Functional enhancement of platelet activation and aggregation by erythrocytes: role of red cells in thrombosis

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Abstract

Platelets expose phosphatidylserine (PS), a component of the prothrombinase complex, on the outer surface of the plasma membrane when activated.[1] The prothrombinase complex catalyzes the conversion of prothrombin to thrombin, and it has been demonstrated that an increase in PS exposure is correlated with an increase in thrombin generation by platelets.[2,3] Similarly, erythrocyte (RBC) activation, or eryptosis, is also characterized by PS exposure on the plasma membrane.[4] Although PS exposure on RBCs is considered a signal for splenic macrophage destruction, eryptosis may allow RBCs to contribute to thrombosis.[4] The aims of this study were to determine whether the addition of RBCs to platelets increased functional platelet aggregation and coagulation properties. A ratio of 4 RBCs to 1 platelet (4:1) was evaluated for aggregation and coagulation compared to platelet control. Platelet aggregation and coagulation properties were evaluated with impedance aggregometry and thromboelastography, respectively. The 4:1 experimental group had significant increases in aggregation and coagulation relative to the platelet control. These results indicate that RBCs increase platelet aggregation and coagulation properties. This suggests that RBCs play a role in diseases traditionally thought of as associated solely via dysregulated platelet activation.

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Introduction

Thrombotic diseases are among the leading causes of death in the United States each year.[5] Increased platelet activation can lead to thrombus formation and occlusion of organs. Of major concern is the coronary or cerebral vasculature, resulting in myocardial infarction or ischemic stroke. Coronary heart disease is the leading cause of death, affecting 1.2 million Americans each year, while stroke, the third leading cause of death, affects approximately 700,000 Americans each year.[6]

Activated platelets are the cells that are conventionally implicated in pathological thrombus formation. Under basal conditions, platelets circulate in an inactivated state characterized by membrane phospholipid asymmetry such that the phospholipid phosphatidylserine (PS) is contained within the inner membrane leaflet. Damage to the endothelium exposes subendothelial substances, such as collagen and tissue factor, which promotes platelet activation and adherence to the damaged endothelium. Platelet activation is characterized by pseudopod formation, exocytosis of cytosolic granules, and exposure of the anionic PS on the outer surface of the plasma membrane. PS exposure forms a procoagulant surface, which allows the assembly of the prothrombinase complex (factors Xa, Va, and calcium). This complex is responsible for catalyzing the conversion of prothrombin to thrombin. Thrombin is a serine protease that converts fibrinogen to fibrin, which further promotes platelet activation. Fibrin contributes to the strength of the thrombus by forming a meshwork around the adhered platelets and other cells passively trapped in the developing clot. It has been demonstrated that an increase in PS exposure is strongly correlated with an increase in thrombin generation.[2,3] In addition to elevated thrombin production, PS exposure has been found to increase platelet adherence to the endothelial membrane, further facilitating thrombus formation.[7] While thrombin may be temporarily elevated during hemostasis, basally elevated thrombin activity is observed in prothrombotic diseases, such as type 2 diabetes, uremia, and sepsis.[2,8,9]

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Although not as well studied as platelet activation, erythrocyte (RBC) activation, or eryptosis, is characterized by PS exposure on the outer plasma membrane.[4] Although PS exposure on RBCs during senescence serves as a signal for splenic macrophage destruction, eryptosis may allow RBCs to participate in thrombosis.[4] A significant correlation has been identified between erythrocyte PS exposure and plasma thrombin levels.[8] PS-exposing erythrocytes have been implicated in several procoagulant hematological diseases, including sepsis, sickle cell anemia, hereditary hydrocytosis, and β-thalassemia.[10-13] PS exposure has also been demonstrated to facilitate erythrocyte adhesion to endothelium, further contributing to thrombus formation.[7] Similarly, impaired erythrocyte senescence in blood bank storage has been shown to facilitate circulatory disorders by an increased adherence to endothelial cells (13).[14] In fact, about 0.5% of the circulating erythrocyte population expresses PS.[15] The active role of RBCs in thrombosis may be implicated in procoagulatory disease states and thus the purpose of this study was to determine if erythrocyte-mediated platelet activation plays an active role in increased aggregation and coagulation.

Methods

Animals

Male Sprague-Dawley retired breeders (300-400g) were used in this study. All experiments were conducted according to the guidelines issued by the Institutional Animal Care and Use Committee and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals. University of Arizona Animal Care and Use Committee, under Protocol 08-105 (Novel Use of a Natural Product for Acute Stroke Therapy).

Cardiac puncture

Male Sprague Dawley rats (N=9) were deeply anesthetized in an induction chamber with 5.0% isoflurane and 1.0 L/minute oxygen. To administer anesthetic gas continuously throughout the procedure, rats were removed from the induction chamber and placed on an isoflurane face mask with 3-5% isoflurane and 1 L/minute oxygen. Cardiac puncture was

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performed by direct insertion of an eighteen gauge needle attached to a 20 ml citrated syringe (4% solution at 1:9 ratio of citrate:blood, Sigma Aldrich) into the left ventricle following a method established in our laboratory.[16,17] Blood was drawn slowly to prevent platelet activation and clot formation.

