Sublethal mechanical trauma alters the electrochemical properties and increases aggregation of erythrocytes

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Abstract

Circulation of blood depends, in part, on the ability of red blood cells (RBCs) to aggregate, disaggregate, and deform. The primary intrinsic disaggregating force of RBCs is derived from their electronegativity, which is largely determined by sialylated glycoproteins on the plasma membrane. Given supraphysiological shear exposure – even at levels below those which induce hemolysis – alters cell morphology, we hypothesized that exposure to supraphysiological and subhemolytic shear would cleave membrane-bound sialic acid, altering the electrochemical and physical properties of RBCs, and thus increase RBC aggregation.

Isolated RBCs from healthy donors (n=20) were suspended in polyvinylpyrrolidinone. Using a Poiseuille shearing system, RBC suspensions were exposed to 125 Pa for 1.5 s for three duty-cycles. Following the first and third shear duty-cycle, samples were assessed for: RBC aggregation; the ability of RBCs to aggregate independent of plasma (“aggregability”); disaggregation shear rate; membrane-bound sialic acid content, and; cell electrophoretic mobility.

Initial shear exposure significantly increased RBC aggregation, aggregability, and the shear required for rouleaux dispersion. Sialic acid concentration significantly decreased on isolated RBC membranes ghosts, and increased in the supernatant following shear. Initial shear exposure decreased the electrophoretic mobility of RBCs, decreasing the electronegative charge from -15.78 to -7.55 mV. Three exposures to the shear duty-cycle did not further compound altered RBC measures.

A single exposure to supraphysiological and subhemolytic shear significantly decreased the electrochemical charge of the RBC membrane, concurrently increasing cell aggregation/aggregability. The cascading implications of hyperaggregation appears to

potentially explain the ischemia–associated complications commonly reported following mechanical circulatory support.

Keywords hemorheology; mechanical damage; red blood cell; subhemolytic; disaggregation; electrophoretic mobility; sialic acid; zeta potential

Introduction

As blood circulates the vasculature, the ability of the red blood cells (RBCs) to form stacks of branched aggregates and three-dimensional structures (i.e., rouleaux) under low shear conditions facilitates important physiological processes that decreases resistance to flow (e.g., axial migration of formed elements), while concurrently augmenting low-shear viscosity (Baskurt et al., 2011). The rate and amplitude of RBC aggregation is modulated by counteracting factors that may be clustered broadly as “pro-aggregating” and “disaggregating” processes. Pro-aggregating forces are largely due to the presence of large molecular weight plasma proteins (Baskurt and Meiselman, 2008) (e.g., fibrinogen), while disaggregating forces are principally generated by mechanical shear and cell-cell electrostatic repulsion (Rampling et al., 2004) (with negatively charged sialylated glycoproteins being the major determinant of such repulsion).

RBC sialic acids (primarily N-acetylneuraminic acids) are located on the plasma membrane within the glycocalyx, and account for up to 90% of the cell’s negative charge (Eylar et al., 1962). Distribution of sialic acids over the surface of the membrane are reported to influence morphology, cell deformability, oxygen carrying capacity, and distribution of intracellular hemoglobin (Huang et al., 2016). With the normal ageing and senescence process of RBCs, the zeta potential (ζ) and the plasma membrane sialic acid content is decreased (Simmonds et al., 2013). Removal of sialic acid from the glycocalyx of RBCs, using the enzyme neuraminidase, was reported to significantly increase cell aggregation (Jan and Chien, 1973).

The process of RBC aggregation is rapid, forming rouleaux within seconds, and is observed to occur *in vivo* in low shear regions, such as within post capillary flow (Baskurt and Meiselman, 2008; Cabel et al., 1997). With sufficiently high shear, however, formed rouleaux are able to disperse and disaggregate, differentiating this reversible process from coagulation

and agglutination (Baskurt and Meiselman, 2013). Despite wall shear flows being too high in arterial circulation for aggregates to form, the shear profile varies radially across the vessel, decreasing to a minimum in the central region of flow (axis), where aggregation may occur (Mazumdar, 1992). This aggregation and axial migration of formed elements facilitates a partial phase-separation, contributing to the existence of a cell-poor layer of plasma at the vessel wall (Fåhræus and Lindqvist, 1931). The cell-poor region facilitates development of a boundary layer with decreased local viscosity and frictional resistance, minimizing wall shear stress and endothelial stimulation. The cell-poor layer also facilitates a “plasma skimming” effect, where side branches of the vasculature principally receive plasma, significantly decreasing hematocrit to ~10% in some capillary networks (Klitzman and Duling, 1979), contributing to lower hemodynamic resistance, and thus also limiting oxygen-rich RBCs (Cokelet and Goldsmith, 1991).

