RESEARCH ARTICLE

Quantitative Detect of Fatigue of Membrane of Erythrocyte in Uniform Shear Field

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An erythrocyte has high deformability. In the shear field, it deforms from biconcave disc to ellipsoid, and make tank-treading motion at the membrane. When the membrane is ruptured by fatigue, contents get out from the inside of the cell (hemolysis). At the fatigue test of the membrane in the shear field, "the shear stress" as "the amplitude" and "the shear rate" × "the exposure time" as "repeat count" are critical parameters. For the quantitative fatigue test, the uniform shear field has been realized between the rotating concave cone and the stationary convex cone. With the rheoscope, deformability is evaluated with shear stress responsiveness and with critical deformation calculated from an exponential curve between the deformation ratio (the ratio between the major axis and the minor axis of the ellipsoidal shape) and the shear stress. Deformability decreases at erythrocytes of high density after shearing. The erythrocytes deformation ratio varies periodically at the double frequency of tank-treading motion of the membrane, when the erythrocyte has the sublethal damage point on the membrane.

Introduction

An erythrocyte has high deformability. The deformability plays an important role to pass through the capillary in the microcirculation *in vivo* [**1**]. It also related to the filterability [**2**]. *In vitro*, the deformability can be traced by slits: between micro-cylindrical pillars [**3**], and between micro-ridges [**4**]. Some erythrocytes deform from biconcave to parachute shape through the capillary *in vivo* [**5**]. The high deformability protects erythrocyte from destruction [**6**]. Destruction of the erythrocyte usually occurs by fatigue after repetitive deformation [**7**]. In the circulatory assist device, destruction of the erythrocyte [**8**] can be accelerated by the unphysiological shear flow field [**9**]. The quantitative relationship between the shear flow and the destruction of the erythrocyte by fatigue could be analyzed by parameters as the mechanical material fatigue test: the amplitude and the number of repetitive cycles. It is important for the several applications: physiological blood flow control, the erythrocyte preservation technology, and the design of the circulatory assist devices.

In the previous studies, erythrocyte destruction in the blood flow was measured. Most of them were highlighted on the instantaneous destruction. Because of adaptation of the biological system, quantitative detection of erythrocyte destruction is not always easy *in vivo*. The accelerated destruction causes anemia. Destructed erythrocytes make clogging in the microcirculation system. In the short time course studies *in vitro*, on the other hand, the very high-speed unphysiological flow like a jet was applied on erythrocytes [**10**].

In the present study, erythrocyte destruction by fatigue in the physiological moderate shear flow has been investigated quantitatively *in vitro*. Several Couette type flow devices have been designed to control the uniform shear field.

Experimental

Materials

In the fatigue test, one milliliter of the fresh human blood sample was collected with anticoagulant of heparin from the author (63 years old, normal male). The sample was sheared in the Couette type of the constant uniform shear field between the concave cone and the convex cone *in vitro*. For the rheoscope study, erythrocytes were collected from one microliter of the fresh human blood of the author with the aid of the centrifugal technique. Human erythrocyte was classified into three groups according to the density by the centrifugal method with the separator of phthalate-ester. Variations were made on the density of the separator between 1.03 g/cm^3 and 1.09 g/cm^3. The heavier section of 10 percent, and the lighter section of 10 percent from the whole sample were used in the test. The middle section of 80 percent was not used in the test. Erythrocytes were dispersed in the saline solution, which includes dextran to increase the viscosity of the medium (0.24 Pa s). Every preparation of the blood sample was performed at 298 K of the room temperature just before each test.

Concavo-convex device

The shearing device was fabricated by the combination between the rotating concave cone and the stationary convex cone (**Fig. 1**) [**11**]. The both cones were made of polymethylmethacrylate. The device was placed in the chamber to control the atmospheric CO_2 content of five percent. The uniform shear rate (γ) between the conical surface of the concave cone and the conical surface of the convex cone is calculated by Eq. 1.