Dilution preparation

A general complete blood count (CBC) was performed to determine the initial number of platelets and eythrocytes (Beckman Coulter). Blood was centrifuged at 150 x g for 18 minutes at 22°C to obtain platelet-rich plasma (PRP). PRP and concentrated RBCs were isolated and analyzed for cell count. PRP was diluted with phosphate buffered saline (PBS) to obtain a final dilution of 400 x 10^3 platelets/µl. RBCs were diluted with PBS to obtain a final RBC:platelet ratio of 4:1. A final cell count was performed to confirm the dilution accuracy.

Impedance Aggregometry

Platelet aggregation was determined using impedance aggregometry. Control samples were prepared by combining five hundred microliters of PRP with 500 microliters of PBS in an aggregometer cuvette (Whole Blood Aggregometer, Chrono-log). The experimental cuvettes contained five hundred microliters of ratio samples combined with 500 microliters of PBS. Samples were warmed in incubation wells at 37 °C for five minutes, and mixed continuously with siliconized stirbars at 900 rpm, according to the Born method.[18] The aggregometer was calibrated by setting impedance traces for PRP and RBCs to 0% aggregation. 20 µl of Calcium chloride (0.2M, Haemoscope) were introduced to each sample to counteract the calcium chelating properties of sodium citrate. Collagen (8 µg/ml, Chronolog) was then added to activate the samples. Aggregation traces were recorded for six and ten minutes following addition of collagen, and the data were analyzed using AGGROlink software. Probes were thoroughly cleaned before introducing the subsequent sample into the incubation wells.

Thromboelastography (TEG)

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Thromboelastography assays were performed using a computerized TEG® coagulation analyzer (Model 5000, Haemoscope Corp., Niles, IL). All analyses were performed with TEG® disposable cups and pins as devised by the manufacturer. Polypropylene and polyethylene pipettes were used to handle reagents and blood. TEG® analyses were performed in PRP and 4:1 (RBC:platelet). For all TEG® analyses, 360 µL of sample was pipette into the prewarmed TEG® cup. Twenty µL of 200-mM calcium chloride was pipette into the prewarmed TEG® cup.

The following TEG® variables were recorded: the maximal amplitude (MA, mm), which is a measurement of maximal strength or stiffness of the developed clot; and the shear elastic modulus strength (SEMS or G, dynes/cm^2), which is a parametric measure of clot firmness expressed in metric units calculated from MA as follows: G = (5000 x MA)/(100 – MA). Furthermore, we calculated the coagulation index (CI), which is an overall measurement of coagulation, using the following equation: CI = (-0.1227 R + 0.00092 K) + (0.1655 MA – 0.0241 α) – 5.022.

Data analysis

Data were collected and stored on spreadsheets (Microsoft Excel). Platelet aggregation and coagulation properties were compared between the control and 4:1 groups with a paired T-test. Summary data were expressed as mean ± SEM. $P \leq 0.05$ was considered statistically significant.

Results

Platelet aggregation

Collagen-stimulated blood was evaluated by impedance aggregometry to measure platelet aggregation. The experimental sample containing 4:1 (RBC:platelets) demonstrated a significant increase in platelet aggregation relative to PRP control. Figure 1A is a representative trace of platelet aggregation in control PRP as well as the 4:1 group. The maximal extent of aggregation is determined by measuring the amplitude of the aggregation curve. The

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amplitude was significantly increased in the 4:1 group compared to PRP control (17.17 ± 2.6Ω vs. 12 ± 1.83Ω; P < 0.005) at six minutes following addition of collagen agonist (Figure 1B). The 4:1 group maintained this increase in aggregation to ten minutes. The amplitude was significantly higher in the 4:1 group compared to the control (20.17 ± 2.73Ω vs. 14 ± 2Ω; p < 0.001) at ten minutes following agonist addition (Figure 1C).

Coagulation properties

Calcium chloride-stimulated blood was evaluated by thromboelastography to quantify coagulation properties. Figure 1A shows a standard representative TEG curve comparing 4:1 to PRP. Maximum amplitude (MA) represents fibrin and platelet aggregation. MA was significantly elevated in the 4:1 (RBC:platelet) group relative to the PRP control (58.52 ± 1.99 mm vs. 52.16 ± 2.79 mm; P < 0.01), as represented in Figure 2B. The clot strength at a standardized time point is described by the A parameter, which was significantly increased in the 4:1 group compared to the PRP control (59.17 ± 2.08 mm vs. 52.54 ± 2.98 mm; P < 0.01; Figure 2C). The A parameter can be transformed into the shear elastic modulus strength (G parameter) for an actual measurement of clot strength. Figure 2D demonstrates that the G parameter was significantly elevated in the 4:1 group relative to the PRP control (7.18 ± 0.56 dyn/cm^2 vs. 5.67 ± 0.6 dyn/cm^2; P < 0.001). The coagulation index (CI) is derived from four TEG parameters to describe the overall coagulation of the sample. The CI was significantly increased in the 4:1 group compared to the PRP control (1.73 ± 0.32 vs. 0.66 ± 0.42; P < 0.01; Figure 2E).

Discussion

Traditionally, erythrocytes are considered oxygen transporters, however, this study suggests that erythrocytes enhance functional coagulation properties and platelet aggregation. Phosphatidylserine exposure on the erythrocyte outer membrane is proposed to be the mechanism of increased thrombosis, via erythrocyte facilitated thrombin production and

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adhesion to the endothelium.[19] Erythocytes have been demonstrated to play a role in coagulation based on RBC number (hematocrit), deformability, and shear rates.[20-22] We show in this study that at a ratio of 4 RBCs to 1 platelet is sufficient to increase functional measurements of aggregation and coagulation. Physiologic concentrations of erythrocytes to platelets are closer to 12:1, suggesting that the contribution of RBCs to coagulation and aggregation may be even greater in-vivo.

