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Effect of erythrocyte aggregation and flow rate on cell-free layer formation in arterioles

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Ong PK, Namgung B, Johnson PC, Kim S. Effect of erythrocyte aggregation and flow rate on cell-free layer formation in arterioles. *Am J Physiol Heart Circ Physiol* 298: H1870–H1878, 2010. First published March 26, 2010; doi:10.1152/ajpheart.01182.2009.—Formation of a cell-free layer is an important dynamic feature of microcirculatory blood flow, which can be influenced by rheological parameters, such as red blood cell aggregation and flow rate. In this study, we investigate the effect of these two rheological parameters on cell-free layer characteristics in the arterioles (20–60 μm inner diameter). For the first time, we provide here the detailed temporal information of the arteriolar cell-free layer in various rheological conditions to better describe the characteristics of the layer variation. The rat cremaster muscle was used to visualize arteriolar flows, and the extent of aggregation was raised by dextran 500 infusion to levels seen in normal human blood. Our results show that cell-free layer formation in the arterioles is enhanced by a combination of flow reduction and red blood cell aggregation. A positive relation ($P < 0.005$) was found between mean cell-free layer widths and their corresponding SDs for all conditions. An analysis of the frequency and magnitudes of cell-free layer variation from their mean value revealed that the layer deviated with significantly larger magnitudes into the red blood cell core after flow reduction and dextran infusion ($P < 0.05$). In accordance, the disparity of cell-free layer width distribution found in opposite radial directions from its mean became greater with aggregation in reduced flow conditions. This study shows that the cell-free layer width in arterioles is dependent on both flow rate and red blood cell aggregability, and that the temporal variations in width are asymmetric with a greater excursion into the red blood cell core than toward the vessel wall.

microcirculation; cell-free layer variation; red blood cell aggregation

IT HAS BEEN LONG ESTABLISHED that red blood cells flowing in a narrow tube are subjected to hydrodynamic forces that favor migration of the cells to the center of the tube (12, 14, 27, 34). This migration leads to formation of a cell-free layer near the vessel wall (14, 29) and is limited by an opposing force due to collisions among red cells that favor cell movement away from the center. The cell-free layer width is defined as the distance from the outer edge of red blood cell core to the luminal surface of the endothelium. As a result, the cell-free layer width represents the dynamic positioning of the outermost red blood cells in the cell core of the flow stream. The forces, as determined by a combination of shear-induced or other relevant forces (dispersive or aggregating forces) acting on the surface of the cells, vary with time and position, leading to temporal variations in cell-free layer width. The magnitude of the forces in the radial direction may also differ for cell-wall and cell-cell interactions that, in turn, may lead to an asymmetric structure of the cell-free layer about its mean width. Due to technical considerations, almost all of the current information on the cell-free layer and underlying processes has been obtained from in vitro studies. The applicability of these findings to the in vivo situation and the complexities of microvascular networks are unclear.

In our laboratory's previous study, we have shown that the width of the cell-free layer in arterioles is dependent on the width of the arteriole (19). In this study, we focus on the effect of changes in flow on the arteriolar network in the presence and absence of aggregation. We chose the arterioles, since they play a principal role in regulating flow to the tissues and are normally responsible for at least 50% of total vascular resistance, primarily by changing vessel diameter. However, any factor that changes resistance in the arterioles could have a significant effect on blood flow to the tissues. Arteriolar resistance would also be determined by a number of rheological parameters that include red blood cell aggregability, tube hematocrit, and red cell flexibility. These factors may vary in individual vessel segments of the network. Due to the heterogeneity in rheological properties of blood flow distribution in the arteriolar network, the extent and characteristics of the cell-free layer formed in different arteriolar vessels may be greatly dependent on the local hemodynamics and rheological conditions.

One of the rheological characteristics listed above, red blood cell aggregability, is a prominent feature in blood of humans and other athletic species, but is not found in nonathletic species (31). This shear rate-dependent rheological property accentuates the non-Newtonian behavior of blood by reversibly promoting the formation of multicellular aggregates at low flow rates. These rheological factors (aggregation and flow rate) are responsible for the enhanced radial migration of red blood cells to the vessel center, leading to an apparent increase in mean cell-free layer width. Many in vitro studies (28, 32, 33, 40) have previously attempted to unravel the functional importance of red blood cell aggregation in relation to changes in mean cell-free layer width. Comparing the perfusion of long vertical glass tubes with human blood and red blood cells suspended in a nonaggregating buffer (saline or albumin) at low pseudoshear rates of $<10\ s^{-1}$ (32, 33), red blood cell aggregation at low shear forces was capable of attenuating the elevation of effective blood viscosity and flow resistance through an increase of cell-free layer width. Utilizing an isolated rabbit mesentery model (28), mean cell-free layer width was also found to increase with the infusion of incremental concentrations (0–4 g/dl) of dextran 70 solution at low-flow velocities (0.8–1.2 mm/s). It was suggested that this change in the mean layer width could be instrumental in

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modulating the overall flow resistance of the vascular network. The above findings suggest that the cell-free layer acts as a lubricating layer that reduces flow resistance, and the extent of this effect may be dependent on the layer width. This would be particularly important in the arteriolar network due to the role of these vessels in flow regulation. Conversely, a recent theoretical study has suggested that variability in the cell-free layer could play a potential role in offsetting any reduction in flow resistance by contributing to additional viscous dissipation (35). Alonso et al. (1) have qualitatively described the geometry of the interface between the cell-free layer and red blood cell core as irregular outer contours, which are more pronounced under reduced flow rather than normal flow state in vertical tubes perfused with human blood. Such variations of cell-free layer width in small tubes are also evident in many other studies (10, 32, 33, 38), where red blood cell aggregation is present. Hence it appears that, not only the mean magnitude, but variations of the cell-free layer width in either the spatial or temporal domain are important when considering any physiological implications relating to cell-free layer characteristics.

Based on earlier in vitro studies, in this study, we test the hypothesis that formation of the cell-free layer in arterioles will not be significantly influenced by red blood cell aggregation under physiological flow conditions due to high shear rates, but the aggregation will enhance the layer formation in low-flow conditions that are found in certain pathophysiological states. Thus we examined the influence of red blood cell aggregation on the cell-free layer characteristics at normal and reduced arterial pressures. To ascertain the hemo-rheological relevance to humans and other athletic species, the degree of red blood cell aggregation was adjusted to levels seen in normal human blood. To better understand how the cell-free layer characteristics are altered by these rheological changes, we carried out detailed analyses on distribution of cell-free layer widths about their mean value, including the tendency for the cell-free layer to extend either toward the vessel wall or into the red blood cell core.

