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Impact of environment on Red Blood Cell ability to withstand mechanical stress

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Abstract. Susceptibility of red blood cells (RBC) to hemolysis under mechanical stress is represented by RBC mechanical fragility (MF), with different types or intensities of stress potentially emphasizing different perturbations of RBC membranes. RBC membrane mechanics were shown to depend on cell environment, with many details not yet understood. Here, stress was applied to RBC using a bead mill with oscillation up to 50 Hz, over durations up to 50 minutes. MF profiles plot percent lysis upon stresses of progressive durations. Supplementing media with polyethylene glycol (PEG) which interacts with the cell membrane, but not Dextran which does not, resulted in higher resistance to hemolysis. Albumin, and to a lesser extent fibrinogen and globulins (at physiological concentrations), significantly increased cell ability to withstand mechanical stress versus with un-supplemented buffer solution and with PEG. This is partly due to changes in rheology, per tests done including (PEG) and Dextran, but is mostly due to cell-protein interaction, noting the effect of pH on RBC MF with albumin but not with buffer. Presence of lipids reduced RBC resistance to potentially hemolytic stress with lypemic plasma effecting lower "protection" from induced hemolysis than essentially fatty-acid free plasma. This effect was less dependent on incubation than on fatty-acid presence during stressing. The reduced propensity for hemolysis afforded by plasma proteins also depended markedly on the speed of the bead, potentially reflecting changes from a predominantly Von Karman trail at lower frequencies to an increasingly disorganized turbulent wake at higher frequencies.

Keywords: Red Blood Cells, mechanical fragility, hemolysis, albumin, fibrinogen, gamma-globulin, fatty acid, plasma, blood storage

1. Introduction

Red Blood Cells (RBC) are subject to significant mechanical stress while in circulation and must be able to sustain extensive deformations without fragmentation. This ability can potentially be compromised by the effects of drugs, medical devices or even storage that can induce pronounced changes in RBC membrane structure (e.g. [3, 6, 12, 19, 39]). Typical ways to assess the ability of RBC to withstand external stresses involve evaluation of hemolysis (complete destruction of a fraction of RBC in the sample studied). While reflective of the terminal cell damage, such a test does not reflect possible sub-lethal damage to RBC, i. e. damage that can lead to delayed hemolysis even under normal physiological conditions. Such sub-hemolytic damage can be potentially evaluated through RBC deformability, which reflects cell membrane ability to deform at sub-lethal stress intensities (e.g. [40, 41]). Alternatively, a different yet related property of mechanical fragility (MF), which reflects cells' propensity to hemolyse under potentially lethal stress, can be used [5, 18]. While MF more directly represents RBC susceptibility to lysis induced by mechanical stress, at the present time, RBC MF testing remains unstandardized, with multiple testing approaches varying in the ways mechanical stress

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is applied [16]. Different types and intensities of stresses employed likely result in different induced perturbations of RBC membrane and thus in the different outcomes of the testing [10].

In addition, mechanical properties of RBC membranes were shown to significantly depend on the cells' environment. In particular, it had been demonstrated that blood plasma can effect a protection of RBC, with cells in that environment typically less susceptible to mechanically-induced lysis than those in saline or similar media [20]. It has also been demonstrated that a significant amount of protein is associated with RBC membranes when in plasma [24]. Such association may modify the RBC membrane response to external mechanical stress. This protective effect observed in plasma seems to a large extent to be due to plasma albumin (e.g. [20], which at a physiological concentration was shown to decrease RBC MF. However, Dextran-40 or ovalbumin failed to increase RBC resistance to induced lysis, possibly due to decreased interaction with the cell membrane [20, 42]. Both long- and short-chain fatty acids (FA), when present in solution, interact with RBC membranes. Increased concentration of long-chain FA has been reported to lower RBC osmotic fragility; however short-chain FA – with a dependence on concentration, chain length and structure – were shown to be overall detrimental to RBC ability to resist osmotic stress [26, 42]. These results suggest that the effect is due to FA incorporation into or disturbing of the RBC membrane lipid bilayer. Membrane cholesterol content, or cholesterol/phospholipid ratio, has also shown to significantly affect membrane properties including deformability and osmotic fragility [11, 15, 27]. Similar observations have been reported for mechanical fragility, which was found to correlate with plasma lactate dehydrogenase (LDH) and triglycerides existing prior to shear (based on a rotating disks approach to applying RBC stress) [29].

Overall, it appears that changes in the dynamic balance that exists between the membrane and the environment can have marked implications on a cell's ability to withstand stress without lysing. Understanding these effects may have implications for performance and interpretation of the results of RBC fragility testing, and by extension, to standardization of mechanical fragility as a metric of RBC membrane stability. Presented here are results aimed to elucidate some aspects of environmental impact of RBC under mechanical stress.

