Important Physical Regulatory Roles of Erythrocytes on Platelet Adhesion Under Blood Flow Conditions.

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Article

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Abstract

Aim: Functional roles of erythrocytes on platelet adhesion to vessel wall under blood flow condition is still to be elucidated.

Methods: Blood specimens containing native, biochemically fixed, or artificial erythrocytes, at various hematocrits were perfused on immobilized von Willebrand factor (VWF) at a shear rate of 1,500 s^{-1}. Number of platelets adhered on VWF within the region of interest (ROI: $5x10^3$ μm^2) was serially measured for 2 minutes using the fluorescent microscopy system. Regression analyses were conducted to evaluate the relationship between the rates of platelet adhesion and the hematocrit values. Computer simulation of platelet adhesion on the wall of von Willebrand factor (VWF) at a shear rate of 1,500 s^{-1} was conducted by solving governing equations with a finite-difference method on K-computer. Calculations were conducted at various hematocrits conditions in the computational domain of 100 μm (x-axis) x 400 μm (y-axis) x 100 μm (z-axis).

Results: Biological experiments demonstrated the positive correlations between the rates of platelet adhesion and hematocrit values in native, fixed, and artificial erythrocytes. ($r = 0.992$, 0.934, and 0.825, $p < 0.05$ for all) The number of platelets adhered after 2 minutes blood perfusion at 24% hematocrit of 221.7 ± 22.6/$5x10^3$ μm^2 (fixed erythrocytes) and 208.0 ± 26.5/$5x10^3$ μm^2(artificial ones), respectively, were comparable to that with native ones of 195.9 ± 28.3/$5x10^3$ μm^2. The simulation results demonstrated the hematocrit dependent increase in platelet adhesion rates (94.3/sec at 10%, 185.2/sec at 20%, and 327.9/sec at 30%, respectively) suggesting the importance of augmented z-axis fluctuation of flowing platelet by erythrocytes as the cause of platelet adhesion.

Conclusions: Our experimental results indicate the importance of the physical roles of erythrocytes inducing wall-normal fluctuations of flowing platelets on their vessel adhesion under blood flow conditions.

Highlights

'What is known on this topic'

- Platelet adhere on the vessel wall expressing von Willebrand factor (VWF) through their glycoprotein (GP)Iba under blood flow conditions.
- Functional roles of erythrocytes on the rates of platelet adhesion on VWF under blood flow conditions are unknown.

'What does this paper add?'.

- Platelet adhesion rates on the wall coated with VWF increase in a hematocrit-dependent manner, regardless of erythrocytes type (native, chemically fixed, or artificial) suggesting the importance of physical rather than bio-chemical roles of erythrocytes on platelet adhesion.

- Computer simulation solving governing equations for blood flow suggest the increase of z-axis fluctuation of flowing platelet by the presence of erythrocytes at higher hematocrits as the cause for higher platelet adhesion rates on VWF wall.

Introduction

Platelets adhere on damaged vessel wall through their glycoprotein (GP)Iba binding with von Willebrand factor (VWF) under blood flow conditions. [1, 2, 3, 4] VWF mediated shear-induced platelet aggregation occurs even in the absence of erythrocytes. [5, 6, 7] However, platelet adhesion on injured vessel wall is known to be influenced by the presence of erythrocytes. [8-10] Indeed, the risk of thrombotic diseases caused by mural thrombi such as myocardial infarction [11, 12] is influenced by hematocrit level. [13, 14] Many biological studies dissecting the mechanism of platelet adhesion under blood flow conditions were conducted in the presence of erythrocytes. [1-3, 15-17] Some reports suggested the importance of biochemical roles of erythrocytes, [18-24] but others supported biophysical roles. [8, 20, 24, 25] Previously published simple model of blood flow suggested the potential importance of near-wall rebounding collisions of platelet in the presence of erythrocytes. [25] However, precise functional roles of erythrocytes for the platelet adhesion and thrombus formation are still to be elucidated.

Here we investigate the mechanism underlining the influence of erythrocytes on the rates of platelet adhesion using a medical-engineering cooperation approach combining large-scale computer simulation with biological experiments.

Methods

1. Biological Experiments

1) Preparation of Blood Specimens

Venous blood specimens were collected from adult volunteers. Our study protocol for drawing blood from human volunteers was approved by the Internal Review Board (IRB) of Tokai University School of Medicine (17R17). Written informed consents were obtained from all participants. All the study subjects were instructed to abstain from drugs known to interfere with platelet function (such as aspirin or $P2Y_{12}$ inhibitors) at least a month preceding the study. The blood samples were immediately transferred into plastic tubes containing 1/10 volume of the specific thrombin inhibitor Argatroban (Mitsubishi Tanabe Pharma Corporation, Osaka Japan) at a concentration of 100 μmol/L. Both erythrocytes and platelets were separated from the blood samples and stored as previously reported.[7, 26]

Biochemically fixed erythrocytes (fixed erythrocytes) were prepared by exposing separated native erythrocytes to paraformaldehyde solution (2%) for 10 minutes. Then, residual paraformaldehyde was washed three times by 10 mM Hepes buffer (0.14 mM NaCl and 10 mM Hepes). Particles of polystyrene-copolymer were prepared as artificial erythrocytes (density 1.05 g/cm^3, diameter 8 μm (Thermo Fisher

scientific, MA, USA). For each experiment, concentrations of native, fixed, and artificial erythrocytes are adjusted with hematocrit values.

2) Measurements of the Number of Platelet Adhered on VWF

A method to calculate the number of platelets adhered on VWF under various blood flow condition in the presence of erythrocytes was established previously. [3, 16] According to previous publication, all the experiments were conducted at blood flow condition achieving the wall shear rate of 1,500 s^{-1}. [16, 27] To clarify whether erythrocytes influence platelet adhesion by their bio-chemical or physical functions, the rates of platelet adhesion on VWF at initial 2 minutes in the presence of native (erythrocytes), chemically fixed (fixed erythrocytes), and artificial erythrocyte (artificial erythrocytes) at various hematocrits were measured.

Reconstitute blood specimens containing native, fixed, and artificial erythrocyte at various hematocrits were prepared in the presence of 200,000/μl of platelets rendered fluorescent by addition of FITC-conjugated antibody against glycoprotein (GP) IIb/IIIa of abciximab. (gifted from Dr. Nakada in Centcore Co., USA)

3) Calculation of the Platelet Adhesion Rate

To calculate platelet adhesion rate, regression analyses, settling the intercept for both x- and y-axis as 0, were conducted between perfusion time and the number of platelets adhered on VWF in the presence of native, fixed, and artificial erythrocytes at various hematocrits. The slope of regression lines in each analysis was interpreted as the rate of platelet adhesion on the VWF wall (number of platelet bound/$5x10^3$ μm^2/second).

