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A low-volume, single pass *in-vitro* system of high shear thrombosis in a stenosis

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ABSTRACT

Introduction: Arterial thrombosis leading to heart attack and stroke requires the rapid accumulation of millions of platelets under pathologically high shear. Previous *in vitro* systems studying platelets typically use endpoints that emphasize platelet-surface effects rather than large-scale platelet-platelet accumulation that precedes occlusion. Further, most platelet tests do not recreate shear rates present during arterial occlusion. We present an alternative flow system to study large thrombus formation under pathologic shear conditions in an anatomic stenosis with reasonable volumes of human blood.

Materials and Methods: An *in-vitro* system using a syringe pump was created to subject low volume (<30mLs), whole blood samples to very high shear rates (>3,500 s^{-1}) through a stenosis. Thrombus was quantified using an optical microscope from initial deposition to large scale accumulation. Images were taken using a high definition camera in real time.

Results and Conclusions: Occlusive thrombus blocks the collagen-coated lumen with millions of platelets using human whole, heparinized blood. Rapid Platelet Accumulation rates in human blood are 4.5 ± 2.4 $\mu m^3/\mu m^2$/min ($n = 21$). There is an initial lag time of 7.4 ± 3.8 min ($n = 21$) before the onset of large scale thrombosis. The rates of platelet accumulation *in vitro* are consistent with the clinical timescale of coronary or carotid artery occlusion. Porcine blood has a faster accumulation rate of 9.6 ± 6.1 $\mu m^3/\mu m^2$/min ($n = 7$, $p < 0.05$) and a shorter lag time of 2.7 ± 0.5 min ($n = 7$, $p < 0.05$). The long lag time for large thrombus formation suggests that some *in-vitro* assays will miss the main mechanism creating thrombotic occlusion.

Introduction

Thrombus formation in atherosclerotic arteries contributes to myocardial infarction (MI) and stroke, the leading causes of death in developed countries. Following plaque cap rupture, platelets may adhere to exposed collagen in the damaged arterial subendothelium in the region of very high shear rates. Subsequently, the platelet thrombosis may grow to fully occlude the vessel with subsequent ischemia and possible death [1]. We have recently described the phenomenon of Rapid Platelet Accumulation (RPA) whereby millions of platelets accumulate under very high shear stenotic conditions that can lead to full occlusion with porcine whole blood [2]. This is in strong contrast to other *in vitro* platelet adhesion studies that typically focus on surface area coverage of thousands of platelets over short time periods without large-scale thrombus formation [3–9]. Many of the parallel plate studies utilize shear rates less than 1,000 s^{-1}. High shear rates in stenotic arteries (3,500 – 300,000 s^{-1}) may strongly affect platelet aggregation, possibly from von Willebrand Factor (vWF) configuration changes in tertiary structure under high shear [10]. Thus, the presence of high shear and shear rate gradient should be a critical factor to studying pathologic arterial thrombosis [11–13].

Previous in-vitro studies of platelet accumulation fall into several method types: platelet aggregometry, cone and plate viscometers, PFA-100®, parallel plate, and gravity fed models. For all of these models, there are varying shear rates, volumes of blood and species used.

Platelet Aggregometry

Aggregometers such as VerifyNow® do not utilize shear and test blood under no/low flow conditions with a stir bar [14]. Thus, this method cannot test for shear induced platelet aggregation [2,15,16].

Cone and Plate Viscometer

Cone and plate viscometers can create uniform or time-varying shear rates [8,17]. However, the small blood sample sustains continuous exposure to potentially activated platelets for long durations of time (minutes) that are very different from the millisecond exposure time of platelets to very high shear in a stenotic artery. Further, platelet aggregation is rarely large. Thus, this test may overemphasize long-duration platelet activation over thrombus accumulation in-vivo [7]. Cone and plate systems may be better for testing normal platelet activation under long-term physiologic conditions rather than the pathologic situation. A more favorable approach for testing platelet accumulation

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in whole blood under high shear would utilize a single pass system where these sustained agonistic conditions do not occur.

PFA-100®

The PFA-100® system uses high shear (>5,000 s^{-1}) and a single pass arrangement [18,19]. In the system, blood is pulled through a capillary tube and over a membrane orifice of imprecise dimensions in both diameter and axial length. The short closure time of <5 minutes could refer to the time to platelet-surface occlusion or potentially embolic occlusion of the membrane [20]. Further, the system does not measure platelet volume deposition over time or large-scale thrombus accumulation.

Parallel Plate

Parallel plate devices can be made with high shear, single pass and use optical measurement of platelet aggregation [21,22]. Due to the *en face* view through flat cover slips, most studies quantify platelet adhesion to the surface measuring only hundreds to thousands of platelets [3,4]. Measurement of subsequent platelet-to-platelet deposition is quite difficult as depth resolution is not as precise. With fluorescence labeling, the measurement of thrombus volume is even more problematic due to saturation, after which additional fluorescence cannot be measured. Hemodynamically, the parallel plate model utilizes a non-anatomic wide aspect ratio that may create secondary flows in the corners of the chamber. This phenomenon is known to create spurious platelet concentrations in the corners. Further, the gap height needed for very high shear is small to achieve with good uniformity over the width. Lastly, most studies in parallel plates terminate within 5 minutes with thrombotic occlusion difficult to define in the rectangular channel [9,23].

