al.*, 2002*a*), and increases in [Hb] increased FMD in end stage renal failure patients (Verbeke *et al.*, 2007). Further, it was recently demonstrated that a ~2% reduction in Hct had no effect on FMD in middle-aged, overweight individuals without any additional co-morbidities (Gnasso *et al.*, 2019). Overall, the relatively small changes in Hct/[Hb] and/or presence of co-morbidities in these latter studies makes reconciliation of these conflicting data problematic. Additionally, none of the aforementioned studies assessing the impact of Hct/[Hb] on FMD provided indices of NO bioavailability or coupled measurements of blood viscosity with appropriate statistical adjustments (Atkinson *et al.*, 2009, 2013) for *shear stress* area under the curve [the stimulus for FMD (Pyke & Tschakovsky, 2007)] in their analyses. Recently, our research group demonstrated that large (~15%) reductions in [Hb] through isovolumic hemodilution of high-altitude Andean natives with excessive erythrocytosis increases FMD (Tremblay *et al.*, 2019*b*), however, whether this is a fundamental relationship that persists in the absence of pathology (e.g.

polycythemia) and without chronic hypoxemia in healthy humans remains unknown. Given these previous results we speculate that reduced Hb-mediated NO scavenging following isovolumic hemodilution would improve FMD irrespective of the potential for a reduced shear stress stimulus. Further, whether the shear stress stimulus is actually reduced is not clear given post-occlusive flow may be elevated due to decrease in resistance consequent of reduced viscosity with hemodilution.

In this study of healthy humans we aimed to comprehensively assess the influence of Hct/[Hb] on FMD. To address our hypotheses we utilized isovolumic hemodilution to reduce Hct and measured arterial NO bioavailability and blood viscosity along with FMD prior to and following this intervention. We hypothesized that FMD would be increased following hemodilution, with this increase occurring independent of any changes in resting steady-state NO bioavailability.

METHODS

Ethical Approval

This study was approved by the Clinical Research Ethics Board of the University of British Columbia (H16-01028). All participants gave written informed consent in English prior to participating. This study conformed to the standards set by the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2) and Declaration of Helsinki, except for registration in a database.

Eleven healthy males were recruited to participate in this study (28±7 years of age; 177±4 cm in height; 72±6 kg in weight; BMI of 23±2 kg $\cdot$ m^{-2}). All participants were recruited at the University of British Columbia's Okanagan campus. Participants were free of cardiovascular, respiratory and neurological disease, and were non-smokers.

137

138 **Protocol Overview**

139 Participants arrived at the laboratory having abstained from alcohol, caffeine and exercise for 24
140 hours, were fasted for a minimum of 4 hours, but drank water *ad libitum*. Half the participants began the
141 protocol at 0600 h and the other half at 1300 h.

142 Upon arrival, participants assumed the supine position and were instrumented with a radial artery
143 and ante-cubital venous catheter (see below). Twenty minutes was provided for subjects to rest
144 following cannulation, at which time the rest of the experimental set-up was performed. Following
145 instrumentation and ~5 min of baseline measurements (heart rate, HR; mean arterial pressure, MAP;
146 stroke volume, SV; cardiac output, CO; and blood sampling) a FMD test was performed.
147 Echocardiography (SV, CO) was performed to determine if isovolumic hemodilution influenced central
148 hemodynamic for the purposing of aiding in interpretation of our study. Whole blood was then
149 transferred into an Anticoagulant Citrate Phosphate Dextrose Solution BLOOD-PACK^{TM} (4R0012MC,
150 Fenwal, USA) through a non-pyrogenic plasma transfer kit (4C2240, Fenwal, USA) to collect up to 450
151 mL of whole blood per BLOOD-PACK. This hemodilution protocol was conducted in two stages aimed
152 at removing/replacing blood in 10% increments (i.e., 10% of whole blood volume per stage). Thus, a
153 total of approximately 20% of whole blood was removed and then replaced with an equal volume of 5%
154 human serum albumin (Alburex® 5%). Repeat arterial blood sampling allowed for the tracking of
155 changes in Hct, which were taken to reflect the extent to which we had successfully removed and
156 replaced whole blood. For example, a 10% reduction in Hct following the removal and replacement of
157 equal volumes of whole blood and 5% serum albumin respectively was inferred to indicate the removal
158 and replacement of 10% of whole blood. The duration of the hemodilution protocol was ~1.5-2 hours.
159 The second FMD test and blood sampling was conducted 30 minutes following completion of the

hemodilution protocol (~4 hours following the baseline FMD). A secondary study assessing FMD prior to and following 4-hours of rest was conducted in a separate cohort as a time control.

Experimental methods

Catheterization

Using sterile technique, a 20G arterial catheter (Arrow, Markham, ON) was advanced into the left radial artery under local anesthesia (Lidocaine, 1.0%) and ultrasound guidance. The radial artery catheter was attached to an in-line and waste-less sampling system (VAMP system, Edwards Life Sciences). This allows for serial blood sampling and the continuous measurement of radial arterial blood pressure (Truwave Transducer, Edwards Life Sciences). An 18G venous catheter (Insyte^{TM} Autoguard^{TM}, Becton Dickinson, USA) was then inserted into an ante-cubital vein (for albumin infusion).