$$\gamma = \omega / \theta \quad (1)$$

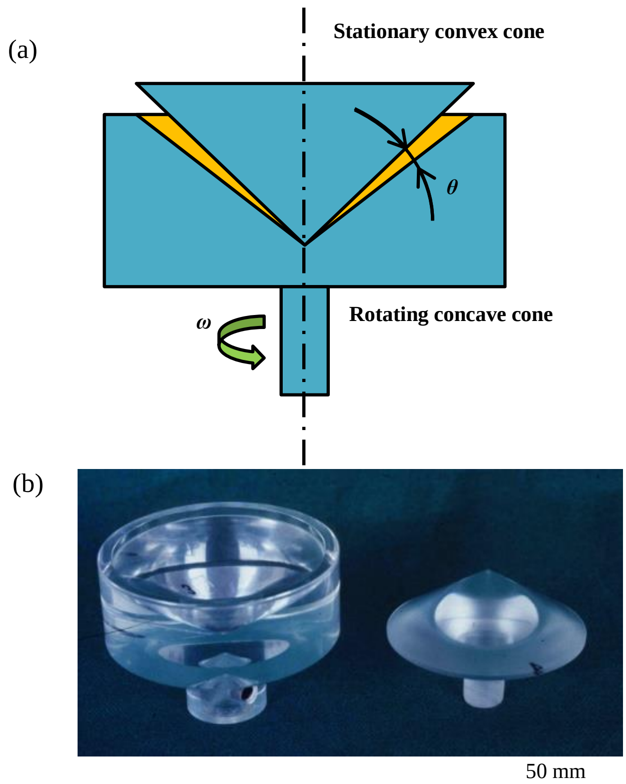

Fig. 1. Uniform Couette type of shear flow field (a) between rotating concave cone (left (b)) and stationary convex cone (right (b)) [**11**].

In Eq. 1, ω is the angular rotating velocity of the concave cone, and θ is the central gap angle between the conical surface of the concave cone and the conical surface of the convex cone. The shear rate (γ) is controlled by adjusting the angular rotating velocity of the concave cone.

Rheoscope

The rheoscope was fabricated by a couple of counter-rotating flat disks. Both disks were made of transparent glass (30 mm of radius, 2 mm thickness) (**Fig. 2**) [**12**]. Each erythrocyte suspended in the medium near the neutral plane between two disks was observed by the inverted phase contrast microscope. The shear rate (γ) in the space between two disks is calculated by Eq. 2.

$$\gamma = \Delta v / d \quad (2)$$

In Eq. 2, Δv is the velocity difference between two disks, and d is the distance between two disks. The velocity v is calculated by the angular velocity of the disk ($\omega/2$) and by the rotating radius at the observation point (r). The shear stress (τ) is calculated by Eq. 3.

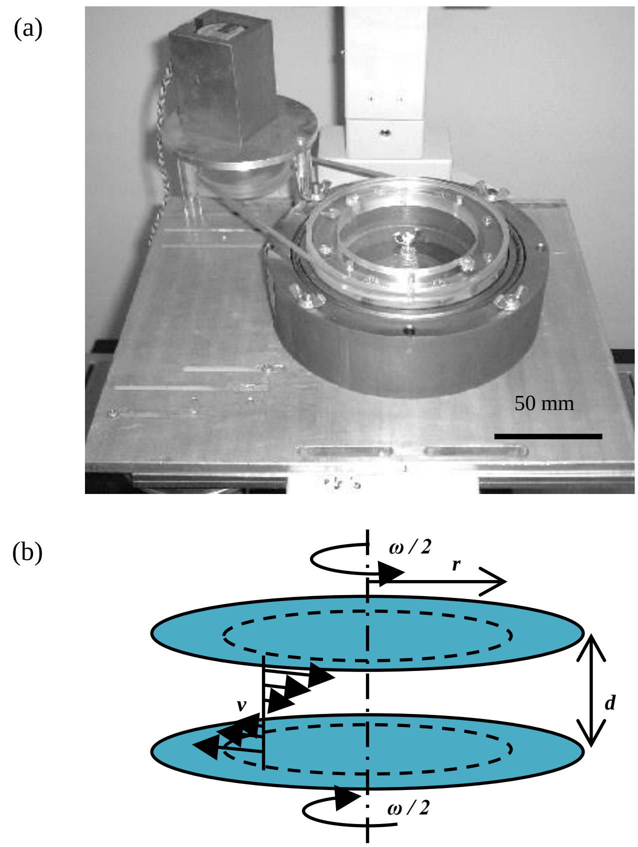

Fig. 2. Rheoscope (a) of counter-rotating parallel discs (b) [**12**].

$$\tau = \eta \gamma \quad (3)$$

In Eq. 3, η is the viscosity of the medium. The viscosity of the media was measured with a cone and plate type of viscometer. Variation was made on the shear stress (τ) between 0.6 Pa and 6 Pa by the control of the rotating speed of the disks at the observation area.

