Red blood cell mechanical stability test

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Abstract. Red blood cells (RBC) are exposed to various levels of shear stress (SS) during their flow in the circulatory system, yet no significant damage occurs if their mechanical stability is not impaired. Alternatively, normal RBC may be damaged during flow in non-physiological environments and under extreme SS (e.g., extracorporeal circulation, ventricular assist devices). The shear-induced damage may result in hemolysis or in altered mechanical properties of RBC that, in turn, reduces the ability of RBC to withstand further damage by SS. An ektacytometer employing a Couette shearing system was used to apply SS at a constant level of 100 Pa for 300 seconds as a model of sub-hemolytic mechanical stress. The degree of cellular damage during and after the application was assessed by diffraction pattern analysis. The area of the diffraction pattern was found to correlate with the number of RBC in the sheared suspension. Monitoring the ellipse area during the application of gradually increasing SS provides the concentration of the remaining intact RBC and can therefore be used to estimate the hemolytic threshold as a measure of RBC mechanical stability. The hemolytic threshold determined after the mechanical stress application was found to be ~150 Pa, while it was ~250 Pa in the same samples before the SS application. Additionally, SS-elongation index curves recorded before and after the application of the sub-hemolytic SS significantly differed from each other, indicating the impairment in deformability following the mechanical stress. The Couette type ektacytometer can be used as a tool to assess the sub-hemolytic damage to RBC in testing the biomedical equipment.

Keywords: Erythrocyte mechanical stability, artificial organs, left ventricular assist device, ektacytometry, hemolytic threshold

1. Introduction

Red blood cells (RBC) are exposed to mechanical stresses during their “everyday life” in various levels of the circulatory system. They respond to this effect by deforming and changing their shape, but they are not “overtly” damaged by these forces under physiological conditions. However, if they are exposed to non-physiological conditions, such as circulating through artificial organs or devices (e.g., stents, artificial heart valves, ventricular assist devices, extracorporeal circulation, hemodialysis machines), they experience damage ranging from impairment in mechanical properties to disruption of cell membrane releasing hemoglobin (i.e., hemolysis) [14]. The most significant factor in an artificial circulatory environment that causes mechanical damage is high levels of shear stress (SS), although other factors (e.g., turbulence, cavitation, contact/collision to foreign surfaces, and collisions between blood cells) may also contribute to damage [9, 14]. The physical and biochemical properties of RBC are also among the determinants of damage, particularly under non-physiological conditions. These properties collectively determine RBC *mechanical stability* [19]. The mechanical stability of RBC may, in turn,

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be altered under various physiological conditions [26] or by exposure to non-physiological conditions (e.g., artificial circulatory environment or blood storage) [14, 28], increasing the susceptibility of RBC to further mechanical damage [17]. Alternatively, hereditary membrane defects (e.g., band 4.2 deficiency), hemoglobinopathies (e.g., thalassemias), enzyme deficiencies (e.g., glucose-6-phosphate dehydrogenase deficiency) are characterized by reduced RBC mechanical stability [1, 2, 7, 20, 21, 27, 29].

Various approaches have been used to test the mechanical stability of RBC (i.e., susceptibility to mechanical damage by effective shear forces), usually based on the detection of the degree of hemolysis under certain shearing condition [22, 24]. The resultant degree of hemolysis following a standardized shearing period is usually expressed as the “mechanical fragility index” (MFI) [12, 24], reflecting the mechanical stability of RBC. The alterations in MFI (hence mechanical stability) reflect the “sub-lethal” or “sub-hemolytic” damage to RBC [14], caused by shear forces which are not sufficiently high enough to cause hemolysis but do induce cellular alterations, including morphological changes [23, 25] and impaired deformability [6, 15, 16].

The level of hemolytic trauma has been a widely used criteria in testing biomedical instrumentation contacting blood for many decades [14] while sub-hemolytic damage to RBC has also been suggested to be used in the testing of such devices [18]. Implementation of the test procedures for artificial organs and biomedical devices based on sub-hemolytic damage may lead to the improvement of the design of such systems, as the sub-hemolytic damage will reflect lower levels of impact on RBC yielding more sensitive data. However, there is still a need for standardized procedures that can be applied under clinical settings to assess the sub-hemolytic damage to RBC. This study explores the possibilities for using a Couette-type ektacytometer for detecting the impairment in RBC mechanical stability.