Hyperaggregability of RBCs may alter flow distributions of cells and near wall conditions, specifically increasing leukocyte and platelet margination and attachment (Nash et al., 2008). *Ex vivo* investigations have indicated that increases in aggregation (at maintained hematocrit) have correlated with low-shear viscosity and the non-Newtonian behavior of blood, with speculation of these findings *in vivo* being negatively correlated with tissue perfusion (Baskurt, 2008). An *in vivo* animal model of chronic hyperaggregation observed decreased wall shear stress which diminished nitric oxide synthesis mechanisms, altering vasomotor tone (Baskurt et al., 2004). Moreover, given the importance of nitric oxide in the prevention of platelet activation and attachment, enhanced aggregation may increase thrombotic risk (Baskurt and Meiselman, 2013).

Mechanical circulatory support devices have evolved such that they adequately meet the functional demands of replaced organs, and achieve current hemocompatibility criteria (Crow et al., 2009; Piccione Jr, 2000; Zhang et al., 2007); however, several significant

complications have been reported with long-term use that are suggestive of blood damage and malfunction, without remarkable hemolysis. This damage, which is due to blood-interaction with non-physiological environments (i.e., contact with foreign surfaces, elevated shear forces, turbulence and cavitation), can cause dangerous complications of altered perfusion and rheological properties of blood (Horobin et al., 2017; McNamee et al., 2017; McNamee et al., 2016b; Ruggeri et al., 2006). The observed complications of hemorrhagic events, thrombotic events, tissue ischemia and anemia, leading to multi-system organ failure (Kirklin et al., 2014), may be partially explained by altered RBC aggregation behavior. Current investigations of blood trauma and mechanical circulatory support have found that acute and chronic exposure may increase low-shear blood viscosity (Kormos et al., 1987) and increase RBC aggregation in hemodialysis (Kameneva et al., 2002; Koppensteiner et al., 1996). Using light microscopy, increases in RBC aggregation have been observed in bovine animal models implanted with ventricular assist devices, with concurrent increases in low-shear viscosity and erythrocyte sedimentation rate (Kameneva et al., 1994).

Given that high shear environments, such as those present within mechanical circulatory support devices, have been identified to induce ultrastructural changes to RBCs, altering morphology (McNamee et al., 2016b), causing cell fragmentation (Watanabe et al., 2007), and disrupting the lipid bilayer (Dao et al., 1993), it is likely that this sublethal mechanical trauma may cleave sialylated membrane glycoproteins responsible for the cells' electrostatic negative charge. The aim of the present study was thus to investigate the aggregation behavior and biochemical/electrochemical properties of RBCs (with emphasis on membrane-bound sialic acid) following short-term exposure to sublethal magnitudes of shear stress.

Materials and Methods

Experimental overview

The electrochemical and rheological properties of RBCs following exposure to acute high-shear environments typical of clinically-relevant mechanical circulatory support were explored in an *in vitro* experimental model of Poiseuille flow. Isolated human RBCs were exposed to 125 Pa for 1.5 s on three repeated duty-cycles; following the first and third duty-cycles, samples were collected to quantify the level of blood trauma. The experimental procedures were conducted and completed within 4 h of venesection. The experimental protocols were reviewed and approved by the Griffith University Human Research Ethics Committee (Protocol number: 2016/712), which conforms with the Declaration of Helsinki.

Subjects and sampling

Twenty healthy men (28 ± 8 yr) volunteered to participate in the present study. After informed consent was obtained, participants were interviewed to determine if they were free from hematological and immune disorders, metabolic or cardiovascular comorbidities, and were not using medications known to influence rheology. Blood was collected from a prominent vein in the antecubital region within 90 s of tourniquet application, using a 21-G needle and syringe, and then immediately transferred into tubes containing $1.8 \text{ mg}\cdot\text{mL}^{-1}$ of K_2EDTA .