Flow Mediated Dilation

Brachial artery FMD was measured in accordance with internationally-accepted guidelines (Thijssen *et al.*, 2011). Briefly, a one-minute recording of baseline arterial diameter and blood velocity was recorded, followed by a five-minute suprasystolic cuff occlusion (220 mmHg). The brachial artery cuff was placed immediately distal to the epicondyles. Vessel imaging was always performed proximal to the cuff. Recording resumed 30-seconds prior to deflation and continued for three minutes post cuff deflation. All measurements were acquired with a 10 MHz multi-frequency linear array probe (15L4 Smart Mark, Teratech, USA) attached to a high-resolution ultrasound machine (Terason uSmart 3300).

The angle of insonation for the acquisition of velocity was 60°. The ultrasound recordings were saved as a video files (Camtasia Studio, Techsmith Co, Ltd, USA) for future analysis using edge-detection software.(Woodman *et al.*, 2001) The video files were anonymized prior to determination of FMD. The within day coefficient of variation for our sonographer is <10% (Tremblay *et al.*, 2019*a*).

Shear stress was calculated as the product of shear rate (4*peak envelope blood velocity / arterial diameter) and whole blood viscosity at a shear rate of 225 s^{-1}. The FMD stimulus was quantified as the shear stress area under the curve (SSAUC) from cuff deflation to peak diameter (Pyke & Tschakovsky, 2007). Reactive hyperemia (3-min) and reactive hyperemia over the first minute post cuff deflation were calculated as mL of blood flow while peak reactive hyperemia was calculated as the peak blood flow rate following cuff deflation (i.e. mL/min) (Limberg *et al.*, 2020; Rosenberry & Nelson, 2020). Antegrade and retrograde shear stress were calculated as shear stress in the forward (positive) and backward (negative) direction, respectively, and mean shear stress as the sum of antegrade and retrograde (time-averaged mean shear stress). The oscillatory shear index (OSI) was calculated as |retrograde shear stress| / (|antegrade shear stress| + |retrograde shear stress|). Blood flow (mL/min) was calculated as peak envelope blood velocity / 2 * (π(0.5*diameter)^2). Vascular resistance was calculated as MAP / blood flow.

Echocardiography

Stroke volume was estimated from the cross sectional area of the left ventricular outflow tract in a parasternal long axis view and pulsed wave Doppler recordings acquired from the apical five chamber image. All images were acquired by a single experienced sonographer (MS) in accordance with the American Society of Echocardiography guidelines (Lang *et al.*, 2015) on a commercially available ultrasound machine (Vivid E9, GE Healthcare, Piscataway, NJ, USA). Three consecutive cardiac cycles

were analysed offline (Echopac, GE Healthcare, Piscataway, NJ, USA) and averaged. Stroke volume was calculated as the product of the velocity-time integral and aortic area, which was then multiplied by heart rate obtained from the lead II electrocardiogram inherent to the ultrasound to derive cardiac output.

Blood Sampling & Analyses

Prior to and following hemodilution, ~1.0 mL of radial arterial blood was drawn into a pre-heparinized syringe (SafePICO, Radiometer, Copenhagen, Denmark) and analyzed immediately at 37°C using a commercial blood gas analyzer (ABL90 FLEX, Radiometer). This analysis included measurement of the partial pressure of arterial oxygen (PaO_2), the partial pressure of arterial carbon dioxide ($PaCO_2$), arterial oxygen saturation (SaO_2), arterial oxygen content (CaO_2), pH, bicarbonate ion concentration [HCO_3^-], [Hb] and Hct. Arterial blood was also analyzed for whole blood viscosity. Arterial blood was drawn into a Lithium Heparin Vacutainer® (Becton Dickinson, USA). Blood viscosity was measured in duplicate within 15 minutes of blood sample acquisition at a shear rate of 225 s^{-1} at 37.0°C with a cone-and-plate viscometer (Model DV2T, Brookfield Amtek, USA) (Baskurt *et al.*, 2009). Further, a separate *ex vivo* study was conducted to determine the influence of hemodilution with 5% human serum albumin on plasma viscosity (see below).

Arterial blood was also drawn into a K_2EDTA Vacutainer® (Becton Dickinson, USA). Whole blood was then immediately centrifuged at 600 g for 10 minutes at a temperature of 4.0°C. Plasma and packed red blood cells were then aliquoted into cryovials, flash frozen in liquid N_2, and stored at -80°C. Samples were then shipped on dry ice to the UK using a commercial clinical science logistics company (Marken, Durham, NC, USA). Temperature tracking indicated samples remained at -78.5°C throughout

transport. There, plasma and red blood cell NO were measured using tri-iodide reductive ozone based chemiluminescence as previously described for our research group (Bailey *et al.*, 2017).

Plasma S-Nitrosothiols (PL-RSNO): Plasma (540 μL) was mixed with 5% acidified sulphanilamide (60 μL) and left to incubate in the dark at 21°C for 15 min to remove NO_2^- before injection into the tri-iodide reagent for direct measurement of RSNO.

Plasma Nitrite (PL-NO_2^-): A separate sample (200 μL) was injected into the tri-iodide reagent for the combined measurement of NO_2^- and RSNO with NO_2^- calculated by subtracting the concentration of RSNO.

Red Blood Cell NO (RBC-NO): The original tri-iodide solution was modified with the addition of potassium ferricyanide [$K_3Fe^{III}(CN)_6$] to limit auto-capture of NO (Rogers *et al.*, 2005). The packed red blood cells were lysed 1:4 in EDTA (0.5 mM; pH corrected to 7.0) and incubated for 5 min on ice (Rogers *et al.*, 2005). A 400 μL sample was then injected into the modified tri-iodide reagent for the measurement of total RBC-NO.