Under basal conditions, erythrocyte membrane phospholipid asymmetry is maintained by three integral membrane transporters: translocase, floppase, and scramblase.[19,23] Translocase is an ATP-dependent transporter that generates and maintains membrane phospholipid asymmetry by transporting phospholipids from the outer to inner membrane.[19] Floppase and scramblase are ATP and calcium dependent transporters, respectively, that transport phospholipids from the inner to outer membrane.[19] Eryptosis is characterized by the structural loss of membrane phospholipid asymmetry and translocation of PS to the outer membrane leaflet of the erythrocyte as a result of increased scramblase activity. While PS exposure targets the cell for degradation by splenic macrophages, it may also have procoagulant implications. Similar to PS exposure on activated platelets, PS translocation to the external membrane leaflet may facilitate the assembly of the prothrombinase complex on the surface of the erythrocyte and result in increased thrombin formation.[24] Thrombin is a potent platelet activator; hence eryptotic cells may not only increase intravascular thrombin levels, but enhance platelet activation. We found an increase in coagulation in erythrocyte-enhanced platelets compared to the platelet control, as evidenced by the maximal amplitude, clot strength, and overall coagulation index. While not mechanistically characterized in the present study, the augmented coagulation in the presence of erythrocytes supports the theory of their procoagulant role in thrombosis.

In addition to increased coagulation, we found an increase in platelet aggregation in the presence of erythrocytes. Previous studies have found correlation between thrombin levels and erythrocytes.[8] Thrombin facilitates the conversion of fibrinogen to fibrin, which forms a mesh-like web around a developing clot. Platelet aggregation is mediated by glycoproteins on the membrane surface, and is reinforced by fibrin. The increased aggregation may be a result

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of this downstream interaction. PS-exposing erythrocytes further contribute to hemostasis by adhering to receptors on the endothelial membrane.[7,20,25,26] Diseases resulting in vascular complications, such as uremia, sickle cell anemia, and malaria, are characterized by PS exposure on erythrocytes.[8,27,28] Increased adhesion may lead to occlusion of microvasculature and atherosclerosis.[16]

Antithrombotic therapy, such as salicylic acid (aspirin; ASA), is widely used to inhibit platelet activation and prevent thrombus formation in patients with prothrombotic vascular diseases. ASA therapy reduces the outcome of serious thrombotic events in approximately 25% of high risk patients.[29] Furthermore, ASA administration has been shown to reduce stroke and myocardial infarction severity.[30,31] However, individuals that experience recurrent thrombotic events while undergoing aspirin therapy may be classified as "aspirin resistant". ASA resistance is thought to be caused by the inability of ASA to exert inhibitory effects on platelet activation.[5] The exact mechanism of ASA resistance is unknown, though blood samples of patients undergoing ASA therapy were consistently found to have enhanced platelet activation and recruitment in the presence of erythrocytes compared to platelets alone.[32] Similarly, we observed an increase in coagulation and platelet aggregation when platelets were enhanced with RBCs.

While eryptosis is an important physiological method for removal of defective or senescent erythrocytes from circulation, it may also be involved in thrombosis. The findings of this study and others suggest that PS-exposing erythrocytes may enhance coagulation and platelet aggregation. Substances such as nitric oxide and erythropoietin have been shown to inhibit eryptosis, which may one day have clinical significance.[33] However, erythrocyte and platelet interactions must be investigated further to determine the exact mechanism and consequences of erythrocyte-mediated platelet activation.

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Figure Legends

Figure 1: Platelet aggregation is significantly increased in the presence of red blood cells.

A. Representative aggregation curves displaying the percent platelet aggregation as a function of time. The red curve represents the control sample containing PRP, while the blue curve represents the 4:1 (RBC:platelet) experimental group. Collagen agonist was added at 2 minutes.

B. Each line represents PRP and 4:1 measurements taken from one animal (N=6). The 4:1 group had a significant increase in aggregation relative to PRP control, as measured by the amplitude of the curve at six minutes ($P<0.005$).

C. Each line represents PRP and 4:1 measurements taken from one animal (N=6). The 4:1 group had a further significant increase in aggregation as compared to PRP control, as measured by the amplitude of the curve at ten minutes ($P<0.001$).

Figure 2: Coagulation properties are significantly increased in the presence of red blood cells.

A. A representative TEG curve with labeled parameters. The inner curve represents PRP clot formation, while the outer curve represents 4:1 clot formation.

B. Each line represents PRP and 4:1 measurements taken from one animal (N=6). Fibrin and platelet aggregation is represented by the MA value. MA was significantly increased in the 4:1 measurements relative to the PRP control ($P<0.01$).

C. Each line represents PRP and 4:1 measurements taken from one animal (N=6). The A describes the clot strength at a standardized time point. The A value was significantly elevated in the 4:1 group as compared to the PRP control ($P<0.01$).

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D. Each line represents PRP and 4:1 measurements taken from one animal (N=6). The G significantly increased for the 4:1 measurements relative to the PRP control, which represents the clot firmness ($P<0.001$).

E. Each line represents PRP and 4:1 measurements taken from one animal (N=6). The CI value significantly increased in the 4:1 measurements relative to the PRP control, which is a descriptor of overall coagulation ($P<0.01$).