Cell preparation

Following blood collection, blood products were isolated by centrifugation at $1500 \times g$ for 10 min. Plasma was then aspirated into a separate microfuge tube and stored until required for RBC aggregation measures. To further isolate RBCs, packed cells were washed twice with isotonic phosphate buffered saline (PBS; $\text{pH} = 7.40$, $290 \text{ mOsmol}\cdot\text{kg}^{-1}$), with use of centrifugation at $1500 \times g$ for 5 min, each time to remove and discard supernatant.

Overcoming the non-Newtonian characteristics of blood is critical for the accurate calculation and quantification of shear stress in Poiseuille flow, thus isolated RBCs were resuspended in a 360 kDa polyvinylpyrrolidinone solution (PVP; 4.93 mPa·s, pH 7.4, 290 mOsmol·kg⁻¹) at a decreased hematocrit of 0.15 L·L⁻¹. The RBC-PVP solution adopted a final viscosity of 5 mPa·s and exhibited predominantly Newtonian properties, facilitating accurate shear stress calculation whilst still exhibiting viscosity typical of normal *in vivo* ranges. Viscosity was measured using a cone-plate viscometer at a temperature reflective of the specific assay/experiment requirements (0.5 DVII+ with CPE40 spindle, Brookfield Engineering Labs, Middleboro, MA). The resuspension of RBC in PVP facilitated investigation of shear damage independent of other factors that may compound cell aggregation (i.e., shear activation of platelets) (Ding et al., 2015).

Application of shear stress

As shear stresses in current mechanical circulatory support devices are predicted to induce less than 150 Pa for residence times ranging 0.04–3.08 s (device dependent) (McNamee et al., 2016b; Selgrade and Truskey, 2012), the wall shear stress magnitude-duration combination (125 Pa for 1.5 s) applied to RBC suspensions was specifically chosen to: i) avoid overt hemolysis, while ii) being representative of clinical blood pumps. Development of the shear stress magnitude and duration of exposure was achieved using a linear Poiseuille shearing system, constructed using a 0.94 m segment of 200 µm I.D. polyethylene tubing. This microtube system was pressurized by synchronized infuse/withdraw dual syringe pumps (NE-8000X, New Era Pump Systems Inc., New York, USA) at a continuous flow rate of 1.178 mL·min⁻¹. The pumping of cell suspensions was continued until all cells had been exposed three times to the shearing condition. Through specific control of various design elements (i.e., flow rate, microtubing diameter and solution composition/viscosity), the present design could investigate sublethal shear damage to the RBC independent of extraneous

compounding forces also observed in current clinical devices (e.g., turbulence, cavitation and recirculation).

Isolation after shear stress

Immediately following the first and third exposure to shear, RBCs were separated from PVP via centrifugation at $1500 \times g$ for 10 min, aspirating and storing the supernatant for determination of cleaved sialic acid. Given PVP suspensions are considered to be pro-aggregating (Başkurt et al., 2000), isolated RBCs were washed twice with isotonic PBS prior to suspension in the desired medium for assessment (i.e., autologous plasma, dextran, PBS); centrifugation was performed at $1500 \times g$ for 5 min.

RBC aggregation and disaggregation shear rate threshold

Isolated RBCs were resuspended in autologous plasma at a standardized hematocrit of $0.4 \text{ L}\cdot\text{L}^{-1}$ for measurement of RBC aggregation. Using the $0.4 \text{ L}\cdot\text{L}^{-1}$ blood sample, 1 mL was injected into a $300 \mu\text{m}$ gap of an annular Couette-type photometric rheoscope (LORCA, MaxSis, Mechatronics, Hoorn, The Netherlands) operating at 37°C . Syllactometry was used to obtain indices of RBC aggregation by measuring magnitudes of laser backscatter generated by blood samples illuminated by laser light from an emitting laser diode (670 nm, 4 mW).

Following the application of a disaggregating shear (500 s^{-1} for 5 s), laser backscatter was measured for samples at stasis over a 120-s period to generate a time-dependent intensity syllectogram, where increased laser backscatter indicated a more disaggregated homogenous blood sample. Through parameterization of this curve, indices reflecting the amount of aggregation over the 120-s period (AI_{120}) and the time required for half-maximal aggregation ($\text{T}_{1/2}$) were calculated. AI_{120} was obtained by calculating the ratio of the area under the laser backscatter-time curve to the sum of the total area under and above the curve.