2. Materials and methods

Blood components (packed RBC and Fresh Frozen Plasma) were obtained from American Red Cross Biomedical Services and stored according to AABB Standards [23]. Human and Bovine Serum Albumins, including essentially fatty acid free, were purchased from Sigma- Aldrich (St. Louis, MO) and RPI Corp (Mt. Prospect, IL); bovine fibrinogen and dextrans were purchased from Alfa Aesar (Ward Hill, MA); polyethylene glycol (PEG) 8,000 was purchased from Hampton Research (Aliso Viejo, CA); γ-globulins and all other chemicals were purchased from Sigma- Aldrich (St. Louis, MO).

2.1. Fragility test

Samples were diluted to total hemoglobin (Hb) concentration of 1.7 g/dl corresponding to about 4% hematocrit, verified by a Hemoglobin 201 system from HemoCue (Angelholm, Sweden), with AS-3 storage buffer, pH 5.8, containing at times additives as specified in the text. The diluted sample was gently agitated and aliquoted into 2 ml low-retention centrifuge tubes at 350 μl per tube. Mechanical stress was applied to RBC samples with the use of a TissueLyser LT (Qiagen, Dusseldorf, Germany) vertical bead mill (at an oscillation frequency of 50 Hz) in the presence of one 7 mm diameter stainless steel ball. Samples from each RBC unit were subjected to such stress at 10 different durations (ranging from 30 sec to 30 min) to ensure a wide range of induced hemolysis levels. The sample holder of the TissueLyser was modified to allow air-cooling while in operation, which resulted

in sample temperature stabilization to within 2 degrees of the start temperature. Un-lysed cells were precipitated by centrifuging the samples for 5 minutes at 5,000 rpm on an Eppendorf 5417C centrifuge (Hauppauge, NY). Aliquots of supernatant were collected and their spectra recorded. All tests were performed at 6°C, in AS-3 storage buffer, pH 5.8 unless indicated otherwise. Such conditions were selected to mimic that of packed RBC storage with AS-3, commonly used storage solution with its low pH value aimed to suppress cell metabolism. Presented data is the result of three independent measurements with per sample SD = 0.03 for these three measurements, except where stated otherwise. Fragility can vary between different samples (e.g. due do donor-to-donor variability or differences in manufacturing process [38]); such sample-to-sample variability indicated where relevant. Figures present representative results obtained for a given RBC sample, with the error bars (±SD) indicating experimental error on three independent measurement for that value.

2.2. Hemolysis assessment

Hemolysis (Hem), both as present in untreated sample and as induced by the bead mill, was determined based on the difference in absorbance at 576 nm, a wavelength of oxygenated Hb maximum, and absorbance at 685 nm, the local minimum for the oxygenated Hb form. It was expressed as a fraction of free hemoglobin (HB^F) relative to total hemoglobin concentration (Hb^T) according to Formula 1 which included the correction for sample hematocrit as detailed by Sowemino-Coker [34].

$$Hem = \frac{Hb^F_{576} - Hb^F_{685}}{Hb^T_{576} - Hb^T_{685}} * (1 - Hematocrit) \quad (1)$$

Total hemoglobin concentration for each diluted RBC sample was determined by subjecting a small (350–400 μL) aliquot to ultrasound for 40 seconds (0.1 second pulses, with 0.2 intervals between pulses, on ice) delivered by a Branson Digital Sonifier 450 (Danbury, CT), at 15% intensity (from the manufacturer specified 400 Watt). In control experiments, such treatment was shown to fully lyse RBC without inducing hemoglobin oxidation. For determining auto-hemolysis (i.e., hemolysis prior to any application of mechanical stress), small (30 μl) aliquots of undiluted samples were centrifuged for 5 min at 5,000 rpm, supernatants were collected, and hemoglobin content was measured spectrophotometrically. Spectroscopic measurements were performed with a NanoDrop N100 spectrophotometer (Thermo Scientific, Waltham, MA).

2.3. RBC fragility profiles

RBC Fragility Profiles are defined here as the incremental (amount exceeding auto-hemolysis) hemolysis in a sample resulting from applied stress of progressively increasing durations. This can optionally be performed at different intensities, for multidimensional profiling. Unlike single-point measurements that use a single stress duration at a single stress intensity (as implemented for example by Raval et al. [30]), MF profiles allow recording of a RBC sample's propensity to hemolyze over a range of applied stress magnitudes (from that resulting in minimal lysis to that resulting in nearly total hemolysis in the tested sample). Such profiles thereby allow multiple fragility-based indexes to be interpolated for separate analyses [2]. Here, a Hem_3 hemolysis parameter (Hem-parameter) was used to represent amount of hemolysis achieved as a result of 3 min-long stress application. This parameter was obtained from best-fit second-order polynomial regression of the experimental data. (All fits shown on the figures are for illustration purposes only.)

