In-Vitro Clot Formation and Failure

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Abstract

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A primary cause of bleeding and mortality after traumatic injury is failure of hemostatic clot formation at wound sites. After trauma, approximately 25-30% of severe injured patients display trauma induced coagulopathy (TIC) which is a multifactorial condition that causes poor clot formation and performance. A decreased level of viscoelastic clot stiffness or strength and rapid clot breakdown by enzymatic degradation are hallmarks of this condition. Clots formed under these conditions are unable to withstand hemodynamic forces of flowing blood, and therefore are likely to repeatedly rupture, leading to progressive bleeding and worsening coagulopathy.

Current research examines clot formation to understand TIC and to guide treatment. However, the most useful and clinically-relevant aspect of clot formation, for TIC, is clot durability (i.e. its resistance to breakdown) instead of the initiating events related to clot formation. Decreased clot viscoelastic firmness is among the first changes taking place during TIC, which can be detected using current viscoelastic monitors including thrombelastograpy (TEG) and rotational thromboelastometry (ROTEM). However, these devices (TEG/ROTEM) are not ideal modalities for assessing clot mechanical properties in trauma conditions due to the amount of time taken to obtain results, poor sensitivity, repeatability, and lack of standardization. Therefore, in this thesis, I examine clot durability and failure characteristics using a novel method to better understand TIC and develop new clinically-relevant point-of-care diagnostic. I developed a system that forms a clot in a small tube and then applies increasing pressure until the clot fails. I show that that clot failure is strongly associated with intrinsic mechanical properties of the clot. I show the effect of fibrinogen, factor XIIIa, platelet count on the in-vitro failure pressure of the clot. The clot failure system shows strong correlation with the maximum clot firmness obtained from ROTEM, suggesting that it could be used to diagnose TIC and inform the clinicians on the pressure at which clots will fail in patients, thus guiding patient care in trauma situations.

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Finally, and most importantly I would like to thank my family for being the strongest support at all times.

CHAPTER 1: Background & Literature Review

1.1 Background

Trauma remains the leading cause of death in the United States for individuals under the age of 45 [20]. In 2011, trauma from injuries and violence accounted for 51.3% of all deaths for this age group, causing an estimated 187,000 deaths—more than all other causes of death for this age group combined. The total number of years-of-life lost due to trauma is greater than all forms of heart disease and cancer combined. Trauma also kills quickly, with as many as 50% of urban and 80% of rural trauma fatalities occurring prior to hospital arrival. Additionally, 25-35% of trauma patients develop coagulopathy, in which the ability of the patient's blood to clot is impaired. Trauma-induced coagulopathy (TIC) is a multifactorial condition that causes poor clot formation and performance. A decreased level of viscoelastic clot stiffness or strength and rapid clot breakdown by enzymatic degradation are hallmarks of this condition. Clots formed under these conditions are unable to withstand hemodynamic forces of flowing blood, and therefore are likely to repeatedly rupture, leading to progressive bleeding and worsening coagulopathy. Patients with TIC have a three to four-fold increase in mortality, and are eight more times likely to die within 24 hours than trauma patients without coagulopathy [21]. Many trauma patients develop coagulopathy before they reach the hospital, creating challenging scenarios for both paramedics and physicians [22]. The consequences of TIC and trauma are dire, both medically and financially. TIC patients are more likely to undergo blood transfusions, require longer stays in intensive care units, are more likely to require ventilators, and have an increased chance of organ failure [23]. It is necessary to diagnose TIC rapidly in order to decrease the burden of this pathology.

Trauma patients currently receive fluid resuscitation in order to achieve tissue perfusion when they have a hemorrhage [24]. However, pushing fluids in the patient puts the individual at risk for re-bleeding because of the increased blood pressure and the risk of bursting blood clots. In trauma, hypotensive resuscitation can be applied, where the pressure is increased but not to the normal level so as to facilitate coagulation and reduce the risk of bursting newly formed clots [24]. Hypotensive resuscitation can cause ischemic brain damage or acidosis. Medical professionals thus have to balance blood pressure to maintain adequate blood pressure without bursting new clots and causing additional bleeding. Measuring clot strength and characterizing clot failure can allow physicians and paramedics to make more informed choices concerning trauma patients and if hypotensive resuscitation should be applied.

1.2 Literature Review

Bleeding is responsible for 30 to 40% of trauma-associated deaths and is a leading cause of death in the initial hours after injury [1]. Blood coagulation is a vital physiological process for preventing hemorrhage via formation of a stable insoluble clot and is necessary to prevent major blood loss and death from a severe injury. Clots are formed initially by a platelet plug that is then reinforced by a fibrin fiber network. However, the depletion and rapid consumption of functional circulating clotting factors after large volume blood loss prevents the formation of robust fibrin networks [2, 3]. The resulting clots are therefore weak and insufficient to stop bleeding. This acquired coagulopathy, known as trauma-induced coagulopathy (TIC), is observed in 25% of trauma patients [2] and is associated with three to four times increased mortality [3]. Therefore, methods to detect trauma-induced coagulopathy are needed in order to augment or restore hemostatic function and prevent hemorrhage-related deaths.

The blood clot is produced at the site of vascular injury by activation of the coagulation cascade and demonstrates viscoelastic behavior primarily because of its main component, fibrin which is a viscoelastic biopolymer produced at the site of vascular injury by the coagulation cascade [4]. Soluble fibrin monomers self-polymerize in a half-staggered, double-stranded manner to form protofibrils, which then associate noncovalently, bundle into fibers, and branch to form a three-dimensional insoluble scaffold after their activation from fibrinogen by thrombin. [5]. Fibrin fibers provide the primary resistance to mechanical deformation, and viscoelastic firmness and clot resilience can both be increased by increasing fibrinogen concentration [21]. Increasing fibrinogen concentration increases clot density, weight, and reduces porosity, thus providing direct resistance to mechanical deformation. Fibrin fibers demonstrate resistance to strain deformation that includes fiber realignment under strain, extrusion of water from within fibers, and ordered unfolding of individual fibrin molecules [5]. The plasma transglutaminase, factor XIIIa, then stabilizes the clot by creating intra- and inter-fiber cross-links through amide bond formation between lysine and glutamic acid residues. The introduction of these cross-links has dramatic effects on the viscoelastic properties of fibrin [7, 8]. Forceful contraction of the fibrin network by platelets adds additional stiffness [22]. These components primarily contribute to overall clot firmness and resilience. Locally activated thrombin enzyme cleaves circulating fibrinogen to form fibrin monomers. These architectural changes contribute to increased clot stiffness. Though the viscoelastic properties of the clot and the various factors, proteins contributing to the formation of the clot are relatively well researched, the failure characteristics of the clot under hydrostatic pressure exerted by the blood, both in-vivo and in-vitro, are not.

Decreased clot viscoelastic firmness is among the first changes taking place during TIC [2], which can be detected using viscoelastic monitors including thrombelastograpy (TEG 5000, Haemonetics Corp, Niles, IL, USA) and Rotational Thromboelastometry (ROTEM, Tem International GmbH, Munich, Germany). TEG and ROTEM have been widely used in a clinical setting for monitoring coagulation during procedures including, trauma, hepatic and cardiac surgeries, which are associated with high risk of massive bleeding [9]. Both TEG and ROTEM measure coagulation by assessing the shear elasticity of blood as it clots; the principles have been described elsewhere but in brief, both employ a vertical pin held in the blood sample, contained within a cup or cuvette. In the TEG, the cup oscillates clockwise and anticlockwise and as the blood clots, fibrin strands and platelet aggregates form between the pin and the inner walls of the cup, resulting in torque on the pin that is monitored and converted to an electrical signal. In ROTEM, an oscillatory force is applied to the pin and the cuvette is held stationary. As the blood clots, the extent of the pin's actual oscillation is reduced and this is measured by the angle of deflection of a beam of light directed at the pin. However, TEG/ROTEM are not ideal modalities for assessing clot mechanical properties in trauma conditions due to the amount of time taken to obtain results, poor sensitivity, repeatability, and lack of standardization. ROTEM/TEG are designed to detect changes in viscoelastic clot behavior in response to sub-maximal rotational forces that are applied slowly and consistently. However, the resulting mechanical behavior of the clot in these tests may not reflect the reality of *in vivo* clot formation and hemostasis. Clot failure and rebleeding at wounds has instead often been described as an abrupt clot failure event [12] rather than a slow process of clot degradation over time. The elastic modulus of individual fibers within a fibrin clot have successfully been measured by instruments such as optical tweezers, however, reliable and repeatable clinical tools for monitoring blood coagulation are still lacking.

Studies of clot failure and rebleeding in animal models have revealed that high-velocity bleeding that correlates with systemic blood pressure is present at major wounds [17]. Therefore, increasing blood pressure by infusing resuscitation fluids or administering medications can cause clot rupture and increase blood loss [14] often as a result of abrupt and catastrophic mechanical clot failure [13]. The systolic blood pressure at which rebleeding occurs (called "pop off" pressure) from vascular aortic injury is reproducible and increasing clot quality by providing clotting activators can increase the blood pressure at which clot failure takes place. [15, 16]. However, the character and mechanics of clot failure in the setting of high-pressure bleeding remains unknown.

Currently, trauma patients receive fluid resuscitation in order to achieve tissue perfusion when they have a hemorrhage [11]. However, pushing fluids in the patient puts the individual at risk for rebleeding because of the increased blood pressure. In trauma, hypotensive resuscitation can be applied, where the pressure is increased but not to the normal level so as to facilitate coagulation [11]. The risk is that hypotensive resuscitation can cause ischemic brain damage or acidosis. Medical professionals thus must balance the patient's blood pressure to provide adequate pressure without bursting the clots and causing rebleeding. Measuring clot strength and characterizing clot failure enables physicians and paramedics to make more informed choices concerning trauma patients and if hypotensive resuscitation should be applied.