Hemolysis ratio

Partial destruction of erythrocyte is quantitatively evaluated by hemolysis ratio. The concentration of plasma free hemoglobin was measured after the shearing test, and the hemoglobin content of plasma was calculated. The hemolysis ratio (R) is defined as the ratio of hemoglobin, which is out of erythrocytes.

$$R = Cp / Ct \quad (4)$$

In Eq. 4, Ct is the total hemoglobin content, which is measured after total hemolysis with aid of the osmosis. The ratio of 0.01 means that ninety-nine percent of hemoglobin is left inside of erythrocytes.

For the shearing test, the sample blood was sheared between the rotating concave cone and the stationary convex cone. The erythrocytes were exposed to the constant uniform shear field for several minutes in the constant environment: 298 K of temperature and five percent of CO_2. The value of the shear rate was adjusted by the rotating speed of the concave cone. The test was repeated with variations of conditions: the shear rate (<1000 s^{-1}), and the exposure time (<120 min).

The results of hemolysis ratio were arranged according to the parameters: “the shear stress” as “the amplitude”, and “the shear rate” × “the exposure time” as “repeat count”. The boundary condition of erythrocyte destruction by fatigue was evaluated at hemolysis ratio of 0.01.

Deformation ratio

The deformation of each erythrocyte is quantitatively evaluated by the deformation ratio (D), which is calculated as the ratio between the length of the major axis (x) and the length of the minor axis (y) of the ellipsoidal shape at the two-dimensional projection image (Eq. 5).

$$D = (x - y) / (x + y) \quad (5)$$

The deformation ratio (D) is zero at circle ($x = y$). The ratio D approaches to one, as the ellipsoid shape elongates ($x >> y$).

Shear stress responsiveness

The relationship between the deformation ratio (D) and the shear stress (τ) was approximated to the exponential curve.

$$D = D_\infty (1 - \exp(-\tau / \tau_0)) \quad (6)$$

The deformation ratio (D) approaches to the critical deformation (D_∞) with the increase of the shear stress (τ). The shear stress responsiveness (τ_0) corresponds to the shear stress to make deformation of 63 percent of the critical deformation (D_∞). Deformability is evaluated with the shear stress responsiveness (τ_0) and with the critical deformation (D_∞) on two groups of erythrocytes: the lighter group, and the heavier group. The higher flexibility corresponds to the higher value of D_∞ and to the lower value of τ_0.

These parameters (D_∞, τ_0) were traced after shearing test: the blood sample was sheared at shear rate of 640 s^{-1} for several minutes (<60 min) in the Couette type of the constant uniform shear field between the concave cone and the convex cone at 298 K *in vitro*.

Results and discussion

Materials

To get the standardized results, the statistic procedure is necessary according to the large number of samples to check on individuality and on time dependence. In the present study, the blood sample was collected only from the same donor. Even the results from the primitive stage has meaning, because the main point of the present study is to find the analogical parameters for basic conceptual methodology of material fatigue test on the erythrocyte membrane.

Deformability

The rheoscope system has been successfully manufactured and used to observe deformation of each erythrocyte from a biconcave disk (**Fig. 3(a)**) to an ellipsoid (**Fig. 3(b-d)**) in the constant uniform shear rate field. The deformation ratio increases as the shear stress increases (**Fig. 3(b-d)**).

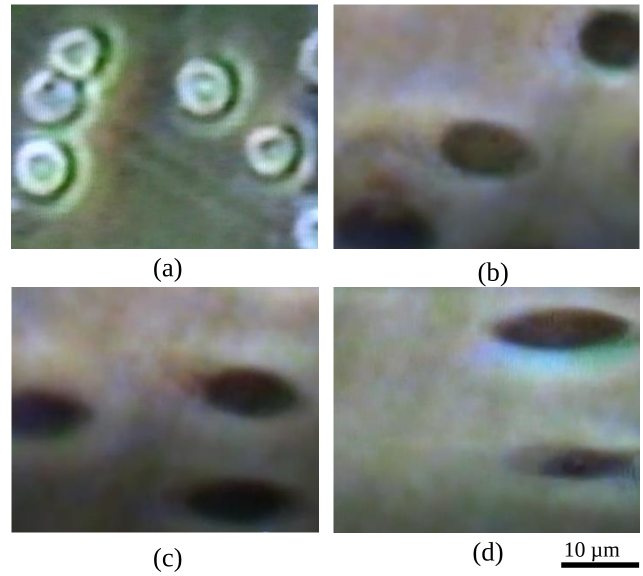

Fig. 3. Erythrocyte deformation in shear stress field: (a) τ = 0 Pa, (b) τ = 1 Pa, (c) τ = 2 Pa, (d) τ = 5 Pa.