MATERIALS AND METHODS

Animal preparation. A total of 11 Wistar-Furth rats, weighing between 190 and 250 g, were utilized in this study. All animal handling and care procedures were in accordance with that outlined in the Guide for the Care and Use of Laboratory animals (Institute for Laboratory Animal Research, National Research Council, Washington, DC: National Academy Press, 1996) and approved by the local Animal Subjects Committee. Pentobarbital sodium (50 mg/kg ip) was used to anesthetize the rat and was further administered during the experiment when needed. During the surgery, the animal was placed on a water-circulating heating pad at 37°C to maintain body temperature. While the jugular vein was catheterized for infusion of anesthetic or dextran 500, the femoral artery was catheterized for blood withdrawals and pressure monitoring. All catheters were heparinized with saline (30 IU/ml) solution to prevent blood clotting. An in vivo metrics 1.5-mm inner diameter (ID) pneumatic cuff was placed around the abdominal aorta to control blood flow in the muscle. The rat cremaster muscle was exteriorized and prepared for study, as previously described (19). Warm Plasma-Lyte A (Baxter), adjusted to pH 7.4, was continuously applied to the surgically exposed muscle to keep it moist. The muscle was cleaned and secured onto a Plexiglas platform whose temperature was maintained at 35°C by a heating element attached beneath it. A probe was placed beside the muscle to monitor the temperature. Finally, the muscle was irrigated with Plasma-Lyte A before being covered with a polyvinyl film (Saran, SC Johnson & Son).

Hematocrit, aggregation, and pressure measurements. Blood sample (~0.1 ml) was withdrawn from the femoral artery for hematocrit and aggregation measurements. Hematocrit was determined with a microhematocrit centrifuge (Readacrit, Clay Adams), while the degree of red blood cell aggregation was measured periodically during the experiment using an aggregometer (Myrenne aggregometer; Myrenne, Roetgen, Germany), based on a 10-s setting. Arterial pressure was continuously recorded with a physiological data-acquisition system (MP 100 System; BIOPAC Systems, Goleta, CA).

Adjustment of red blood cell aggregation level and flow rate. Red blood cell aggregation level was elevated to healthy human levels by infusion of dextran 500 (average molecular mass 460 kDa; Sigma) dissolved in saline (6%). A total of 200 mg/kg body wt was infused over the course of 1–2 min to achieve a plasma-dextran concentration of ~0.6%. An additional blood sample was then withdrawn for hematocrit and aggregation level determination ~10 min after the dextran infusion. To reduce flow rate in the microcirculatory vessels of the muscle, the pneumatic cuff pressure was increased with an air-filled syringe to lower the femoral artery pressure to ~40 mmHg and maintained by manual adjustment. The arterial pressure was monitored and allowed to stabilize upon reduction. After physiological or rheological measurements at reduced flow rates, the cuff pressure was released to allow the arterial pressure to return to the normal pressure range.

Experimental protocol. Initially, an arterial blood sample was withdrawn from the femoral artery to measure control values of hematocrit and aggregation level. The rat was then mounted on the microscopic stage, and an unbranched region of an arteriole with an inner diameter ranging from 20 to 60 μm was chosen for the study based on the criteria of stable flow, clear focus, and good contrast of the image. An intravital microscope (Ortholux II, Leitz) was used in conjunction with a water immersion objective (×40, Olympus) and a long working distance condenser (Instec, Boulder, CO) with numerical apertures of 0.7 and 0.35, respectively. In addition, a blue filter (model no. 59820, Spectral Physics, Stratford, CT) with peak transmission of 400 nm and spectral bandpass of 300–500 nm was used to enhance light absorption by the red blood cells so as to improve the contrast between the cells and background. A high-speed video camera (FASTCCAM ultima SE, Photron USA) was utilized to record blood flow in the arteriole at a frame rate of 4,500/s. Dextran 500 was administered to elevate the red blood cell aggregation level, and the recording procedure was then repeated. The above protocol was repeated at reduced flow condition, and a reduced frame rate of 2,250/s or 1,125/s was used instead for video recording. A total of 4,500 frames were obtained for one side of a vessel, which corresponded to a video recording time of 1 s for normal flow and 2 or 4 s for reduced flow conditions.

Cell-free layer width measurement. A detailed description of the cell-free layer width determination was presented in an earlier study (19) and will not be repeated here. Briefly, an analysis line is positioned perpendicularly to the vessel wall using the same procedure described in our laboratory's earlier study (19). Cell-free layer width is defined as the distance from the edge of the red blood cell core to the inner wall of the vessel. The latter was determined based on criteria defined by previous studies (16, 36), while the former was obtained by applying the Otsu's method (18, 19). The spatial resolution of this cell-free layer measurement was ~0.4 μm with the current system. Whenever possible, the layer width was determined on both sides of the vessel.

Temporal variations of the cell-free layer width. A typical example of temporal variation of the cell-free layer width obtained on one side of an arteriole is shown in Fig. 1. To better interpret the variability of the cell-free layer, the layer widths were investigated in terms of their distribution on either side of their mean value, defined as either toward the vessel wall or into the red blood cell core. Shown as the dashed

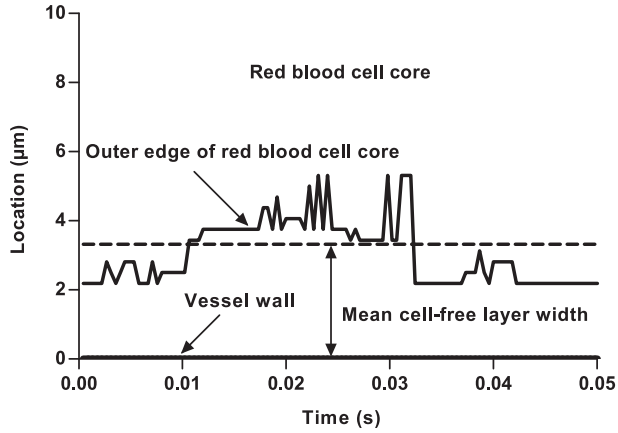

Fig. 1. Example of temporal variation of cell-free layer width in a 47.1-μm inner diameter (ID) arteriole. Dynamic variations of the cell-free layer width can be observed on both sides of its mean (dashed line), extending either toward vessel wall, or into the red blood cell (RBC) core.

line in Fig. 1 is a reference line to divide cell-free layer width variations into the two different groups. Dynamic variations in frequency distribution and magnitude of the cell-free layer width on the two sides of this reference line were separately analyzed. Cell-free layer width distributions on either side of this reference line were compared based on the two key parameters, namely the frequency of the layer width distributions and their corresponding magnitudes of deviation in opposite radial directions from the reference line.

Mean cellular velocity and pseudoshear rate. The centerline velocity was obtained from the high-speed video recordings by the dual-window method via a video sampler (model 204A, Vista Electronics) and velocity correlator system (model 102BC, Vista Electronics) (2, 17, 44). Mean cellular velocity (V) was approximated from the centerline velocity in the arteriole with a correction factor of 1.6, whereas the pseudoshear rate ($\dot{\gamma}$) was determined by using the relation: $\dot{\gamma} = V/D$, where D is the vessel diameter. It would be important to note that this correction factor is more appropriate for estimation of flow velocities where radial velocity profiles are nearly Poiseuille parabolas, such as that found typically in high-flow conditions. Under circumstances in which red blood cell aggregation is enhanced and flow velocity is low, the velocity profile would become blunter, and the use of the correction factor could tend to underestimate the mean cellular velocity and pseudoshear rate (30).