In this thesis, I introduce a fluidic device to form a blood clot and measure the pressure at which it fails. Ideally this information can be used to diagnose TIC and provide a reference blood pressure that should not be exceeded while resuscitating the patient due to the risk of re-bleeding and worsening coagulopathy. I demonstrate the proof of concept of the device and show that fibrinogen and factor XIIIa concentration as well as platelet count impact the in-vitro clot failure pressure. The clot failure pressure correlates well with maximum clot firmness obtained from ROTEM, suggesting that the measurements could be used to diagnose TIC.

CHAPTER 2: Experimental Methodology

2.1 Experimental Setup

Clot failure was determined by inducing a clot of standardized size and shape within a small silicon tube. The tube was connected to a syringe pump (KDS 200, KD Scientific Inc. MA) and a pressure transducer. After clot formation, the syringe pump was used to apply increasing air pressure to the clot. The pressure was measured continuously using a pressure transducer (PX40-15G5V, Honeywell Inc.) linked to a data acquisition system (NI myDAQ, National Instruments Inc.). The resulting output was a pressure vs. time curve. Silicone tubing (Cole-Parmer Instrument Company, LLC) with an inner diameter of 800 microns was used to approximate the inner diameter of a large artery. Silicone tubing connections were secured by Luer-lock connectors to ensure a leak proof system. The syringe pump and a 3 ml syringe applied increasing air pressure to the clot until the clot abruptly failed out of the open end of the tube which was exposed to atmospheric pressure. The applied air pressure was measured continuously until failure pressure (FP) was reached, defined as the maximum pressure obtained before abrupt clot failure and associated decrease of pressure. Figure 1(a) shows an experimental setup schematic and figure 1 (b) shows an image of the experimental setup.

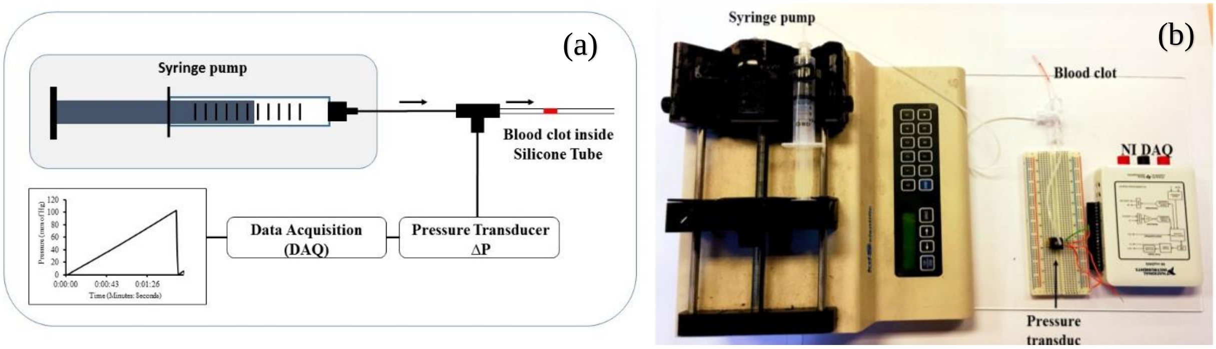

Figure 1. (a) Schematic of the experimental setup. The clot was formed in a silicone tube which was connected to a syringe pump and pressure transducer through a three-way connection. The syringe pump applied increasing air pressure on the clot which was measured by the pressure transducer connected to a data acquisition system. (b) Image of the experimental setup.

2.2 Activators

Thrombin, factor IIa in the coagulation cascade (Stago Diagnostica, NJ), and calcium chloride were used to activate the citrated plasma, blood and fibrinogen solutions. Experiments were conducted to find the effect of thrombin on the failure pressure of the clot and the optimal concentration of thrombin to be used. The concentration of thrombin did not affect the failure pressure, it increased the rate of clot formation with the increase in concentration. The thrombin concentration was fixed at 1 unit/ml. Calcium chloride was added to replenish the calcium in the citrated blood before being introduced into silicone tube. The calcium concentration used was 0.2 M, which is used to perform the extrinsic pathway activation EXTEM test on the ROTEM.

2.3 Clot Formation

Two methods of clot formation were explored. The first method is premixing the clot activators with the blood or blood products. The second method is coating the inner diameter of the tubing with thrombin and calcium to functionalize the area. When premixed, the liquid activator was mixed directly with the liquid sample using a syringe just prior to introducing the mixed sample into the silicone tube. When coating the tubing wall, liquid activators were introduced into the tube by a syringe, over a desired length, and allowed to dry overnight so that inner surface of the tube was coated with a standardized amount of thrombin over a predetermined area. Figure 2 shows the steps involved in functionalizing the inner walls of the silicone tube. Unmixed liquid test sample was then taken up into the tube by a syringe until 10 mm of sample was in contact with the functionalized area and clot was allowed to form for at least 10 minutes. Both methods provided similar results and the second method was used to conduct the experiments because of its ease of use and repeatability.

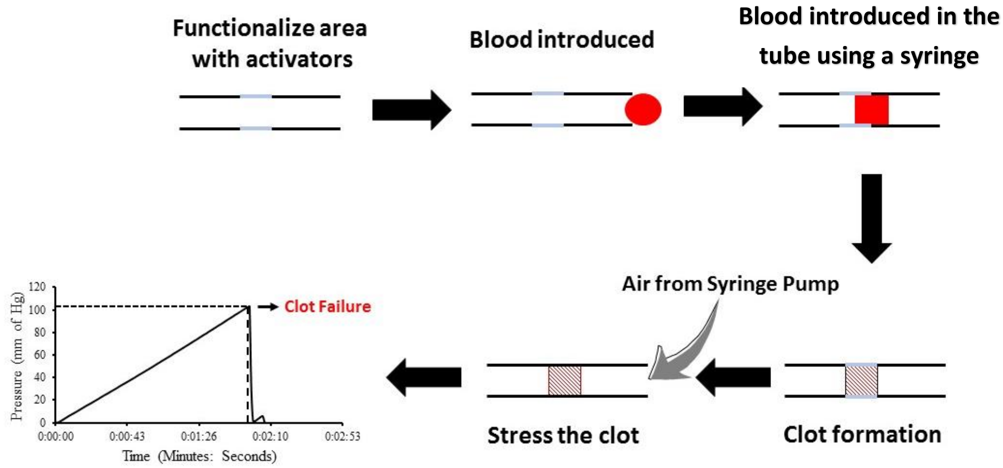

Figure 2. Schematic of the process of in-vitro clot formation by coating the tube with activators. The inner diameter of the silicone tube was functionalized by coating it with Thrombin (activator). The test sample was then introduced into the tube using a syringe until it comes in contact with the functionalized area and clot was allowed to form. The clot was then stressed using the syringe pump to obtain the failure pressure.

2.4 Experimental Parameters

I conducted several sets of experiments to determine the conditions that will yield reproducible clot formation and failure pressure. Figure 3. shows the typical pressure curve generated by the setup as the clot is stressed using the syringe pump. The syringe pump ramps up the pressure linearly till there is a discernible drop in the pressure which indicates the clot failure. The peak pressure in the graph is identified as the Failure Pressure indicating the clot failure under the hydrostatic pressure of the fluid. Figure 4. shows a plot of the failure pressure as a function of the time provided for the clot to form. The failure pressure increases with clot formation time and then asymptotes at times greater than 7 minutes. Therefore, all clots were allowed to form for at least 10 minutes prior to testing. This allowed ample time for the clot to reach a stable structure before external pressure was applied. Figure 5. shows the failure pressure as a function of the initially formed clot length. This data shows that the failure pressure increases with the clot length. A standardized length of 10mm was selected for all experiments. We tested the effect of the syringe pump flow rate, however, we found to no dependence on the failure pressure (data not shown). A standard flow rate of 1 milliliter per minute was used to conduct all experiments. Thrombin (FibriPrest, Diagnostica Stago, Asnières sur Seine, France) and calcium chloride were used to induce clot formation in all experiments. The thrombin concentration was standardized at 1 unit/ml within the sample as it increased the rate of clot formation and did not affect the failure pressure.

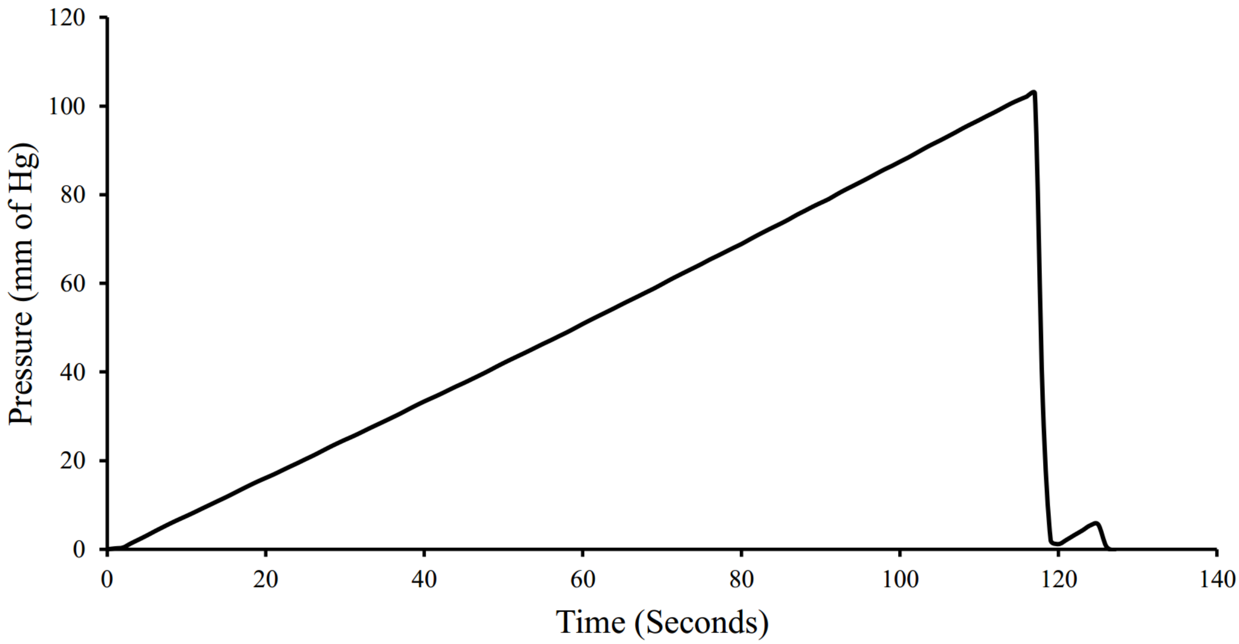

Figure 3. Typical pressure versus time curve generated by the system. using the syringe pump and air as the fluid to stress the clot. The pressure increases will the clot is stressed until the clot breaks at 117 seconds and the pressure rapidly decreases to zero. The peak pressure in the graph is identified as the failure pressure (FP), in this case 102.8mm of Hg, indicating the clot failure under fluid pressure.