After the measurement of RBC aggregation, the magnitude of shear rate sufficient to prevent the formation of aggregates was detected (i.e., the disaggregation shear rate; γ_{thr}). Following shearing sufficient for complete disaggregation (i.e., 500 s^{-1}), laser backscatter intensity was recorded at 14 predefined shear rates between 40 s^{-1} and 800 s^{-1} . The difference between the laser backscatter intensity at a given shear rate and the high-shear period for disaggregation was calculated across all shear rates, and analyzed for the local minima. The magnitude of shear at which the difference in laser backscatter was minimal indicated the critical level of shear rate that prevented RBC aggregation, and thus the disaggregation shear rate.

RBC aggregability

To examine the intrinsic tendency of RBCs to form aggregates independent of extrinsic plasma factors (i.e. RBC aggregability), RBCs were resuspended in a standard pro-aggregating solution (3% dextran 70 kDa in PBS) adjusted to $0.4\text{ L}\cdot\text{L}^{-1}$ hematocrit. RBC aggregability is typically measured using $50\text{ }\mu\text{L}$ of the RBC-dextran suspension injected into a cone-plate shearing system (Myrenne RBC Aggregometer, Myrenne GmbH, Roetgen, Germany) operating at room temperature (22°C) (Baskurt, 2007; McNamee et al., 2016a). The cone-plate shearing system generates shear by rotating a cone with an angle of 2° against a stationary glass plate. Following an initial disaggregating shear of 600 s^{-1} for 10 s, infrared light transmission was measured for samples at stasis over a 120-s period to produce a syllectogram. Similar to the laser backscatter-time curve, parameterization of the light transmission-time curve provided indices of AI_{120} and $\text{T}_{1/2}$. Increased magnitudes and rates of aggregation were indicated by increases in AI_{120} and decreases in $\text{T}_{1/2}$, respectively.

RBC electrophoretic mobility

Determination of the change in zeta potential (ζ) and cell charge following exposure to shear required $5\text{ }\mu\text{L}$ of isolated RBCs to be suspended in 30 mL of PBS in a custom-developed

electrophoretic mobility chamber. Using a regulated DC power supply (MP3087, Powertech Systems Equipment Corp., CA, USA) at 30 V and 80 mA, a field strength of $203 \text{ V}\cdot\text{m}^{-1}$ was produced within the chamber between two platinum electrodes. Measurements were performed at room temperature (22°C), and the velocity at which RBCs travelled towards the cathode was recorded using an Olympus IX70 inverted microscope (Olympus, Melville, NY) with an optiMOS high-speed camera (QImaging Corporation, Surrey, Canada) and standardized for optical viewing depth. The velocity was recorded for a minimum of 15 cells across a minimum of 20 s for a subset of randomly-selected subjects ($n=5$). Electrophoretic mobility was determined by the quotient of the measured velocity of a tracked cell ($\mu\text{m}\cdot\text{s}^{-1}$) for a given field strength ($\text{V}\cdot\text{cm}^{-1}$). The zeta potential (ζ) of RBCs in each group was calculated using the Henry equation (Equation 1) with the Smoluchowski approximation:

Equation 1. $\zeta = \frac{\eta \times \mu}{\varepsilon}$

where η was the dynamic viscosity and ε was the dielectric constant of the buffer, and μ was the electrophoretic mobility of the cells.

Preparation of RBC ghosts

To isolate the red cell membrane from its cytosolic contents and hemoglobin, RBC ghosts were prepared for each experimental group. The protocol, previously described by Dodge et al. (1963) requires isolated RBCs to be washed with a hypotonic phosphate buffer solution (sodium phosphate dibasic; Na_2HPO_4 ; prepared at a pH of 8) and centrifuged at $20,000 \times g$ for 40 min. Following the initial centrifugation, the supernatant solution (containing released cytosolic components) was aspirated and removed before the RBC ghost pellet was washed twice with hypotonic buffer solution (centrifugation was at $20,000 \times g$ for 20 min).