All calculations were performed using Origin/Peak Analysis software. All chemicals were of the highest purity available from MilliporeSigma. Whole blood [NO] was subsequently calculated as: ((1-Hct) x plasma [NO]) + (Hct x RBC [NO]).

Time control flow-mediated dilation experiment

To account for changes in FMD that may occur during supine rest, we enrolled 11 male participants (age: 28±6 years; height: 180±4cm; weight: 76±9kg) into a time control study (separate cohort from primary study). Participants had their brachial FMD assessed as described in the main experimental protocol. Following 20-minutes of supine rest, a baseline FMD test was conducted. Subjects then rested for 4 hours, with their upper body at a 45° incline. Following this period, the supine position was re-assumed for 20-minutes prior to the post-test FMD. Blood pressure (brachial sphygmomanometer), heart rate, and blood viscosity (Cone and plate viscometer) were also measured prior to and following the resting period (as described previously).

***Ex vivo* blood viscosity experiment**

To determine the influence of whole blood replacement with 5% human serum albumin on plasma viscosity a follow up *ex vivo* study was conducted. Data from 7 young healthy adult males was included (age: 27±3 years; height: 176±6 cm; weight: 71±6 kg).

Blood was collected from the medial ante-cubital vein into two lithium heparin Vacutainers ® (Becton Dickinson, USA). Blood from each tube was measured under the following conditions:

Tube 1: Control blood

1) Whole Blood: Whole blood viscosity was measured.
2) Plasma: Whole blood was centrifuged at 600 g and 4°C for 10 minutes. Viscosity of the separated plasma was then measured.

Tube 2: Hemodiluted blood

1) Whole Blood: 4 mL of whole blood was transferred to a conical tube and 1 mL of 5% human serum albumin was added (i.e. 20% hemodilution). This blood was thoroughly mixed and subsequently whole blood viscosity of the diluted blood was assessed.
2) Plasma: Blood that had been diluted by 20% was centrifuged at 600 g and 4°C for 10 minutes. Viscosity of the separated plasma was then measured.

For all samples, viscosity was measured in duplicate and within 30 minutes of blood sample acquisition at a shear rate of 225 s^{-1} at 37.0°C with a cone-and-plate viscometer (Model DV2T, Brookfield Amtek, USA). Hematocrit was also measured to confirm the magnitude of hemodilution.

Statistical Analyses

Statistical analyses were completed in SPSS (IBM, V24). All pre- to post-hemodilution comparisons were made using a paired two-tailed t-test. To account for differences in FMD stimulus, testing was also performed with SSAUC included as a covariate in linear mixed model analysis. Furthermore, allometric scaling was performed to account for differences in baseline diameter within participants. Briefly, the diameter change on a logarithmic scale (ln(peak diameter) - ln(baseline diameter)) was assessed as the outcome variable in a linear mixed model with logarithmically-transformed baseline diameter included as a covariate (Atkinson & Batterham, 2013; Atkinson *et al.*, 2013). The time control experiment followed the same statistical analyses described above. For the *ex vivo* blood viscosity experiment, data were compared pre to post hemodilution using two-tailed paired t-tests.

RESULTS

Resting Hematological and Hemodynamic variables

Following hemodilution, [Hb] (13.9±0.7 vs 11.4±0.6 g/dL; P<0.01) and Hct (43.7±2.3 vs. 34.8±1.7 %; P<0.01) both decreased by 18±2%, which coincided with a 22±3% reduction in arterial whole blood viscosity (3.54±0.24 vs. 2.80±0.22 cP; P<0.01; **Figure 1A&B**). Due to the reduction in [Hb], CaO_2 was reduced by 18±2% (**Figure 1C)**, while PaO_2 (95±7 vs. 92±4 mmHg; P=0.08) and SaO_2 were unaltered (97.7±0.7 vs. 97.6±0.4 %; P=0.59). Whole blood [NO] was reduced by 32±17% following hemodilution (135.3±42.0 vs. 89.0±18.2 nmol/L; P<0.01)(**Figure 1E**). This was due to a 22±22% reduction in plasma [NO_2^-] (105.7±37.0 vs. 79.9±21.1 nmol/L; P=0.01)(**Figure 1F**), 16±55% reduction in plasma [RSNO] (6.5±3.2 vs. 4.4±2.0 nmol/L; P=0.03)(**Figure 1G**), and 37±17% reduction in total red blood cell [NO] (151.0±67.6 vs 89.1±30.2 nmol/L; P<0.01)(**Figure 1H**). There was an increase in $PaCO_2$ (39.9±1.5 vs. 41.6±1.8 mmHg; P<0.01), decrease in [HCO_3^-] (25.2±1.2 vs. 24.4±0.8 mmol/L; P=0.01), and decrease in pH (7.41±0.01 vs. 7.38±0.02; P<0.01) following hemodilution.

Hemodilution did not alter MAP (99.8±6.6 vs. 97.0±7.2 mmHg; P=0.15). Similarly, HR (62±13 vs. 64±12 bpm; P=0.26), SV (61.9±11.8 vs. 64.3±15.3 mL; n=8; P=0.52) and CO (3.74±0.75 vs. 3.95±0.81 L/min; n=8; P=0.52) were unaltered following hemodilution.

