COMBINED EFFECT OF SHEAR STRESS AND SECONDHAND SMOKE ON PLATELET ACTIVATION AND AGGREGATION

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COMBINED EFFECT OF SHEAR STRESS AND SECONDHAND SMOKE ON PLATELET ACTIVATION AND AGGREGATION

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Abstract: Platelets play important role in hemostasis (physiological), thrombosis and atherosclerosis (pathological). Changes in functional responses of platelets, due to presence of biochemical and biomechanical agonists, can contribute in onset and progression of cardiovascular diseases (CVD). Secondhand smoke (SHS) and altered shear stress in the vasculature are considered as biochemical and biomechanical risk factors for CVD. Though previous studies have investigated the effects of these two factors on change of platelet functions, none of the study have focused on how SHS and altered shear stress together can modulate platelet activation and aggregation. The main objective of this study was to investigate the combined effect of SHS and altered shear stress on platelet functions. Experiments were conducted *in vitro* on platelets by applying constant (1 and 3 Pa) or physiologically relevant dynamic (normal and elevated) shear stress in a cone-plate shearing device for 60 min, with or without the exposure of SHS (smoke of 1 cigarette/5 L). Platelet activation was quantified by platelet surface P-selection expression using flow cytometry. Following the same procedure, platelet surface GPIbα and GPIIb expressions were also measured. Platelet aggregation parameters were measured from TRAP induced platelet aggregation using Chrono-log aggregometer. Simultaneously, Thromboxane B_2 generation was quantified using a sandwich ELISA (enzyme immunoassay) approach. Results from this study indicated that, SHS enhanced shear induced platelet activation represented by enhanced platelet surface P-selectin expression. SHS with elevated constant (3Pa) shear stress significantly increased P-selectin expression. However, platelet surface GPIbα and GPIIb expressions remained unaffected in presence of the combined exposure. In addition, SHS and shear stress combinedly altered platelet aggregation response. Combined exposure of SHS and shear stress had no significant effect on accumulated Thromboxane B_2. Thus, the observations of this study indicated that, exposure to environmental SHS can potentially cause detrimental effects on the cardiovascular system by enhancing platelet activation, especially in patients with CVD.

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CHAPTER I

INTRODUCTION

Cardiovascular disease (CVD), characterized as any disorder that affects the cardiovascular system, is the number one killer disease worldwide as well as in the USA. According to the World Health Organization's (WHO) estimation in 2008, around 17.3 million people died of CVD, which is about 30% of global death[1]. According to the statistics conducted by American Heart Association (AHA) in 2009, 1 of every 3 deaths in the USA was caused by CVD[2].

Atherosclerosis is the most common type of CVD. Atherosclerosis is a complex long term process of lipid accumulation in the sub-endothelial layer of blood vessels. Atherosclerosis causes hardening of arteries and changes the geometry of the blood vessel by narrowing the vessel's inner perimeter. Atherosclerosis can result in partial or complete occlusion of a blood vessel, which hinders the required blood flow into the downstream tissues and leads to scarcity of oxygen and nutrition in those tissues.

Clinical observations have highlighted that atherosclerotic lesions preferentially localize to regions with complex vasculature. Vessels with complex geometry produce complex hemodynamic shear stress, which is considered as a risk factor for

atherosclerosis development[3]. The presence of atherosclerotic plaque further disturbs local shear stress conditions. Altered shear stress plays a significant role in platelet activation, which is critical in coagulation and thrombosis[4-6].

Smoking increases the risk of CVD. Second hand smoke (SHS) is capable of imposing deleterious effects on the cardiovascular system[7]. Previous studies have reported that short term exposure to SHS can cause adverse effects on cardiovascular system[8]. Exposure to SHS alone can increase the risk of CVD about 30%[9-11]. SHS can also significantly affect platelet activation[12].

It is well established that both disturbed hemodynamic shear stress and secondhand smoke could affect platelet activation[12;13]. However, the combined effect of dynamic shear stress and SHS on platelet behavior has not been determined.

The hypothesis of the present study is that the combined effect of altered shear stress and secondhand smoke could further enhance platelet activation and aggregation compared to their individual effect. The hypothesis will be tested through the following specific aims.

Specific Aim 1: To study the effect of dynamic shear stress and SHS on platelet surface protein expression by measuring platelet surface P-selectin, GPIbα and GPIIb expression using flow cytometry.