2. Materials and methods

2.1. Blood samples and preparation of red blood cell suspension

Venous blood samples were obtained from healthy volunteers, aged between 24 to 57 years, anticoagulated with ethylenediaminetetraacetic acid (EDTA, 1.8 mg/mL) [3]. Blood samples were diluted (1/200) in a 6% solution of high-molecular weight polyvinylpyrrolidone (PVP, MW = 360 kDa) in isotonic-phosphate buffered saline (PBS, pH = 7.4). The viscosity of the PVP solution was 29.3 mPa.s.

2.2. Mechanical stress model for red blood cell suspensions

Dilute RBC suspensions in 6% PVP solutions were exposed to 100 Pa SS continuously for 300 seconds in a co-axial cylindrical shearing system that is a part of the laser diffraction ektacytometer (LORCA MaxSis, Mechatronics, Hoorn, The Netherlands). Both inner and outer cylinders were made of transparent material, with a gap of $\sim 350 \mu\text{m}$ between them. The outer cylinder rotates at a calculated rotational speed to generate the desired SS in the sample within the gap. A laser beam (670 nm wavelength) is projected perpendicular to the rotation axis and the diffraction pattern generated on the screen on the opposite side is captured by a CCD camera and analyzed by the attached computer, which also controls the rotation of the outer cylinder of the co-axial system.

RBC elongation indexes (EI) were calculated during the application of SS by using the long and short axes of the elliptical diffraction patterns (a and b, respectively) as $\text{EI} = (a - b)/(a + b)$.

2.3. Assessments of sub-hemolytic mechanical trauma

The sub-hemolytic damage induced by the mechanical stress protocol described in Section 2.2. was assessed by two approaches:

- RBC EI were determined as a function SS between 0.3 to 50 Pa before and after the application of mechanical stress. Half-maximal shear stress ($SS_{1/2}$) and maximum EI (EI_{max}) were calculated using the Lineweaver-Burk approach and non-linear curve fitting [4]. The $SS_{1/2}/EI_{max}$ ratio was calculated as a normalized measure of $SS_{1/2}$ [5].
- RBC suspensions before and after the mechanical stress protocol were exposed to SS that logarithmically increased between 100 to 360 Pa in 16 steps, with five second exposure time at each SS level. The diffraction pattern parameters (i.e., short and long axis of the ellipse) were obtained using the data from 50 measurements that were completed within ~ 2 seconds following each SS step. These data were used to calculate the area of the elliptical diffraction pattern in order to estimate the RBC concentration in PVP solution within the gap (see Section 2.4). The mean of fifty estimates at each SS was used as the data point corresponding to a given SS level for each experiment.

2.4. Estimation of red blood cell concentration in suspensions under shear stress

In the preliminary experiments, RBC concentration was adjusted to various levels ranging from $6.2 \times 10^8/\text{ml}$ to $5.3 \times 10^9/\text{ml}$ by diluting whole blood with autologous plasma. The diluted samples were further diluted with 6% PVP solution as described in Section 2.1 in order to yield final RBC concentrations between $3.2 \times 10^6/\text{ml}$ and $2.7 \times 10^7/\text{ml}$. Diffraction patterns for these RBC-PVP solution suspensions having various RBC concentrations were recorded within the first five seconds following the application of 100 Pa SS. The area of diffraction patterns was calculated as $a.b.\pi$ where a and b are the long and short axis of the elliptical pattern. The RBC concentration - ellipse area data were fitted to the following non-linear regression equation (Fig. 1):

$$\text{RBC}(10^6/\text{ml}) = 4.52 - 2.0 \times 10^{-3}A + 4.2 \times 10^{-7}A \quad (1)$$

where “A” is the area of diffraction pattern in pixels; $R^2 = 0.994$. The non-linear regression equation was then used for the estimation of RBC concentrations in suspensions during the time course of SS application.