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Figures

Figure 1A

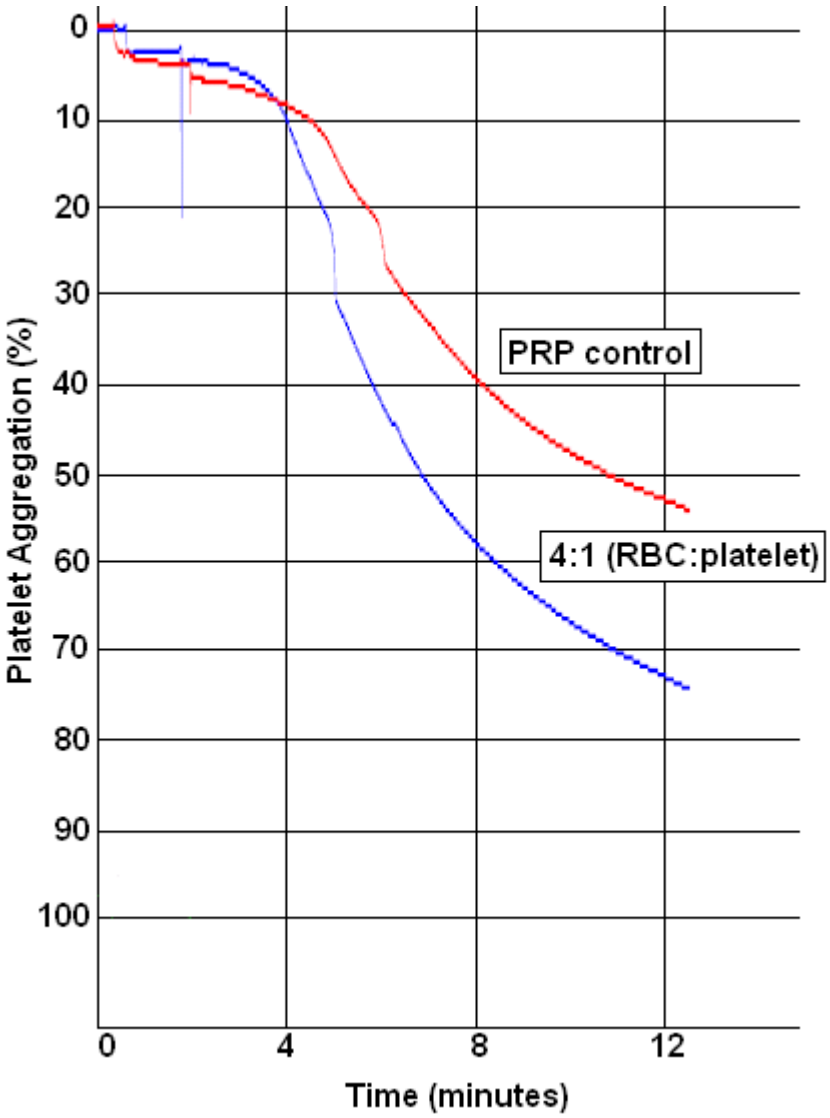

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Figure 1B