4) Statistical Analysis for Biological Experiments

All numerical data are expressed as mean ± SD unless otherwise specified.

For comparison of number of platelets adhered on VWF wall after 2 minutes blood perfusion under different conditions, one-way analysis of variance (ANOVA) was conducted. The differences among groups of data were assessed by Newman-Keuls post-hoc test when ANOVA suggested statistical difference. A two-sided p value of 0.05 was considered to denote statistical significance.

1. Numerical Simulation

1) Simulation System

Numerical simulations of blood flow in the presence of platelet and erythrocytes were conducted using the method we developed previously .[28 29] Briefly, the system is consisted of Newtonian fluid and the vesicles are constructed with hyper-elastic membrane. The fluid was considered incompressible. The cell membranes were assumed to have no thickness, but the elastic tension was considered. The governing

equations for incompressible Navier-Stokes equations coupling with fluid-structure interaction were solved by means of a finite-difference method. The erythrocytes and the platelets were treated as vesicles coupled with fluid-structure interaction. Platelet adhesion process to the vessel wall was analyzed by multiscale modeling of coupling continuum scale finite difference method with the molecular scale Monte Carlo method. [30] The adhesion of platelets to the vessel wall is caused by the protein-protein bindings (GP1ba proteins on the platelet binding with VWF proteins on the vessel wall.). This protein-protein binding process is modeled using the concept of transition state theory and evaluated by Monte Carlo simulation, solving the equations for the stochastic process of each biding. [30] Biochemical characteristics, such as release of ADP are not included in this simulation system.

Computational domain was set to the region with 100 mm (x-axis) x 400 mm (y-axis) x 100 mm (z-axis). VWF modelled to bind with platelet GPIba was settled at z wall of the computational domain to mimic the biological experiments. As the initial condition, 8,632 platelets were settled in the computational domain ($2.158 \times 10^3/\mu L$). Periodical boundary condition was imposed and pressure gradient was added to realize wall shear stress of 1,500 s^{-1} for the fluid viscosity of $1.2e^{-3}$ Pa.s in the blood flows in x-direction. This pressure gradient was set as a constant value throughout the various hematocrit conditions

2) Deformability of Erythrocytes

A spherical vesicle in this system was prepared to subject to an unbounded shear flow. Material property of erythrocytes was given as previously reported. [28] Fluid mechanical dimensionless numbers in our system were Reynolds ($Re = \rho\dot{\gamma} d2/(4\mu)$) and the capillary ($Ca = \mu\dot{\gamma} d/(2Es)$) numbers, where ρ denote density, d denotes the vesicle diameter, $\dot{\gamma}$ the shear rate, μ represent viscosity, and *E*s is the surface elastic modulus. In the present study, the Reynolds number was fixed at Re = 0.01, while the capillary number was variable. Both mean velocity (cm/s) and apparent relative viscosity of the fluids were calculated under various hematocrit conditions.

3) Evaluation of the Number of Platelets Adhering on the Vessel Wall

The number of platelets adhered on the vessel wall were counted using simulation results in the region of 400 μm × 100 μm for each second. Total number of adhered platelets after 2 minutes perfusion was calculated for comparison with biological experiments. All the simulations were conducted on K computer. (RIKEN, Kobe, Japan)

Results

1. Biological Experiments

Platelet adhesion increased with high hematocrit level regardless of the types of erythrocytes (Native, Fixed, and Artificial Erythrocyte)

To distinguish the biochemical and biophysical effect of erythrocytes on initial platelet adhesion, we carried out a series of experiments by perfusing blood on fixed VWF wall in the presence of various types of erythrocytes (native, chemically fixed, or artificial) at various hematocrit values. As shown in Fig. 1, number of platelets bound within the ROI (5 x $10^3/\mu m^2$) increased linearly with perfusion time in the presence of native erythrocytes at various hematocrit levels. Similar relationships were shown in the presence of fixed and artificial erythrocytes (Fig. 2 and Fig. 3). The rates of platelet adhesion were constantly greater at higher hematocrit conditions regardless of the type of erythrocytes used (native, chemically fixed, or artificial) (Table 1).

Fig. 4 shows the number of platelets adhered on VWF after 2 minutes perfusion in the presence of native, fixed, and artificial erythrocytes at a hematocrit of 24%. The number of platelet bound on VWF in the presence of native erythrocytes of 195.9±28.3/5x10^3 μm^2 was not different from 221.7±22.6/5x10^3 μm^2, and 208.0±26.5/5x10^3 μm^2 in the presence of fixed and artificial erythrocytes, respectively ($p>0.05$ for all comparison).

The regression co-efficient between the rates of platelet adhesion and the hematocrit levels with native, fixed and artificial erythrocytes were 0.992, 0.934, and 0.825, respectively (Fig. 5.)

2. Numerical Simulation

Numerical simulation of platelet adhesion to the vessel wall in the presence of erythrocytes at various hematocrits shows importance of z-axis fluctuation.

Next, to gain theoretical insight on our finding, we performed a computer simulation. Fig 6 and corresponding movie shows the distribution of erythrocytes (red vesicles) and platelet (yellow vesicles) in the simulation domain (x axis: 100 mm, y axis: 400 mm, and z-axis: 100 mm) with hematocrits of 20%. As an initial condition (Fig 6A), erythrocytes and platelets were distributed randomly and uniformly. When blood starts flowing, erythrocytes become deformed to the discoid shape in the flow direction (Fig 6B, 6C, and 6D). Erythrocytes move toward the center region of the channel due to Fåhræus–Lindqvist effect. [31]. The mean velocity of erythrocytes and platelets decreased in higher hematocrits in the fixed pressure gradient (Table 2). Platelets, of which volume is relatively smaller than an erythrocyte, move along interspaces of erythrocytes. When the distance between a platelet and –z wall becomes close, the bond between VWF and GPIba is formed and the platelet adheres to the wall as shown as blue dots in panel B, C, and D in Fig. 6.

Fig 7 shows time dependent increase in the numbers of platelets adhered on the vessel wall in the presence of erythrocytes at hematocrits of 0%, 10%, 20%, and 30%. Platelet adhesion rates in the presence of erythrocytes at hematocrits of 10, 20, and 30% were apparently higher in initial phase up to 60 ms. Then, the adhesion rates stabilized in each condition. The number of platelets adhered to the vessel wall increased with hematocrit values both in the initial phase and the stable late phase. Platelet adhesion rates at stabilized phase later than 60 ms, in conditions with hematocrit value of 10%, 20%, and 30%, were 94.3/sec, 185.2/sec, and 327.9/sec, respectively.