Neither mean (39.6±17.1 vs. 52.7±31.0 mL · min^{-1}; P=0.23), antegrade (52.0±17.5 vs. 66.3±26.2 mL · min^{-1}; P=0.15) or retrograde (-12.4±8.6 vs. -13.6±11.2 mL · min^{-1}; P=0.51) brachial artery blood flow were altered following hemodilution. Likewise, forearm vascular resistance (3.03±1.47 vs 2.74±2.14 mmHg · mL^{-1} · min^{-1}; P=0.52) and shear stress patterns were unaltered following hemodilution (**Figure 2A**), which was reflected in an unaltered brachial artery OSI (0.19±0.10 vs. 0.18±0.14; P=0.80).

Reactive hyperemia responses

Reactive hyperemia responses prior to and following hemodilution are presented in **Table 1** and **Figure 2B**. Total reactive hyperemia (ml; +57.8±77.7%; P=0.02) and reactive hyperemia within one-minute post cuff deflation (ml; +42.9±50.1%; P=0.02) were increased following hemodilution, while changes in the peak reactive hyperemia (ml/min) following hemodilution did not reach statistical significance (+24.8±35.1%; P=0.056; **Table 1**). The resulting SRAUC for the brachial artery was elevated following hemodilution (19,314±7,642 vs. 34,410±19,159; P=0.02; **Figure 2C**); however, given the lower viscosity post hemodilution, SSAUC was not significantly altered pre to post hemodilution (686±286 vs. 943±525; P=0.12; **Figure 2B&D**).

Flow mediated dilation

Brachial FMD was increased by ~160% following hemodilution (3.8±2.1 vs 9.7±4.5%; P<0.01). Inclusion of SSAUC and baseline diameter as covariates did not alter the observed changes, with the adjusted FMD still increasing by ~120% from 4.2±3.0 to 9.2±3.0% (P<0.01) (**Figure 2E**). As the time to peak dilation of the brachial artery was longer following hemodilution (**Table 1**), we also standardized SSAUC (to 60-seconds following cuff deflation) to further assessed changes in FMD (SSAUC-60; **Table 1**). With inclusion of baseline diameter and SSAUC-60 as covariates the increase in FMD with hemodilution persisted (3.9±0.9 vs. 9.5±0.9%; P<0.01).

Time Control Flow-mediated Dilation Experiment

Neither mean arterial pressure (MAP; 81±4 vs. 84±7 mmHg; P=0.42) nor heart rate (56±11 vs. 53±11; P=0.14) were different pre-to post the time control rest period. Blood viscosity was also unaltered (3.88±0.30 vs 3.88±0.44 cP; P=0.99; **Figure 3A**). Mean shear was reduced from 133±83 to 67±31 s^{-1} (P<0.01), antegrade shear was reduced from 147±79 to 91±34 s^{-1} (P=0.01), while retrograde shear increased from -13±14 to -24±23 s^{-1} (P=0.03; **Figure 3B**). Shear rate was reported given that viscosity was not altered in the time control period. Following the time control period, SRAUC was reduced from 31167±8483 to 24647±6456 (P=0.04) but FMD remained unaltered (8.2±3.7 vs. 7.5±3.8%; P=0.50; **Figure 3D**). Indeed, even following inclusion of the SRAUC stimulus as a covariate (**Figure 3C**), there was no alteration in FMD following the four hour time control period (8.0±3.7 vs. 7.7±3.7%; P=0.76).

Ex Vivo Blood Viscosity Experiment

Hematocrit was reduced from 44.9±2.4% to 35.9±2.4% following *ex vivo* hemodilution, which represented a 20.10±0.01% change. Viscosity data are presented in **Table 2**. Hemodilution with 5% human serum albumin reduced plasma viscosity from 1.33±0.04 to 1.17±0.5 cP (P<0.001).

DISCUSSION

This study demonstrates that acute reductions in Hct following isovolumic hemodilution increase brachial artery FMD in healthy humans. The observed increase in brachial FMD occurs independent of the impact of Hct on shear stress and despite a reduction in resting whole blood, plasma, and red blood cell NO bioavailability. These findings are evidenced by an unaltered SSAUC following hemodilution as well as reductions in whole blood, plasma, and red blood cell NO bioavailability. Collectively, these data point towards hemoglobin scavenging of NO as an important factor in the regulation of FMD in healthy humans.

Comparison to previous studies

Previous studies have demonstrated an inverse correlation between [Hb] and FMD across cohorts of healthy (Madsen *et al.*, 2006) individuals and those with chronic kidney disease (Yilmaz *et al.*, 2009; Sonmez *et al.*, 2010). However, interventional studies conversely support the notion that reductions in [Hb] impair FMD in hemochromatosis patients (Giannattasio *et al.*, 2002*b*), while increases in [Hb] improve FMD in end stage renal failure (Verbeke *et al.*, 2007). Only recently did we consistently demonstrate, both *between* and *within* participants, that a lower [Hb] is associated with higher FMD in an Andean highlander population with polycythemia (Tremblay *et al.*, 2019*b*). However, the collectively equivocal nature of these previous studies is complicated by the differing patient populations tested and the variable Hct values these populations exhibit. For the first time in healthy humans, utilizing experimental hemodilution to reduce Hct under normoxic laboratory conditions, our study provides confirmatory evidence that a lower Hct is related to a higher FMD. We further extend these findings, to demonstrate that this increase in FMD occurs despite reductions in resting steady state NO

bioavailability (whole blood, plasma, and red blood cell) and statistical covariation for the SSAUC stimulus. That there were no changes in central hemodynamics (i.e. SV & CO) following isovolumic hemodilution and our time control data indicate changes in FMD were unrelated to a prolonged period of rest both strengthen our finding of increased FMD following hemodilution.