Fig. 1. The nonlinear relationship between the area of the diffraction pattern and RBC count in suspensions sheared at 100 Pa. The solid line represents the polynomial fitted to the data (Equation 1), $R^2 = 0.994$.

2.5. Statistics

Data are presented as mean $\pm$ standard error. One or two-way ANOVA tests were used for the comparisons between “before” and “after” measurements, followed by a Bonferoni post-test where appropriate. RBC deformability parameters $SS_{1/2}$, $EI_{\max}$ and $SS_{1/2}/EI_{\max}$ ratio were compared with paired t tests. Non-linear curve fitting was performed by Graphpad 4.0 software package.

3. Results

3.1. Alterations in EI during the application of mechanical stress

Figure 2 presents a typical result of EI against time during the application of 100 Pa to RBC suspended in the PVP solution. EI slightly increased ($\sim 4\%$) reaching a maximum in 30.7 ± 2.0 sec. EI then decreased and approached a minimum during the next 30–40 sec, the mean time for the minimum being 67.0 ± 4.2 sec. EI slightly increased in the latter phase and tended to decrease in the last 200 seconds; at the completion of the 300 sec exposure to 100 Pa SS, EI was $89.7 \pm 2.1\%$ of the initial value (i.e., EI at the start of the measurement).

3.2. Red blood cell deformability before and after the mechanical stress

EI-SS curves obtained for the RBC suspensions before and after the mechanical stress application indicated a significant impairment in RBC deformability (Fig. 3). EI levels measured before and after the 300 sec application of 100 Pa shear stress were found to be significantly different at 0.3 Pa and also above 1.65 Pa. $SS_{1/2}$ values were increased and $EI_{\max}$ was decreased, the latter change being statistically significant ($p < 0.001$); the decrease of $EI_{\max}$ and increase in the $SS_{1/2}/EI_{\max}$ ratio thus reflects the impairment in RBC deformability following the mechanical stress application (Fig. 4).

3.3. Detection of hemolytic threshold before and after mechanical stress application

RBC counts in suspensions exposed to logarithmically increasing SS from 100 to 360 Pa estimated as described in Section 2.4. are shown in Fig. 5. RBC concentration in the suspensions which were not

Fig. 2. The typical time course of RBC elongation index (EI) during the application of 100 Pa for 300 sec.

Fig. 3. Shear stress (SS) - elongation index (EI) curves for RBC before and after the application of 100 Pa SS for 300 sec. The data are mean $\pm$ standard error for 10 experiments. For some data points error bars remain within the symbol. Difference between “before” and “after”: * $p < 0.05$; *** $p < 0.001$, assessed by two-way ANOVA followed by Bonferoni post test.

Fig. 4. Parametric analysis of elongation index-shear stress curves. a) Shear stress at half maximal deformation ($SS_{1/2}$). b) Maximum elongation index (EI_{max}). c) $SS_{1/2}/EI_{max}$ ratio. Data are presented as mean $\pm$ standard error for 10 experiments. Difference from “before”: ** $p < 0.01$; *** $p < 0.001$.

exposed to mechanical stress prior to testing remained unchanged until the applied shear stress reached ≈ 250 Pa, following which significant decrements were found at each SS increment above this value. The decrements in the RBC concentration (estimated based on the decrease of ellipse area) were utilized as the indicator of hemolysis: the threshold for hemolysis for RBC prior to being exposed to the mechanical stress model (see Section 2.2) was 250 Pa. RBC count-SS relations were markedly different for RBC previously exposed to 100 Pa for 300 sec: 1) estimated counts began to decrease at a lower SS; 2) the

Fig. 5. Estimated RBC counts in suspensions exposed to gradually increasing shear stress between 100 to 360 Pa. The data are mean $\pm$ standard error for 10 experiments. Difference from the 100 Pa data (the first data point for each set): * $p < 0.05$; ** $p < 0.01$; assessed by one-way ANOVA followed by Bonferoni post-test.

threshold for hemolysis was ≈ 150 Pa and thus approximately 40% lower than for cells not previously exposed to 100 Pa SS.