Statistical analysis and data presentation. Most of the statistical analyses, including regression fits and 95% confidence intervals of the experimental data, were performed using a statistical software package (Prism 4.0, Graphpad). In addition, the two-sample Kolmogorov-Smirnov test was utilized to test for statistical differences between distributions of experimental groups. Unpaired *t*-tests were used to determine differences in experimental parameters between normal and dextran-treated animals. For data that do not follow a normal distribution, both a nonparametric test (Mann-Whitney test) and unpaired *t*-tests were used to compare the means. To compare three or more experimental groups, a one-way ANOVA test and post-Bonferroni and Tukey multiple-comparison tests were utilized to determine the statistical significance of differences between experimental groups. All physiological and rheological data are reported as means ± SD. For all statistical tests and regression fits, $P < 0.05$ was regarded as statistically significant.

RESULTS

Systemic parameters. Normal arterial pressure for normal and dextran-treated rats was 116.3 ± 11.6 and 107.8 ± 6.2 mmHg, respectively. After cuff inflation, arterial pressure was 42.6 ± 7.3 mmHg for normal rats and 42.8 ± 6.8 mmHg for dextran-treated rats. Systemic hematocrit was 40 ± 2 and 38 ± 1% before and after dextran infusion, respectively. We found no significant difference in arterial pressures or hematocrits before and after dextran infusion. Red blood cell aggregation index (M) given by the aggregometer was 0.0 (no aggregation) before dextran infusion, but it rose to 11.9 ± 3.0 after dextran infusion, which was similar to levels reported for healthy humans (24, 43). In the normal flow condition, the mean red blood cell velocities were 5.9 ± 1.1 and 6.5 ± 1.2 mm/s before and after dextran infusion, respectively. With flow reduction, the velocity dropped to 1.2 ± 0.9 mm/s before dextran infusion and 1.4 ± 0.8 mm/s after dextran infusion. There was no significant difference in the mean cellular velocity before and after dextran infusion during normal and reduced flow protocols.

Relation between mean and SD of cell-free layer widths. The effects of red blood cell aggregation and flow rate on the relationship between mean value of the cell-free layer width and its SD are shown in Fig. 2. The SD of the cell-free layer widths was plotted against the corresponding mean values for both control (M = 0) and dextran-treated (M = 11.9 ± 3.0) groups under normal and reduced flow conditions. A strong positive correlation ($P < 0.005$) was consistently found between the two statistical parameters for all conditions. How-

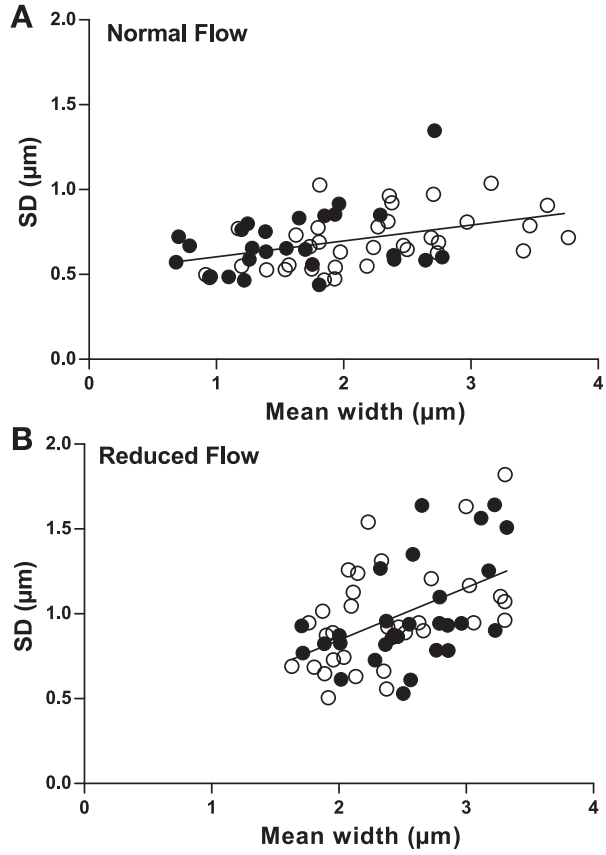

Fig. 2. Relationship between standard deviation (SD) and mean value of the cell-free layer width before (○) and after (●) aggregation induction. *A*: normal flow condition. *B*: reduced flow condition. Linear regression fits ($y = 0.09x + 0.51$, $R^2 = 0.16$ for normal flow, and $y = 0.31x + 0.23$, $R^2 = 0.24$ for reduced flow) are used for combined groups, since no significant effect of dextran infusion is found in either flow condition.

ever, no significant effect of dextran infusion on this relationship was found for both normal and reduced flows.

Effect of aggregation and flow reduction on mean and SD of cell-free layer widths. Pseudoshear rate in arterioles at normal arterial pressure was 204.1 ± 40.9 and 311.4 ± 49.3 s^{-1} before and after dextran infusion. After reduction of arterial pressure, the pseudoshear rate dropped to 76.7 ± 31.9 and 105.2 ± 27.3 s^{-1} before and after dextran infusion, respectively. Figure 3, *A* and *B*, shows the values of mean cell-free layer width and its SD normalized by vessel radius before and after dextran infusion under normal and reduced flow conditions. A significant increase ($P < 0.05$) in normalized mean and SD of cell-free layer width was found with flow reduction in the dextran-treated groups. Although a higher normalized mean and SD were also apparent after dextran infusion at reduced flow, no statistical significance of this effect was found, which might be due to the wide range of flow velocities associated with this flow condition. This was reflected in the large SDs (>25.9% of mean values) for the pseudoshear rates in reduced flow conditions, indicating that flow velocity in arterioles might be nonuniformly reduced by the arterial pressure drop. Thus, to carry out a more thorough examination of red blood cell aggregation effect on the formation of the cell-free layer, we divided the data of mean width and its SD in reduced flow conditions into three different pseudoshear rate groups based on their mean values (<50, >50 and <300, and >300 s^{-1}). After the classification, the pseudoshear rates for all of the flow conditions became 16.9 ± 6.1, 140.7 ± 59.5, and 320.8 ± 11.5 s^{-1} before dextran infusion and 17.0 ± 6.4, 129.6 ± 89.3, and 317.5 ± 11.6 s^{-1} after dextran infusion, respectively. In presentation of the data in Fig. 3, *C* and *D*, a set of combined pseudoshear rates (17.0 ± 6.1, 135.4 ± 74.8, and 318.7 ± 11.3 s^{-1}) was utilized for the three pseudoshear rate groups, since no significant differences were found between pseudoshear rates before and after aggregation induction. As shown in Fig. 3*C*, in the absence of red blood cell aggregation, there was no significant effect of pseudoshear rate on the normalized mean cell-free layer width, whereas, after induction of aggregation, a significant increase ($P < 0.001$) in the normalized mean width was observed when pseudoshear rate was reduced to 17.0 ± 6.1 s^{-1}. As shown in Fig. 3*D*, a significant increase ($P < 0.001$) in the normalized SD of the cell-free layer width was also found under aggregating condition with the same reduction of pseudoshear rate. As expected, at the lowest pseudoshear rates (17.0 ± 6.1 s^{-1}), magnitudes of both the normalized mean and SD of the layer width significantly increased ($P < 0.02$) after dextran infusion.