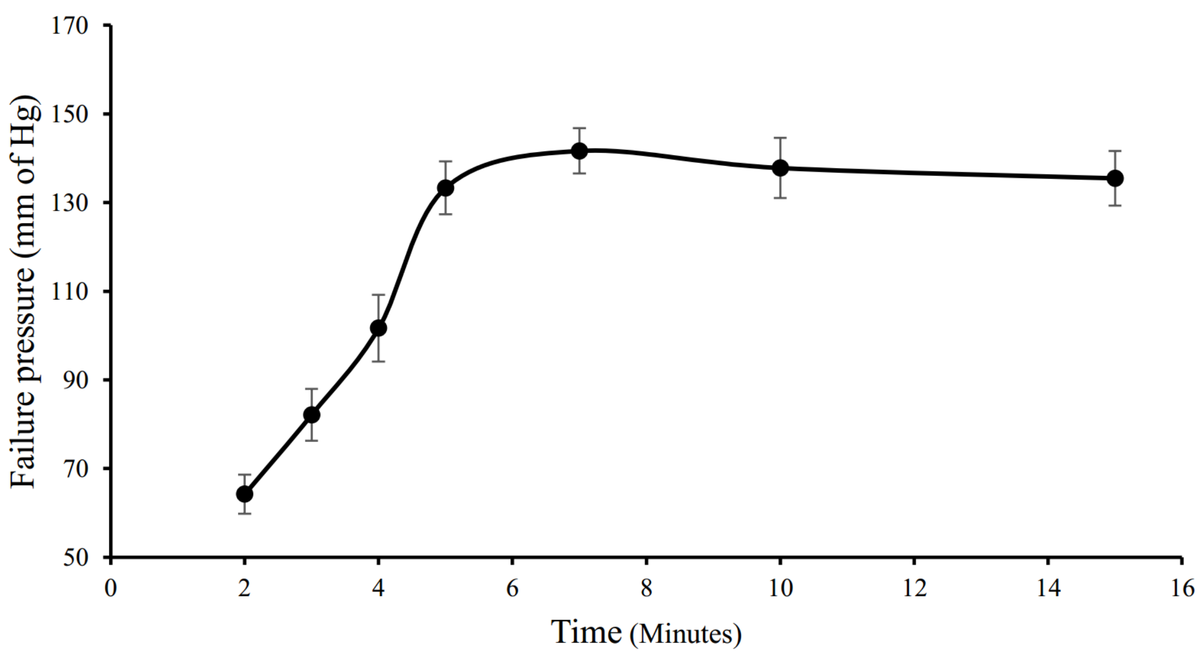

Figure 4. Failure pressure vs. time for clot formation time. As expected the Failure pressure increases with the clotting time till it plateaued out at approximately 8 minutes. Therefore, all clots were allowed to form for 10 minutes prior to testing. Each experimental value represents the mean value from a set of ten experiments with error bars that correspond to 95% confidence intervals.

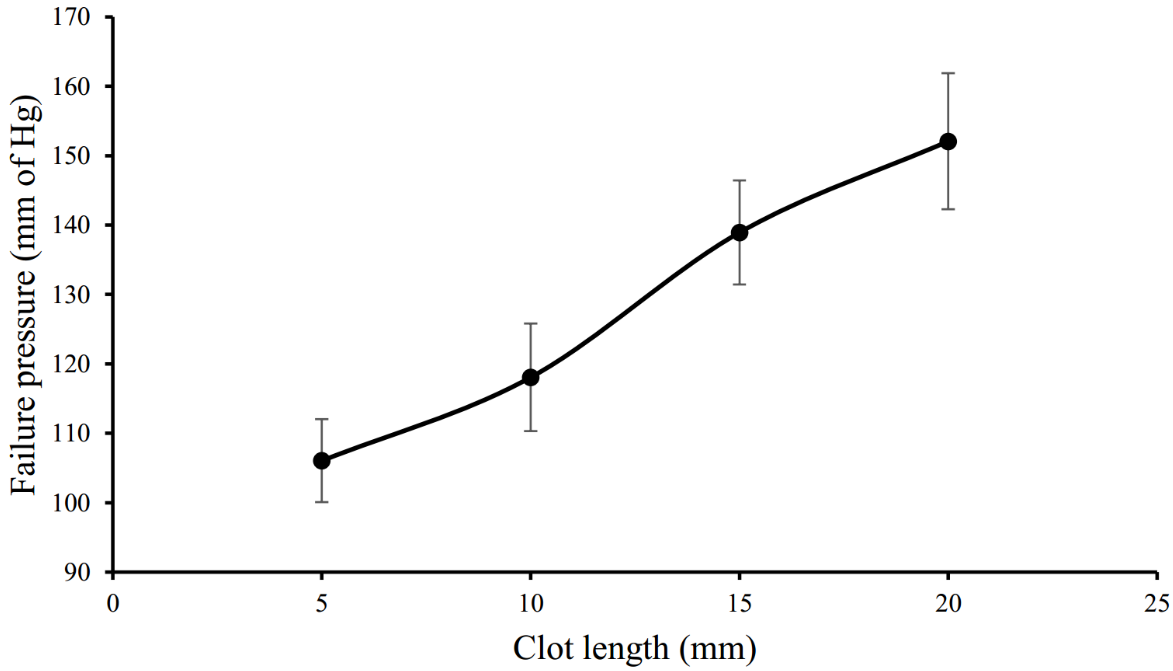

Figure 5. FP vs. Clot Length: Effect of length on FP. As expected FP increases with the increase in clot length. The clot length was standardized at 10 mm. Each experimental value represents the mean value from a set of ten experiments with error bars that correspond to 95% confidence intervals.

Calcium chloride was added to replenish the calcium in citrated blood. The final calcium concentration used was 0.2 M, which is the same concentration used for ROTEM experiments.

CHAPTER 3: Results

3.1 Visualization of Clot Failure

The in-vitro clot formed inside the tube is stressed hydrostatically until failure. In order to understand the in-vitro clot failure mechanism, a high speed camera (v711 Phantom, Vision Research, Wayne, NJ) coupled with a microscope (Nikon AZ-100) was used. The clot failure event was observed under this setup to gain valuable insights into the clot failure event. Figures 7 - 9 show the images captured by the camera. The clot appears to counter-intuitively fail at the center rather than the walls. The clot failure occurs in three steps: Initial Compression, Bubble formation (air pocket), Catastrophic failure. Both the front and the back meniscus of the clot were observed under magnification. Figure 6. shows the schematic of the clot inside the tube and the position of the front clot meniscus (Leading edge) and back clot meniscus (Trailing edge).

Figure 7a shows the initial clot meniscus position and shape. Figure 7b shows the onset of the air pocket but also the fact that the meniscus has shifted to the left under the effect of the hydrostatic pressure of the air.

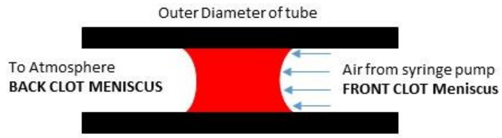

Figure 6. Schematic of the clot inside the tube.

This is the first step of clot failure process and the longest step. After initial compression of the clot meniscus under the effect of hydrostatic pressure, the second stage is the onset of bubble formation (air pocket) where the air is entrapped in the clot at its weakest point, forming a bubble. This process of clot meniscus developing an air pocket is similar to the propagation of crack in solid surfaces. This step begins with the formation of a defect on the clot meniscus under the effect of pressure and this defect undergoes massive change in size, entrapping more of the oncoming air (figure 7b – 7d), until the clot structure can no longer handle the hydrostatic pressure and fails catastrophically as shown in figure 7e. The bubble formation (on-set of air pocket) step (figure 7b) is shown in detail in figure 8 under 10x magnification. The clot develops a defect at the center under the hydrostatic pressure and this defect propagates to form an air pocket inside the clot, shown in figure 8.