Number of platelet adhered on the vessel wall at 2 minutes perfusion in the presence of erythrocytes at 10, 20, 30% hematocrit were calculated as 8343, 22304, and 44088/per 400 μm × 100 μm ROI, respectively. It is of notes that we did not implement any chemical interactions in this simulator. Thus, the results further support the importance of physical roles of erythrocytes.

3. Combined Analysis

Direct comparison between biological experiments and computer simulation shows similarity.

Fig. 8 shows the direct comparison between number of platelets adhered with 5 x 10^3 μm^2 by 2 minutes perfusion of blood in the presence of various erythrocytes (native, fixed and artificial) at various hematocrit levels. Results with computer simulation calculation of the number of platelets adhered on VWF wall on 5 x 10^3 μm^2 by 2 minutes perfusion of 96, 258, and 510/per 5 x 10^3 μm^2 in the presence of 10, 20, and 30% hematocrits of erythrocytes, are also shown. Hematocrit dependent increase in the number of platelets in the area of 5 x 10^3 μm^2 were comparable in biological experiments and computer simulation with various erythrocytes condition. Regression analysis revealed that the number of platelets bound within 5 x 10^3 μm^2 with 2 minutes perfusions were expressed as 4.88 x hematocrit values ($r=0.992$, $p=0.0016$) in the presence of native erythrocytes and 15.68 x hematocrit values ($r=0.988$, $p=0.001$) in computer simulation.

Discussion

Here, we show in both, biological experiments and computer simulation, that platelet adhesion on VWF under a blood flow condition increase depending on hematocrit levels. Our experimental results suggest that influences of erythrocytes on platelet adhesion are not depend on their bio-chemical function, but on physical presence. Computer simulation solving Navier-Stokes equations coupled with fluid-structure interaction provided similar hematocrit-dependent increase in the rate of platelet adhesion gave theoretical support on our biological experimental findings. It suggests the importance of the near-wall access of platelets by central migration of erythrocytes in flowing blood which was established by Fahraeus R in early 20th century. [32, 33]

The most important biological role of erythrocytes is to transport oxygen from lung to peripheral organ. Erythrocytes themselves have active metabolic activities. [34] The cellular energy metabolisms are mediated by substances known to influence platelet activation such as adenosin 5'-tri and di phosphate. (ATP and ADP).[35-37] ADP is known as strong activator for platelets. [38, 39] There are several previous publications demonstrating the regulatory role of erythrocytes for platelet activation.[19, 24] However, our results suggest that bio-chemical role of erythrocytes on platelet adhesion rate under blood flow condition is limited as compared to their bio-physical roles.

Our results assessing the effect of erythrocytes on platelet adhesion here is focusing only for platelet adhesion under shear rate of 1,500 s^{-1}. Previous publication demonstrated that the binding between

activated GPIIb/IIIa and fibrinogen/VWF occurred only under wall shear rate lower than 750 s^{-1}. Platelet may still be activated by ADP released from erythrocytes, [35] but the rate of platelet adhesion was not influenced in this experimental condition. Our results can be interpreted as focusing only on the initial phase of platelet adhesion occurring within 2 minutes of blood perfusion. Thus, our present results are not conflicting with the previous publication indicating the important role of ADP for stabilization of platelet adhesion and thrombi because the previous ones focus more about later phase of blood perfusion. [4 17]

In the parallel plate flow chamber, blood flow between 2-glass plates is assumed to follow Hagen–Poiseuille equation. [40, 41] The fact of axial accumulation of erythrocytes in the flow chamber were previously demonstrated. [42] Yet, the relationship between axial accumulation of erythrocytes and the rate of platelet adhesion at the wall has yet to be clarified. Here computer simulation with the use of full Eulerian Fluid-Structure Inter-action (FSI) solver [43 44] showed increased wall-normal fluctuations of flowing platelets induced by axially accumulated erythrocytes as the cause of platelet adhesion to the wall. All the basic equations to solve the structure and position of erythrocytes and platelet under blood flow condition was simulated by finite difference volume-of-fluid scheme with fractional step algorithm by high performance computer K. Indeed, both fluid-structure and fluid-membrane interaction were handled with Eulerian numerical approach. [44 44] Biological validity of this model was confirmed by shape changes in flowing erythrocytes in capillary circulation in mice. [45] As confirmed by simulation results shown in this paper, higher platelet adhesion rates in the presence of erythrocytes at higher hematocrits should reflect increased grade of z-axis fluctuation of platelet present close to the VWF wall.

There are a few methodological limitations on this study. First, biological experiments were conducted only at one wall shear rate condition of 1,500 s^{-1}. The impact of the presence of erythrocytes on the rates of platelet adhesion at the wall may differ at other wall shear rate conditions. Second, the number of platelets were counted manually, but not automatically. We might have missed some platelet that once bound on VWF but did not stay for a long period. However, these limitations do not influence our main results of higher rate of platelet adhesion in the presence of higher concentration of erythrocytes.

In conclusion, we show here the importance of the physical effect of erythrocyte for platelet adhesion rates on the wall under blood flow condition. The increased platelet adhesion rates with higher hematocrits could be explained by axial accumulation of erythrocytes and increased z-axis fluctuation of platelet close to vessel wall.

Declarations

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Tables

Table 1. Rates of Platelet Adhesion on VWF Wall (/5x10^3/μm^2/min) Calculated from Regression Analysis Shown in Fig. 1, 2, and 3.

Hematocrit (%)	Erythrocytes (r=0.99)	Biochemically Fixed Erythrocytes (r=0.93)	Artificial Erythrocyte Mimetic (r=0.83)
4	7.2	8.9	10.5
8	21.4	30.1	38.0
12	55.0	64.1	53.4
24	108.3	129.9	118.2
36	156.8	120.7	82.5

Table 2. Mean Velocity and Apparent Relative Viscocity of the Fluids in the Presence of Erythrocytes at Various Hematocrits.