Effect of aggregation and flow reduction on dynamics of cell-free layer formation. As shown in Fig. 4*A*, no significant relation was found between the mean cell-free layer width and frequency of cell-free layer deviations occurring toward the vessel wall for all of the four experimental groups (normal and dextran-treated groups at normal and reduced flows). The slopes of their individual regression fits or combined regression fit were not significantly different from zero. The effect of red blood cell aggregation and flow reduction on the frequency of cell-free layer variations occurring toward the vessel wall was shown in Fig. 4*B*. Similar mean frequency of cell-free layer

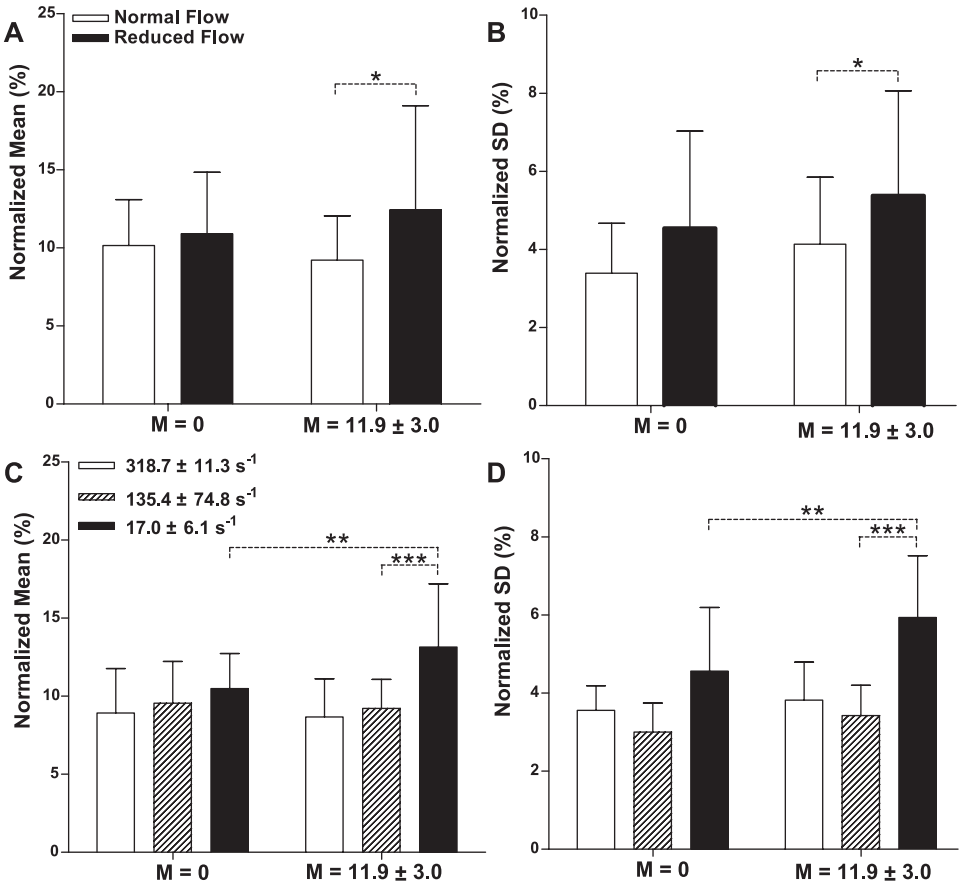

Fig. 3. *A* and *B*: normalized mean value and SD, respectively, of cell-free layer width variations at normal and reduced flow conditions, before and after aggregation induction. *C* and *D*: normalized mean value and SD, respectively, of cell-free layer width variations at different pseudoshear rates, before and after aggregation induction. M, aggregation index. *$P < 0.05$, **$P < 0.02$, and ***$P < 0.001$.

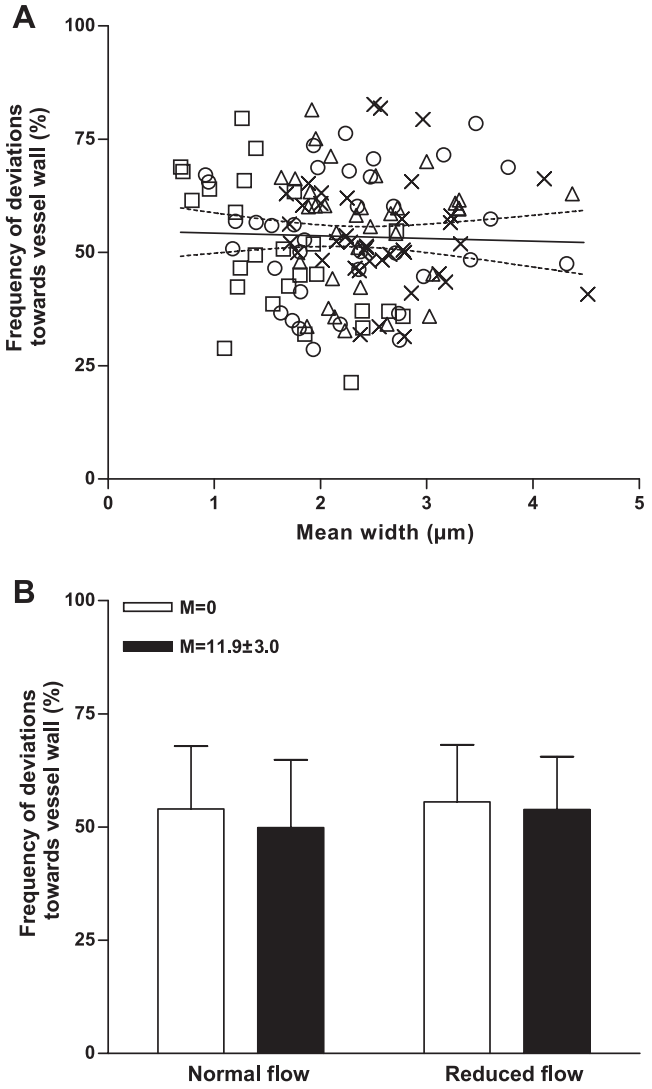

Fig. 4. Frequency of cell-free layer width deviations toward vessel wall. *A*: relation between the frequency of cell-free layer width deviations toward vessel wall and mean cell-free layer width. The different rheological conditions are represented by ○, normal flow (M = 0); △, reduced flow (M = 0); □, normal flow (M = 11.9 ± 3.0), and ×, reduced flow (M = 11.9 ± 3.0). No significant relation is found for all conditions. *B*: effect of flow rate and RBC aggregation on the frequency of cell-free layer width deviations toward vessel wall. There is no flow rate or aggregation effect on frequency of cell-free layer width deviations.

variations (~50%) was found between all groups, suggesting that the tendency for cell-free layer width to extend either toward the vessel wall or into red blood cell core might be independent of these two rheological parameters in the arteriolar blood flow.