Determination of sialic acid content

Sialic acid concentration was measured using the modified spectrophotometric periodate-resorcinol method (Spyridaki and Siskos, 1996), before and after exposure to shear using 20 μ L of isolated RBC membrane ghosts and 500 μ L of the PVP supernatant (indicating cleaved sialic acid). After initial oxidation of total sialic acid with periodic acid (IO_4^- ; 0.04 M), samples were mixed with an acidic resorcinol solution containing resorcinol (0.06 % w/v), HCl (10 M), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.1 M), and H_2O . Formation of the sialic acid chromophore was catalyzed by heating samples to 98°C for 5 min; stabilization was subsequently achieved via rapid cooling and addition of tert-butanol. Following stabilization for 3 min at 37°C, light absorbance was measured against a sample blank at 625 nm using a microplate reader (FLUOstar Omega, BMG LABTECH, Offenburg, Germany). Sialic acid concentration was determined using a calibration curve created from known standard solutions ranging 20-200 μ M. To facilitate a comparison between amount of sialic acid removed from RBCs and gained in the supernatant, following determination of concentration of sialic acid, the absolute change in mass of sialic acid was calculated and reported.

Statistical analyses

Data were confirmed to be parametric using the Shapiro-Wilk test. All data were investigated with a one-way ANOVA with repeated measures applying Bonferroni corrections. Statistical analyses were performed using Prism 7 (Graphpad Software Inc., La Jolla, CA), where significance was determined at an alpha of 0.05. Unless otherwise stated, data are presented as mean $\pm$ standard error.

Results

RBC aggregation and RBC aggregability

Indices obtained from syllectometry that characterize the magnitude and rate of RBC aggregation at stasis for RBCs previously exposed to shear are presented in Figure 1.

Following exposure to 125 Pa for 1.5 s, the magnitude of RBC aggregation in plasma measured over 120 s (i.e., AI_{120} ; Figure 1A) significantly increased ($F=33.35$, $p<0.001$). The rate of RBC aggregation in plasma also significantly increased following shear exposure, as indicated by decreased $T_{1/2}$ (Figure 1B; $F=33.77$, $p<0.001$). Congruent with the RBC aggregation in plasma, RBC aggregability in a standardized plasma-free solution (3% dextran 70 kDa in PBS) significantly increased with initial exposure to shear (Figure 1C; $F=13.01$, $p<0.001$). The rate of RBC aggregability also increased, indicated by a significantly decreased aggregation halftime (i.e., $T_{1/2}$; Figure 1D; $F=15.94$, $p<0.001$). For all measures, no additional changes were observed subsequent to the initial exposure to shear.

Figure 1. Aggregation of red blood cells (RBCs) resuspended in autologous plasma (PLA), and a plasma-free medium (DX), at a standardized hematocrit ($0.4 \text{ L} \cdot \text{L}^{-1}$) following exposure to shear (125 Pa for 1.5 s). The magnitude of RBC aggregation in plasma measured at stasis over 120 s (AI_{120} ; Panel A) significantly increased following exposure to shear, while the halftime for aggregation in plasma ($T_{1/2}$; Panel B) significantly decreased. Exposure to shear increased the magnitude of RBC aggregability in a plasma-free medium measured over 120 s (i.e., AI_{120} ; Panel C), while the measured halftime significantly decreased (i.e., $T_{1/2}$; Panel D). *, significantly different to unsheared control (i.e., “0” shear exposures), $p < 0.05$.

RBC disaggregation shear rate threshold

The critical level of shear rate required to induce disaggregation (γ_{thr}) of blood samples previously conditioned with 125 Pa for 1.5 s, is presented in Figure 2. Following exposure to shear, the disaggregation shear rate significantly increased ($F=8.93$, $p < 0.001$) from 158.7 ± 25.1 to $240.0 \pm 23.0 \text{ s}^{-1}$ (one exposure) and $242.7 \pm 19.2 \text{ s}^{-1}$ (three exposures). Subsequent to the initial change following exposure to shear, successive exposures to shear did not significantly alter the disaggregation shear rate.

Figure 2. The critical level of shear rate required to induce disaggregation (γ_{thr}) of red blood cells (RBCs) determined for blood samples exposed up to three times to conditioning shears (125 Pa for 1.5 s). *, significantly different to unsheared control (i.e., “0” shear exposures), $p < 0.05$.