Specific Aim 2: To study the effect of dynamic shear stress and SHS on platelet aggregation induced by thrombin receptor activator peptide (TRAP).

Specific Aim 3: To investigate the effect of dynamic shear stress and SHS on Thromboxane A_2/B_2 generation from activated platelets.

Results obtained from this study could improve our understanding of platelet functional changes caused by altered dynamic shear stress and SHS, as well as the potential mechanisms associated, which may lead to new preventive or therapeutic solutions to atherosclerosis and other cardiovascular diseases.

CHAPTER II

BACKGROUND

2.1 Platelets

Platelets are non-nucleated discoid shaped cells circulating through the blood. These cells are produced from megakaryocytes in the bone marrow. Platelet count is generally maintained in the range of 150,000-350,000 per µL of blood. They have approximate dimensions of 3 µm by 0.5 µm and remain functional in the circulation for about 10 days. After this duration, dead platelets are removed and blood is replenished with new platelets.

Platelets have a tri-laminate cytoskeletal system that gives platelets their discoid shape and helps in enduring blood flow induced shear stress. The cytoskeletal lamina is comprised of a phospholipid bilayer, with inclusion of glycolipids, glycoproteins and cholesterol[14]. Platelets' cytoplasm contain normal cellular organelles like mitochondria, lysosomes, endoplasmic reticulum, and platelet specific granules. Although platelets are non-nucleated cells, they contain some megakaryocyte derived mRNA which can synthesize some protein[15]. Regardless of this capacity, most of the proteins required for various physiological processes are present either in the storage granules or on the

platelet membrane. Platelets contain two types of granules: α-granules and dense bodies. Between these two granules, α-granules are larger and most abundant in platelets. Approximately 50 to 80 α-granules are present in each platelet[16]. Contents of the α-granules consist of several proteins including von Willebrand Factor (vWF), fibrinogen, fibronectin, vitronectin, thrombospondin, P-selectin, platelet factor 4, platelet derived growth factor (PDGF), and coagulation factor V. On the other hand, dense bodies contain ADP, ATP, calcium (Ca^{2+}), serotonin and pyrophosphate[17]. The platelet membrane contains some glycoproteins such as GPIb-IX-V, GPIIb/IIIa, GPV and GPVI that regulate some of the platelet functions like adhesion and aggregation[15].

The major function of platelets is maintaining hemostasis. Apart from hemostasis, platelets are also involved in inflammatory responses which may lead to atherosclerosis, thrombosis and other cardiovascular diseases[18]. Hemostasis consists of several processes including coagulation, platelet adhesion, activation and aggregation. Hemostasis is a series of processes working in parallel to keep blood within the body by formation of blood clots, dissociation of clot, and healing of injured blood vessels. During vascular injury, the endothelial cell lining of the blood vessel wall becomes disrupted and the subendothelium is uncovered. Platelets start to become tethered and to roll along the endothelial cell surface using the platelet surface receptor P-selectin[19]. Adhesive molecules like collagen present in the subendothelium associate with plasma vWF (a multimeric protein) to mediate platelet adhesion. The platelet membrane receptor GPIb-IX-V interacts with vWF to promote adherence to the vessel wall[20]. Multiple binding sites of vWF facilitates multiple platelet capturing and cause firm platelet adhesion. Another receptor, GPVI, also mediates platelet attachment to subendothelial collagen.

GPIb-IX-V to vWF interaction transduces a conformational signal and activates platelets initiating a series of complex processes. Conformational change from this interaction activates platelet membrane receptor GPIIb/IIIa, induces granular secretion, translocation of phospholipid, rearrangement of platelet cytoskeleton (leading to shape change), increases in surface area and formation of pseudopods. Phospholipid translocation moves negatively charged phospholipid from the inner surface to the outer surface of platelet membrane and provides an anionic surface for Ca^{2+} binding and coagulation. Thromboxane A_2 (TXA_2) and ADP/ATP, released by activated platelets, are capable of mediating further activation, and recruitment of neighboring platelets from the blood circulation. Following initial platelet adhesion, activated platelet surface receptor GPIIb/IIIa and GPIa/IIa bind to fibrinogen and collagen respectively, stabilizing initial adhesion[21]. Fibrinogen has two binding sites for the platelet GPIIb/IIIa receptor that enable platelet-platelet binding, forming platelet aggregates. Figure 2.1 shows the initial platelet adhesion, activation related secretion, and stable aggregate formation.