Ht [%]	Red blood cell		Undeformable sphere	
	Mean velocity [cm/s]	Apparent relative viscosity	Mean velocity [cm/s]	Apparent relative viscosity
0	2.50	1.00	2.50	1.00
10	2.02	1.24	1.56	1.60
20	1.83	1.37	1.16	2.15
30	1.49	1.68	0.729	3.42

Figures

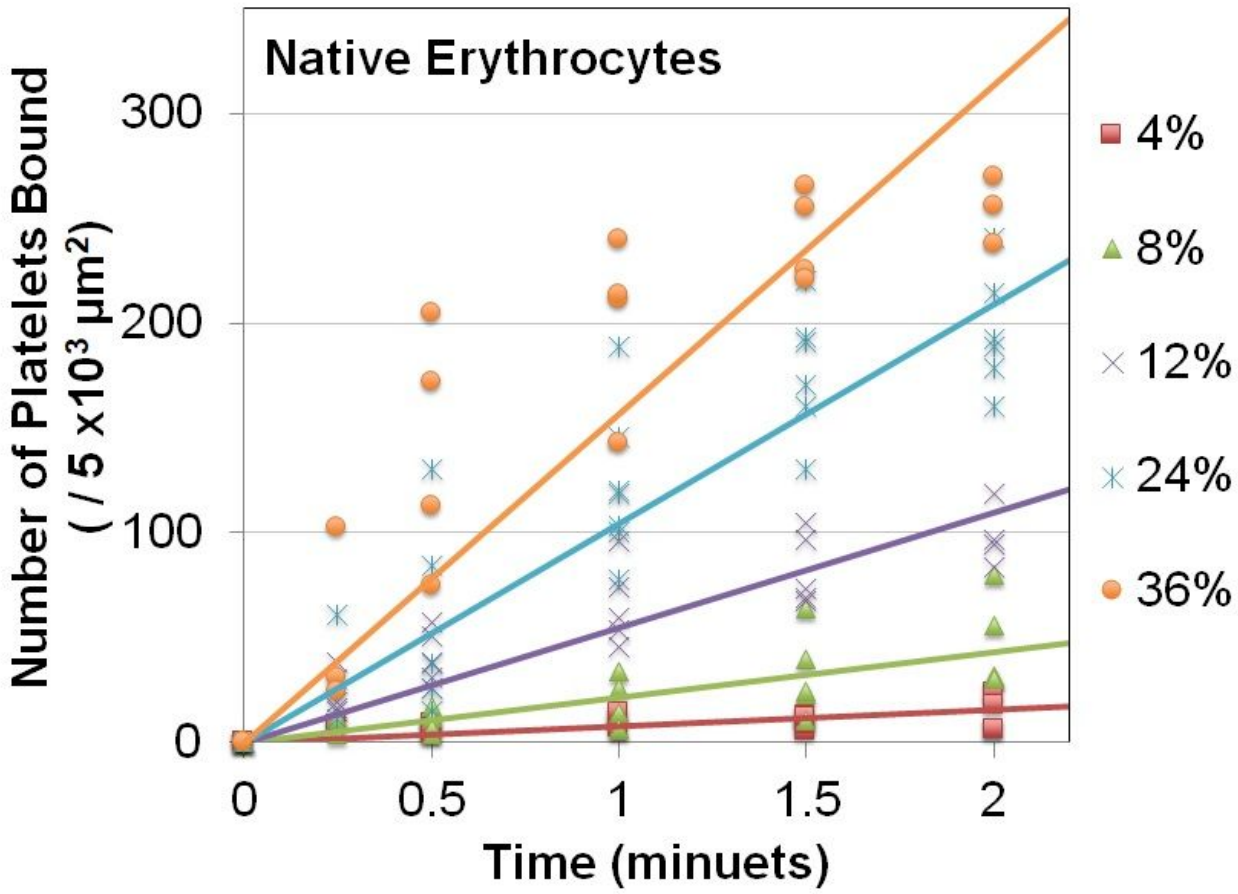

Figure 1

The Numbers of Platelets Adhered within 5x103 µm2 of VWF Wall in the Presence of Native Erythrocytes at Various Hematocrits. Numbers of platelet adhered on the 5 x 103/µm2 area of VWF wall, counted in each 30 second (0.5 minute), using reconstituted blood containing native erythrocytes at hematocrits of 4, 8, 12, 24, and 36%, at platelet concentration of 200 x103/µL of native platelet. The solid line represents the linear regression.