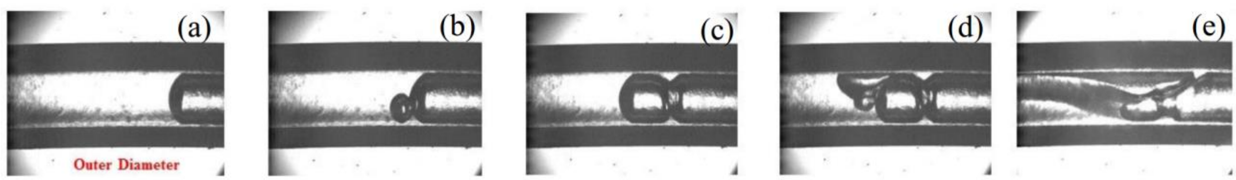

Figure 7. Front clot meniscus observed under 4x magnification: The failure is shown as a five step procedure from the initial meniscus to failure. The figures show all the stages of the clot failure process, (a): Initial clot meniscus position (syringe pump switched on). (b): Initial compression and bubble formation (70 seconds after the syringe pump is switched on and the pressure has roughly increased to 59 mm of Hg, (c): Propagation of the bubble (defect) in the clot (85 seconds after the syringe pump switched on and the pressure is roughly 73 mm of Hg, (d): Formation of secondary bubble and propagation of the bubble (115 seconds of the syringe pump switched on at a pressure of roughly 10 mm of Hg, (e): Catastrophic failure (119 seconds after the syringe pump switched on at the failure pressure of 103 mm of Hg*).

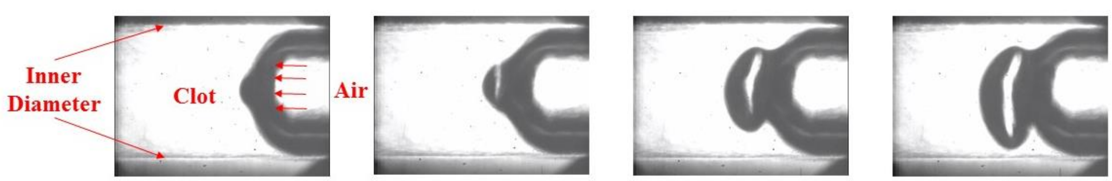

Figure 8. On-set of air pocket (bubble formation) at the center of the clot (front clot meniscus) observed under 10x magnification.

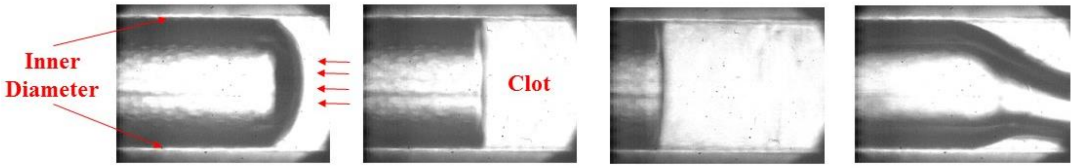

Figure 9. Representation of the behavior of the Trailing edge of the clot (back clot meniscus) under 10x magnification. The trailing edge is being pushed out of the tube as the clot fails.

It was observed that the process slows down significantly after the initial bubble formation (onset of air pocket), which may be the effect of strain hardening. In some cases, bubble formation step is followed by secondary air pockets formation. These air pockets travel through over length of the clot before the clot fails catastrophically under pressure. Figure 9. shows the back clot meniscus being pushed out of the tube as the leading edge of the clot undergoes the various stages of failure. The back meniscus changes shape from a curved front to a straight front, during the initial compression step. Gradually, as the air pocket at the front clot meniscus grows in size the trailing edge of the clot is pushed out and eventually clot is pushed of the tube during catastrophic failure.

3.2 Initial Proof-of Concept

Initial proof-of-concept experiments were performed using swine plasma, diluted with saline solution to achieve desired concentration, to ascertain if the assay could differentiate between normal and diluted plasma. Figure 10 shows the failure pressure as a function of the volume fraction (i.e. 100% means the clot are formed by 100% plasma and 50% means the clots are formed by a mixture with 50% plasma and 50% saline buffer.) Each data point is the average of ten clot samples. Similar solutions were tested in ROTEM to determine the maximum clot firmness (MCF) of the various plasma concentrations. The setup successfully distinguished between diluted and undiluted plasma, with the failure pressure (FP) increasing with increasing volume fraction of plasma.

The failure pressure from the assay was plotted against the MCF from the ROTEM to achieve a linear correlation with R^2 value of 0.96 as shown in Figure 11. Following the initial proof-of concept experiments, various experiments were conducted to test the effect of various functional clotting factors on the failure pressure and check the efficacy of the novel diagnostic assay. After the successful demonstration of proof-of-concept, experiments were planned to determine components of clot formation primarily responsible for mechanical clot failure under stress. We titrated fibrinogen concentration, platelet concentration, Factor XIIIa concentration to determine their individual effects on clot failure characteristics. To demonstrate the effect of fibrinogen, a simple solution of bovine fibrinogen reconstituted in buffer (44 mM Hepes titrated to pH 7.4 in deionized H_2O) was first used to determine the isolated effect of fibrinogen concentration on FP. Fresh citrated porcine plasma spiked with increasing concentration of human fibrinogen (Sigma Aldrich, St. Louis, Missouri USA) was used to determine the contribution of fibrinogen in plasma to FP. Human Factor XIIIa (Sigma Aldrich, St. Louis, Missouri USA) was added to porcine plasma to determine the effect of fibrin crosslinking on FP.

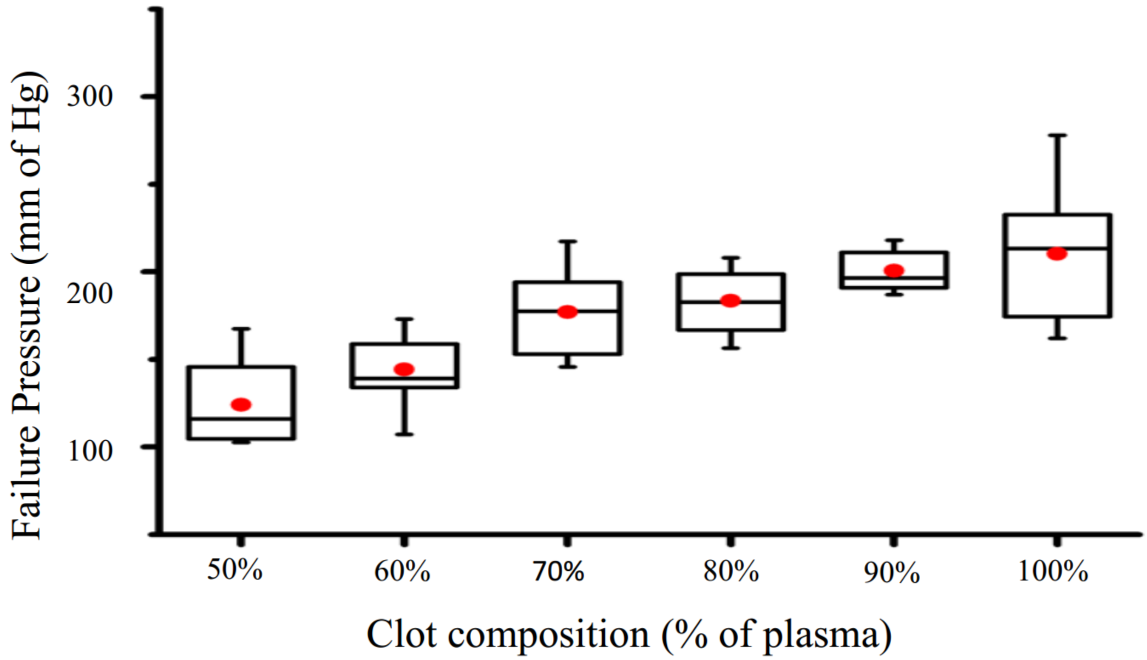

Figure 10. Clot Failure pressure for various clot composition. FP increases with the plasma volume fraction.

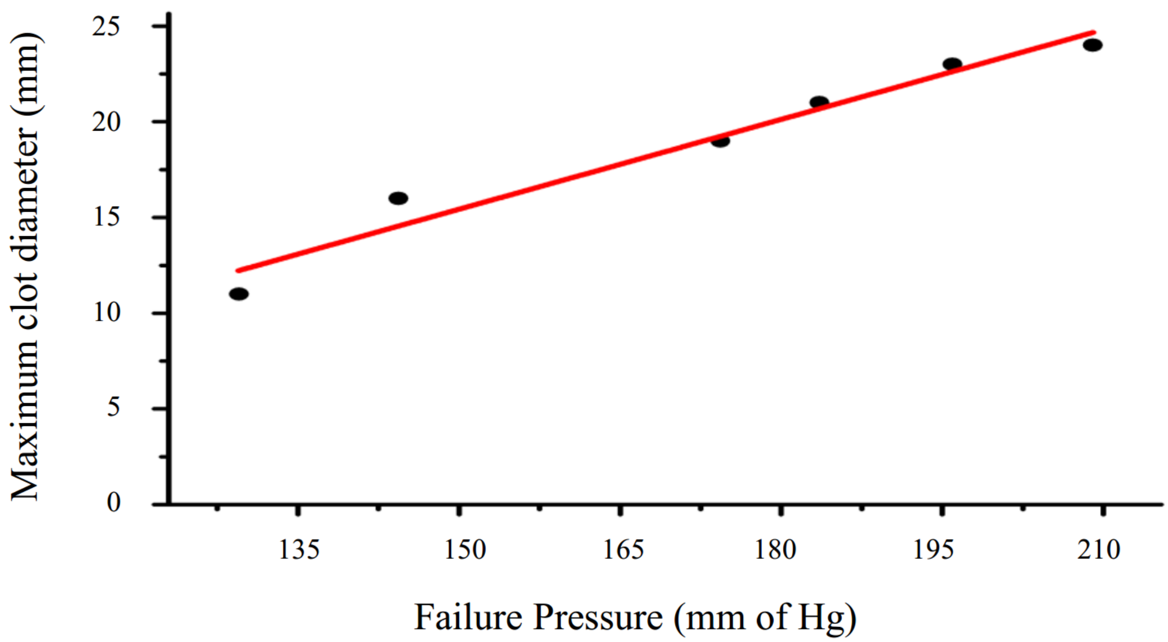

Figure 11. Correlation between FP and ROTEM MCF. The varying clot firmness was obtained by varying the dilution of the swine plasma. FP and MCF show strong correlation with a R^2 value of 0.96.