Potential mechanism(s) of improved FMD

Previous work has aimed to partition the influence of blood viscosity from Hb on blood flow regulation by differential manipulations of plasma viscosity during hemodilution. Indeed, in pre-clinical models when hemodilution is produced with both a low and high viscosity plasma expander, vasodilation occurs with increased plasma viscosity whereas vasoconstriction ensues when plasma viscosity is unaltered (Tsai *et al.*, 1998, 2005). This vasodilation following administration of a high viscosity plasma expander is associated with a greater shear stress-mediated formation of NO (Tsai *et al.*, 2005). However, in the current study, hemodilution with 5% human serum albumin reduces plasma viscosity (**Table 2**) concurrent to reduced whole blood viscosity (**Figure 1B**), which points to reduced hemoglobin scavenging versus increased plasma dependent shear mediated NO production as the mechanism by which hemodilution increases FMD in humans. In further support of this notion is the reduction in plasma NO_2^- following hemodilution (**Figure 1F**). This reduction in NO_2^- likely indicates a reduction in NOS activity in the resting state (Lauer *et al.*, 2001; Kleinbongard *et al.*, 2003), which would be expected to impair – not improve – FMD. Importantly, such a reduction in NOS activity in the resting state may not necessarily reflect the potential for endothelial NOS dependent activation in the context of elevated shear stress (see next paragraph). The SSAUC stimulus was not different between the pre- and post-hemodilution FMD protocols, indicating the stimulus for endothelial NOS mediated NO production was also likely not different.

Previous investigation examining an *in situ* lung perfusion model demonstrated similar potentiation of NO mediated vasodilation with lower Hct (i.e. [Hb])(Deem *et al.*, 1998) to that of the current study. The potentiated vasodilation observed by Deem *et al.*, was validated as an NO dependent scavenging mechanism effect via NOS blockade (Deem *et al.*, 1998). Similar to the present study, potentiated vasodilation occurred without a simultaneous increase in blood [NO_2^-], highlighting that the potential role of scavenging is related to constraint of transiently liberated NO not detected by 'steady-state' blood measurements (in the study by Deem *et al.*, 1998 this NO production was measured through analysis of expirate)(Deem *et al.*, 1998). Therefore, while it was not possible to *directly* quantify alterations in NO scavenging *in vivo*, the evidence for this physiological phenomenon (Deem *et al.*, 1998; Azarov *et al.*, 2005) coupled with the link between FMD and NO (Green *et al.*, 2014), suggest the observed increase in FMD herein is due in large part to reductions in Hb scavenging of the NO that is transiently produced following the post-occlusive shear stimulus.

Previous *in vitro* work demonstrated a tripling of NO's half-life when Hct was lowered from 50% to 15% (Azarov *et al.*, 2005). However, interpretation of our data require further consideration of important *in vivo* hemodynamic factors. Reductions in Hct increase the cell free layer at the abluminal wall of microvessels (Sriram *et al.*, 2011), which is related to the axial migration of red blood cells that occurs under conditions of laminar flow (Goldsmith & Mason, 1961). This would be expected to impose a greater diffusional limitation for Hb scavenging of NO and increases its half-life following hemodilution (Kim-shapiro *et al.*, 2006). Moreover, the important influence of the unstirred layer surrounding red blood cells is also another potential factor associated with Hct mediated alterations in NO scavenging by Hb (Kim-shapiro *et al.*, 2006). While our data suggests reduced NO scavenging may underpin the observed increases in FMD with isovolumic hemodilution, the specific factors that

412 contribute to this effect and their relative importance in larger vessels such as the brachial artery (not
413 microvessels in which these relationships are defined) are not discernible in the present study.

414

415 *Experimental Considerations*

416 Recent work has highlighted the importance of considering blood viscosity across a range of shear
417 rates that reflect *in vivo* hemodynamics for the accurate quantification of shear stress and corresponding
418 implications for vasodilation. Indeed, as shear rate increases, blood viscosity decreases as a function of
419 the non-Newtonian nature of whole blood (i.e. shear thinning) (Leo *et al.*, 2019). In the present study,
420 whole blood viscosity was determined at a single shear rate of 225 s^{-1}; however, if a shear thinning
421 profile had been applied to each participant and incorporated into their SSAUC calculation, one would
422 expect the SSAUC post-hemodilution to be comparatively lower than that reported herein given the
423 increase in SRAUC following hemodilution (i.e. a greater level of shear thinning than pre-
424 hemodilution). A potentially lower SSAUC than that reported would be reflected in an even greater
425 FMD post-hemodilution following adjustment for the SSAUC stimulus. Further, it is important to
426 address that females were not tested in the present study and future work should aim to determine the
427 influence of [Hb] on FMD in females. Given the observed role of [Hb] on FMD, and that the impact of
428 NOS inhibition on NO scavenging has been demonstrated as dependent upon Hct (or [Hb])(Deem *et al.*,
429 1998) raises the possibility that variability in the proportion of FMD that is attributed to NO (Green *et*
430 *al.*, 2014) may be potentially due in part to variability in Hct within these study cohorts.