Bound and unbound sialic acid

The concentration of sialic acid (SA) for 20 μL of RBC membrane ghosts (Figure 3A) and cleaved into 500 μL of the supernatant (Figure 3B) is presented following shear exposure. To facilitate comparison, calculated changes in mass of SA relative to control (ΔSA) are displayed in Figure 3C for RBC bound SA (SA_{RBC}) and unbound SA in the supernatant ($\text{SA}_{\text{unbound}}$). The concentration of membrane-bound sialic acid (i.e., SA_{RBC}) significantly decreased with exposure to 125 Pa for 1.5 s ($F=12.38$, $p < 0.001$), while the concentration of SA measured in the shearing supernatant (i.e., $\text{SA}_{\text{unbound}}$) significantly increased ($F=37.18$, $p < 0.001$). No significant changes were detected between the first and third exposure to shear.

Figure 3. The concentration of sialic acid (SA) measured in 20 μL of isolated red blood cell (RBC) membrane ghosts (SA_{RBC} ; Panel A) and released into 500 μL of shearing supernatant ($SA_{unbound}$; Panel

B) following exposures to $125 \text{ Pa} \times 1.5 \text{ s}$. Panel C reports the change in mass of SA following exposure to shear. *, significantly different to unsheared control (“0”), $p < 0.05$.

Electrophoretic mobility and zeta potential

The electrophoretic mobility (EPM) of RBCs exposed to shear is illustrated in Figure 4. EPM significantly decreased following first exposure to shear ($F=331.3$, $p < 0.001$). The calculated mean zeta potential was $-15.78 \pm 0.31 \text{ mV}$ for unsheared control RBCs (“0” number of exposure); one exposure to shear induced a significantly less negative zeta potential of $-7.55 \pm 0.21 \text{ mV}$. Following this initial change, subsequent exposures to shear maintained the less electronegative zeta potential ($-7.52 \pm 25 \text{ mV}$); however, no further cumulative effect was detected.

Figure 4. The electrophoretic mobility for red blood cells (RBCs) suspended in PBS following exposure to shear (125 Pa for 1.5 s). Data collected from a subset of randomly-selected subjects ($n=5$).

*, significantly different to unsheared control (“0”), $p < 0.05$.

Discussion

The salient findings of the present study indicate that exposure of RBCs to shear stress, representative of that observed in mechanical circulatory assist devices, induces: i) increased magnitude and rate of RBC aggregation; ii) increased tendency of RBCs to aggregate in a standardized plasma-free medium (i.e., aggregability); iii) increased amount of shear required to disaggregate formed rouleaux; iv) cleaved membrane-bound sialic acid into the surrounding media; and, v) lowered electrostatic charge of RBCs (observed via decreased electrophoretic mobility), and thus also the cells' zeta potential. The collective findings present, for the first time, evidence that supraphysiological shear exposure may remove membrane-bound sialic acid from the glycocalyx of RBCs, leading to increased cell aggregation. Given that RBC aggregation and disaggregation are important parameters for the regulation of microcirculatory blood flow, these results may explain, in part, the elevated incidence of secondary complications leading to multi-system organ failure in patients receiving mechanical circulatory support.

Recent investigations of subhemolytic damage to blood have predominantly focused on morphological changes, rather than alterations to the physical and electrochemical properties which explain cell behavior in various environments (micro- and macro-vascular circulation). The present study, for the first time, reports that supraphysiological, subhemolytic shear stress exposure (representative of the transit within mechanical circulatory support devices) increased the magnitude and rate of RBC aggregation. These novel findings are congruent with clinical data, which highlight that circulatory support-induced blood trauma increases low-shear blood viscosity, and erythrocyte sedimentation rate (Kameneva et al., 1994; Kormos et al., 1987); RBC aggregation is a primary determinant of these clinical markers (for a review, see Simmonds et al. (2013)). The present investigation extends beyond these prior studies, however, by shearing RBCs in a medium independent of plasma and other formed

elements, which facilitated our investigation into the discrete responses of RBCs to the specific flow conditions.

While isolated RBCs were sheared prior to being resuspended in autologous plasma, the increased cellular aggregation in the present study may originate from decreased disaggregating factors intrinsic to the sheared cells (i.e., surface charge density), or increased pro-aggregating factors resulting from interactions of sheared blood cells with other unsheared formed elements. It is plausible that damaging stimuli (such as shear exposure), for example, may cause the translocation of phosphatidylserine to the RBC surface membrane, altering subsequent interactions of RBCs with leukocytes and platelets (Borst et al., 2012). To determine whether interactions between RBCs and other formed elements of blood may explain the present findings, we also investigated the tendency of RBCs to aggregate independent of plasma factors, and observed similar trends of increased RBC aggregability following shear exposure. These findings suggest that factors intrinsic to RBCs, independent of extrinsic factors, are altered following supraphysiological shear stress exposure and thus explain shear-induced promotion of cell aggregation.