Frequency distribution of cell-free layer variations. To better understand the relative magnitudes of cell-free layer width deviations from their mean values, the width data from 13 arteriolar vessels were grouped into bins, according to their distances from the vessel wall, as shown in Fig. 5. The box plot in the figure is further utilized to describe various distribution parameters (mean, median, 25th percentile, 75th percentile, and maximum values) of the cell-free layer widths. The magnitudes of these parameters indicated in the box plots are given in Table 1. This is important, since the respective distributions failed to pass the normality tests ($P < 0.05$), which indicates that the associated cell-free layer width values are not distributed symmetrically about their mean value. Thus the mean and SD would not provide information regarding this distribution (13). Asymmetry in distribution is, in part, evident in the deviation of median cell-free layer width value from its corresponding mean, which appears to be particularly prominent under reduced flow conditions. The dispersion of cell-free layer widths in the distribution can be further revealed utilizing the defined percentile points. The 25th, 50th (median), and 75th percentile points showed an overall shift in individual cell-free layer widths toward larger values from the vessel wall after dextran infusion and flow reduction. As shown in Fig. 5, for all experimental groups, the cell-free layer width generally extended with larger magnitudes into the red blood cell core than toward the vessel wall. This disparity in magnitudes of the deviations became significantly greater ($P < 0.05$) in the reduced flow condition after dextran infusion (Fig. 5*D*). This was evident in the larger range of deviations (2.73–10.0 μm: mean-maximum value) of cell-free layer width variations from the layer mean toward the red blood cell core under such rheological condition compared with other test conditions (1.92–6.54 and 1.79–7.19 μm for normal and dextran-treated rats, respectively, at normal arterial pressure and 2.43–8.99 μm for normal rats at reduced arterial pressure). A slight increase in mean cell-free layer width was correspondingly observed with larger possible deviations of the layer width into the red blood cell core. On the other hand, there were no significant differences in the distribution of cell-free layer width variations toward the vessel wall among all four groups, as the range of cell-free layer width variations was smaller.

In addition, the fraction of deviations with magnitudes >1.5 μm on either side of the mean value were computed to demonstrate how the cell-free layer variations could be affected by the asymmetric nature of the blood flow environment under different rheological conditions. For normal rats, the cell-free layer width variations in terms of their magnitude of deviation from their mean value showed an increase in the frequency of deviations with magnitudes >1.5 μm in either direction, from 0.4 to 12.0% (toward the vessel wall) or from 6.7 to 14.2% (into the red blood cell core) with flow reduction. This effect became more pronounced (4.5 → 17.8% toward vessel wall and 8.4 → 24.1% into red blood cell core) after aggregation induction and flow reduction.

DISCUSSION

The cell-free layer in arterioles may impose significant physiological influence by altering flow resistance (10, 37, 40), material exchange between red blood cells and tissues or organs (39), and other important rheological parameters, including wall shear stress and nitric oxide (NO) diffusion (3, 25). All of these parameters are highly dependent on the cell-free layer width, which can exhibit dramatic variations when subjected to rheological changes. Based on our results shown here, in nonathletic species where red blood cell aggregation is generally absent, flow rate may play a major role in cell-free layer formation, whereas, in athletic species where the aggregation is present, this important rheological property can become a dominant factor in affecting the layer formation, in particular, under low-flow conditions. The flow pattern of red

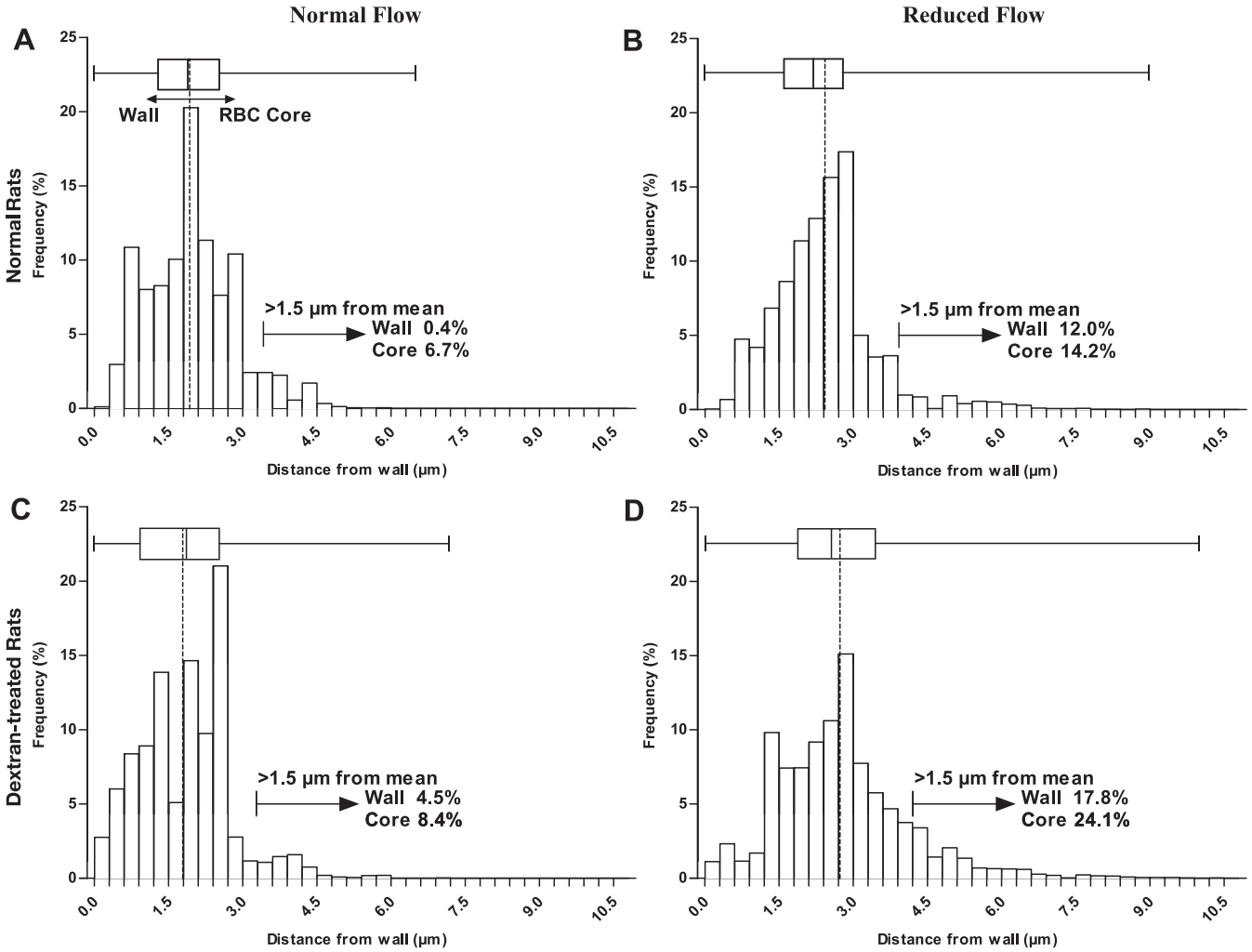

Fig. 5. Frequency distribution of cell-free layer variations from the vessel wall. A box plot is utilized in each condition to describe the distribution. A dashed line indicates the mean width of cell-free layer, while the solid line in the box corresponds to the median cell-free layer width value. *A* and *C*: normal and dextran-treated rats in normal flow condition, respectively. *B* and *D*: normal and dextran-treated rats in reduced flow condition, respectively. Cell-free layer widths deviate with larger magnitudes into RBC core than toward the vessel wall about the mean layer width. This disparity is significantly enhanced ($P < 0.05$), especially with dextran treatment in reduced flow condition. The total frequency of the cell-free layer widths on each side of the mean are considered as 100%. The reduction of flow increases the frequency of cell-free layer deviations with magnitudes >1.5 μm toward both the vessel wall and the RBC core. All units are in μm.

blood cells in the flow stream can be significantly affected by the presence of aggregates, which will, in turn, alter cell-free layer formation.