431 There is considerable debate surrounding the notion that hyper versus hypo-viscosity may be more
432 appropriate for optimal cardiovascular function (Forconi & Gori, 2009; Salazar Vázquez *et al.*, 2010).
433 While the specifics of this debate are beyond the scope of this investigation, it is important to note that

much of the pertinent literature is limited by the use of volume expanders that do not properly mimic the complex functions of Hb (e.g. NO scavenging). Further, human studies that are considered in this debate, as previously mentioned, do not appropriately account for the collective influence of Hct on shear stress and NO [e.g. (Giannattasio *et al.*, 2002*a*)]. Thus, moving forward, it is clear that consideration of viscosity in isolation is inappropriate and that a view for how the integrated influence of changes in blood viscosity, NO bioavailability, and Hb scavenging coalesce to regulate FMD as demonstrated herein requires consideration in the hyper- versus hypo-viscosity debate.

CONCLUSION

Our study demonstrates that acute reductions in hemoglobin concentration increase brachial artery flow-mediated dilation in humans. This increase occurs while accounting for alterations in the shear stimulus for flow-mediated dilation and despite reductions in plasma [NO] following hemodilution. Therefore, hemoglobin scavenging of NO appears to be an important factor in the regulation of flow-mediated dilation and constrains endothelium-dependent NO-mediated vasodilation in otherwise healthy humans.

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Data availability statement

The data are available from the corresponding author upon reasonable request.

Author contributions

Conception or design of the work: RLH, DMB, PNA; Acquisition or analysis or interpretation of data for the work: RLH, JCT, BSS, GBC, DN-F, MMT, AP, MS, CAH, DMB, DJG, DBM, PNA; Drafting of the work or revising it critically for important intellectual content: RLH, JCT, BSS, GBC, DN-F, MMT, AP, MS, CAH, DMB, DJG, DBM, PNA; Final approval of the version to be published: RLH, JCT, BSS, GBC, DN-F, MMT, AP, MS, CAH, DMB, DJG, DBM, PNA

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TABLES

Table 1. Brachial hemodynamics prior to and following isovolumic hemodilution

	Pre	Post	P-value
Baseline Diameter (mm)	4.61±0.37	4.51±0.38	**0.03**
Peak Diameter (mm)	4.78±0.36	4.95±0.41	**0.04**
Delta Diameter (mm)	0.17±0.09	0.44±0.19	**<0.01**
Time to Peak Diameter (sec)	42.0±15.7	72.1±45.1	**0.03**
Response SSAUC-60	806±225	841±267	0.66
Reactive hyperemia total (mL)	21178±7248	31570±13231	**0.02**
Reactive hyperemia peak (mL/min)	378±88	464±153	0.056
Reactive hyperemia 1 min (mL)	13201±3560	18180±6510	**0.02**

SRAUC, shear rate area under the curve; SSAUC, shear stress area under the curve. Significance testing was conducted with a two tailed paired t-test.

Table 2. Impact of hemodilution on whole blood and plasma viscosity

	Whole Blood Viscosity			Plasma Viscosity		
Subject	Tube 1: Control	Tube 2: Diluted	P-value	Tube 1: Control	Tube 2: Diluted	P-value
1	4.28	3.12		1.37	1.16	
2	3.89	2.98		1.29	1.15	
3	4.54	3.45		1.32	1.14	
4	4.17	3.18		1.28	1.18	
5	4.05	2.95		1.37	1.23	
6	4.28	3.28		1.38	1.22	
7	4.22	3.07		1.32	1.10	
Mean	**4.20**	**3.15**	**<0.001**	**1.33**	**1.17**	**<0.001**
STDEV	**0.20**	**0.17**		**0.04**	**0.05**	