The relationship between the deformation ratio (D) and the shear stress (τ) was successively approximated to the exponential curve (**Fig. 4(a)**). The critical deformation (D_∞) is higher at the lighter erythrocyte than at the heavier erythrocyte. The shear stress responsiveness (τ_0) is lower at the lighter erythrocyte then at the heavier erythrocyte.

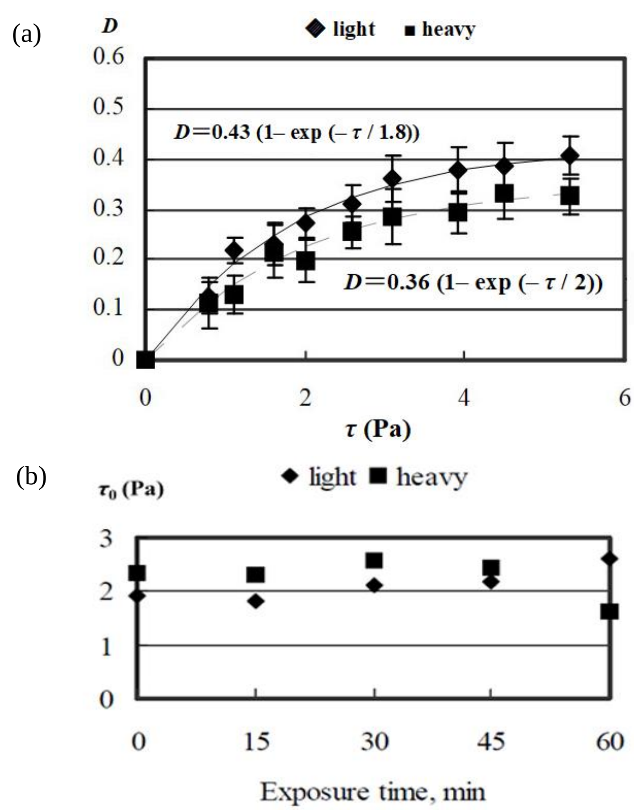

Fig. 4. (a) Deformation ratio (D) (each mark shows the mean value, each bar shows the standard deviation) vs. shear stress (τ) with approximated exponential curve (Eq. 6), n=10, (b) τ_0 vs. exposure time: light (rhombus), heavy (square) [**12**].

Even after exposure to the shear field of 640 s^{-1} for one hour, erythrocyte is deformed from biconcave to ellipsoid. In the lighter group, deformability is maintained for one hour at erythrocyte (**Fig. 4(b)**). In the heavier group, on the other hand, the shear stress responsiveness (τ_0) decreases after one hour of exposure to the shear field. The decrease corresponds to the sublethal damage. Reynolds number of the flow between two disks has been kept small by several parameters: the small distance between two disks, the low rotating speed of the disks, and the high viscosity of the medium.

Sublethal damage of the membrane

The content came out through the crack of the membrane, after erythrocyte destruction (**Fig. 5(a)**). The segments of the membrane were floating in the medium after the destruction of erythrocytes. Some of the segments were adhered to the membrane of the erythrocyte. The segment as the marker of the position of the membrane showed the tank-treading motion [**13**] of the membrane in the shear field (**Fig. 5(b)**) [**14**]. The frequency of the tank-treading motion increases in proportion to the shear rate.

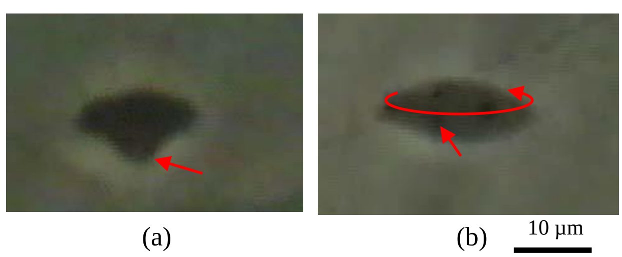

Fig. 5. (a) Contents (arrow) are leaked out from inside of erythrocyte, (b) tank-treading motion (arrow) of the membrane of erythrocyte is traced by marker (arrow) [**15**].