As many previous studies utilized general statistical parameters in particular, SD to quantify the variability of the cell-free layer width, which may obscure important information due to the non-Gaussian behavior of the layer, a new approach was made in this study to carry out more comprehensive examination of the cell-free layer characteristics. We analyzed the cell-free layer variation in terms of its frequency and magnitude of deviations from its mean width, which would provide an insight into how the layer variations are influenced by the asymmetric nature of its surrounding physical environment, defined by the vessel wall, plasma, and red blood cells.

Table 1. *Distribution parameters for box plots shown in Fig. 5*

	Normal Flow		Reduced Flow	
	Normal Rats	Dextran-Treated Rats	Normal Rats	Dextran-Treated Rats
Mean	1.92	1.79	2.43	2.73
Median	1.88	1.88	2.23	2.56
25th percentile	1.25	0.94	1.61	1.88
75th percentile	2.50	2.50	2.81	3.44
Maximum	6.54	7.19	8.99	10.00

All values are in μm.

Relation between mean and SD of cell-free layer widths. The relationship between the mean cell-free layer width and SD is of interest, since a larger mean cell-free layer width may be associated with an increase in SD of the layer width, as reported in previous studies (1, 15). A theoretical study has shown that variations of the cell-free layer may cause an additional viscous dissipation (35). Our results, shown in Fig. 2*A*, confirmed the existence of a positive correlation between mean cell-free layer width and SD at normal arterial pressure

for both nonaggregating and aggregating conditions, which was consistent with that shown in our previous study in arterioles (19). In addition, the present study was extended to examine this relationship in reduced flow conditions, and a significantly enhanced positive correlation ($P < 0.01$) was found with flow reduction for both aggregating and nonaggregating conditions, as shown in Fig. 2*B*. However, dextran treatment itself did not seem to exert a significant influence on this relationship. This was expected, since SD of the cell-free layer width increases with its mean value, regardless of aggregation, as reported in our earlier study (19). The positive correlation between mean layer width and SD suggests that any reduction in wall shear stress due to the presence of the cell-free layer in the arterioles is likely to be countered by an opposite increase in the shear stress caused by greater variation of the cell-free layer. In both athletic and nonathletic species (regardless of aggregation tendency), the extent of this opposing effect would be more prominent under pathological reduced flow conditions, since the slope of the linear relation shown in Fig. 2 became steeper in the reduced flow condition.

Effect of aggregation and flow rate on mean and SD of cell-free layer widths. In previous studies (6, 7), pseudoshear rates of < 5 s^{-1} were required to observe significant effects of aggregation on axial migration of red blood cells in venules of the rat spinotrapezius muscle. This was mainly attributed to the branching nature of vascular network defined by short vessel segments between bifurcations, which favors the frequent infusion of red blood cells and aggregates from the side branches and interferes with cell-free layer formation. In the arterioles, by contrast, the diverging flow pattern at successive branching points of the network is less likely to disrupt cell-free layer formation, and prominent layer formation could still occur, despite the higher pseudoshear rates that are expected with the similar reduction of arterial pressure. Indeed, in this study, with reference to the nonaggregating condition, pseudoshear rates of larger magnitudes (40.9 ± 32.4 s^{-1}), which correspond to mean cellular velocities of ~1.2 mm/s, were obtained in the arterioles, with arterial pressure reduction to ~40 mmHg compared with pseudoshear rates of 6 ± 4 s^{-1} (mean cellular velocities of ~0.25 mm/s) in the venules when arterial pressure was lowered to ~50 mmHg (6). Despite an eightfold higher pseudoshear rate in the arterioles than that required for prominent layer formation in the venules, a reasonable extent of layer formation was found in the arterioles, which further substantiated the notion that layer formation is highly dependent on the network flow topography.

The cell-free layer widths of ~1.5–3.5 μm obtained in this study under nonaggregating and reduced flow conditions are in agreement with that of ~2 μm obtained by Maeda and coworkers in arterioles (25–35 μm ID) of the rabbit mesentery under similar flow conditions, where the mean cellular velocity was comparable to ours (28). In their study, mean cell-free layer width was found to increase by ~1 μm at 0.8–1.2 mm/s (mean cellular velocity) after dextran 70 infusion (4 g/dl). This increase in mean width of the cell-free layer may be due mainly to accelerated axial migration of red blood cells in the presence of aggregation (20, 39). A corresponding increment in relative flow resistance (flow resistance of red blood cell suspension normalized by the flow resistance of suspending medium alone) was mitigated with this increase in mean layer width, demonstrating the possible flow lubricating impact of the cell-free layer in the microvascular network, particularly with the influence of red blood cell aggregation. Similarly, a significant increase in normalized mean cell-free layer width by ~2.6%, which corresponds to a mean cell-free layer width increase of 0.7–0.9 μm in arterioles (25–35 μm ID), was found in our study (Fig. 3*C*) at 17.0 ± 6.1 s^{-1} after dextran 500 infusion. Our results show that cell-free layer formation in the arteriolar network is enhanced by a combination of flow reduction and red blood cell aggregation. Hence, in the event of pathological flow, defined by low-flow rates in the arterioles, a thicker cell-free layer could lead to enhanced plasma skimming, resulting in heterogeneous red blood cell distributions in the capillary network and reduction in functional capillary density, as reported in our laboratory's earlier study (21).

As reported in our laboratory's previous study (22), dextran 500 infusion to raise the aggregation level in rats to those seen in normal human blood increases plasma viscosity (from 1.29 ± 0.03 to 1.53 ± 0.08 cP). The plasma viscosity increase could provide some resistance for the motion of red blood cells in the suspending medium, which might decrease red blood cell aggregation, resulting in retardation of cell-free layer formation process. Therefore, increased plasma viscosity may diminish the effect of aggregation on the cell-free layer width at lower flow rates. However, the significant cell-free layer formation found under aggregating condition at low pseudoshear rates in this study suggests the predominant influence of red blood cell aggregation over plasma viscosity effects on the layer formation under these flow conditions.