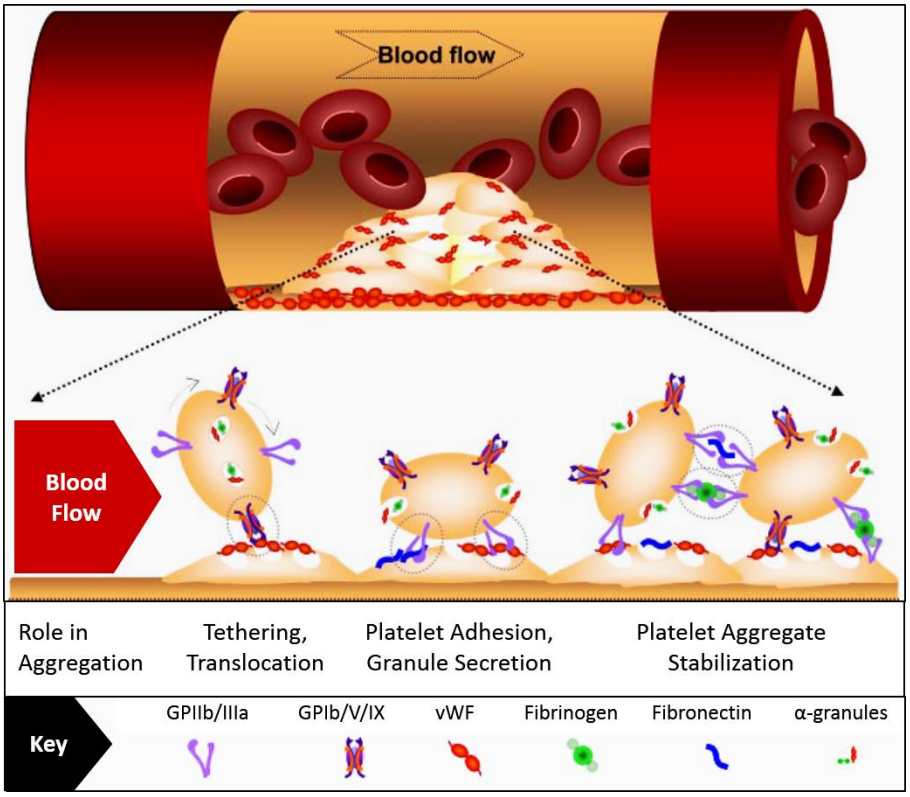

Figure 2.1: Platelet adhesion, activation and aggregation under blood flow[22].

Platelets can become activated in the presence of both biochemical and mechanical agonists. Important biochemical agonists are ADP, serotonin, thrombin (formed during coagulation), collagen and TXA_2. Disturbed shear stress generated in diseased blood vessels or in complex vascular structures are termed as mechanical agonists[4].

2.2 Hemodynamic Shear Stress

Hemodynamic shear stress generated by the flowing blood within the vasculature, is the most relevant mechanical force that can modulate platelet functions. Shear stress generated in blood vessels can induce intra-platelet and inter-platelet cell signaling, resulting in platelet activation, adhesion and aggregation. Platelet thrombus formation is

also modulated by physiological shear stress that can become unregulated in the presence of pathological shear stress conditions[5]. The presence of geometrical complexities like bifurcation, twisting and tapering in vascular regions alters the normal shear stress patterns, which may induce platelet activation. Activated platelets in the circulation pose a risk of atherosclerotic lesion formation, as they can secrete alpha granules and dense bodies packed with different adhesion molecules, coagulation factors (factor V), and generate microparticles[17]. Presence of atherosclerotic plaque in vessel lumens reduces vessel diameter, further disturbing the shear stress profile. Due to the effect of altered shear stress on platelet function and on cardiovascular disease progression, study of the effect of altered shear stress on platelet activity is of interest to many researchers.

Hemodynamic shear stress is the mechanical shear force exerted by blood flow on the vascular wall and on the circulating blood cells. Considering blood as a Newtonian fluid, the generated shear stress can be calculated by the following relation:

$$\tau = \mu \frac{du}{dy}$$

where, τ is the generated shear stress, μ is the effective dynamic viscosity of the fluid, and $\frac{du}{dy}$ is the velocity gradient towards the flow direction.

Under physiological condition, the generated shear stress within healthy vasculature is between 10 to 30 dyne/cm^2. Whereas, under pathological conditions, such as in the presence of atherosclerotic plaque in a stenosed vessel, shear stress can increase up to 380 dyne/cm^2 [23]. Numerous studies have been conducted to investigate how altered shear stress can induce platelet functional changes like activation and aggregation[3;24].

