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Shear induced damage of red blood cells monitored by the decrease of their deformability

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Abstract

Shear-induced damage of Red Blood Cell (RBC) is an imminent problem to be solved for the practical application of artificial organs in extra corporeal circulation, as it often happens and affects physiological homeostasis of a patient. To design and operate artificial organs in a safe mode, many investigations have been set up to correlate shear and shear-induced cell damage. Most studies were focused on hemolysis i.e. the extreme case, however, it is important as well to obtain a clear understanding of pre-hemolytic mechanical damage. In this study, the change in deformability of RBC was measured by ektacytometry to investigate the damage of RBC caused by shear. To a small magnitude of pre-shear, there is little difference, but to a large magnitude of pre-shear, cell damage occurs and the effect of shear becomes significant depending on both the magnitude and imposed time of shearing. The threshold stress for cell damage was found to be approximately 30 Pa, which is much less than the threshold of mechanical hemolysis but is large enough to occur *in vitro* as in the extra corporeal circulation during open-heart surgery or artificial heart. In conclusion, it was found and suggested that the decrease of deformability can be used as an early indication of cell damage, in contrast to measuring plasma hemoglobin. As cell damage always occurs during flow in artificial organs, the results as well as the approach adopted here will be helpful in the design and operation of artificial organs.

Keywords : cell damage, ektacytometry, red blood cell deformability, extra corporeal circulation, artificial organ, LORCA

1. Introduction

The red blood cell (RBC) has a biconcave shape in the static state, but can easily deform and elongate under flow to facilitate oxygen transfer and to reduce flow resistance. This characteristic of RBC has been called deformability. Due to deformability of RBC and flexibility of the vessel wall, normal cells are not mechanically fragmented in the circulation, even though they experience significant stresses during circulation or extensive deformation in passing through the capillaries that have much smaller dimensions (Lenormand *et al.*, 2001). However, when RBC flows in the bypass to extra corporeal cardiovascular devices, it experiences high shear stresses above the pathological level (Table 1).

At such extreme conditions, RBC cannot sustain its elongated shape and can lose its membrane elasticity, which is called mechanical damage. This condition may lead to membrane damage, or ultimately hemolysis depending on the degree of damage. Mechanical hemolysis is defined as the release of hemoglobin into plasma due to the breakup of cell membrane induced by external mechanical forces. It causes a serious trouble and restricts the practical application of artificial organs in a human body. In this regard it is important to reduce such an undesirable phenomenon. Researches have been intensively carried out to understand the mechanism of mechanical hemolysis and to find the quantitative correlation of shear stress and the mechanical

Table 1. The approximate range of shear stresses in human vessel and artificial organs

	Approximate shear stress ranges (Pa)
Healthy human vein	0.1 – 1
Healthy human artery	1 – 20
Human artery at stenotic lesion	20 – 50
Artificial blood pump or heart valve	1 – 1000

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hemolysis (Grigioni *et al.*, 1999; Sallam *et al.*, 1984).

It is essential to operate the artificial devices in a safe mode to ensure that the maximum shear stress is below the threshold stress of hemolysis. The threshold shear stress leading to mechanical hemolysis, obtained from various *in vivo* and *in vitro* experiments, is known to be in the range of 150 – 400 Pa (Sallam *et al.*, 1984; Sharp and Mohammand, 1998). Some investigators have suggested correlation equations for hemolysis, based on the assumptions that the amount of hemoglobin released by the flow is a function of the magnitude of shear stress and the exposure time of RBC to the flow field (Sharp and Mohammand, 1998).

Though it is important to reduce hemolysis as well as membrane damage, most of studies have been focused on hemolysis because its quantitative analysis is more easier than that of membrane damage. But it is clear that RBC membrane could be damaged by external stresses prior to hemolysis and the damage affects RBC metabolism. Since RBC deformability is highly correlated with the integrity of the cell membrane, it can be considered as a sensitive indicator of membrane damage.