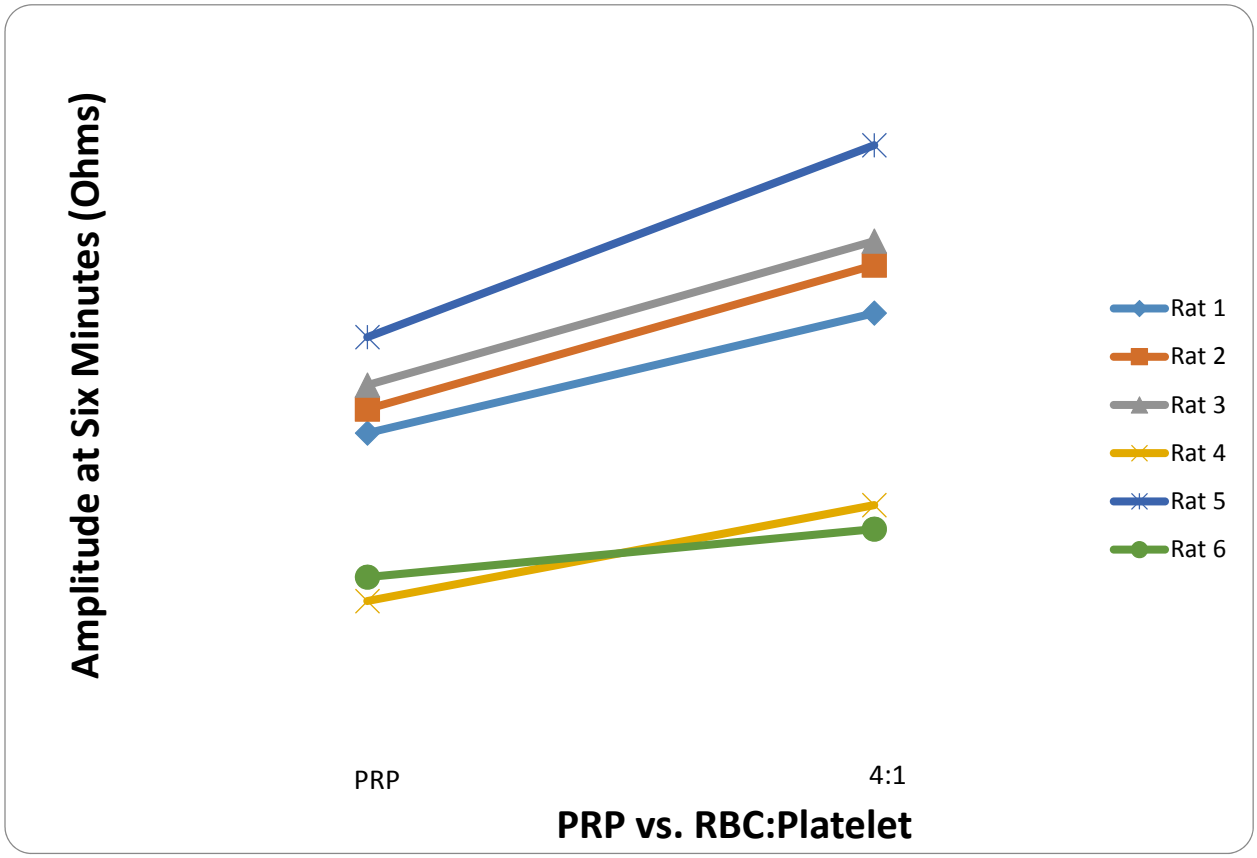

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Figure 1C

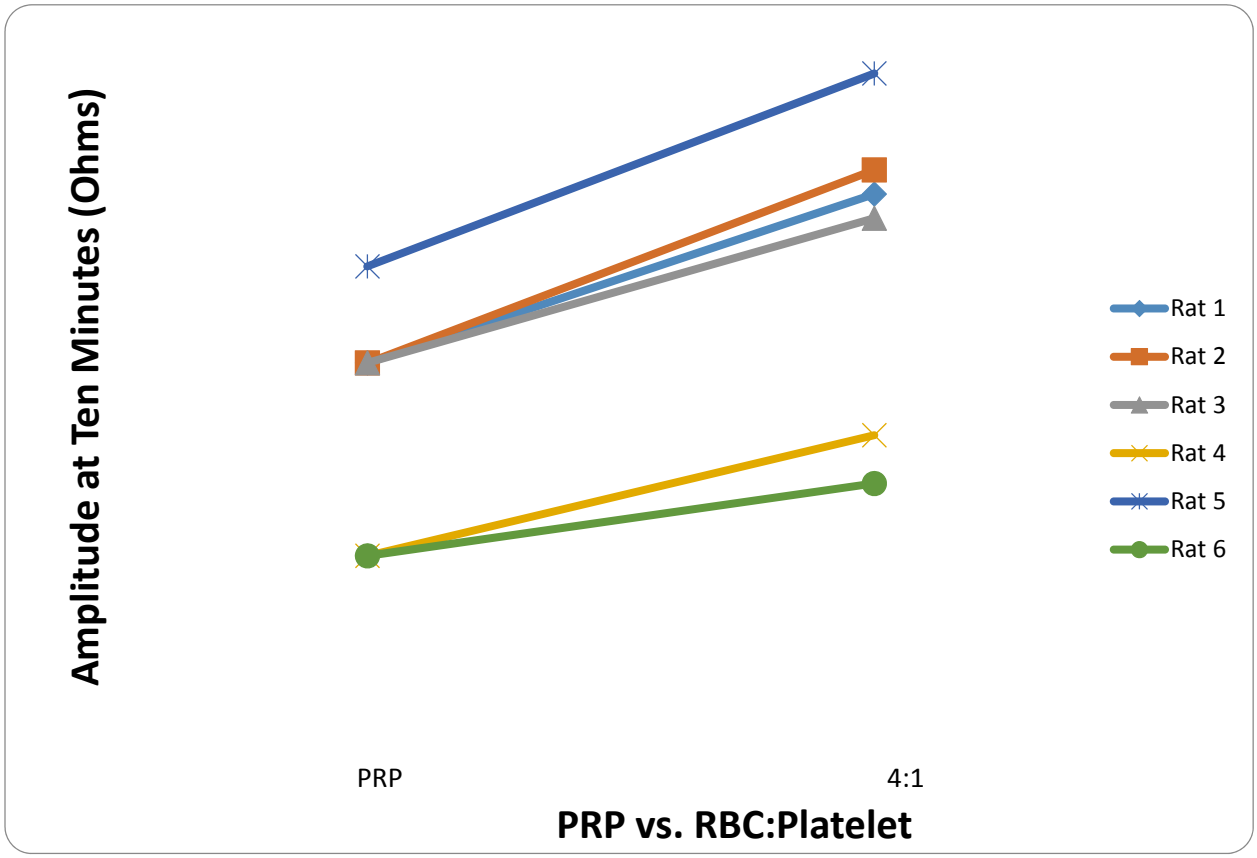

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Figure 2A