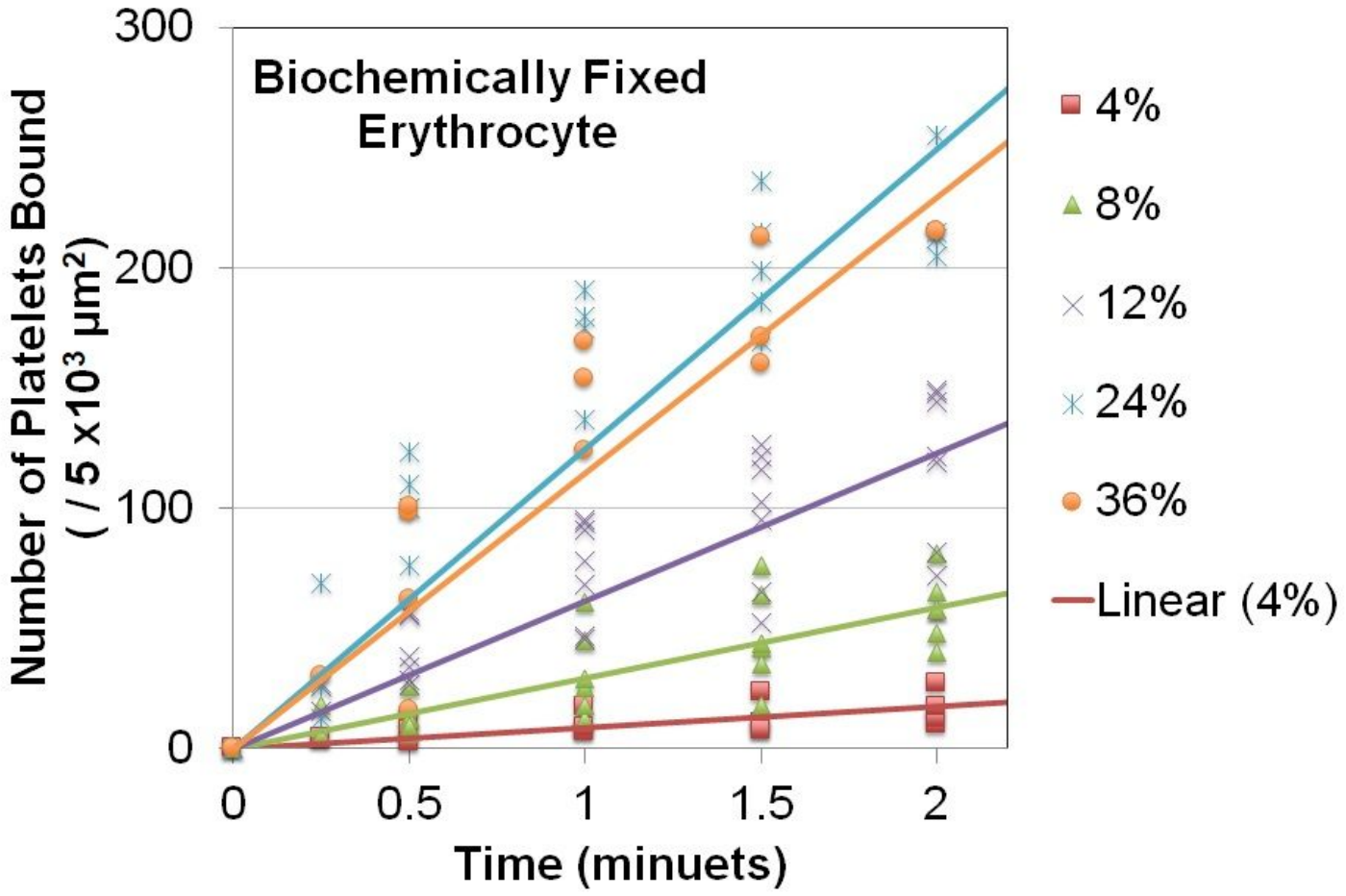

Figure 2

The Numbers of Platelets Adhered within 5x103 µm2 of VWF Wall in the Presence of Bio-Chemically Fixed Erythrocytes at Various Hematocrits. Numbers of platelet adhered on the 5 x 103/µm2 area of VWF wall, counted in each 30 second (0.5 minute), using reconstituted blood containing biochemically fixed erythrocytes at hematocrits of 4, 8, 12, 24, and 36%, at platelet concentration of 200 x103/µL of native platelet. The solid line represents the linear regression.

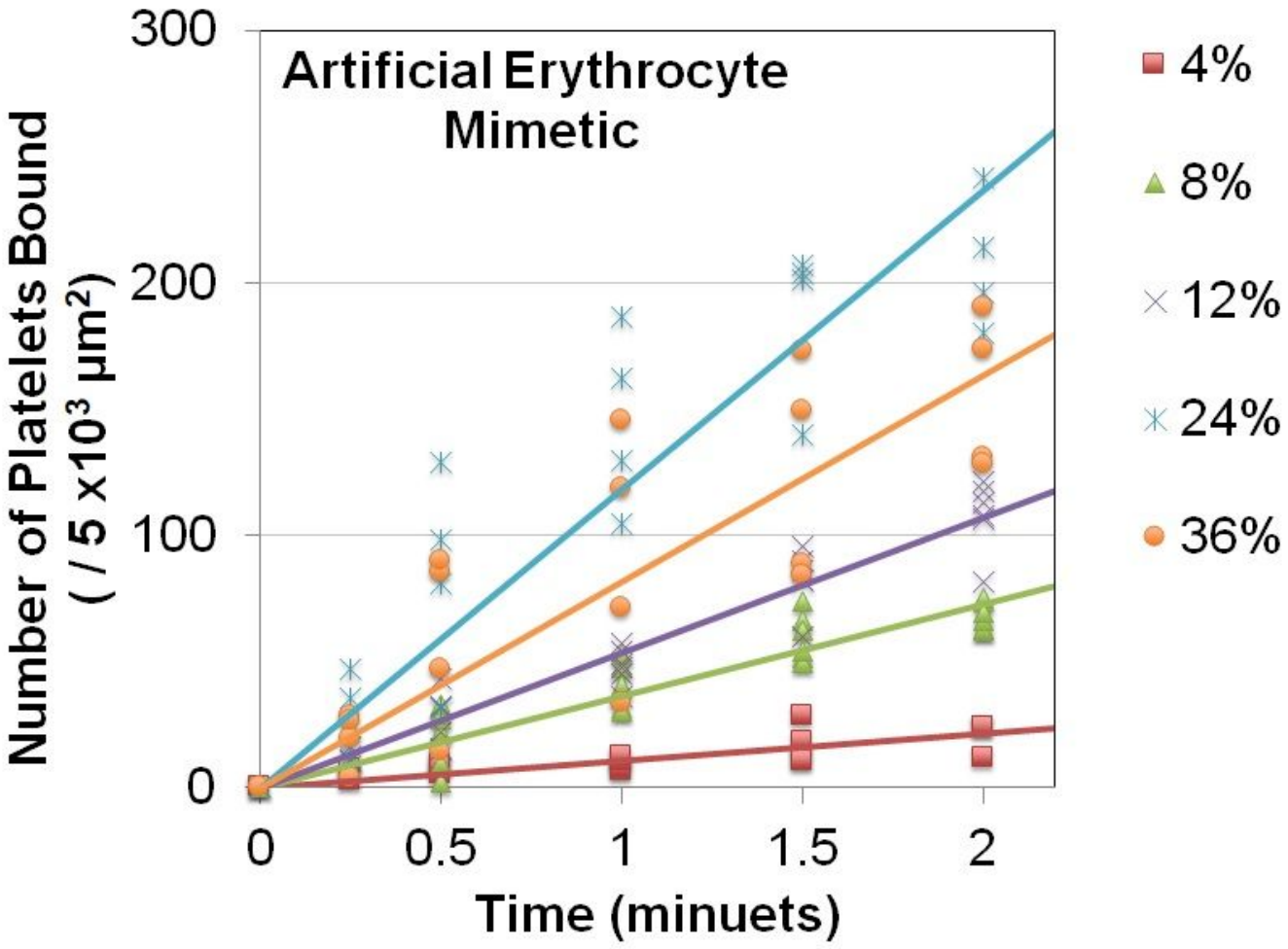

Figure 3

The Numbers of Platelets Adhered within 5x103 µm2 of VWF Wall in the Presence of Artificial Erythrocytes at Various Hematocrits. Numbers of platelet adhered on the 5 x 103/µm2 area of VWF wall, counted in each 30 second (0.5 minute), using reconstituted blood containing artificial erythrocyte at hematocrits of 4, 8, 12, 24, and 36%, at platelet concentration of 200 x103/µL of native platelet. The solid line represents the linear regression.