Standard methods of assessing RBC aggregation typically examine laser backscatter-time curves while cells are at stasis. Using the same hardware, the disaggregation shear rate threshold was explored by measuring changes in laser backscatter in shear flow, from 40 – 800 s^{-1} . As displayed in Figure 2, the magnitude of shear required to disaggregate formed rouleaux (without augmenting cellular deformability) significantly increased following initial shear exposure, and was maintained for subsequent exposures to shear. These data indicate that exposure to subhemolytic magnitudes of shear stress increases the amount of aggregation in shear flow, and the magnitude of subsequent shear required to sufficiently disaggregate formed rouleaux. If aggregates fail to disperse in circulation, the resistance of cells entering smaller vessels may increase, propagating a heterogenous distribution across capillary beds

(Johnson, 1971). While increased aggregation increases the likelihood of RBC stasis in smaller capillaries, blood flow can be preserved via rerouting RBC via thoroughfare/preferential channels (i.e., capillaries with larger diameter) (Mchedlishvili et al., 2002), partially bypassing smaller capillaries and thus potentially disturbing local tissue oxygenation.

In the present study, the electrophoretic mobility of RBCs significantly decreased following exposure to conditioning shear, indicating a decreased negative cell charge (i.e., less negatively charged). As electrostatic repulsion of RBCs is a primary disaggregating force (Schmid-Schönbein et al., 1980), this finding likely explains the increased aggregation, aggregability, and shear required for disaggregation observed following the conditioning shear. One of the primary contributors to the negative charge of the RBC is the abundant distribution of sialic acid across the cell surface (Eylar et al., 1962), accounting for ~90% of the cell's net negative charge and hydrophobicity (Mehdi et al., 2012). High shear environments have been reported to induce ultrastructural changes to RBCs, causing changed morphology (McNamee et al., 2016b), cell fragmentation (Watanabe et al., 2007), and disruption of the lipid bilayer (Dao et al., 1993). Given the significant surface alterations that occur to the cell membrane of RBCs with subhemolytic trauma, it is likely that supraphysiological shear stress cleaved sialic acid from the cell membrane, decreasing the electrostatic surface charge and thus also the disaggregating force intrinsic to the glycocalyx.

The removal of sialic acid from the RBC membrane has previously been correlated with normal cell ageing/senescence processes resulting in decreased negative surface charge of the cell (Hadengue et al., 1998; Simmonds et al., 2013); this process may be accelerated with the enzyme neuraminidase (Jan and Chien, 1973). While shear stress has not previously been reported to cleave sialic acid, Figure 3 presents that shear exposure decreased RBC membrane-bound sialic acid, and increased sialic acid content in the suspending media.

Figure 3C confirms that, when concentrations of sialic acid are standardized as mass lost/gained, the change in sialic acid content in the supernatant matches the amount removed from the RBC membrane. These data indicate that membrane-bound sialic acid can be removed in high shear conditions and thus may explain some of the physical changes in RBCs following supraphysiological shear exposure.

The impact of shear removal of membrane-bound sialic acid content may also explain the decreased RBC deformability and increased membrane rigidity observed in other studies (Horobin et al., 2017; McNamee et al., 2017; McNamee et al., 2016b; Simmonds et al., 2014; Simmonds and Meiselman, 2017). As sialic acid is negatively ionized, and cytosolic hemoglobin is positively charged, membrane-bound sialic acid is proposed to be an important regulator of hemoglobin distribution in the cytoplasm, thus also playing a role in maintaining the oxygen carrying capacity of RBCs (Huang et al., 2016). Moreover, as sialic acid is bound to band 3, it has been observed to influence cell morphology and membrane deformability (Huang et al., 2016). Further studies will elucidate the role of sialic acid in maintaining cell morphology, and will contribute to the development of numerical models of the dynamic balance between pro-aggregating and disaggregating forces.