Various methods to measure RBC deformability have been suggested since its clinical importance became apparent. They include filtration method (Fisher *et al.*, 1992), micropipette aspiration (Brody *et al.*, 1995; Discher *et al.*, 1994), microchannel method (Kaneta *et al.*, 2001; Kikichi *et al.*, 1994; Sutton *et al.*, 1997), optical tweezers (Bronkhorst *et al.*, 1995; Huruta *et al.*, 1998) and so on. Major drawbacks of these methods are poor reproducibility and time-consumption. Recently, the Laser-assisted Optical Rotational Cell Analyser (LORCA), a laser-based ektacytometer was developed as a technique that provides quantitative and reproducible results, which overcomes the disadvantages of others (Hardeman *et al.*, 2001).

In this study, we apply high shear to induce mechanical damage of RBC and investigate the deformability change of unsheared and pre-sheared cells with the LORCA. Also we correlate the mechanical membrane damage with the amount of shear stress by changing the magnitude of shear stress as well as the imposed time.

2. Experimental section

2.1. Materials

Human blood, obtained from healthy volunteers upon informed consent, was drawn into K_3EDTA vacutainer tube to prevent coagulation. 25 μl of blood was diluted in 5 ml of medium solution. For the medium solution, 0.14 mM polyvinylpyrrolidone (PVP; M.W. = 360,000, Sigma-Aldrich) was dissolved in phosphate buffered saline (PBS) solution and stirred at room temperature. The viscosity of this (Newtonian) medium was determined as 31 mPa · s (37°C) and its osmolality was measured as 290 mOsm. Because of this increased viscosity of the medium, i.e. about 6 times that of blood, more stress can be transferred to the cell inducing pronounced cell deformation as well as its orientation along the flow direction which is crucial for the generation of the laser diffraction pattern. Moreover, the hyperviscous condition is relevant here to increase the stress to the level that exists in an extra corporeal cardiovascular device (Ditenfass, 1968; Kameneva *et al.*, 1999).

2.2. Instruments

In this study, a commercial ektacytometry instrument (LORCA™, Mechatronics, the Netherlands) has been used to measure RBC deformability (Hardeman *et al.*, 1987; Hardeman *et al.*, 2001). It gives a quantitative measure of average cell deformability. A laser beam traverses a suspension of blood cells with perpendicular direction to the shear flow between two concentric cylinders and generates a diffraction pattern on a small projection screen. Both concentric cylinders of the couette system are made of glass. The outer cylinder ("cup") rotates and induces shear flow, and the inner cylinder ("bob") can be moved vertically with a lever. The gap between two cylinders is 0.3 mm. The diffraction pattern is projected on a screen monitored by a CCD-video camera, linked to a frame grabber integrated with the computer. It is easy to impose large shear, enough to provoke membrane damage, and subsequently to investigate the deformability following the standard protocol.

The RBC orients along the flow direction when it experiences shear, and it changes its shape gradually from biconcave towards a prolate ellipse as shear stress increases. This is accompanied by a transition from a circular into an elliptic diffraction pattern, which is oriented perpendicular to the direction of the elongated cell (Huruta *et al.*, 1998; Mohandas *et al.*, 1980). The elongational index (EI) is a dimensionless number defined to quantify the degree of elongation, which is a measure of RBC deformability. It is calculated from the diffraction pattern and is defined as $(A - B)/(A + B)$, where A and B are the length of major and minor axis of the ellipse, respectively. The experimental procedure is as follows: (a) to establish the deformability of unsheared RBC, measure a standard deformation curve by increasing shear stress from 0.1 Pa to 30 Pa, (b) impose controlled shear stress to RBC, more precisely to the cells dispersed in PVP medium for a designated time to induce RBC damage (pre-shearing), (c) measure the deformability of pre-sheared cells, (d) and compare the deformability of unsheared and sheared cells. In procedure (b), the pre-sheared stress was controlled by rotating inner cylinder.