To test the contribution of platelets, fresh citrated porcine blood was first centrifuged to platelet-rich plasma (PRP) by centrifugation at 500 rpm for 15 minutes. The PRP was removed and the remaining sample centrifuged again at 5,000 rpm for 15 minutes to obtain platelet-poor plasma (PRP). The platelet count in PRP was then obtained using a VetScan blood cell counter (Abaxis, Union City, CA USA) and remixed with PPP to obtain the desired platelet count. Samples from each solution were also tested for maximal clot firmness (MCF) using ROTEM with extrinsic pathway activation (EXTEM).

3.3 Effect of Fibrinogen

Bovine and Human Fibrinogen was purchased from Sigma – Aldrich (St. Louis, Missouri). The bovine fibrinogen was defrosted to 37^0C and diluted in a buffer solution of 44 mM Hepes titrated to pH 7.4 in deionized H_2O, to achieve the desired concentration. The second set of experiments involved adding the human fibrinogen to Pig plasma to test the effect of increasing fibrinogen levels in plasma on the failure pressure of the clot. Same samples were tested in ROTEM thromboelastometer (Pentapharm GmbH, Munich, Germany) to obtain the Maximum clot firmness (MCF). Figure 12. shows the effect of Bovine fibrinogen on the failure pressure. The failure pressure increases with the concentration of fibrinogen, thus the in-vitro clot gets stronger as expected. There is significant increase in the failure pressure with fibrinogen concentration suggesting that the setup is very sensitive to the presence of fibrinogen in the blood or plasma. The failure pressure was then plotted against the MCF (figure 13.) to check for correlation.

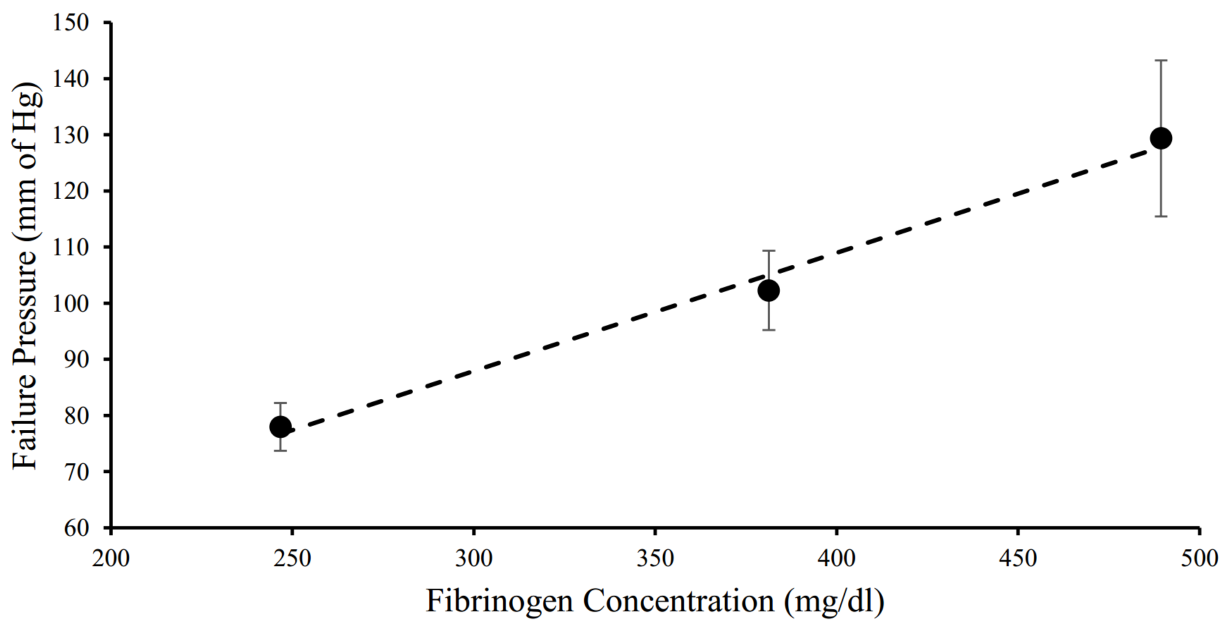

Figure 12. Effect of Fibrinogen: Failure Pressure vs. Bovine Fibrinogen concentration in HEPES buffer. As expected the FP increases with the increase in the fibrinogen concentration, indicating stronger clots. Each experimental value represents the mean value from a set of ten experiments with error bars that correspond to 95% confidence intervals. The plot shows strong correlation between FP and fibrinogen concentration with R^2 = 0.9911, suggesting that fibrinogen level primarily affects the FP value.

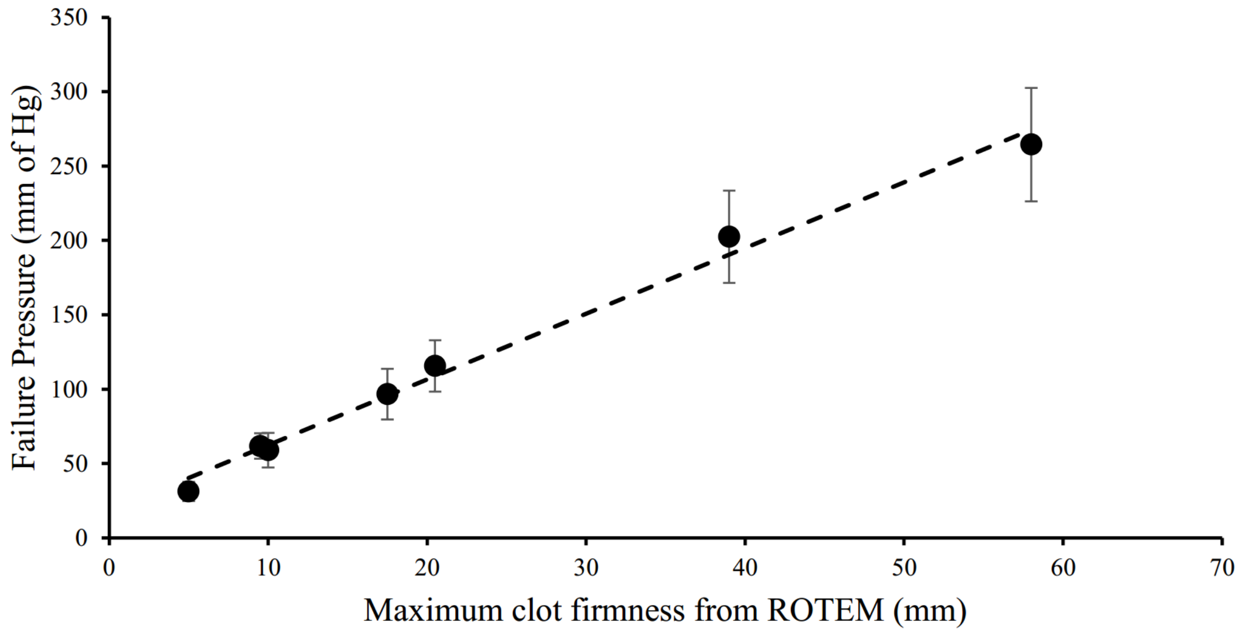

Figure 13. Correlation between FP and MCF: Bovine fibrinogen with HEPES buffer. Each experimental value represents the mean value from a set of ten experiments with error bars that correspond to 95% confidence intervals. Strong correlation between FP and MCF with $R^2 = 0.991$

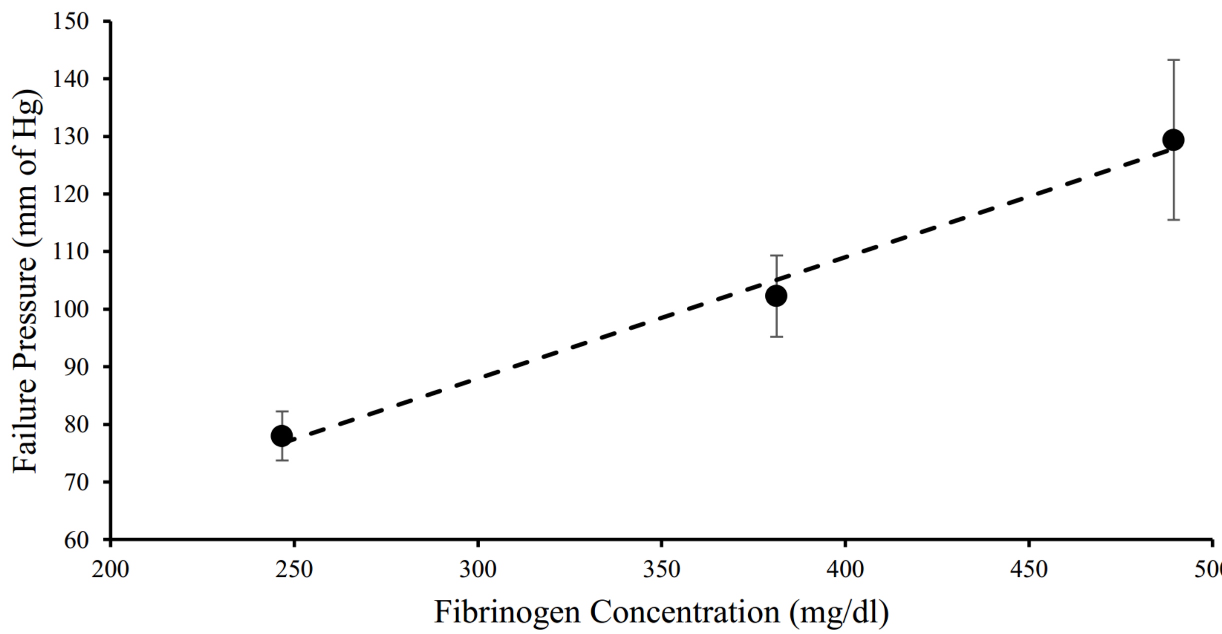

Figure 14. Effect of Fibrinogen: Failure pressure vs. Human fibrinogen concentration added to swine plasma. Each experimental value represents the mean value from a set of ten experiments with error bars that correspond to 95% confidence intervals. FP and Fibrinogen concentration show strong correlation with $R^2= 0.9911$.