4. Discussion

RBC can withstand to SS up to 300 Pa without hemolysis [22]. The 100 Pa SS applied for 300 s during the mechanical stress protocol in this study was thus selected to induce a sub-hemolytic damage. The time course of EI during the application of 100 Pa SS followed a typical pattern, the overall change in EI at the end of the 300 s of application corresponding to a $\sim 10\%$ decrement. The pattern of the change in EI under SS seems to be similar to that reported by An et al. for RBC ghosts under 75 Pa SS; the rate of the change in EI in their study was accepted as a measure of the impairment of membrane mechanical stability [1]. This pattern of alterations in EI under SS has been related to the progressive fragmentation of RBC, yielding more spherical and less deformable vesicles [13, 19]. In the present study, the degree of alterations in EI did not reach to the levels reported by An et al. [1], as intact RBC suspensions were used herein.

The impairment in RBC deformability following the application of the mechanical stress protocol was also obvious with the EI measured under SS between 0.3 to 50 Pa. Despite $\sim 10\%$ decrement in EI under 100 Pa, the analysis of EI-SS curves indicate very significant impairments in deformability, evident by 60% increment in $SS_{1/2}/EI_{max}$ parameter. Lee et al. reported that the alterations in EI indexes of pre-sheared RBC were determined by both the magnitude and the exposure time of SS [17]. It should be noted that the magnitude of SS that results in significant deterioration in RBC deformability assessed by the determination of SS-EI curves was lower compared to the hemolytic threshold [22], therefore ektacytometric measurement of EI appears to be a much more sensitive parameter to detect mechanical trauma to RBC.

Hemolysis has been the most widely used indicator of blood trauma in artificial circulatory systems, owing to the simplicity of the test [11, 22, 30]. Hemolysis under given mechanical stress conditions has also been used as an indicator of altered mechanical stability of RBC [8, 12, 24, 28]. The previously proposed methods to assess the mechanical stability of RBC utilized mechanical stress models which were not well standardized. It has been demonstrated in this study that the Couette type ektacytometer can be used to determine the hemolytic threshold in terms of SS within several minutes.

The detection of hemolytic threshold utilized herein was based on a novel approach to the analysis of diffraction patterns during ektacytometry. It has been observed that the area of the diffraction pattern decreases progressively during the application of high levels of SS, the relative magnitude of the change in the area of the pattern being much more prominent than the change in EI. The area of the diffraction pattern was found to be independent of the briefly applied SS above a certain level (i.e., 100 Pa) and with a given number of RBC per unit volume of the suspension in viscous PVP solution. However, a very strong correlation was found between the area of diffraction pattern and the RBC concentration in the suspension used for ektacytometry. It was also observed that the resulting non-linear regression can be used to estimate the concentration of RBC in any suspension used for ektacytometry (or the remaining number of intact RBC per unit volume of suspension) based on the diffraction pattern area. These observations are also supported by the theoretical approach presented by Feinberg et al. [10].

Based on these considerations, the SS which is just sufficient to induce hemolysis was estimated by monitoring the diffraction pattern area (and estimating the remaining RBC concentration), during the application of progressively increasing SS. The hemolytic threshold was found to be around 275 Pa for normal RBC. It has also been demonstrated that the hemolytic threshold was shifted to significantly lower levels (to ~150 Pa) following the application of mechanical stress protocol (i.e., 100 Pa applied for 300 sec), compared to the control measurement (i.e., prior to the application of mechanical stress). The reduced hemolytic threshold thus serves as an indicator of sub-hemolytic damage of RBC caused by the mechanical stress application.

Determination of in hemolytic threshold as an indicator of sub-hemolytic damage can be implemented as a routine method for testing biomedical devices in contact with blood. It should be noted that the safety tests for blood-contacting biomedical instruments based on this approach should indicate very low levels of trauma, and therefore this new method may stimulate engineering approaches to improve such instruments in terms of generating more physiological shearing conditions.

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