Brown *et al*. studied functional changes of platelets by applying constant shear stress (50, 100 and 250 dyne/cm^2) to measure platelet granule release and aggregation. Observations from their study included simultaneous platelet activation and aggregation at shear levels above 50 dyne/cm^2, platelet lysis starting at shear levels of 100 dyne/cm^2, and platelet fragmentation when the shear stress reaches 250 dyne/cm^2 [25]. Sakariassen *et al*. utilized a higher shear stress (315 dyne/cm^2) exposure for shorter duration (0.075 sec) and observed platelet activation within even this short period of shear exposure. In addition to that, these authors also concluded that prolonged shear exposure duration or a rapid increase in shear stress magnitude results in enhanced platelet activation[26]. In a study conducted by Hellums *et al*., an increased level of Ca^{2+} release from platelets and platelet aggregation was observed over 100 sec under shear stress exposure of 30, 60, 90 and 120 dyne/cm^2. This study established that both shear stress magnitude and shear exposure time have important role in platelet activation[4]. This fact was further supported by Ramstack *et al*., who applied relatively higher shear stress varying between 300-1000 dyne/cm^2 for a duration of 25-1650 msec, and found significant platelet activation at higher shear stress levels applied for lesser time. Summarizing the data from various studies an inverse relationship between shear stress magnitude and shear exposure time can be established, indicating both high and low shear stress can induce platelet activation when they are utilized to shear platelets for shorter and longer durations respectively[27;28].

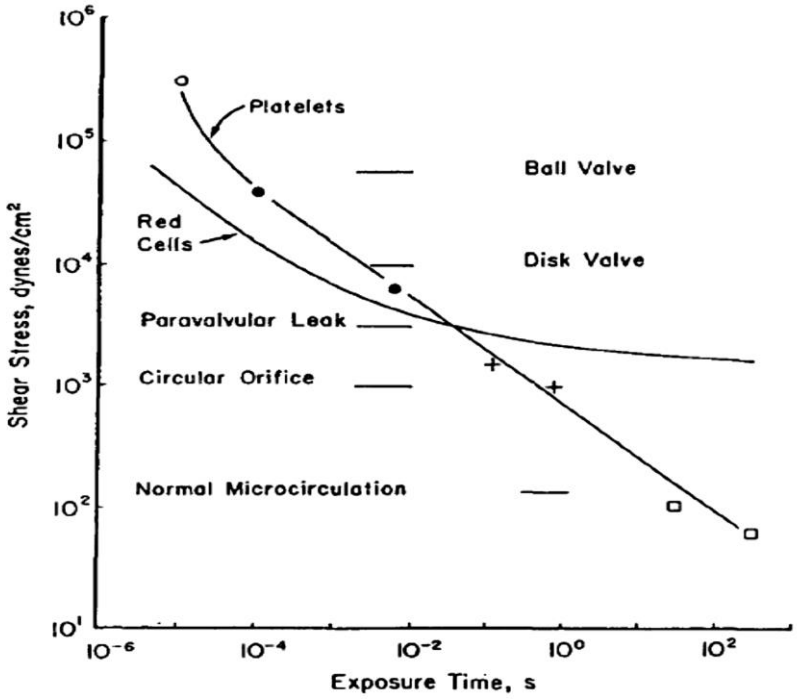

Figure 2.2: Relation between shear stress and exposure time for platelet activation (serotonin release) and hemolysis (red blood cell lysis) from summarized data of previous studies[4].

Though all these studies have investigated the effects of shear stress on platelet activation, most of them have utilized constant shear stress to activate platelets, which is not physiologically relevant. Rubenstein *et al*. used pulsatile shear stress varying between 4 to 40 dyne/cm^2 for 40 min to study the effect of more physiologically relevant dynamic shear stress on platelet activation and aggregation. Enhanced platelet activation was observed in response to a dynamic shear stress waveform with a transient high shear of 40 dyne/cm^2, compared to constant shear of 4.75 dyne/cm^2. Enhanced platelet aggregation was also observed with dynamic waveforms having a peak of 40 dyne/cm^2 [13].