3. Results and discussion

In order to clarify the influence of the amount of

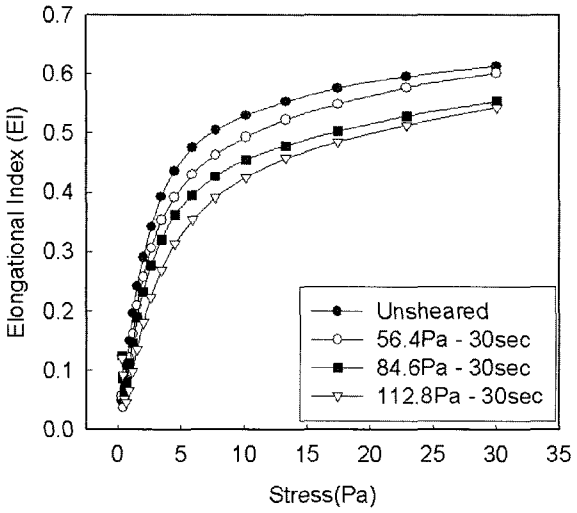

Fig. 1. Deformation curves of unsheared (control) red blood cells and cells presheared for 30 sec as a function of applied pre-shear stress.

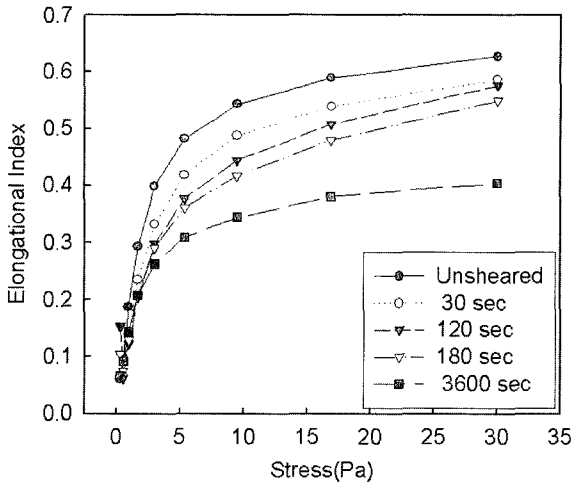

Fig. 2. Deformation curves of unsheared (control) red blood cells and cells pre-sheared at 56.4 Pa as a function of imposed pre-shearing time.

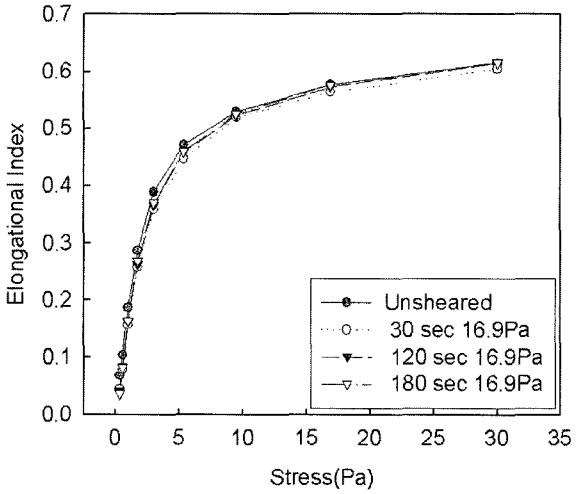

Fig. 3. Deformation curves of unsheared (control) red blood cells and cells pre-sheared at 16.9 Pa for 30, 120, 180 seconds, respectively.

mechanical stress on RBC deformability, the cells were pre-sheared for 30 seconds at the indicated stress of 56.4 Pa, 84.6 Pa, 112.8 Pa respectively, prior to deformability measurement. In general, EI increases fast at low shear stress and approaches a limiting value at high shear stresses. This originates from the elasticity of cell membrane and the cells inherent characteristic that tends to sustain the cell volume. The deformation curve of pre-sheared cell, however, is different from that of control, unsheared cells (Fig. 1). When 56.4 Pa of pre-shear was applied, the EI value saturates at about 0.6, which is a little smaller than that of unsheared cells. However, when the pre-sheared stress was as large as 112.8 Pa, the EI value decreases more than 15% compared to the unsheared cells, which means that RBC deformability is significantly affected by shear.