Evaluation of the impact of storage at high fatty acid concentration on RBC MF was performed by comparing MF after prolonged incubation in lypemic plasma or plasma cleared by ultracentrifugation when tested in either of those plasma types. Packed RBC were precipitated, supernatant replaced with

either lypemic or clear plasma, and stored at 4°C for 1, 3 or 6 weeks. After the storage, the RBC were again precipitated with initial plasma replaced by either lypemic or cleared plasma, and then subjected to MF testing.

2.4. Statistical analysis

Data is presented in the form of sample mean and standard deviation (SD), 95% CI and range, where appropriate. One sample and paired Student *t*-test or signed rank test with a two-tailed *p*-value of 0.05 was used to test for statistical significance. Repeated measures mixed model approach [25] was utilized to test the differences between measurements from the same samples under different conditions, Tukey-Kramer adjustment was used to account for multiple comparisons where appropriate. The analysis was done using SAS 9.4 (SAS Institute, Cary, NC) [33].

3. Results

Human serum albumin at physiological concentration (40 g/L) effected a marked decrease in RBC MF, compared to saline or storage solution such as AS3 (Fig. 1, Traces 1 and 2). No significant variability was detected between bovine and human fatty acid free (free fatty acids <0.007%) albumins, nor between such albumins from different manufactures (Sigma vs. RPI). However, non-fatty acid free HSA (free fatty acids 0.09%) exhibited elevated (by about 30%) hemolysis, compared to the fatty acid free HSA (data not shown).

Unexpectedly, when suspended in plasma, RBC had much smaller protection against mechanical stress from the phenomenon described above. It was noted that the decrease in MF observed in plasma varied between the plasma samples. In particular, lypemic plasma provided a much smaller protection against mechanical stress. The same plasma with lower free lipid content (cleared through ultracentrifugation at 100,000 g for 1 hour) imparted significantly higher protection from applied mechanical stress to RBC, resulting in lower measured MF (Fig. 1, Traces 3 and 4).

Table 1 shows the variability in reduction of RBC MF due to supplementation of storage solution with a physiological concentration of albumin (40 g/L) using induced hemolysis at 3 minutes of applied stress as a metric (Hem_3). With the average value of 30 percent of that in AS3 (statistically significant

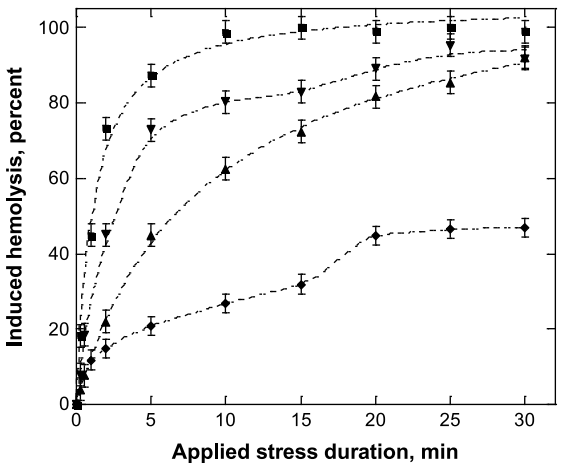

Fig. 1. Hemolysis induced by applied mechanical stress in RBC suspended in 1) AS3 (■); 2) AS3 containing 40 g/L BSA (♦); 3) lypemic (▼), and 4) plasma cleared by ultracentrifugation (▲).

Table 1
Variability in induced hemolysis of RBC after 3 minutes of applied mechanical stress. (Shown as hemolysis in the media with the supplement as a fraction relative to hemolysis observed in un-supplemented AS3)