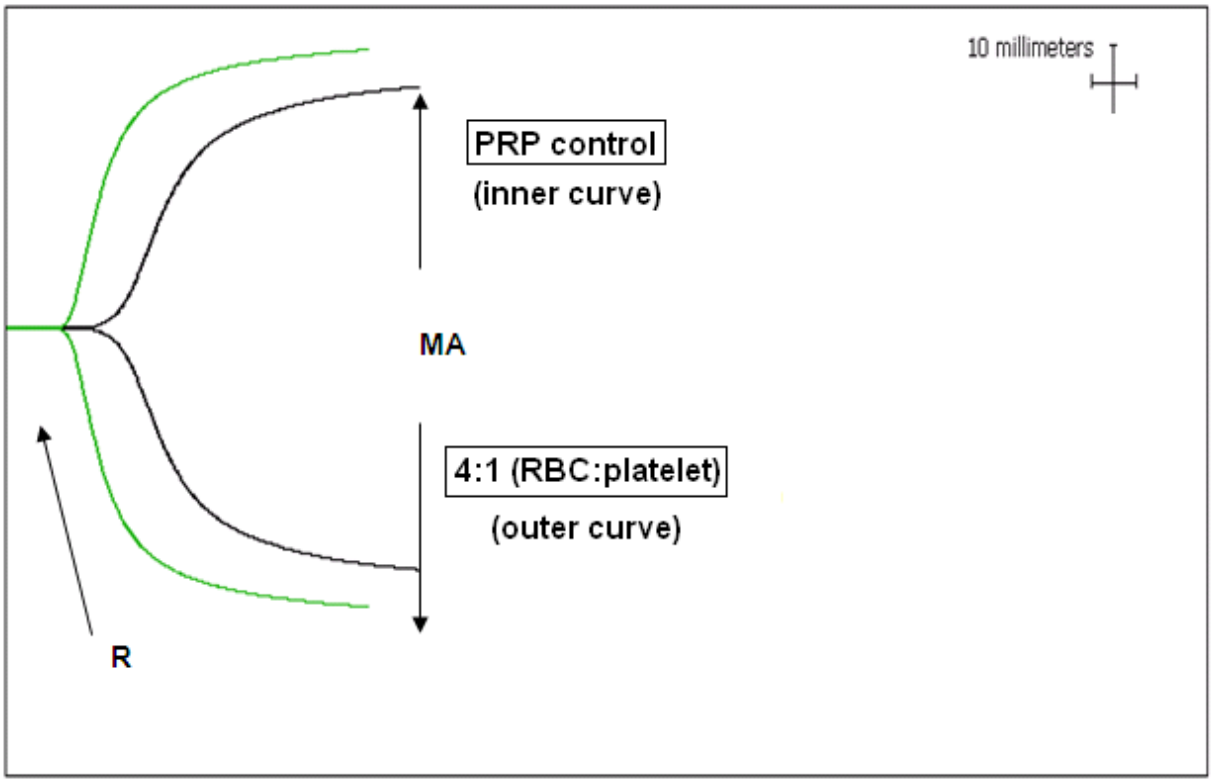

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Figure 2B

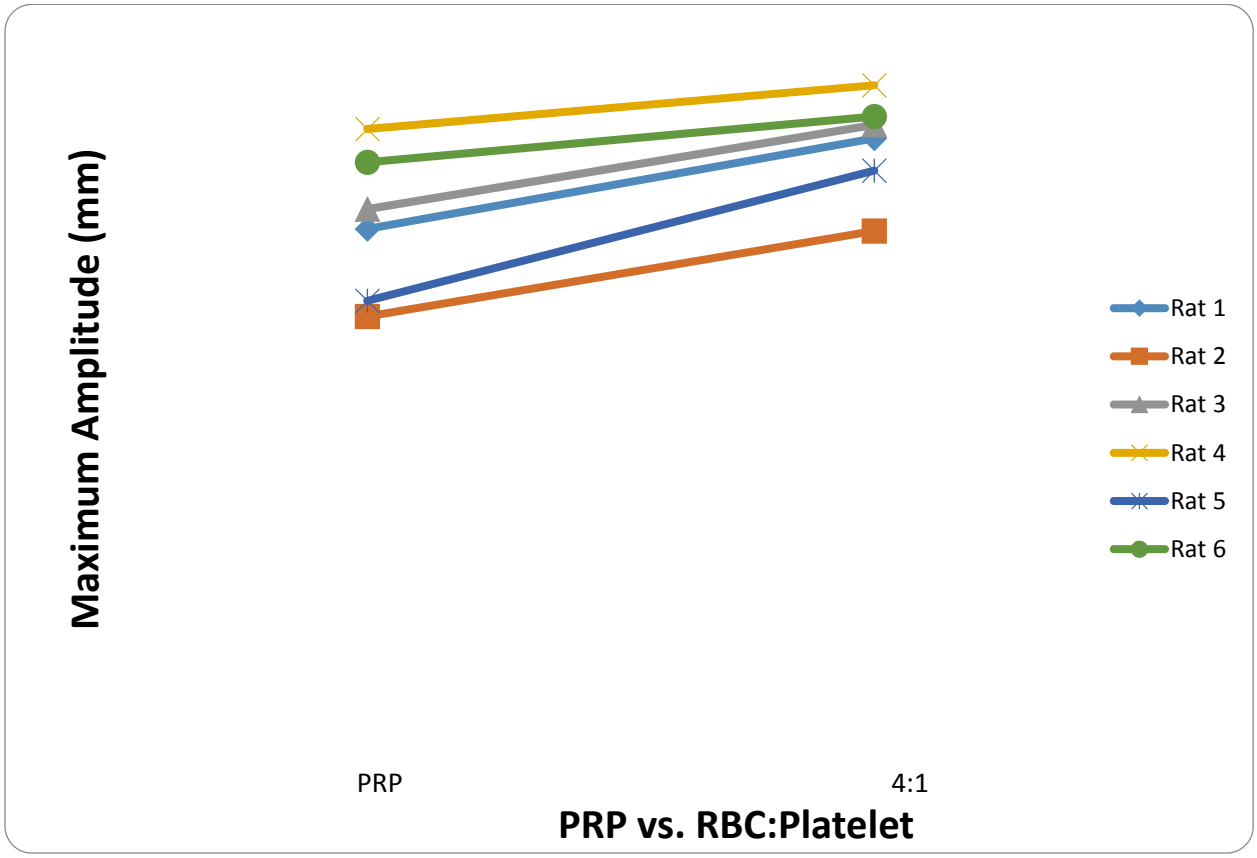