When the imposed time of pre-shearing is increased, a similar behavior is observed. Fig. 2 shows the results when the pre-shear of 56.4 Pa was imposed for 30, 120, 180, 3600 seconds, respectively. The deformability becomes reduced as the imposed time increases (or equivalently, as the total strain is increased). It implies that there exists cell damage due to shear, which increases with imposed time. When 56.4 Pa of pre-shear was applied, the EI value after 3600 seconds is approximately half of the unsheared cells. These results (Fig. 1 and Fig. 2) imply that the cell damage is a function of the amount of shear as well as the duration of shearing. Even though we did not prove in this study, this deformability-reducing phenomenon is an irreversible process. When the pre-sheared cell is measured after sufficient relaxation time, we obtain the same level of reduced-deformability curve.

When the pre-shear stress is as small as 16.9 Pa, however, RBC deformability does not change significantly, up to the duration of 180 seconds (Fig. 3). So there seems to exist a threshold stress, which is the onset of cell damage. In order to standardize the effect of shear, the difference between EI values of unsheared (EI_{un}) and pre-sheared (EI_{pre}) cells has been calculated as the percentage of the EI value of the unsheared cells : $(EI_{un} - EI_{pre})/EI_{un} \times 100\%$. This is called as the shear effect, and can be related to cell damage. The shear effect increases with the amount of pre-shear stress. The shear effect after 120 seconds of pre-shearing is shown in Fig. 4. In the low shear stress region (below 30 Pa of pre-shear stress), the shear effect is not significant. But in the stress level above 30 Pa, the shear effect increases rapidly. Therefore, after a shearing history

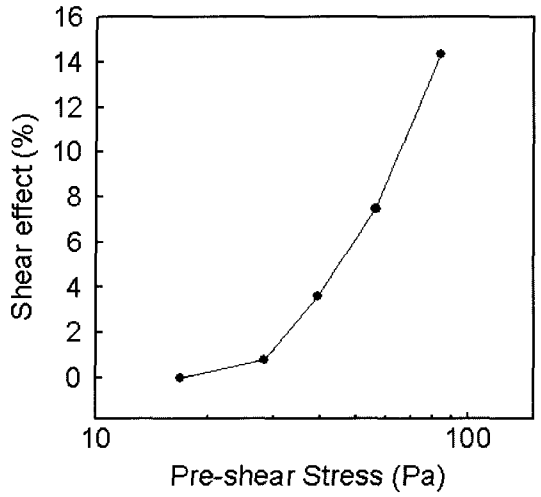

Fig. 4. Shear effect (%) as a function of pre-sheared stress applied for 120 sec. Shear effect is defined as: $[(EI_{un} - EI_{pre})/EI_{un}] \times 100\%$.

of 120 sec, 30 Pa may well be regarded as a threshold of the mechanical cell damage. The concept of threshold has been suggested to explain hemolysis. As is natural, the threshold of cell damage is much less than that of hemolysis, which is about 150 Pa (Sallam *et al.*, 1984; Sharp and Mohammand, 1998).

Recently, Mizuno *et al.* (2002) reported the decrease of RBC deformability induced by continuous exposure to shear stress. They demonstrated the decrease of RBC deformability in an indirect manner by measuring the viscosity increase of the RBC suspension. In the present study, however, we measure the decrease of cell deformability by a direct method. The result that deformability is closely related with shearing time as well as the magnitude of shear, provides a useful information to some operations like cardiopulmonary bypass surgery with extra corporeal circulation (Hirayama *et al.*, 1986). Blood is exposed to anomalous mechanical stress during the operation. If RBC passes or stays at the region above the threshold stress, RBC deformability will decrease as time passes and it can change the mechanical properties of RBC and will meet an immediate or delayed hemolysis (Kaneta *et al.*, 2001).

There are some factors affecting RBC deformability as reviewed and summarized in the literature (Hardeman *et al.*, 1994; Mokken *et al.*, 1992). One is the large surface area to volume ratio, which is inherent to the biconcave disc shape (Brody *et al.*, 1995). The surface area to volume ratio of the normal RBC is 40% larger than that of a sphere with the same volume. Excess surface area allows the cell to deform easily as it passes through the circulatory system. Membrane structure that consists of bilayers of phospholipid (Lenormand *et al.*, 2001; Needham *et al.*, 1990) and the viscosity of the intracellular hemoglobin solution (Dintenfass, 1968) also affect the cell deformability. When these factors are influenced by a physiological mechanism, the cell adjusts to the environment and its deformability changes very sensitively (Hardeman *et al.*, 1994; Mokken *et al.*, 1992).