		$\frac{Hem_3^{Medium}}{Hem_3^{AS3}}$, percent		
Medium	N	Mean (SD)	95% CI of the mean	Range
AS3 (pH 7.4) supplemented with 40 g/L BSA	40	30 (13)	26–34	16–70
Fresh frozen plasma	9	67 (14)	56–77	45–88
Fresh frozen plasma after ultracentrifugation	8	39 (10)	30–47	30–60

difference with $p < 0.001$), the range was 16 to 70 percent reduction in MF. Similarly, the protective effect of normal fresh frozen plasma (FFP) was also found to be variable, with reduction in RBC MF significantly lower than that in albumin ($p < 0.001$ for differences between FFP and AS3, and between FFP and AS3 with albumin). Ultracentrifugation further reduced fragility in each of plasma samples starting to approach that observed in AS3-albumin medium ($p = 0.049$ as compared to AS3 with albumin). Such reduction in RBC MF was also highly variable for different plasmas. With average decline of MF to about 40 percent of that in AS3, the difference between regular and clear FFP RBC MF (due to lipid's precipitation after ultracentrifugation) was 25 (SD = 12, 95% CI 15–35) percent with the range between 1 and 44 percent. This change was statistically significant with $p < 0.001$. All differences remained statistically significant with and without of the assumption of normality for data distribution, as tested with either a *t*-test or a signed-rank test. Repeated measures ANOVA was performed with mixed effects model to account for correlated data, where each blood sample was repeatedly tested in different media. Tukey-Kramer method was used to adjust for multiple comparisons of the means.

The impact of storage at high fatty acid concentration on RBC MF was evaluated by comparing MF after prolonged incubation in lypemic plasma or plasma cleared by ultracentrifugation when tested in either of those plasma types. This procedure allowed separating the effects of extra lipids on RBC MF due to prolonged storage from the effect they might have if present during mechanical stress application. Results (Fig. 2) clearly show that lipid content during stress application affected the

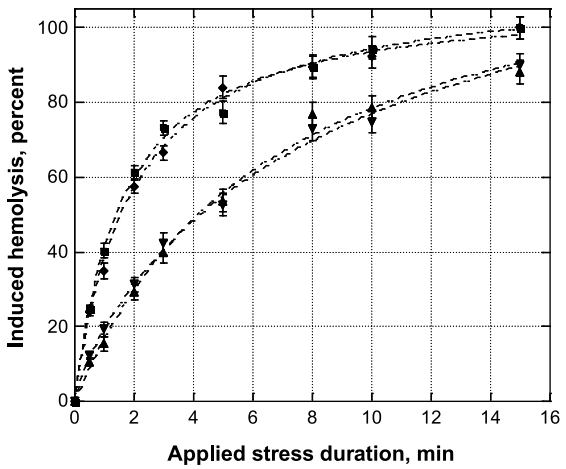

Fig. 2. Hemolysis induced by applied mechanical stress in RBC after 2 weeks incubation in lypemic plasma and tested in 1) the same plasma (■) or in 2) plasma cleared by ultracentrifugation (▼); or after 2 weeks incubation in cleared plasma and tested in 3) lypemic plasma (♦) or in 4) cleared plasma (▲).

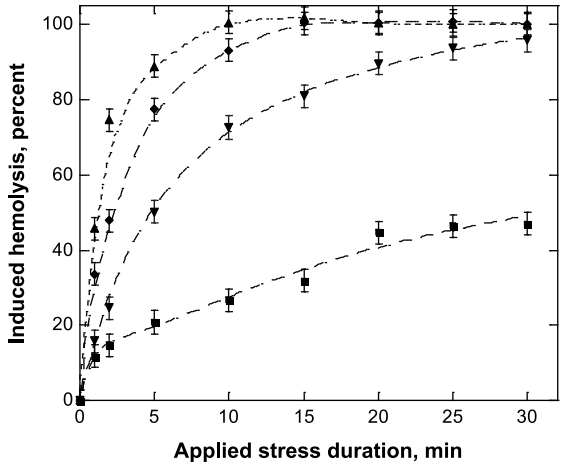

Fig. 3. Hemolysis induced by applied mechanical stress in RBC suspended in AS3 supplemented with: 1) no supplement (▲); 2) 0.4 g/L BSA (♦); 3) 4 g/L BSA (▼); and 4) 40 g/L BSA (■). 50 Hz bead oscillation frequency.

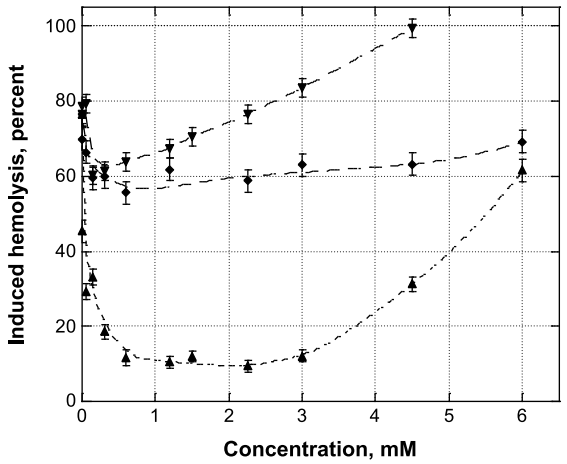

Fig. 4. Changes in induced hemolysis in RBC suspended in AS3 supplemented with variable amounts of 1) BSA (▲), PEG 8K (♦), and PEG 20K (▼) as a fraction of hemolysis in AS3 without supplements. 50 Hz bead oscillation frequency, 3 minutes of stress application.

magnitude of protection (decrease in MF) plasma had on RBC, while storage conditions (in terms of lypemic or cleared plasma) did not influence RBC MF. Similar results were observed at all durations of storage (up to 6 weeks).