A very strong correlation was obtained between the two, with a R^2 value of 0.991. Figure 14. shows the effect of human fibrinogen added to pig plasma. Again the results are in strong agreement with the previous observation.

The experiments with both human and bovine fibrinogen, showed a strong effect of fibrinogen on the failure pressure from the setup. It is shown in section 3.1 that the clot fails at the center and not at the boundary which shows that the clot is failing due to the disruption of the internal structure under the hydrostatic pressure. Fibrin, formed by the action of thrombin on fibrinogen, forms an insoluble scaffold which provides the primary resistance to mechanical deformation. Increasing the fibrinogen concentration leads to more fibrin which in turn leads to increased clot density, reduced porosity and a stronger scaffold structure, thus, increasing the mechanical integrity of the clot. As a result, the failure pressure is increasing with the increase in the fibrinogen concentration and showing a strong correlation with the fibrinogen concentration.

3.4 Effect of Factor XIII

Factor XIII was purchased from Sigma – Aldrich (St. Louis, Missouri). Factor XIII was defrosted to 37^0C and added to the Pig plasma to test the effect of Factor XIII on failure pressure. Factor XIII cross links with fibrin fibers and increases the stability of the fibrin structure, thus, strengthening the internal clot structure. Thus, increasing the Factor XIII concentration will lead to greater stability of the clot and increase in the resistance to deformation. As a result, it was expected that the failure pressure will show a significant increase in Factor XIII concentration. This is corroborated by figure 15 which shows almost fifty percent increase in FP with addition of 20 microliter/ml of factor XIII to the Pig plasma.

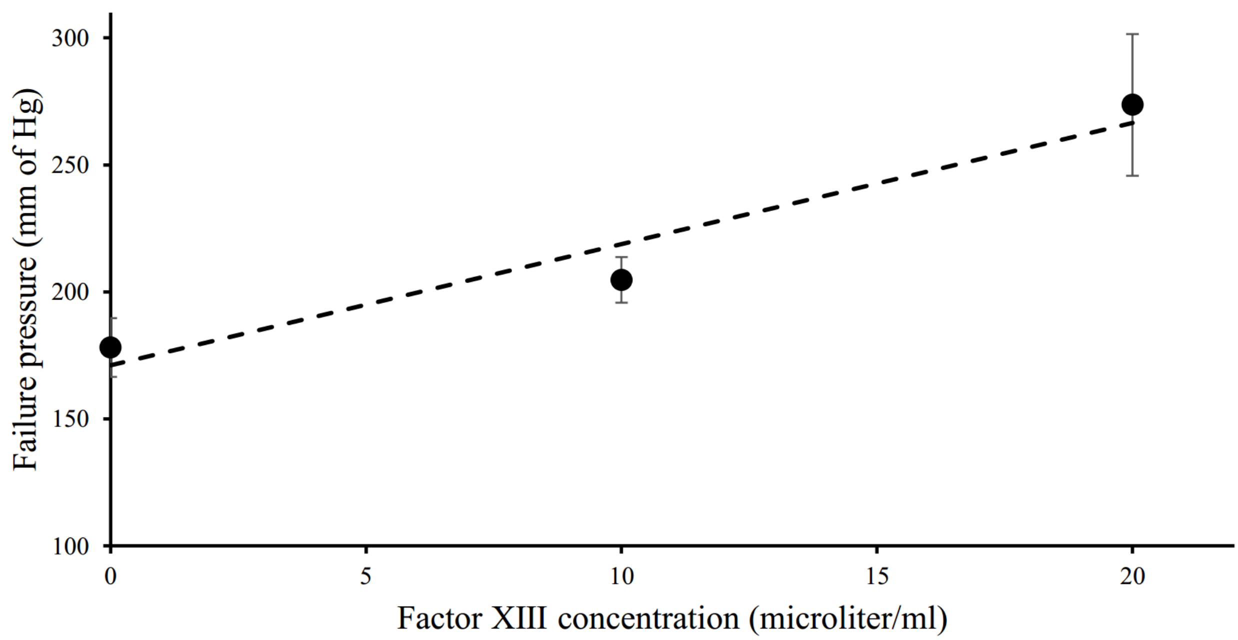

Figure 15. Effect of Factor XIII. FP increases with the increase in the factor XIII concentration. Each experimental value represents the mean value from a set of ten experiments with error bars that correspond to 95% confidence intervals. Strong correlation between FP and Factor XIII concentration with $R^2 = 0.9388$.

3.5 Effect of Platelet count

Swine blood was spun down into Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP). Plasma count was obtained using a VetScan Cell counter. The plasmas were tested on the experimental setup to obtain the effect of Platelet count on the failure pressure. Figure 16. shows the effect of platelet count on the failure pressure. Failure pressure is not significantly affected with the increase in platelet count, as a change of approximately 30 mm of Hg is observed for a platelet count change of sixty thousand and can be attributed to the fact that Thrombin is used as the activator which directly activates the fibrinogen in the blood.

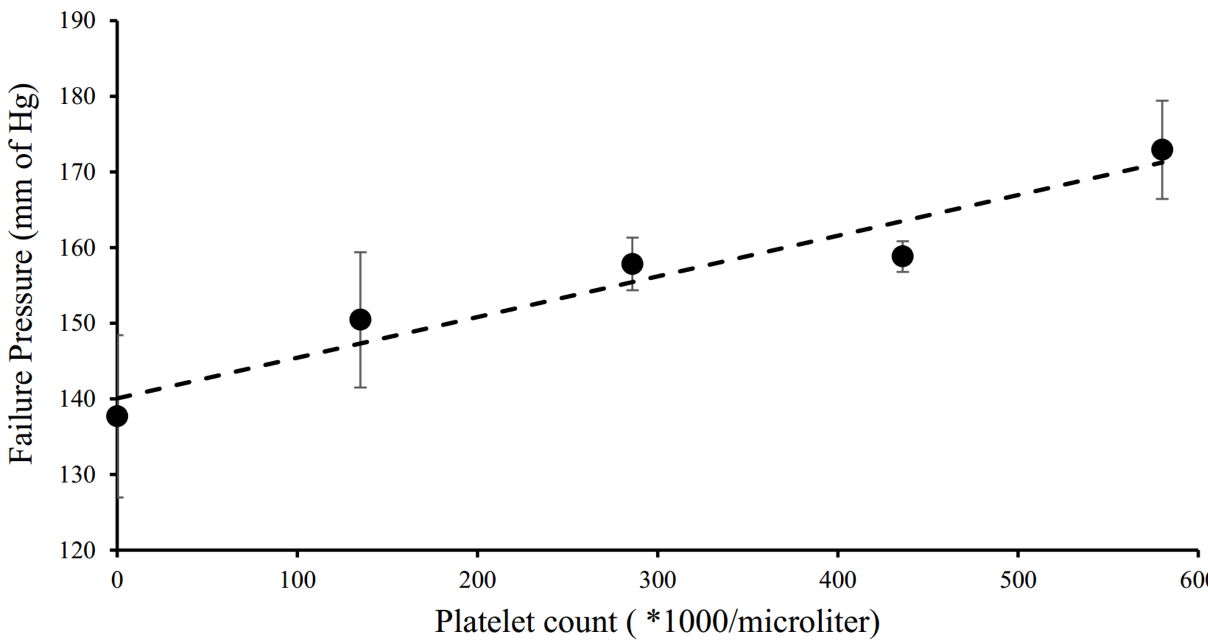

Figure 16. Failure Pressure vs. Platelet count: PRP vs. PPP. Each experimental value represents the mean value from a set of ten experiments with error bars that correspond to 95% confidence intervals.

3.6 Clot Failure During Traumatic Hemorrhagic Shock (Whole Blood)

Blood samples were also obtained from a convenience sample of leftover blood samples from an ongoing porcine study of multiple traumatic injuries with hemorrhagic shock and FP was measured serially at baseline (BL) prior to injury, and again after injury and during hemorrhagic shock at 30 minutes (R30) and 60 minutes (R60). ROTEM MCF by EXTEM activation and standard coagulation tests including prothrombin time, activated partial thromboplastin time, and fibrinogen concentration by the modified method of Clauss were also performed on the same samples using the STArt-4 coagulometer (Diagnostica Stago, Asnières sur Seine, France). The porcine protocol was part of a study investigating the effects of fibrinogen concentrate infusion on blood loss after multiple injuries with internal bleeding from an aortic tear injury. Experimental approval was obtained from the local animal use committee prior to experiments taking place. Briefly, domestic swine were fully anesthetized using isoflurane to surgical plane anesthesia and traumatic brain injury, femur fracture, and aortic tear were inflicted to reproduce multiple injuries with internal bleeding. Animals were randomized to receive fibrinogen concentrate or saline volume control to investigate its effects on blood loss and the blood used in this study was obtained as leftovers from blood sampled serially over time to monitor for coagulopathy. Swine blood was collected from ongoing multi-trauma animal model at intervals of thirty minutes. The base point on the x-axis denotes the baseline measurement. In addition to obtaining the failure pressure, MCF was obtained from ROTEM. It is clear from the figure 17. that the failure pressure can be used to detect coagulopathy with a lower failure pressure corresponds to coagulopathy. ROTEM is also able to detect coagulopathy with the decrease in the MCF value, but the percent change in failure pressure is more than the percent change in MCF value. The coagulopathy can be attributed to the loss of functional clotting factors in the blood on account of blood loss, which in turn affects the failure pressure parameter obtained from the setup.