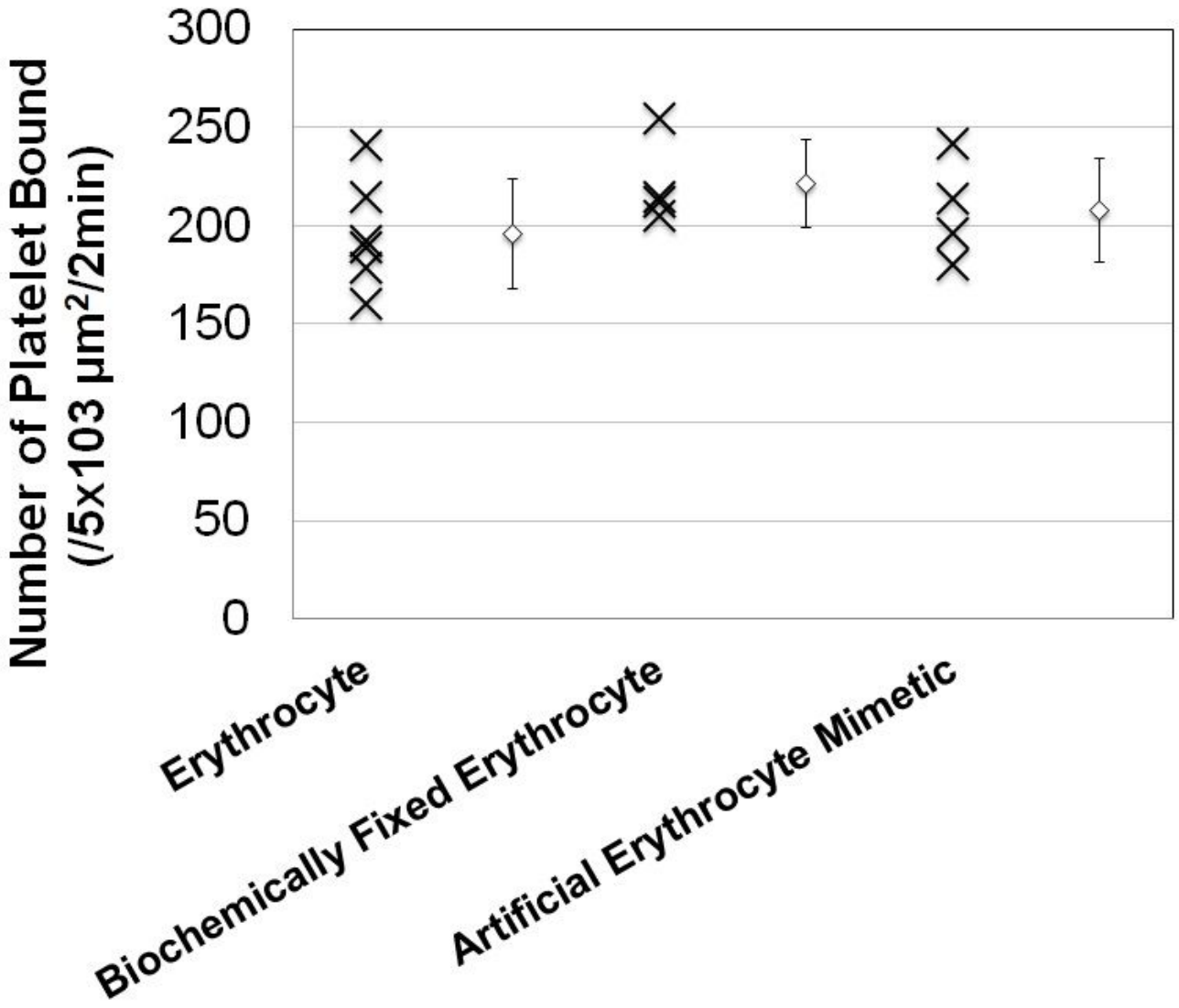

Figure 4

Numbers of Platelet Adhered within 5x103 μm2 of VWF Wall at 2 Minutes Perfusion of Reconstitute Blood Including Native, Fixed, or Artificial Erythrocytes at Hematocrit of 24%. Numbers of platelets adhered on 5x103 μm2 area of VWF wall at 2 minutes perfusion in the presence of native, fixed and artificial erythrocyte at hematocrit of 24%. Each x shows the actual measured value. Each indicate mean value. The lines indicate standard deviation.

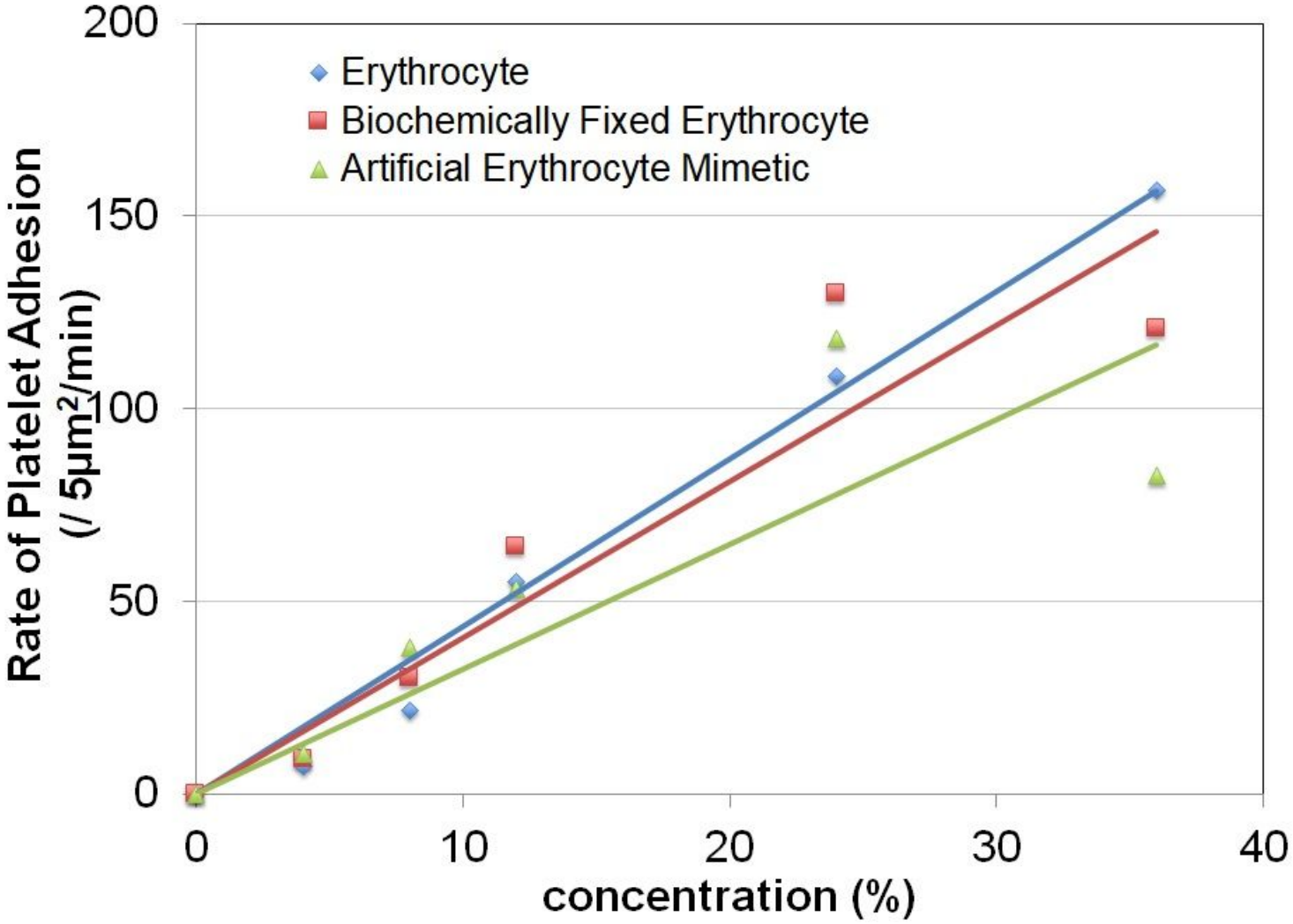

Figure 5

The Relationships between the Hematocrits of Native, Fixed, and Artificial Erythrocyte and the Rate of Platelet Adhesion at VWF Wall.