Gravity-Driven Stenosis Model

We have previously developed a hemodynamic model with a stenotic test section that mimics the shape of an atherosclerotic plaque [24]. The flowing whole blood created an occlusive thrombus under arterial conditions; however, this hemodynamic system required 240mLs of whole blood for each experiment [2,24]. The requirement of 240mLs of blood for human experimentation is impractical for most clinical studies.

In general, the platelet testing models above exhibit wide disparities in shear rate conditions, blood volume, and endpoints. Direct comparisons are confounded by differences in blood treatment and species. Aggregometers may use whole blood in the case of the VerifyNow® or PRP in the case of light transmittance aggregometry [25,26]. Parallel plate models may use PRP or fluoresced/labeled whole blood [27–29]. As the presence of RBC's may strongly affect platelet mobility near the wall in arteries, the least amount of manipulation of whole blood is preferred. A system of platelet-platelet thrombus formation under pathologic hemodynamics should be available for human blood.

To this end, a new stenosis model of occlusive thrombosis has been designed that utilizes reasonably low volumes (30mLs) of whole blood with shear rates similar to diseased atherosclerotic arteries. Major advantages of the new system are that the shape of the tubular stenosis is anatomically realistic creating hemodynamic conditions of shear rate gradients, elongational flow, very high shear rates, short exposure times, and margination of platelets likely present in disease. A syringe pump apparatus allows a significant reduction of whole blood volumes needed for large-scale thrombus formation. The time course of thrombus growth is measured for human blood using direct optical visualization and the resultant thrombus is characterized by histology. The human blood results are also compared to porcine blood for the same system.

Materials and Methods

Experimental Setup: Hemodynamic Flow Pump Apparatuses

An *in-vitro* flow system was designed to create high, pathologic shear rates with relatively low total blood volumes (Fig. 1A). Whole, lightly heparinized (3.5 IU/mL) blood was perfused past a pressure transducer (Harvard Apparatus, South Natwick, MA) using a syringe pump to maintain constant flow (KD Scientific, Holliston, MA) [2]. The use of the syringe pump required less than 30 mLs of whole blood to create large thrombus volumes. For comparison, a gravity-fed system was run as previously described with blood flowing through PVC plastic tubing (Baxter Healthcare Corporation, Deerfield, IL) with an inner diameter of 3 mm to the test section from a raised platform, past a pressure transducer and through the test section (Fig. 1B) [2,24]. The gravity-fed system requires 240 mL of whole blood to form large thrombus volumes.

During the flow experiments, the supply blood was placed on an Orbit LS orbital mixer (Labnet, Woodbridge, NJ) to prevent blood separation. All blood was used within 8 hours of harvest.

The test section was constructed from glass tubes with an original inner diameter of 1.5 mm. A variety of hour-glass shaped stenoses were made by a professional glass blower with a severity ranging from 75 to 92% (n = 34) by diameter for all experiments. The gap diameter at the apex ranged from 375 to 120 μm. The transparent glass test section was visualized under a low-power microscope.

Initial flow rates for the syringe pump experiments are defined using Poiseuille assumptions for the relationship of shear rate and flow rate for laminar, fully-developed flows:

$$\dot{\gamma} = \frac{32 \cdot Q}{\pi \cdot d^3} \quad (1)$$

where d is the smallest inner diameter in the test section and $\dot{\gamma}$ is 3,500 s^{-1} at the apex (Eq. (1)). A minimum shear of 3,500 s^{-1} is chosen to exceed the level of shear induced platelet activation [30]. As the throat diameter, *d*, was not constant, the flow rate, *Q*, was varied between 0.04 – 0.75 mLs/min to achieve an initial shear rate of 3,500 s^{-1}. Note that our upstream Reynolds number was less than 3 making the Poiseuille assumption reasonably good. Computational fluid studies show that the flow does not separate in the test section under these conditions. Reynolds number in the throat was under 46

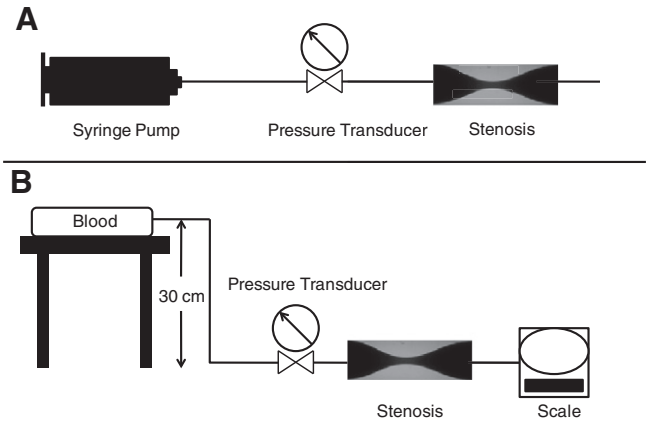

Fig. 1. (A) The syringe pump experiment required a maximum of 30mLs of blood for experimentation to occlusion. Whole blood is driven by a syringe pump, past a pressure transducer, through the collagen coated test section, and onto a scale where weight measurements were taken in real time. (B) Gravitationally fed experiments required 240mLs of blood placed on a platform 30 cm above the stenosis. During experimentation, whole blood flowed from the platform, past a pressure transducer, through the collagen coated test section, and onto a scale. Both systems are single pass.