While the present study demonstrates that RBC sialic acids can be removed with a single exposure to supraphysiological sublethal shear, no further compounding of this effect was observed during repeated exposures. Of note, similar trends of increasing RBC aggregation/aggregability (Figure 1) and decreasing cell electrophoretic mobility (Figure 4) were observed, providing a secondary confirmation of the sialic acid change in response to shear exposure. Collectively, these data indicate that the accumulation of blood trauma may occur rapidly (i.e., < 1 s duration), causing the cells' charge to significantly decrease to half of its initial level. Given enzymatic (neuraminidase) treatment may remove up to 90% of cell charge (Eylar et al., 1962), the initial decrease in charge (followed by a plateau) appears to

indicate: i) further shear accumulation is required to remove the remaining sialic acid, or ii) the remaining sialic acid is less sensitive to shear damage (i.e., possibly located within the cytosol, or the inner monolayer of the RBC membrane, thus “protected” from shear). Although sialic acids are ubiquitous in many tissues of the body, its presence within the cytosol or on the inner monolayer of the RBC membrane remains to be identified. Moreover, while sialic acids are predominantly located on glycophorin A (~75 %), they are also located on band 3, glycolipids and other glycophorins of the cell membrane (de Oliveira and Saldanha, 2010), which may vary in junctional attachment sites. The findings of the present study, may suggest that the relative strength of the sialic acid junctional attachments, or the integrity of the O-linked and N-linked oligosaccharides containing sialic acid is varied (Aoki, 2017), with some attachments being more susceptible to shear.

The collective findings of the present study indicate that RBC’s electrochemical charge and aggregation behavior are altered following the initial exposure to supraphysiological shears. The present magnitude-duration combination of shear exposure, while being below the hemolytic threshold, supports previous estimates of the threshold shear required for sublethal damage (Simmonds and Meiselman, 2017). Given accumulation of sublethal damage occurs rapidly (i.e., during the first exposure to high shear), these data bear substantial implications for patients acutely receiving mechanical circulatory support. Irrespective of short-duration transits, blood passing through mechanical circulatory support devices may rapidly become damaged. The implications for post-surgical tissue perfusion are of interest, given that even once “off-pump”, the continued circulation of sublethally damaged RBCs and associated impaired blood fluidity is likely to persist until complete cell turnover (up to 120 days). The hyperaggregation of RBCs and associated sluggish low-shear blood flow may cause increased leukocyte and platelet margination/attachment (Nash et al., 2008), diminished nitric oxide synthesis altering vasomotor tone (Baskurt et al., 2004), and increased thrombotic risk

(Baskurt and Meiselman, 2013); potentially contributing to the prevalence of complications (e.g., hemorrhage, thrombosis, tissue ischemia, anemia, etc.) that ultimately lead to multi-system organ failure and death (Kirklin et al., 2014). Given patients indicated for mechanical circulatory support are typically at elevated risk of altered blood fluidity (due to comorbid chronic disease and inflammation), future mechanical circulatory support should be designed to limit magnitudes of shear exposure to levels that do not alter the electrochemical and other physical properties of blood cells.

The present study was designed specifically to explore the effects of elevated shear stress on RBC physiology in the absence of other factors known to influence cell viability typical of rotary blood pumps – e.g., complex and turbulent flow – thus caution is required for extending the present experimental insights to clinically-utilised blood pumps and current clinical practice. Moreover, the present study sheared RBCs in PVP to facilitate cell properties to be explored following shear exposure, independent of other blood cells (platelets, leukocytes) and plasma factors; while it is predicted that high shear would have similar effects in plasma, the magnitude of such an effect may be lessened due to the so-called “protective” qualities of plasma (Kameneva et al., 1997). Nevertheless, the findings of the present study may assist the development of current numerical models of blood damage, assisting the advancement of circulatory support device design. Moreover, the present findings emphasize that further investigation of RBC aggregation and blood damage is warranted in clinical populations receiving mechanical circulatory support.

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Highlights:

- Sublethal mechanical damage alters the physical properties of RBCs
- Shear exposure cleaved membrane-bound sialic acid, decreasing RBC electronegativity
- Altered cell charge likely explained increased RBC aggregation
- Shear exposure increased the force required to disperse formed aggregates

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Sublethal mechanical trauma alters the electrochemical properties and increases aggregation of erythrocytes

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