By tracking the trajectory of labeled individual red blood cells under both nonaggregating and aggregating conditions, Bishop et al. (5) reported in both conditions a trend of increasing SD in the radial position (~1 μm) of the cells in postcapillary venules (45–75 μm ID), with decreasing mean cellular velocity ranging from ~12.5 to 0.1 mm/s. By applying this result to the aggregating condition in the present study by relating it to the outermost cell of the red blood cell core that defines the cell-free layer width, an increase in mean magnitude of the layer width SD by < 0.5 μm would be expected, with reduction of mean cellular velocity from 6.5 to 1.4 mm/s, which is comparable to that of 0.25–0.75 μm obtained with arterioles (20–60 μm ID) in the present study. However, the direct quantitative comparison of these two results would be limited by the different topographies of the vascular network and flow conditions, as noted above, although a similar trend of red blood cell movements was observed in both studies. The greater overall variation (higher SD) of the cell-free layer width obtained with reduction of cellular velocity shown in Fig. 3*D* would lead to higher viscous dissipation at regions of

Table 2. *Frequency of cell-free layer deviations >1.5 μm at reduced flow*

	Without Consideration of a Glycocalyx Layer		With Consideration of a 0.5-μm Glycocalyx Layer	
	Normal Rats	Dextran-Treated Rats	Normal Rats	Dextran-Treated Rats
Toward red blood cell core	14.2	24.1	15.4	24.1
Toward vessel wall	12.0	17.8	11.3	16.3

All values are in %.

slow blood flow in the arteriolar network seen in pathological condition, as reported by Sharan and Popel (35).

Cell-free layer variations in terms of frequency distribution and magnitude of deviation from its mean width. A more in-depth analysis of the cell-free layer width variation showed that the frequency of the layer width deviations extending either toward the vessel wall or into the red blood core is independent of the mean width (Fig. 4*A*). Figure 4*B* suggested that the cell-free layer width has similar tendency to extend in both radial directions away from its mean, and this phenomenon is unaltered by rheological changes in the form of red blood cell aggregation and flow rate in the arteriolar blood flow. However, Fig. 5 shows that these two rheological parameters instead exert their influence on the magnitudes of cell-free layer deviations in the radial direction from the mean layer width. Increased frequency of deviations >1.5 μm were found with reduction of flow either toward the vessel wall or into the red blood cell core from the mean value of the cell-free layer width, and this effect was pronounced in the presence of aggregation. In particular, Fig. 5*D* shows that the presence of red blood cell aggregates in the flow stream can greatly influence the dynamics of the cell-free layer variation, such that layer widths deviate with larger magnitudes away from their mean, especially into the red blood cell core, compared with the case (Fig. 5*B*) without aggregation. This suggests that thicker cell-free layer formation may lead to larger deviations of the layer width into the red blood cell core. Conversely, it is likely that the tendency to display greater inward radial deviations of cell-free layer widths into red blood cell core could potentially contribute to both greater overall mean cell-free layer widths and SDs at reduced flows in the arteriolar vessels.

Potential influences on oxygen and NO diffusion. The direct consequence of the cell-free layer formation is an increase in mean physical distance between the endothelium and red blood cells in the arteriolar flow stream. Since the arterioles constitute a site of significant oxygen delivery to the tissues (8, 41), the presence of a wider plasma layer with its relatively low oxygen solubility would reduce oxygen delivery at this site. Furthermore, this diffusion barrier reduces any direct scavenging of NO produced in the endothelium by hemoglobin encapsulated within the flowing red blood cells, leading to a preservation of NO bioavailability in the arterioles for vessel diameter regulation by promoting local flow-induced vasodilatory responses (9, 23, 42). In contrast, recent studies (4, 45) have reported findings suggesting opposite explanations for the influence of enhanced red blood cell aggregation on NO bioavailability. Although cell-free layer thickness was not measured in those studies, their results indicated downregulation in NO-synthesizing mechanisms in endothelial cells due to red blood cell aggregation and associated decrement in wall shear stress.

Consideration of glycocalyx layer. The current cell-free layer measurement includes the glycocalyx layer at the surface of the endothelial cells. This limitation would result in an overestimation of the cell-free layer width magnitude in our study by ~0.5 μm, based on previous estimations reported on microvascular glycocalyx thickness (11, 26). If we correct all of the layer width measurements based on the assumption of a 0.5-μm glycocalyx layer, there will be a slight change in the frequency distribution. As shown in Table 2, with consideration of the glycocalyx layer for reduced flow conditions after this correction, a decrease in frequency of deviations with magnitudes >1.5 μm toward the vessel wall would be observed both before (from 12.0 to 11.3%) and after aggregation induction (from 17.8 to 16.3%). This is expected due to the shift of the reference line shown in Fig. 1 closer to the wall as a result of the decline in mean cell-free layer width, since layer variations within the distance of 0.5 μm from the arteriolar wall, which possess large deviations from the mean width, are now interpreted as 0 μm in width magnitude from the wall. In contrast, no discernible trend in frequency change of deviations with magnitudes >1.5 μm toward the red blood cell core is found, although there would be a slight increase (from 14.2 to 15.4%) in frequency before aggregation induction. As a result, with consideration of the glycocalyx layer, the disparity in the distribution of cell-free layer variation with magnitudes >1.5 μm between red blood cell core and vessel wall would increase by 1.9 and 1.5% before and after aggregation induction, respectively.

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DISCLOSURES

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REFERENCES

1. **Alonso C, Pries AR, Gaehtgens P.** Time-dependent rheological behavior of blood at low shear in narrow vertical tubes. *Am J Physiol Heart Circ Physiol* 265: H553–H561, 1993.
2. **Baker M, Wayland H.** On-line volume flow rate and velocity profile measurement for blood in microvessels. *Microvasc Res* 7: 131–143, 1974.
3. **Baskurt OK, Meiselman HJ.** RBC aggregation: more important than RBC adhesion to endothelial cells as a determinant of in vivo blood flow in health and disease. *Microcirculation* 15: 585–590, 2008.
4. **Baskurt OK, Yalcin O, Ozdem S, Armstrong JK, Meiselman HJ.** Modulation of endothelial nitric oxide synthase expression by red blood cell aggregation. *Am J Physiol Heart Circ Physiol* 286: H222–H229, 2004.
5. **Bishop JJ, Popel AS, Intaglietta M, Johnson PC.** Effect of aggregation and shear rate on the dispersion of red blood cells flowing in venules. *Am J Physiol Heart Circ Physiol* 283: H1985–H1996, 2002.
6. **Bishop JJ, Popel AS, Intaglietta M, Johnson PC.** Effects of erythrocyte aggregation and venous network geometry on red blood cell axial migration. *Am J Physiol Heart Circ Physiol* 281: H939–H950, 2001.
7. **Bishop JJ, Popel AS, Intaglietta M, Johnson PC.** Rheological effects of red blood cell aggregation in the venous network: a review of recent studies. *Biorheology* 38: 263–274, 2001.
8. **Briceno JC, Cabrales P, Tsai AG, Intaglietta M.** Radial displacement of red blood cells during hemodilution and the effect on arteriolar oxygen profile. *Am J Physiol Heart Circ Physiol* 286: H1223–H1228, 2004.
9. **Butler AR, Megson IL, Wright PG.** Diffusion of nitric oxide and scavenging by blood in the vasculature. *Biochim Biophys Acta* 1425: 168–176, 1998.
10. **Cokelet GR, Goldsmith HL.** Decreased hydrodynamic resistance in the two-phase flow of blood through small vertical tubes at low flow rates. *Circ Res* 68: 1–17, 1991.
11. **Damiano ER.** The effect of the endothelial-cell glycocalyx on the motion of red blood cells through capillaries. *Microvasc Res* 55: 77–91, 1998.
12. **Devendran T, Brandhuber M, Schmid-Schonbein H.** Axial migration of RBC and the influence of cell flexibility and aggregation. *Bibl Anat* 13: 95–96, 1975.