The magnitude of RBC protection against mechanical stress, expressed as a decline in observed RBC MF, was significantly dependent on serum albumin concentration. Profiles of RBC MF at selected BSA concentrations are shown in Fig. 3. Maximum protection (i.e. minimum fragility) was observed in the range of 20 – 200 g/L (0.3 – 3 mM) BSA (Fig. 4). Interestingly, the physiological albumin concentrations, typically given as 35 – 50 g/L, reside in the range that supports maximum resistance to hemolysis induced by mechanical stress. At both lower and higher BSA concentrations, RBC were much more susceptible to stress-induced hemolysis.

Other proteins, besides albumin, could potentially contribute to the decrease in RBC MF observed in plasma. Supplementation of AS3 with albumin, fibrinogen or γ-globulins at physiological concentrations resulted in significant reduction of RBC MF ($p < 0.001$; $p = 0.002$ for γ-globulins). On average,γ-globulins exhibited about a 15 percent decrease in RBC MF; markedly less than that caused

Table 2

Changes in hemolysis after 3 minutes of applied mechanical stress due to supplementation of the medium by BSA, γ-globulins, and fibrinogen[1]. (Shown as hemolysis in the media with the supplement as a fraction relative to hemolysis observed in un-supplemented AS3)

	$\frac{Hem_3^{Medium}}{Hem_3^{AS3}}$, percent					
	Physiological concentration[2]			Equimolar Concentration[3]		
Media supplement	N	Mean (SD)	95% CI of the mean	N	Mean (SD)	95% CI of the mean
γ-globulins	7	84 (8)	77–92	7	93 (10)	85–102
fibrinogen	6	47 (8)	38–56	8	48 (8)	41–56
BSA	6	18 (3)	14–21	8	75 (12)	64–85
γ-globulins and BSA	5	18 (4)	14–23	5	80 (14)	65–95
fibrinogen and BSA	5	14 (3)	10–17	5	38 (9)	26–50

[1]At each concentration, each RBC sample was tested with and without all supplements as listed in the table above. Media was adjusted to pH 7.4; [2]Physiological concentration: γ-globulins: 20 g/L; fibrinogen: 400 mg/L; BSA: 40 g/L; [3]Equimolar concentration: 12 μM.

by either fibrinogen (ab. 50 percent) or albumin (over 80 percent). With both albumin and fibrinogen present in the solution at the same time, RBC MF declined even further (Table 2). This decrease in MF was statistically significant with $p = 0$ using paired *T*-test. For albumin-γ-globulin pair, no statistically significant change ($p = 0.8$ compared to albumin alone) was observed.

Another approach is to look at the magnitudes of RBC MF changes at equimolar protein molecules interaction with cell membrane concentrations that, barring second order effects, will ensure a level comparison for potential protein molecule interactions with cell membrane. Concentration of 12 μM was selected as that, still within the physiological range of fibrinogen. Note, that this concentration is significantly lower than physiological for both γ-globulins (ca. 11-fold) and albumin (ca. 8-fold). In such per-mole terms, effect of γ-globulins was practically negligible ($p > 0.1$ comparing to MF in AS3), and while supplementing with either albumin or fibrinogen resulted in lower MF ($p < 0.001$), albumin was less effective (Table 2). With both albumin and γ-globulin present, again, fragility remained unchanged relative to that in albumin alone ($p > 0.7$). With both albumin and fibrinogen present, there was further decline in RBC MF (a decrease of about 10 percent in mean values as compared to fibrinogen alone), ($p = 0.06$); however being highly variable, this change did not reach significance in paired *t*-test.