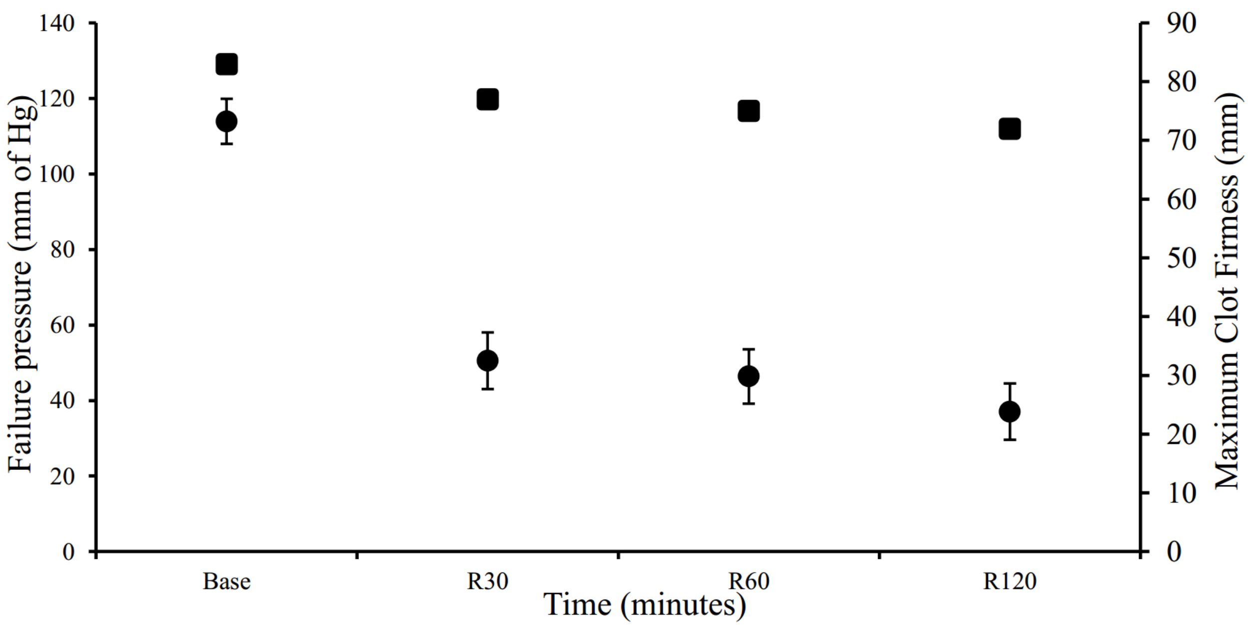

Figure 17. Coagulopathy detection. Multi-trauma pig model failure pressure (circles) and maximum clot firmness (squares) plotted against time of survival. Each experimental value represents the mean value from a set of ten experiments with error bars that correspond to 95% confidence intervals.

Figure 18. plots the failure pressure against the survival time from the swine trauma model. The failure pressure decreased significantly from the baseline after the pigs were subjected to multiple traumatic injuries hemorrhagic shock. These injuries led to a rapid blood loss which resulted in the onset of coagulopathy because of the loss of functional clotting factors. The base line failure pressure was significantly higher than the failure pressure at R30 and R60 for all the pigs. It is clear that the device can detect coagulopathy with the failure pressure going down considerably with the onset of coagulopathy.

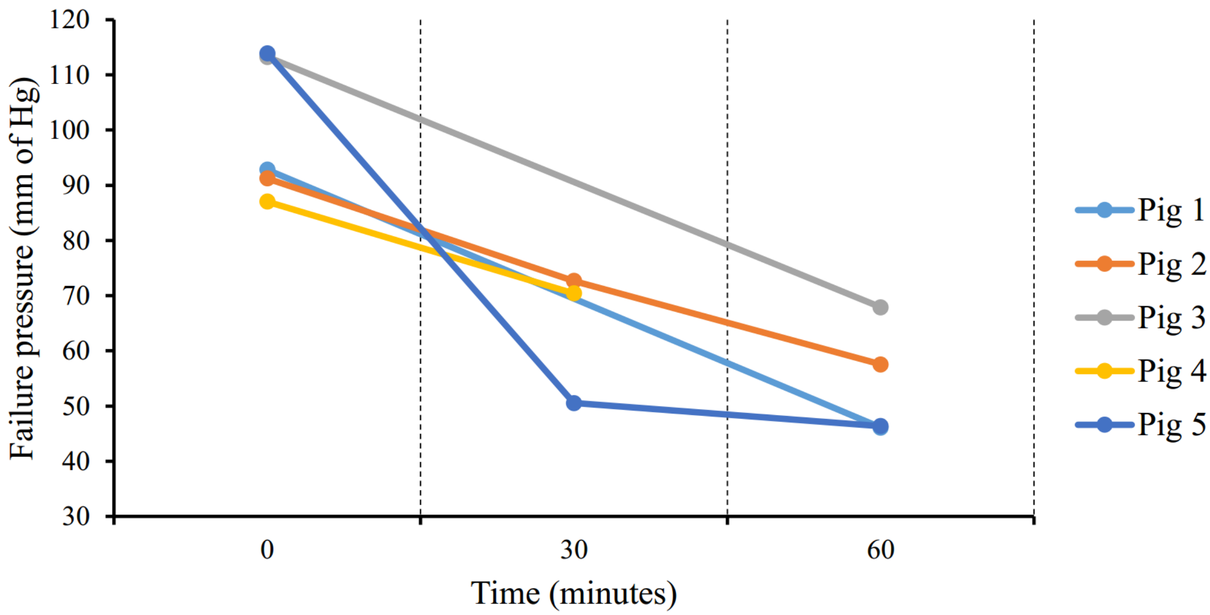

Figure 18. Failure Pressure vs Survival Time for Multi-trauma swine model: The failure pressure decreases significantly with the onset of coagulopathy in the swine model. X-axis denotes the survival time with multiple trauma induced at R0 which induces coagulopathy. The decrease in FP can attributed the loss of functional clotting factors in the blood after the trauma is induced in the pigs signifying that the device can detect coagulopathy in the swine model. Five sets of FP vs survival time is shown in this graph with all showing significant drop in FP with coagulopathy.

The table in figure 18. shows the fibrinogen levels, platelet count, prothrombin time (PT) and activated partial thromboplastin time (aPTT) at various intervals the blood was collected. The table suggests that FP is not only affected with the fibrinogen level as FP decreased significantly from baseline to R30 even though there is an increase in the fibrinogen level for Pig 2. This can be attributed to the fact that FP is affected by other functional factors as well.
Figure 19. shows the consolidated plot of FP and MCF against the fibrinogen level from the multi-trauma pig experiments. The fibrinogen level from all the pigs at baseline, R30 and R60 were plotted against the respective FP and MCF. There appears to be a definite trend of increasing FP with the increase in fibrinogen. Again, the percent change in FP with change in fibrinogen is greater than the percent change in MCF, thus making this device more sensitive to the change in fibrinogen than ROTEM. Figure 20. shows the consolidated plot of FP and MCF against the platelet count from the multi-trauma pig experiments. No definite trend is observed between FP and the platelet count.

From the above results, the setup successfully distinguished between diluted and non-diluted blood, effect of fibrinogen, factor XIII and detected coagulopathy in the swine model.

Table 1. Coagulopathy detection: table shows the Fibrinogen concentration (mg/dl), Platelet count (/nanoliter), Prothrombin Time and Partial Thromboplastin Time at intervals of 30 minutes for each pig.

	0	30	60	
Fibrinogen	276.9			Pig 1
Platelet Count	231			
PT	12.1			
aPTT	30.9			
Fibrinogen	206.7	209	229	Pig 2
Platelet Count	285	159	137	
PT	12.1	11.9	12.4	
aPTT	15.6	15.9	15.7	
Fibrinogen	263.4	222.5	272.7	Pig 3
Platelet Count	339	247	189	
PT	13.5	13.1	12.9	
aPTT	32	25.3	32.3	
Fibrinogen	183.5	215.5		Pig 4
Platelet Count	374	169		
PT	12.5	12.5		
aPTT	28	23.5		
Fibrinogen	262.1	134.6	125.5	Pig 5
Platelet Count	488	320	209	
PT	14.0	13.3	13.4	
aPTT	30.3	24.1	22.2	