The shear effect will partly be related with the change of membrane property. The membrane consists of lipid bilayer with embedded globular proteins and is linked to a skeleton of spectrin networks. Upon the application of shear flow, RBC orients and elongates to the saturated state. The junction point of band 3 proteins (Miznuno *et al.*, 2002) might be broken so that the formation of cellular fragments may cause a reduction in membrane area, and the cells cannot elongate as normal.

Thus, cell fragmentation may be the origin for the decrease of EI value. Fig. 5 shows diffraction patterns for normal and damaged cells. The diffraction pattern of damaged cells is of diamond shape in contrast to the ellipsoidal shape of unsheared cells. The diamond shape is regarded as a superposition of circular and ellipsoidal patterns, which means that the deformable cells coexist with less

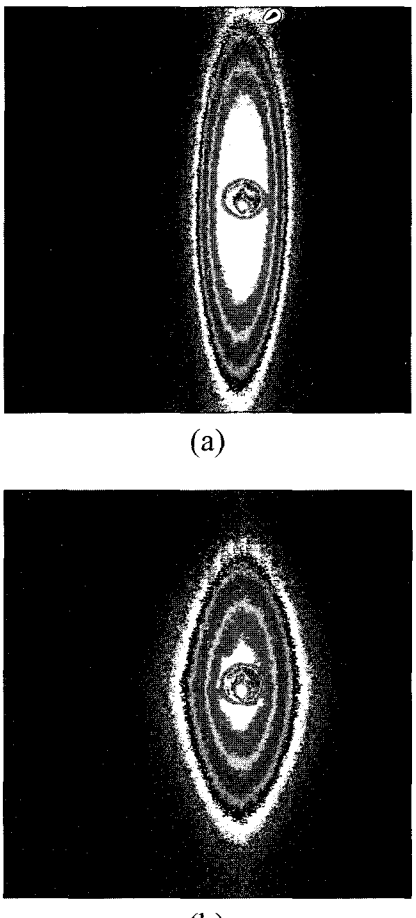

Fig. 5. Diffraction patterns of unsheared (a) and pre-sheared RBC (b) at 30 Pa. The pre-shear stress was 56.4 Pa and pre-shearing time was 3600 seconds.

deformable damaged rigid cells. A small portion of fragmented cells is also expected for damaged cells as evidenced by the reduction of light intensity. As the light diffracted by small fragments cannot be projected on the screen, the reduction of light intensity may well be a sign of cell fragmentation. Small fragments were also observed in an optical microscopy.

Even though the shear effect was quantitatively analyzed and explained in terms of the factors described above, our understanding is still fragmentary. There is no clear understanding on true physics of membrane damage as well as on the translocation of biocomponents through the cell membrane before and after damage. In addition, it is not certain whether the damaged cell recovers its deformability or not, by any means. The effect of complex flow field is not complete as well. Even though we have studied the effect of pure shear flow on the cell deformability, the flow is in fact a mixed flow combined both shear and extensional flow even in a human body. It is also well known that the deformability of a deformable body is more influenced by extensional flow than shear flow (Fuller and Leal, 1980). So it would be important as well to understand the effect of elongational flow on the cell deformability. We hope that the results as well as the approach adopted here will be helpful in the design and operation of artificial organs.

4. Conclusions

In this study, we have measured RBC deformability, using a laser diffraction technique and investigated the damaging effect of shear on cells by controlling the shear stress. Though the mechanism responsible for shear-induced decrease of deformability is not clearly understood, it was confirmed that cell damage reduces deformability, and is related with the magnitude of imposed shear and duration of shearing time. It was suggested that the deformability change could be used as an indication of prehemolytic cell damage. Also we suggested a membrane damage threshold as one of the additional factors for designing and controlling artificial organs.

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