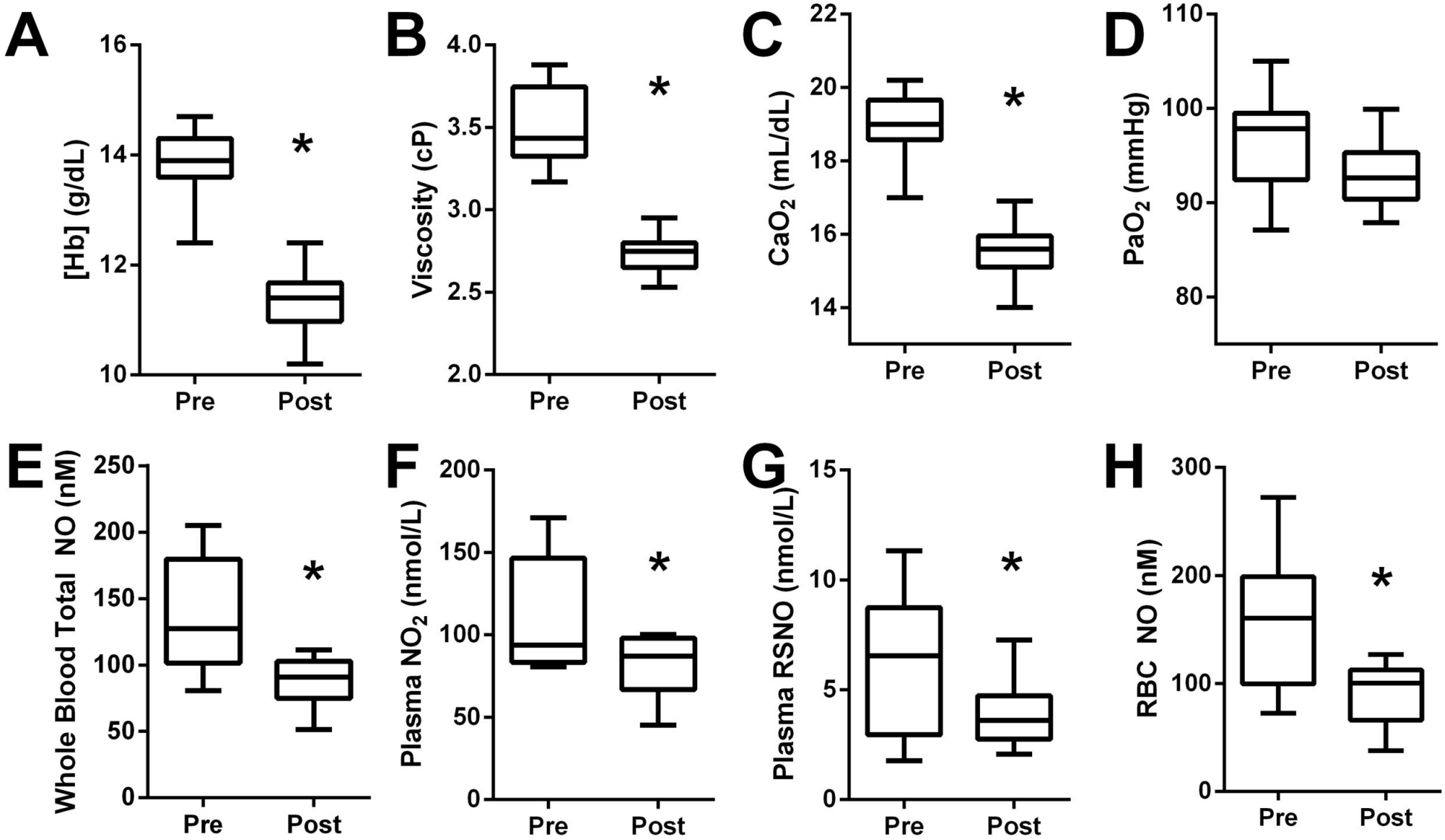
Significance testing was conducted with a two tailed paired t-test.