Influence of rheological effects can be experimentally evaluated by supplementing RBC suspensions s with Dextran or polyethylene glycol (PEG). Supplementation of AS3 with Dextran 20 KDa, 40 KDa or 75 KDa at 0.6 mM, concentration corresponding to physiological concentration of albumin, resulted in RBC MF values similar to that in un-supplemented AS3 (Table 3). Changes in fragility were not statistically significant ($p > 0.05$) for 20 and 75 KDa Dextrans, although reached significance for 40 KDa Dextran ($p = 0.014$). For all three Dextrans, RBC MF was found to be essentially independent of polysaccharide's concentration in the range from 0.006 to 6 mM (zero slope was within the experimental error of the slope of simple linear regression of concentration dependence of RBC MF for all three Dextrans, data not shown). However, statistically significant changes (at $p = 0.01$) in RBC MF were observed when AS3 was supplemented with PEG of either 8 kDa or 20 kDa at 0.6 mM (Table 3). In both cases, the effect was much less pronounced than with BSA or plasma. Decrease in observed RBC MF (i.e. increased resistance to applied mechanical stress) was broader for PEG 8 kDa, and had the maximum (corresponding to a minimum in induced hemolysis) at about 0.6 – 1 mM, compared to 0.01–0.6 mM observed in PEG 20 kDa (Fig. 4).

Table 3
Changes in hemolysis after 3 minutes of applied mechanical stress due to supplementation of the medium by Dextran or PEG. (Shown as hemolysis in the media with the supplement as a fraction relative to hemolysis observed in un-supplemented AS3)

	$\frac{Hem_3^{Medium}}{Hem_3^{AS3}}$, percent $N=3$	
Media supplement, 0.6 mM	Mean (SD)	Range
Dextran, 20 KDa	94 (7)	87–100
Dextran 40 KDa	94 (1)	93–95
Dextran 75 KDa	103 (9)	93–110[1]
PEG, 8 KDa	62 (7)	56–70
PEG, 20 KDa	68 (6)	65–74

[1]Percent hemolysis over a hundred means that hemolysis was higher in sample with supplemented medium, than it was in un-supplemented AS3.

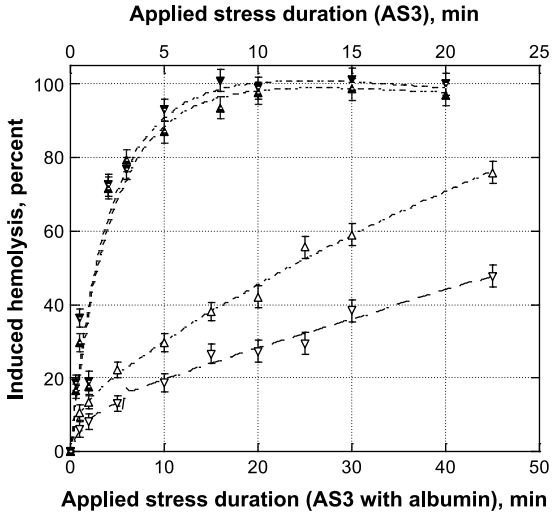

Fig. 5. Hemolysis induced by applied mechanical stress in RBC at different pH values of the suspension: RBC suspended in AS3 at 1) pH 5.5 (▼) and 2) pH 7.4 (▲); suspended in AS3 supplemented with 40 g/L BSA at 3) pH 5.5 (▽) and 4) pH 7.4 (△). 50 Hz bead oscillation frequency.

Changes in RBC MF due to changes in pH of the medium with and without supplementation by albumin can potentially probe the importance of protein interaction with the cell membrane. With cells suspended in AS3 no significant changes in RBC MF were detected.in the range of pH values from 5.5 to 7.4. However, supplementation of AS3 medium with albumin not only resulted in an overall decline of observed RBC MF (increased resistance to stress), but the magnitude of the effect was also found to be pH dependent (Fig. 5).

As can be anticipated, induced hemolysis increased with the increase of oscillation frequency of the ball movement, at a set stress duration (Fig. 6). Non-linearity of this dependence can imply effects of other factors beyond the number of times the ball traverses the length of the chamber in a given time (as represented by oscillation frequency). Indeed, in addition to the number of oscillations of the ball, oscillation frequency can also affect the speed of the ball movement through the medium, which in turn would affect the properties of the flow around the ball. Figure 6, insert, shows the dependence