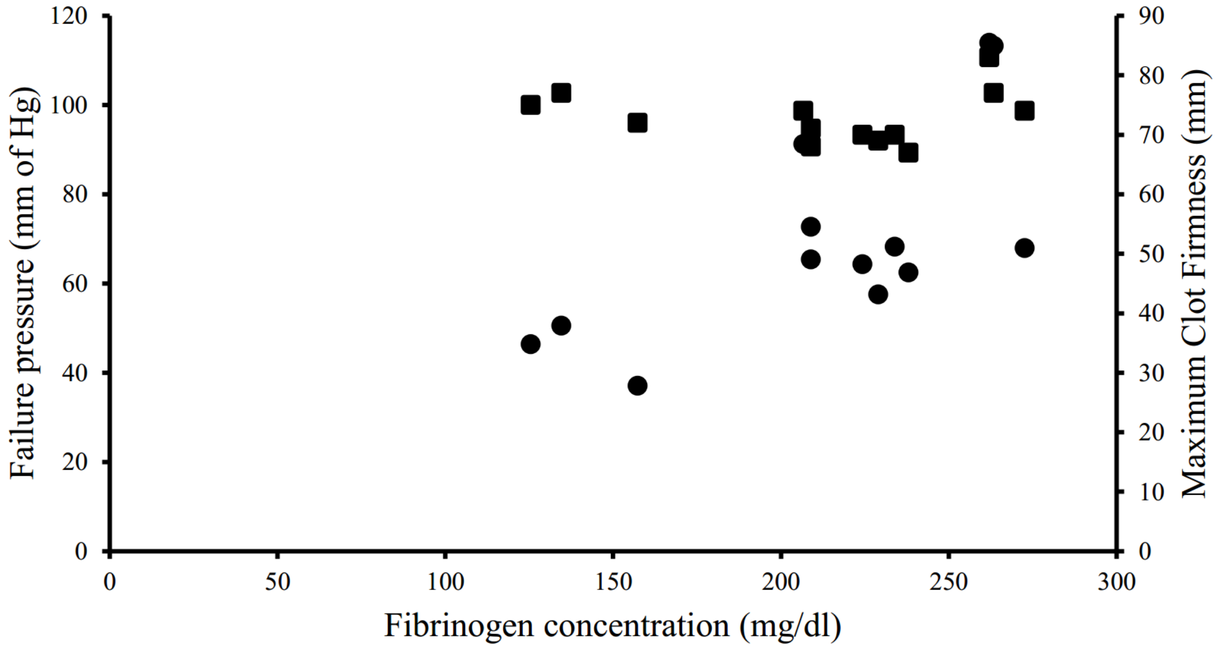

Figure 19. Failure Pressure (circles) and Maximum Clot Firmness (Squares) vs. Fibrinogen level. Multiple trauma studies using whole blood. Fibrinogen level of all the pigs at baseline, R30 and R60 was obtained and plotted against the respective FP and MCF. A definite trend is observed with FP increasing with the increase in Fibrinogen level. FP appears to be more sensitive to the Fibrinogen level than the MCF as the percent change in FP with the change in Fibrinogen level is greater than the percent change in MCF.

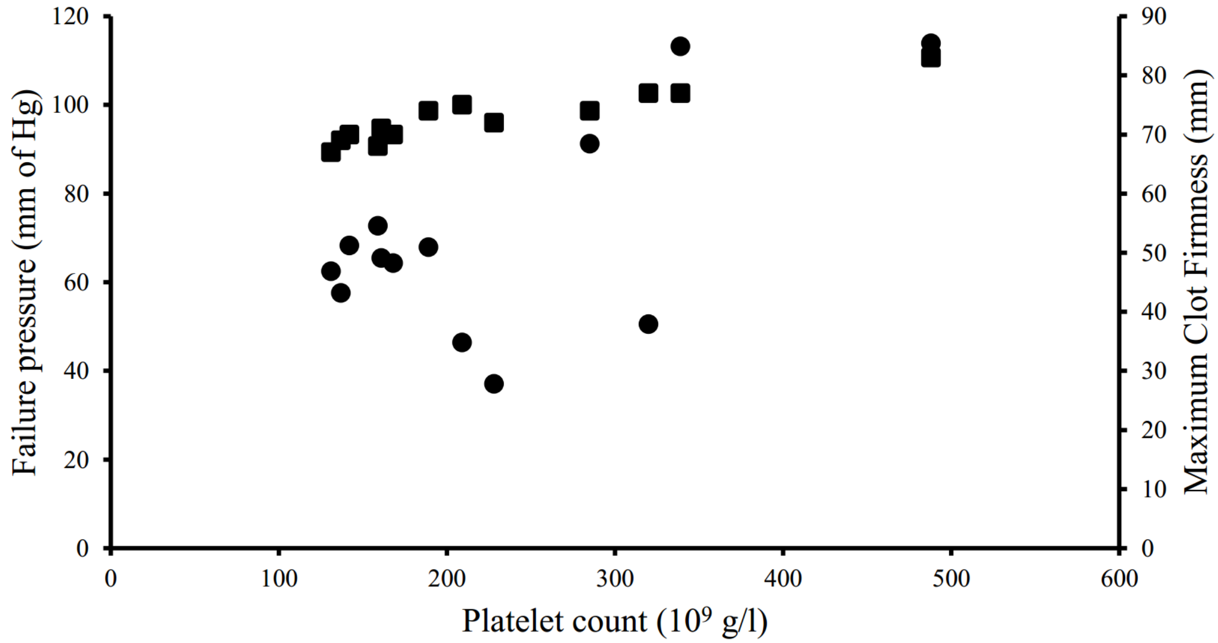

Figure 20. Failure Pressure (circles) and Maximum Clot Firmness (squares) vs. Platelet count: Multiple trauma studies using whole blood. Platelet Count of all the pigs at baseline, R30 and R60 was obtained and plotted against the respective FP and MCF. No definite trend is observed between FP and Platelet count, reaffirming that FP is not significantly affected by platelet count. This can be attributed to the fact that Thrombin was used as an activator which directly activates the fibrinogen in the blood.

CHAPTER 4: Discussion & Summary

The primary findings of this study are that catastrophic failure of clots contained within a rigid tube takes place by mechanical failure within the clot material. The resulting failure was also strongly associated with fibrinogen concentration and Factor XIIIa concentration, both of which are primary determinants of mechanical clot firmness.

Clots contained within our device appear to fail by air pocket propagation within the clot when exposed to increasing air pressure. This process is similar in appearance to propagation of a crack in solid surfaces, and involves initial small crack formation that leads to secondary crack formation and further propagation until catastrophic mechanical failure occurs.

The failure pressure increased consistently with increasing fibrinogen and Factor XIIIa concentration. This is a logical result given that the clots tended to fail in our device by intrinsic disruption of the internal structure of the clot and these components are primary determinants of internal clot structure. Fibrin fibers provide the primary resistance to mechanical deformation, and viscoelastic firmness and clot resilience can both be increased by increasing fibrinogen concentration [20]. Increasing fibrinogen concentration increases clot density, weight, and reduces porosity, thus providing direct resistance to mechanical deformation. Fibrin fibers and individual molecules also demonstrate resistance to strain deformation that includes fiber realignment under strain, extrusion of water from within fibers, and ordered unfolding of individual fibrin molecules [6] Therefore, it is likely that the denser and tighter fibrin network afforded by increasing fibrinogen concentration was responsible for the increased FP. Increasing Factor XIIIa concentration has similar effects on fibrin clot structure that emphasize a decrease in porosity due to crosslinking [21]. More surprisingly, platelet count had a limited effect on FP in our study. Platelets contribute greatly to initial clot formation and clot stiffness when measured using TEG or ROTEM [22]. However, they may play less of a role in supporting fibrin network structure after clots are mature and when supramaximal forces are applied. While initial clot formation may be more sensitive to the effects of platelets, clot failure appears to be more dependent upon fibrin fiber network integrity.

We observed that MCF and FP decreased similarly during traumatic hemorrhagic shock in the porcine trauma model. While this result is too preliminary to provide definitive conclusions, it does agree with published data regarding TIC. Clot firmness measure by ROTEM is the first parameter to decrease and its decrease is associated with increased mortality and blood transfusion requirements after injury [2]. In addition, fibrinogen is the first blood coagulation protein to reach critically low levels during surgery with ongoing blood loss [20]. Factor XIII decreases later after injury, but its loss has been associated with unexpected bleeding during surgery (REF). Our results suggest that clot failure pressure may correspond to the severity of TIC and that measuring clot failure characteristics may provide important and relevant information to the clinician caring for injured patients.

During trauma care, fluids are infused to restore blood pressure and tissue perfusion is lost during hemorrhagic shock [11]. However, increasing vascular hydrostatic blood pressure also puts the patient at risk for clot failure and rebleeding from wounds. Therefore, a strategy of keeping the blood pressure low (or hypotensive resuscitation) is currently used when definitive control of bleeding from wounds is not possible. Our results suggest that, in addition to blood pressure, fibrin clot integrity may also be a strong determinant of clot failure and rebleeding during trauma resuscitation. Data from animal bleeding models [18] and clinical experience support a strong role for the importance of maintaining fibrinogen concentrations and clot firmness during trauma resuscitation [19].

The clot failure device is a simplified model of clot failure within a rigid tube, that does not necessarily represent clot failure in vivo that takes place in 3 dimensions and involves cyclical blood pressure and multiple tissues present at vascular wounds. The trauma data is also limited and intended to only be hypothesis generating.

The results are promising with the device successfully detecting coagulopathy in swine models, showing the effect of fibrinogen and factor XIII and differentiating between diluted and non-diluted plasma. From the results it is clear that current device with Thrombin as the activator doesn't show a significant effect of platelet count. Further experiments are required using the multiple trauma swine model to check if FP can be used to predict blood loss. This can be achieved by plotting FP against the blood loss in each individual pig and checking for a trend. Due to lack of data points using the current experiments this could not be achieved. If this is achieved, it can add to the device's ability to provide a predictive diagnosis using a small sample of the patient's blood but a lot more data needs to generated before this can be achieved.

In the current study, Thrombin was used as the primary activator which directly activates the Fibrinogen in the blood. As a result, no significant effect of platelet count was observed. Collagen is the main structural protein in the extracellular space in the various connective tissues in animal bodies. As the main component of connective tissue, it is the most abundant protein in mammals [25] making up from 25% to 35% of the whole-body protein content. Collagen is the primary activator in the coagulation cascade [26], which comes in contact with the blood after a blood vessel is injured leading to clot formation. It will be helpful to test the effect of Collagen as an activator on FP and check if there is a trend between Collagen and FP. It is expected that using Collagen the clot formation time in the device will increase significantly, thus making it unsuitable to be used in a point-of-care test.

Further, miniaturizing the current bench-top prototype is one of the possible next steps in addition to more experimentation. By fabricating a Microfluidic prototype and conducting experiments to check for the efficacy of the device, this approach will be one step closer to a point-of-care device detecting coagulopathy and thus, guiding patient care.

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