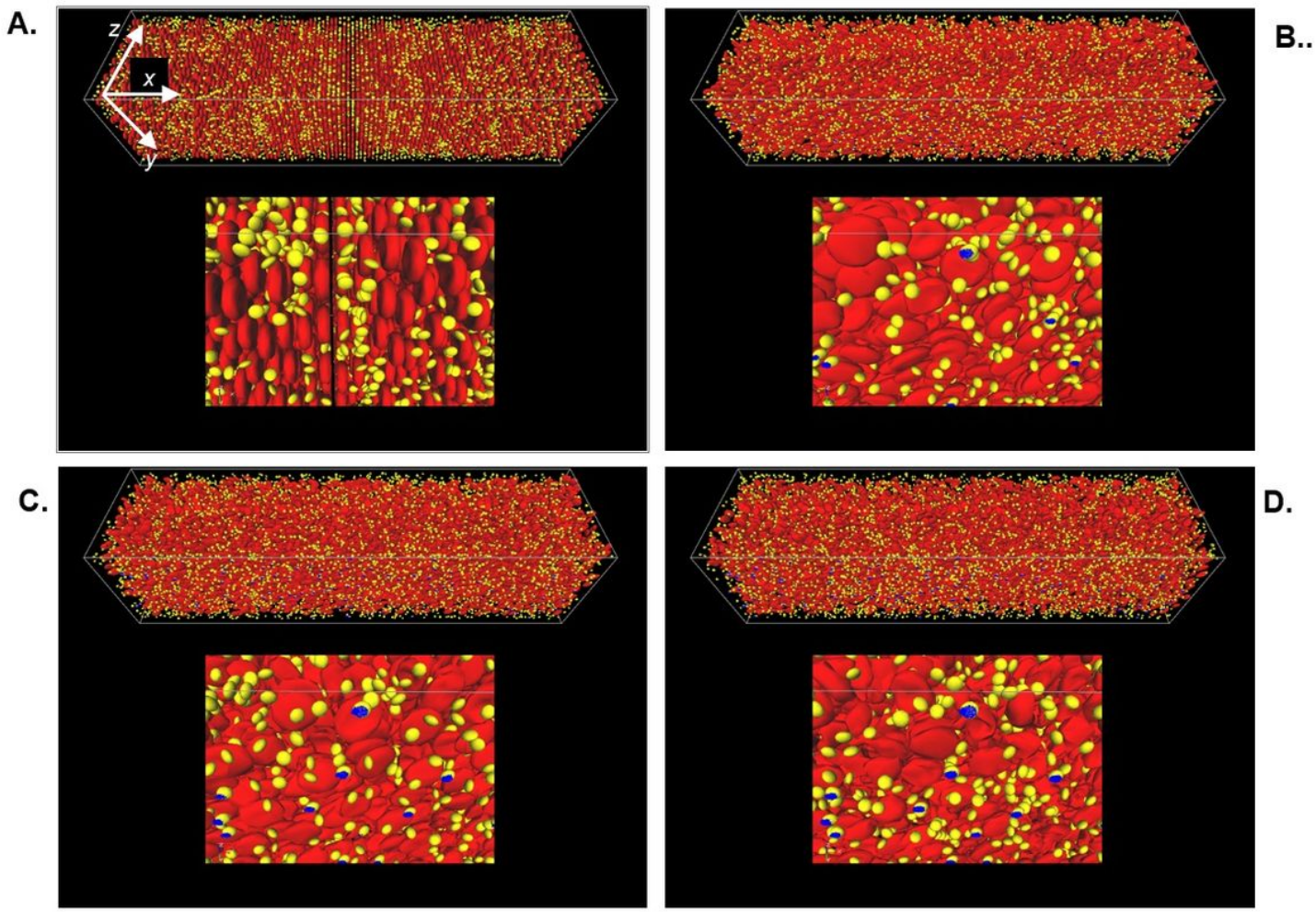

Figure 6

Results of Computer Simulation of the Positions of Erythrocytes and Platelets in the Presence and Absence of Blood Flow. Representative visualization of erythrocytes (red particles) and platelets (yellow particles) distribution at hematocrit 20% before starting blood flow (A), at 3 seconds blood perfusion (B), 6 seconds blood perfusion (6) and 12 seconds blood perfusion (D). The bonds between platelet GPIba and VWF is shown in blue.