During the tank-treading motion the local area of the membrane changes repetitively because of the curvature change on the surface of the ellipsoid. The repetitive change causes fatigue of the membrane. The cyclic deformation was observed at some ellipsoid after exposure to the shear flow field for hours. The period of the cyclic deformation is half of the tank-treading motion. The damage point of the membrane can cause the cyclic deformation according to the change of the curvature of the surface of the ellipsoid. The cyclic deformation of erythrocyte shows the sublethal damage of the membrane.

Hemolysis ratio

The plasma free hemoglobin corresponds to the leakage of the content through the crack of the membrane of the erythrocyte. The destruction of erythrocyte was quantitatively measured by hemolysis ratio calculated by the plasma free hemoglobin concentration *in vitro*. The membrane of the erythrocyte can be resealed after sublethal damage. Erythrocyte after leakage of hemoglobin is called ghost. The oxygenation function of erythrocyte depends on quantity of hemoglobin inside. The value of "hemolysis ratio" can trace the functional loss ratio of erythrocyte including the partial destruction of erythrocyte. As the shear stress becomes lower, the product value of "shear rate" times "exposure time" at the hemolysis ratio of 0.01 is extended (**Fig. 6**).

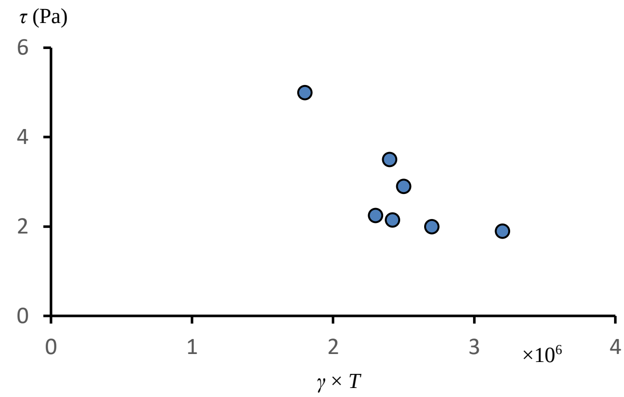

Fig. 6. Relationship between "shear stress (τ)" and "shear rate (γ)" × "exposure time (T)" at hemolysis ratio (R) of 0.01 [**11**].

Concavo-convex device

Couette type of flow has advantages to generate the uniform shear rate field. Conventional Couette flow devises have secondary flow like Taylor vortex by the centrifugal effect: the concentric cylinder system with the rotating inner cylinder, and the rotating cone and the stationary plate system. The corner of the stationary plate has the stagnation area, where the shear rate is not the same as the main area. The effectivity of these conventional devices is enough to measure viscosity of non-Newtonian fluid, but not enough to apply the whole uniform shear rate field to erythrocytes in the suspension.

The erythrocyte destruction by fatigue has quantitatively controlled by Couette type of the shearing device, in which erythrocytes suspended in the medium between the rotating concave cone and the stationary convex cone. The secondary flow by the inertial effect decreases in the device with the rotating concave outer cone compared with the device with the rotating convex inner cone. The slope of the concave cone decreases the centrifugal effect by balance of the force. Every erythrocyte has been successfully exposed to the constant uniform shear flow field in the medium between the rotating concave cone and the stationary convex cone.

Conclusion

Both deformation and destruction of erythrocyte in the shear field has been quantitatively evaluated by the fabricated Couette type of flow devices. Erythrocyte destruction by fatigue is governed by "the shear stress" as "the amplitude" and "the shear rate" × "the exposure time" as "repeat count". The sublethal damage of the membrane of the erythrocyte can be detected by repetitive deformation of ellipsoidal shape at the double frequency of the tank-treading motion of the membrane. The sublethal damage can also be detected by decrease of the shear stress responsiveness. The quantitatively analytical results of the property of the membrane of the erythrocyte as the material fatigue test might contribute to several fields: the physiological blood flow control, the erythrocyte preservation technology, and the design of the circulatory assist devices.

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Keywords

Erythrocyte membrane, fatigue, tank-treading motion, hemolysis, shear flow.

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