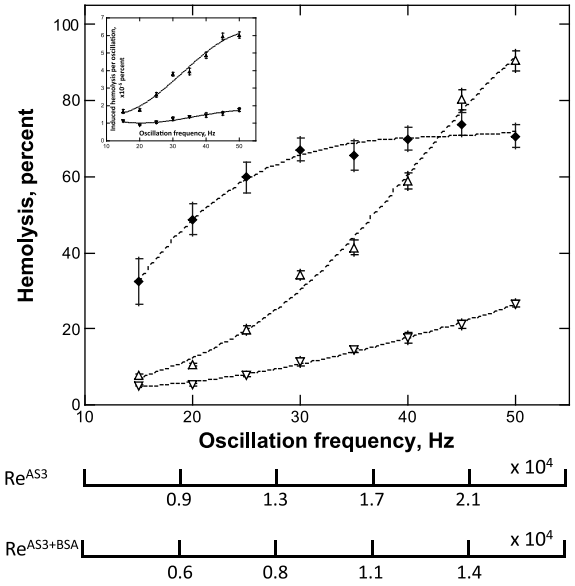

Fig. 6. Dependence of induced hemolysis on oscillation frequency after stress application for 5 minutes. RBC in AS3 (Hem_{AS3}, △) and in AS3 supplemented with 40 g/L BSA ($Hem_{AS3+BSA}$, ▽). Protective effect of albumin ((Hem_{AS3}-Hem_{AS3+SA})/Hem_{AS3}, ♦). Estimated Reynolds numbers for AS3 (Re^{AS3}) and for AS3 supplemented with albumin ($Re^{AS3+BSA}$) were calculated for this experiment's geometry using dynamic viscosities (for 10°C) of 1.3×10^{-3}N s/m^2 for AS3 solution, and of 2.010^{-3}N s/m^2 for AS3 supplemented with albumin [13], with both assumed to be Newtonian fluids. Insert: Percent of induced hemolysis in a single oscillation, as a function of oscillation frequency.

of induced hemolysis per single oscillation, supporting the ball speed importance for resultant cell lysis. The difference between hemolysis induced in the absence of and in the presence of 40 g/L BSA, which represents the amount of protection afforded by the protein presence, also exhibited a marked oscillation frequency dependence increasing to about 70 percent at frequencies over 35 Hz.

4. Discussion

While plasma had been reported to have a protective effect on RBC (reducing MF) when the stress was applied using bead agitation [9, 20], rotating disks [28], filtration [1], propeller mixer [42], and tube flow or a system like Fleisher hemoresistometer [17], it was notably not observed with a jet (orifice) type system [7]. The impact of γ-globulins, which significantly reduced RBC fragility when the stress was applied using rotating disks [28] or filtration [1], was much smaller when using bead agitation [9], and was fairly insignificant in our own experiments as detailed above.

Nichols and Williams reported a reduction of RBC MF in the presence of albumin as well as fibrinogen and γ-globulins at physiological concentrations, with γ-globulins among the three proteins effecting maximum protection against shear stress induced by a rotating disc system [28]. Similarly large protective effects of γ-globulins were observed when RBC were stressed using small pore filtration [1]. However, γ-globulins provided only marginal protection (10 to 15 percent), compared to albumin, against induced hemolysis when stress was applied using agitation of small beads [9]. In our method, with stress induced by a single ball moving at high frequency through the media within an enclosing tube, γ-globulins also provided much smaller protection than either albumin or fibrinogen. The disparity of results could possibly arise from differences of approaches employed, and thus potentially from qualitative as well as quantitative differences in mechanical stresses applied.

In both physiological and equimolar conditions, which differ in BSA concentration, but not that of fibrinogen, the protective effect seems to increase when both proteins were present in the medium. The effect was not fully additive even at lower BSA concentration, as the cumulative reduction in fragility was less that what would be anticipated if proteins' actions were fully independent. That could potentially reflect a limitation of binding sites available for membrane-protein interaction combined with competitive binding between the proteins, which become more significant at increased BSA concentration. That hypothesis correlates with the previous reports which indicated, in RBC aggregation contexts, the capacity of albumin to displace bridging molecules such as fibrinogen from binding sites on the cell membrane [31].

Potential importance of albumin binding to the RBC membrane for protection against mechanical stress is further supported by our observation that fatty acid free albumin was more effective in increasing RBC resistance to hemolytic mechanical stress. Albumin is well known to bind free fatty acids [35], with such binding potentially able to affect its interaction with cell membranes. Relevantly, presence of fatty acids during stress application, but not during storage, was found to reduce RBC MF. Cell recovery had been demonstrated recently to occur in even low concentration (1 percent) HAS solution, with such effect not observed in plasma [32]. Albumin propensity to bind with a wide variety of agents, including fatty acids present in plasma, had been proposed as a possible contributing factor to that difference (e.g. through reduction of available binding sites for protein interactions with the RBC membrane). Similar causes could account for as observed in our experiments, much smaller protection against stress effected by plasma, as compared to albumin alone. Presented results demonstrated significant role of fatty acids in increasing RBC propensity to hemolyze. It is possible that in this case as well, albumin interaction with RBC membrane was inhibited by plasma fatty acids.