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Figure 2C

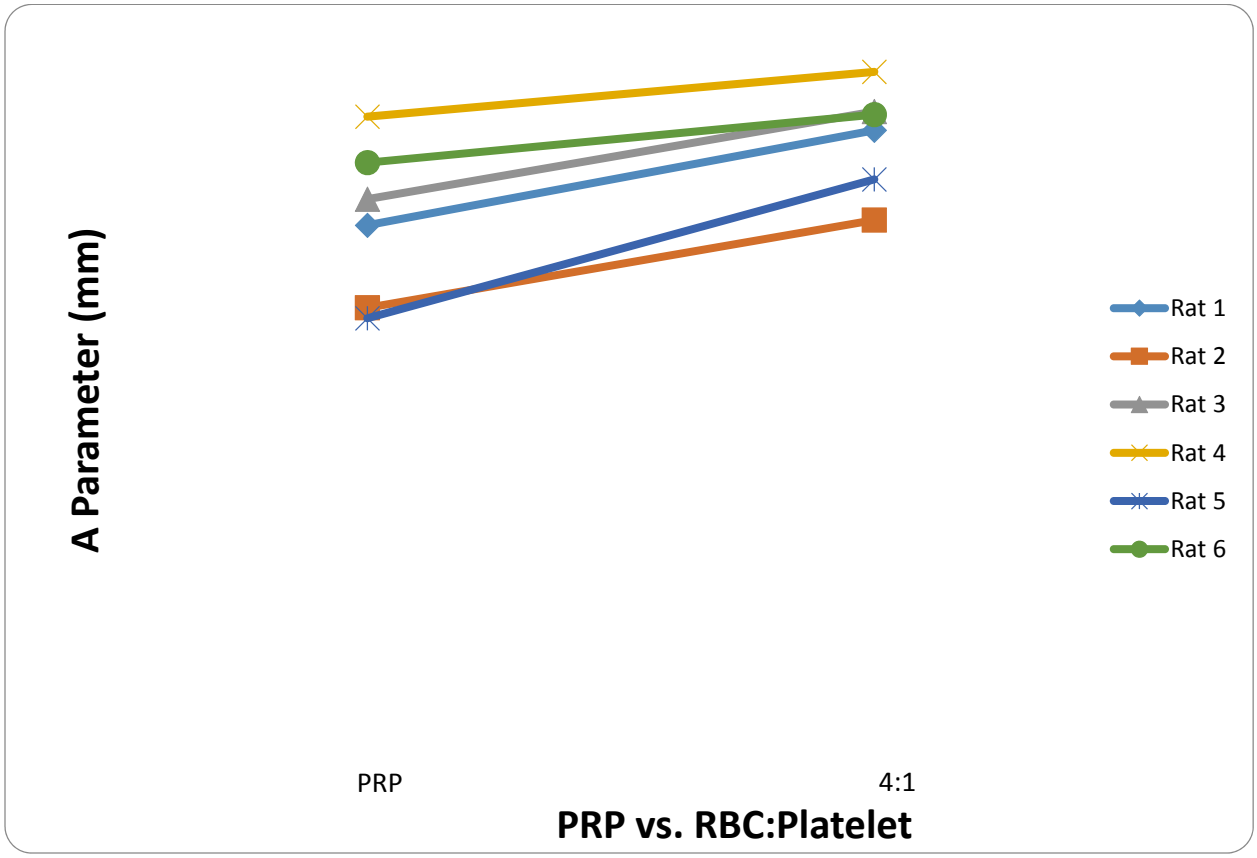

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Figure 2D