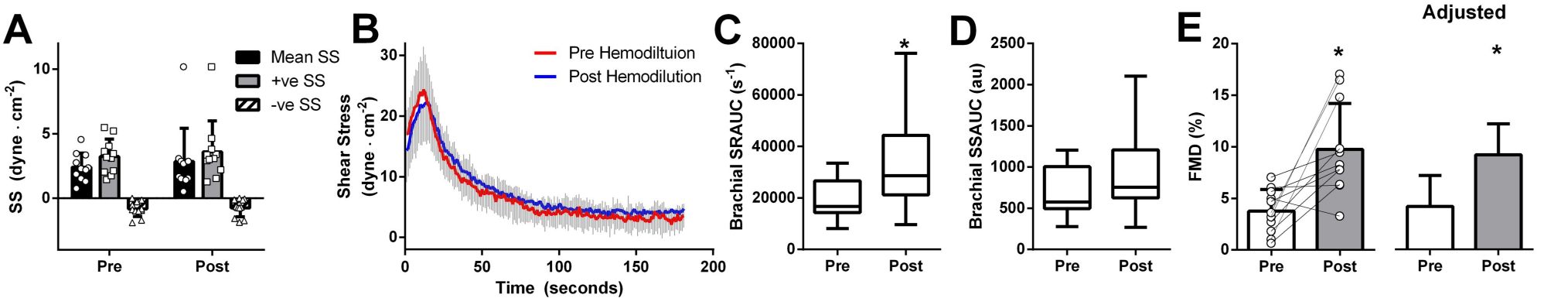
FIGURES

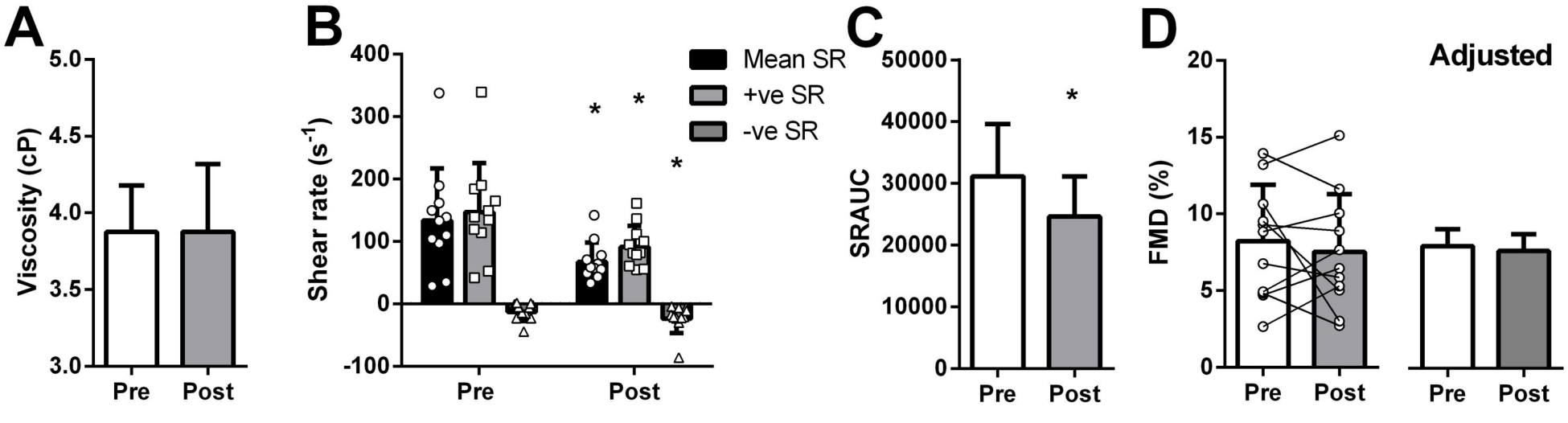
Figure 1. Blood gases and nitric oxide metabolites prior to and following hemodilution. All panels depict box and whisker plots displaying quartiles. Hemodilution reduced hemoglobin concentration ([Hb]), viscosity, and arterial oxygen content (CaO_2), while the partial pressure of arterial oxygen (PaO_2) was unaltered. The concentration of whole blood nitric oxide (NO; nitrite, *S*-nitrosothiols, and heme-bound NO) was reduced along with plasma nitrite (NO_2^-), plasma *S*-nitrosothiols (RSNO) and total red blood cell NO (nitrite, *S*-nitrosothiols, and heme-bound NO). *denotes a significant difference from pre- to post-hemodilution, $P<0.05$. A paired two tailed t-test was used for all comparisons in this figure. For Panels A-D, n=11 while for Panels E-H, n=10.

Figure 2. Shear stress patterns and flow-mediated dilation prior to and following hemodilution. Panel A depicts resting shear stress (SS) patterns pre- and post-hemodilution. Panel B depicts the shear stress response to cuff release with pre-hemodilution denoted in the red line and post-hemodilution the blue line. The grey vertical lines represent standard deviation. Panel C depicts box and whisker plots (quartiles) for shear rate area under the curve (SRAUC) while panel D depicts the shear stress area under the curve (SSAUC) pre- and post-hemodilution. Notably, brachial SRAUC was increased post-hemodilution but SSAUC was not. This highlights the importance of incorporating viscosity measures into the shear stimulus for flow-mediated dilation (FMD). Panel E depicts the FMD response pre- and post-hemodilution along with the covariate adjusted FMD data. *denotes a significant difference pre- to post-hemodilution, $P<0.05$. A paired two tailed t-test was utilized to compare shear stress (Panel A), brachial SRAUC (Panel C), brachial SSAUC (Panel D), and FMD% (Panel E – left side). For the "Adjusted" FMD% in Panel E, the diameter change on a logarithmic scale (ln(peak diameter) - ln(baseline diameter)) was assessed as the outcome variable in a linear mixed model with logarithmically-transformed baseline diameter as well as SSAUC included as covariates. N=11 for all comparisons.

Figure 3. Time control flow-mediated dilation study. Panel A depicts whole blood viscosity prior to and following the time control rest period. Panel B depicts resting shear patterns prior to and following the time control rest period. Panel C depicts shear rate area under the curve (SRAUC), while Panel D depicts FMD. A paired two tailed t-test was utilized to compare viscosity (Panel A), shear rate (Panel B), brachial SRAUC (Panel C), and FMD% (Panel D – left side). For the "Adjusted" FMD% in Panel D, the diameter change on a logarithmic scale (ln(peak diameter) - ln(baseline diameter)) was assessed as the outcome variable in a linear mixed model with logarithmically-transformed baseline diameter as well as SSAUC included as covariates. N=11 for all comparisons.


A
[Hb] (g/dL)
16
14
12
10
*
Pre
Post
B
Viscosity (cP)
4.0
3.5
3.0
2.5
2.0
*
Pre
Post
C
CaO2 (mL/dL)
22
20
18
16
14
*
Pre
Post
D
PaO2 (mmHg)
110
100
90
80
Pre
Post
E
Whole Blood Total NO (nM)
250
200
150
100
50
0
*
Pre
Post
F
Plasma NO2 (nmol/L)
200
150
100
50
0
*
Pre
Post
G
Plasma RSNO (nmol/L)
15
10
5
0
*
Pre
Post
H
RBC NO (nM)
300
200
100
0
*
Pre
Post


A
SS (dyne · cm-2)
10
5
0
Mean SS
+ve SS
-ve SS
Pre
Post
B
Shear Stress (dyne · cm-2)
30
20
10
0
Pre Hemodiltuion
Post Hemodilution
0
50
100
150
200
Time (seconds)
C
Brachial SRAUC (s-1)
80000
60000
40000
20000
0
*
Pre
Post
D
Brachial SSAUC (au)
2500
2000
1500
1000
500
0
Pre
Post
E
Adjusted
FMD (%)
20
15
10
5
0
*
*
Pre
Post
Pre
Post


A
Viscosity (cP)
5.0
4.5
4.0
3.5
3.0
Pre
Post
B
Shear rate (s-1)
400
300
200
100
0
-100
Mean SR
+ve SR
-ve SR
*
*
*
Pre
Post
C
SRAUC
50000
40000
30000
20000
10000
0
*
Pre
Post
D
Adjusted
FMD (%)
20
15
10
5
0
Pre
Post
Pre
Post