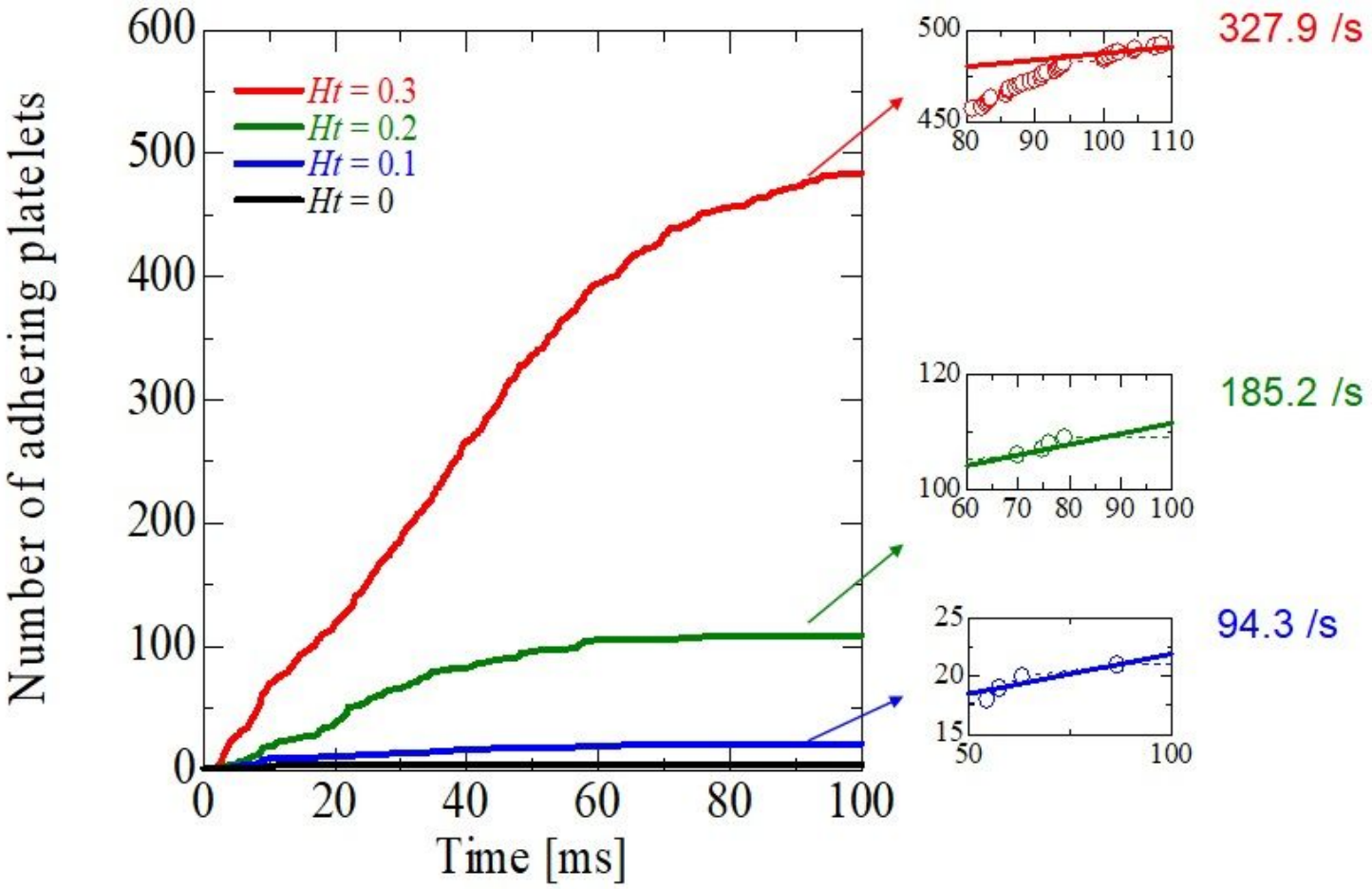

Figure 7

The Numbers of Platelets Adhered within Calculated Domain in the Presence of Erythrocytes at Various Hematocrits Time-dependent changes in the number of platelets adhere on VWF wall, calculated by numerical simulation at hematocrits of 10, 20, 30%.

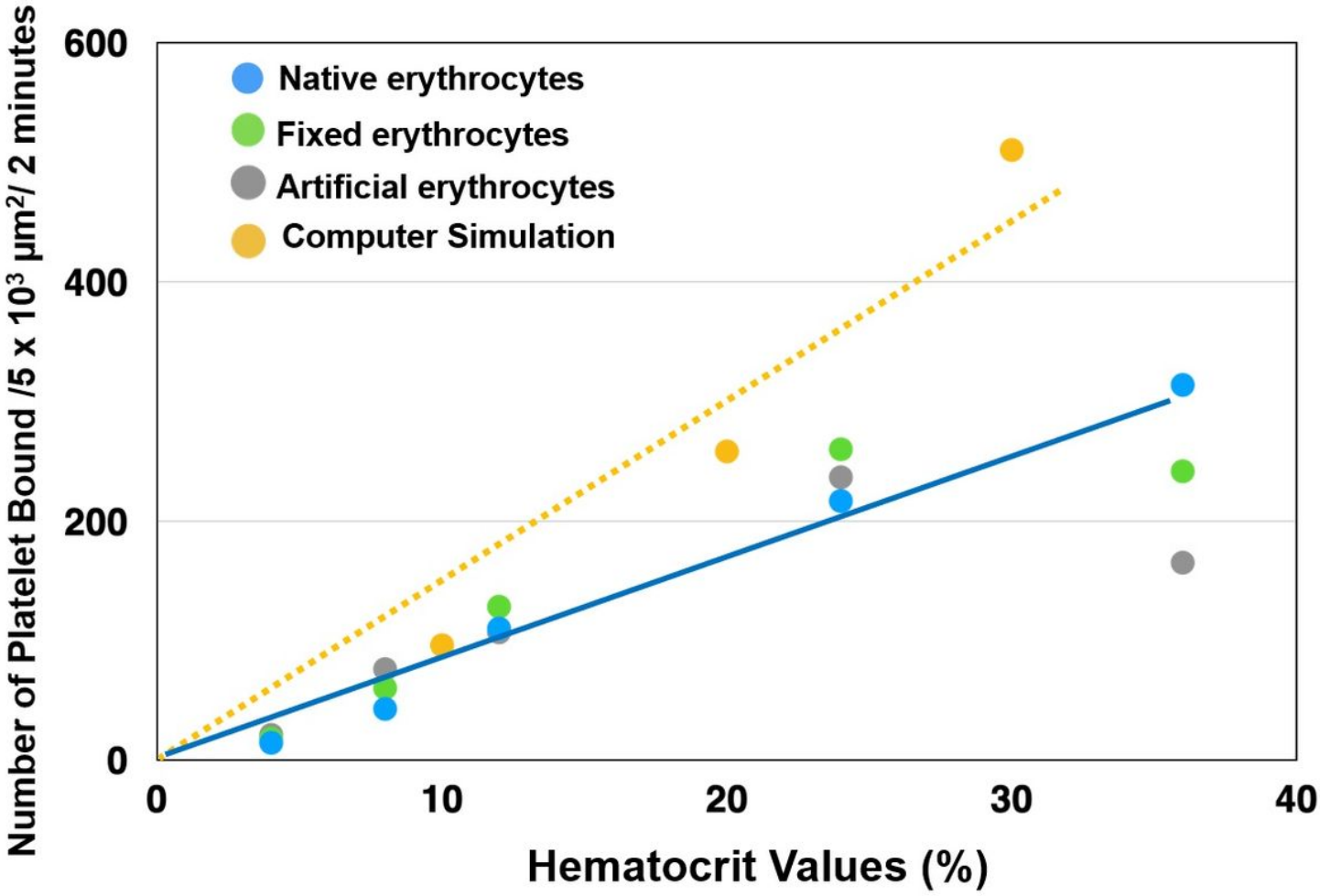

Figure 8

Direct Comparison between Biological Experiments and Computer Simulation The number of platelets adhered within 5x103 µm2 area of VWF wall after 2 minutes blood perfusion in biological experiments and computer simulation. The solid blue line represent the regression line demonstrating the relationship between hematocrit values and number of platelet bounds in the presence of native erythrocytes. Yellow dotted line represents the regression results between hematocrit and the number of platelets bound in the computer simulation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- erythrocytes.mov