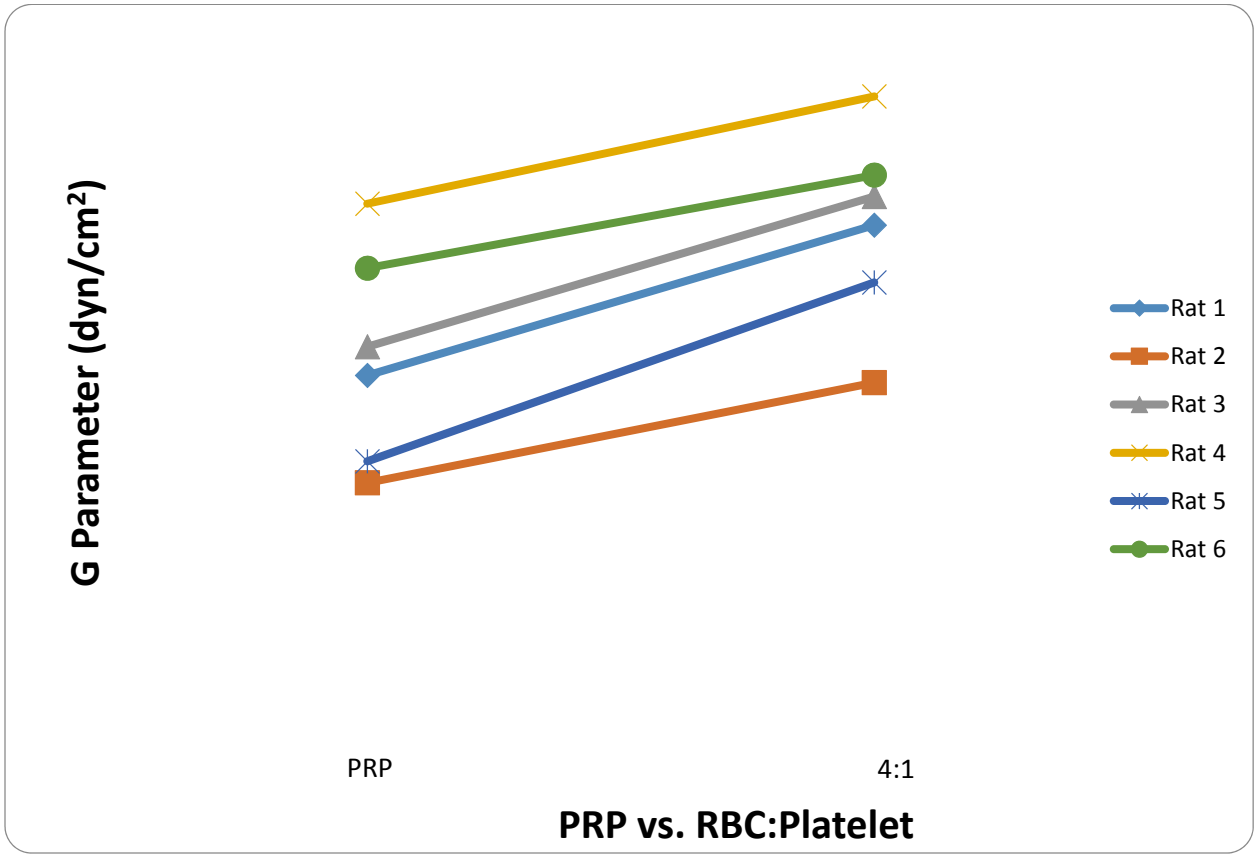

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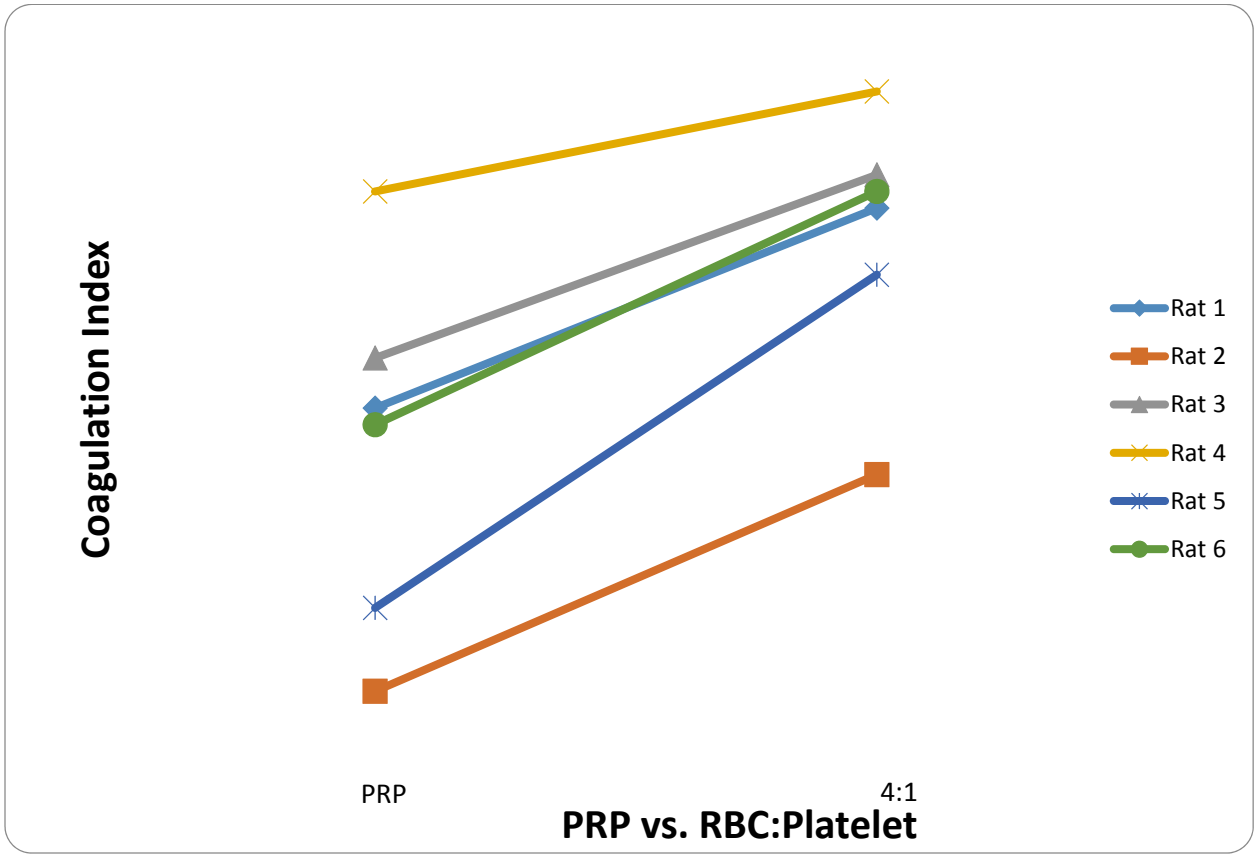
Figure 2E



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