13. **Glantz S.** *Primer of Biostatistics*. New York: McGraw-Hill, Medical Publishing Division, 2001.
14. **Goldsmith HL.** The Microcirculatory Society Eugene M. Landis Award lecture. The microrheology of human blood. *Microvasc Res* 31: 121–142, 1986.
15. **Goldsmith HL.** Microscopic flow properties of red cells. *Fed Proc* 26: 1813–1820, 1967.
16. **Gretz JE, Duling BR.** Measurement uncertainties associated with the use of bright-field and fluorescence microscopy in the microcirculation. *Microvasc Res* 49: 134–140, 1995.
17. **Intaglietta M, Silverman NR, Tompkins WR.** Capillary flow velocity measurements in vivo and in situ by television methods. *Microvasc Res* 10: 165–179, 1975.
18. **Kim S, Kong RL, Popel AS, Intaglietta M, Johnson PC.** A computer-based method for determination of the cell-free layer width in microcirculation. *Microcirculation* 13: 199–207, 2006.
19. **Kim S, Kong RL, Popel AS, Intaglietta M, Johnson PC.** Temporal and spatial variations of cell-free layer width in arterioles. *Am J Physiol Heart Circ Physiol* 293: H1526–H1535, 2007.
20. **Kim S, Ong PK, Johnson PC.** Effect of dextran 500 on radial migration of erythrocytes in postcapillary venules at low flow rates. *Mol Cell Biomech* 6: 83–92, 2009.
21. **Kim S, Popel AS, Intaglietta M, Johnson PC.** Effect of erythrocyte aggregation at normal human levels on functional capillary density in rat spinotrapezius muscle. *Am J Physiol Heart Circ Physiol* 290: H941–H947, 2006.
22. **Kim S, Yang S, Lim D.** Effect of dextran on rheological properties of rat blood. *J Mech Sci Technol* 23: 868–873, 2009.
23. **Lamkin-Kennard KA, Jaron D, Buerk DG.** Impact of the Fahraeus effect on NO and O_2 biotransport: a computer model. *Microcirculation* 11: 337–349, 2004.
24. **Lee BK, Alexy T, Wenby RB, Meiselman HJ.** Red blood cell aggregation quantitated via Myrenne aggregometer and yield shear stress. *Biorheology* 44: 29–35, 2007.
25. **Liao JC, Hein TW, Vaughn MW, Huang KT, Kuo L.** Intravascular flow decreases erythrocyte consumption of nitric oxide. *Proc Natl Acad Sci U S A* 96: 8757–8761, 1999.
26. **Long DS, Smith ML, Pries AR, Ley K, Damiano ER.** Microviscometry reveals reduced blood viscosity and altered shear rate and shear stress profiles in microvessels after hemodilution. *Proc Natl Acad Sci U S A* 101: 10060–10065, 2004.
27. **Maeda N.** Erythrocyte rheology in microcirculation. *Jpn J Physiol* 46: 1–14, 1996.
28. **Maeda N, Suzuki Y, Tanaka J, Tateishi N.** Erythrocyte flow and elasticity of microvessels evaluated by marginal cell-free layer and flow resistance. *Am J Physiol Heart Circ Physiol* 271: H2454–H2461, 1996.
29. **McHedlishvili G, Maeda N.** Blood flow structure related to red cell flow: determinant of blood fluidity in narrow microvessels. *Jpn J Physiol* 51: 19–30, 2001.
30. **Pittman RN, Ellsworth ML.** Estimation of red cell flow microvessels: consequences of the Baker-Wayland spatial averaging model. *Microvasc Res* 32: 371–388, 1986.
31. **Popel AS, Johnson PC, Kameneva MV, Wild MA.** Capacity for red blood cell aggregation is higher in athletic mammalian species than in sedentary species. *J Appl Physiol* 77: 1790–1794, 1994.
32. **Reinke W, Gaehtgens P, Johnson PC.** Blood viscosity in small tubes: effect of shear rate, aggregation, and sedimentation. *Am J Physiol Heart Circ Physiol* 253: H540–H547, 1987.
33. **Reinke W, Johnson PC, Gaehtgens P.** Effect of shear rate variation on apparent viscosity of human blood in tubes of 29 to 94 microns diameter. *Circ Res* 59: 124–132, 1986.
34. **Schmid-Schoenbein H, Wells R.** Fluid drop-like transition of erythrocytes under shear. *Science* 165: 288–291, 1969.
35. **Sharan M, Popel AS.** A two-phase model for flow of blood in narrow tubes with increased effective viscosity near the wall. *Biorheology* 38: 415–428, 2001.
36. **Smith ML, Long DS, Damiano ER, Ley K.** Near-wall micro-PIV reveals a hydrodynamically relevant endothelial surface layer in venules in vivo. *Biophys J* 85: 637–645, 2003.
37. **Soutani M, Suzuki Y, Tateishi N, Maeda N.** Quantitative evaluation of flow dynamics of erythrocytes in microvessels: influence of erythrocyte aggregation. *Am J Physiol Heart Circ Physiol* 268: H1959–H1965, 1995.
38. **Suzuki Y, Tateishi N, Soutani M, Maeda N.** Flow behavior of erythrocytes in microvessels and glass capillaries: effects of erythrocyte deformation and erythrocyte aggregation. *Int J Microcirc Clin Exp* 16: 187–194, 1996.
39. **Tateishi N, Suzuki Y, Cicha I, Maeda N.** O_2 release from erythrocytes flowing in a narrow O_2-permeable tube: effects of erythrocyte aggregation. *Am J Physiol Heart Circ Physiol* 281: H448–H456, 2001.
40. **Tateishi N, Suzuki Y, Soutani M, Maeda N.** Flow dynamics of erythrocytes in microvessels of isolated rabbit mesentery: cell-free layer and flow resistance. *J Biomech* 27: 1119–1125, 1994.
41. **Tsai AG, Johnson PC, Intaglietta M.** Oxygen gradients in the microcirculation. *Physiol Rev* 83: 933–963, 2003.
42. **Vaughn MW, Kuo L, Liao JC.** Effective diffusion distance of nitric oxide in the microcirculation. *Am J Physiol Heart Circ Physiol* 274: H1705–H1714, 1998.
43. **Vaya A, Falco C, Fernandez P, Contreras T, Valls M, Aznar J.** Erythrocyte aggregation determined with the Myrenne aggregometer at two modes (M0, M1) and at two times (5 and 10 sec). *Clin Hemorheol Microcirc* 29: 119–127, 2003.
44. **Wayland H, Johnson PC.** Erythrocyte velocity measurement in microvessels by a two-slit photometric method. *J Appl Physiol* 22: 333–337, 1967.
45. **Yalcin O, Ulker P, Yavuzer U, Meiselman HJ, Baskurt OK.** Nitric oxide generation by endothelial cells exposed to shear stress in glass tubes perfused with red blood cell suspensions: role of aggregation. *Am J Physiol Heart Circ Physiol* 294: H2098–H2105, 2008.