Differences in the impact of changes in pH on RBC MF, in AS3 with vs. without supplementation with albumin, provides further confirmation. It has been reported that BSA, similarly to HSA, undergoes reversible conformational isomerization as a function of pH – with five different isomers having been identified over the pH range from less than three to over eight [8]. Change in pH from 5.5 to 7.4, as used in the present work, results in a conformational shift from the N-form (Native; pH 5–7) to the B-form (Basic; pH 7–8.5), likely changing albumin's interaction with RBC membranes and thus potentially affecting its ability to protect RBC membranes against shear stress.

While supplementing with PEG increase RBC resistance to induced hemolysis, consistent with previous report [22], supplementation with Dextran did not affect RBC MF similarly to what was reported previously [21, 22]. The disparity between these results can be interpreted in terms of a potential for interaction between the additive (dextran or PEG) and the RBC membrane. While dextran would be expected to be essentially inert in that regard, the ability of PEG to bind to and modify RBC membranes is well documented [4, 14].

Bead milling, especially at high frequencies, results in high turbulence as a ball propagates through the medium. Such flow is likely to be of a different type from the laminar or intermediate laminar/turbulent Taylor vortex flows observed with rotating plates [28], flow through the length of a capillary tube [21], or in concentric rotating cylinder systems [36]. In that regard, certain aspects of bead mill induced flow, particularly relating to entering/exiting the annulus (in the case of a ball that is large relative to tube diameter), could create pressure difference induced stress contributions akin to those observed upon entering and exiting a capillary or passing through an orifice [21, 37] or a filtration system [1]. While the geometric configuration of the flow will be different, in principle the ball's movement results in similar changes in the cross-sectional area of the flow – i.e., a decrease followed by an increase in cross-sectional area, as flow passes either through a capillary or around a ball. In our bead mill setup, the difference in diameter between the ball and tube creates an annular gap of about 1 mm, which may limit the inferences that can be drawn from our Reynolds calculations (Fig. 6).

Oscillation frequency-related changes in the ball's speed, and the resultant changes to the flow, can be characterized in part using Reynolds numbers, which can be estimated for the geometry used in the experiments under the assumption of Newtonian fluids for both AS3 and AS3 supplemented with BSA media (Fig. 6). For moderately low Reynolds numbers ($Re < 10^5$), the boundary layer over the front face of a sphere can be expected to be laminar. The flow accelerates through the gap between the ball and the tube wall, slowing as it passes the midpoint. At lower frequencies (Reynolds numbers $10^2 < Re < 10^5$), the flow likely forms a Von Karman trail with a boundary layer separating away from the ball's surface and forming vortexes in the lee side that are shed downstream. As the frequency (and thus ball speed) increases, in AS3 without supplementation, the flow becomes more turbulent with the development of a disorganized wake ($Re \sim 1 \times 10^3 - 5 \times 10^3$). A laminar wake pocket (where fluid is trapped) could also form at the back of the ball; with further increase in flow speed, such a pocket would diminish, with the turbulent wake occupying a larger portion of the back of the ball. The onset of a fully turbulent boundary layer would not be expected until $Re \sim 10^5$, which is significantly higher than the Reynolds numbers estimated for this experiment's setup (Fig. 6). Due to the higher viscosity of a BSA-supplemented medium, the ball's movement likely generates a Von Karman trail through the whole range of the frequencies used, but likely without generating the turbulent wake as well (as in the un-supplemented media).

Thus it can the hypothesized that the changes in the magnitude of the protective effect of albumin, over the range of frequencies used, at least partly reflects changes in flow behavior from a predominantly Von Karman trail at the lower frequencies (less than 25 Hz) to an increasingly more turbulent disorganized wake at the higher frequencies. Experimentally observed changes in the efficiency of BSA-mediated RBC protection indicate it reaches a maximum at about 25–35 Hz, corresponding to Reynolds numbers of $1 \times 10^5 - 1.5 \times 10^5$ for the unsupplemented AS3 medium (see Fig. 6). That would imply that while albumin does offer protection to RBC against stress-induced hemolysis when stress is due largely to vortexes associated with a Von Karman trail, such protection is even more pronounced for a disorganized turbulent wake. That would in turn suggest that the magnitude of protection offered by albumin or other plasma proteins could be potentially used to differentiate between mechanical stresses induced by different types of flow. If validated, that may allow the possibility of perturbing different structural components of cell membranes, thus allowing better probing of membrane response to mechanical stress.

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Personal interest disclosure

Authors Tarasev and Chakraborty are employed by Blaze Medical Devices, LLC. that develops methods and systems for assessment of Red Blood Cells mechanical fragility. Light and Davenport have no conflict of interest to disclose.

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