Several techniques have been used experimentally to apply shear stress to platelets. A modified *in vitro* cell shearing device has been proposed by Blackman *et al*.

based on the cone-plate viscometer principle, which is able to apply not only constant shear stress but also complex shear waveforms[29]. In the current study the modified design was adopted to devise a cone-plate hemodynamic shearing device, which was used to investigate platelet responses under different shear stress conditions. The shear stress generated by this device is uniformly distributed throughout the fluid flow region and can be controlled precisely using a programmable controller. The hemodynamic cone-plate shearing device facilitates the application of dynamic shear stress waveforms obtained from numerical models of arterial flow.

2.3 Secondhand Smoke (SHS)

Second hand smoke (SHS), also termed environmental tobacco smoke (ETS), is mainly side stream smoke from the smoldering end of a cigarette. Tobacco smoke contains more than 4000 chemical compounds, among which approximately 250 compounds are known to be either toxic or carcinogenic[8;30]. SHS exposure is a critical risk factor that has no threshold for safe level of exposure (implying that the risk associated will be zero only at no SHS exposure). SHS exerts deleterious effect on physiological systems including the cardiovascular system[8]. SHS exposure affects inflammatory responses and atherosclerotic plaque progression, increasing the risk of pathological consequences by 30 percent[9-11]. Whincup *et al*., in a 20 year prospective study of passive smoking and coronary heart disease, found that risk of heart disease produced by passive smoking is nearly similar to that of light active smoking[31].

SHS exposure hampers the equilibrium condition of normal hemostasis[32] by modulating platelet function[33]. The impact of SHS on platelets is reported to be as potent

as that of mainstream smoke[12]. Studies have indicated that platelet activation and aggregation increases with SHS exposure leading to unregulated thrombus formation[34]. Unregulated thrombus formation and the presence of activated platelets in the circulation contribute to formation, and growth of atherosclerotic plaque[6;35]. The first study quantifying platelet activation by SHS was conducted by Benowitz *et al*. Human subjects (smokers and non-smokers) were exposed to SHS for 20 min. In this study, enhanced platelet activation was found in non-smokers exposed to SHS compared to control (non-smokers not exposed to SHS)[36]. Several animal studies have also indicated that passive smoking contributes to atherosclerotic plaque growth and decreased bleeding time by enhancing platelet activities (activation, adhesion) [37-39]. Rubenstein *et al*. conducted an *in vitro* study, and exposed platelets to 12 dyne/cm^2 of shear stress and SHS (at the concentration of smoke from 1 cigarette per 5 L of washed platelets) for up to 30 min in a circulation flow loop, and observed increased platelet activation[12].

In summary, although numerous studies have been conducted to investigate the effects of shear stress and SHS on platelet functions separately, not many studies have examined the combined effects of dynamic shear stress and SHS on activation and aggregation of platelets.

2.4 Platelet Surface Protein Expression

Platelet surface proteins play a significant role in modulation of platelet functions. Protein expression is subject to change in the presence of different agonists, as various agonists induce different level of platelet activation. As previously mentioned, hemodynamic shear stress and SHS are two such factors that can cause changes in

platelet function, leading to various levels of surface protein expression. Therefore in our study protein expression of platelets subjected to shear stress and SHS was used for quantifying platelet activation. Common techniques for assessment of platelet surface protein expression include flow cytometry[40;41] and enzyme linked immunosorbent assay (ELISA)[42]. Flow cytometry is the most widely used method for platelet surface protein quantification, and this technique was used in this study.

2.4.1 P-selectin Expression

P-selectin is the largest glycoprotein of the selectin family at 140 kDa molecular weight. Under normal conditions P-selectin remains stored in the membrane of α-granules[43]. Upon activation, P-selectin of α-granules redistributes on the platelet surface as a result of degranulation[44]. Activated platelets express approximately 10,000 P-selectin receptors on their surface[45]. Among all of the platelet surface glycoproteins, P-selectin is considered to be the most reliable activation marker, due to its overexpression during platelet activation[46;47].

P-selectin is involved in the rolling and tethering of platelets on endothelium[48]. P-selectin plays a major role in adhesion by assisting rolling of platelets[49] and by recruiting more platelets. Along with cell-cell adhesion, P-selectin also initiates positive feedback which further activates endothelium. P-selectin is also considered to have an important function in platelet-platelet aggregation, and to aid in stable GPIIb/IIIa-fibrinogen binding[50]. It is also important in developing large stable platelet-leukocyte aggregates[51]. Under pulsatile shear stress at stenosed/atherogenic regions, P-selectin is involved in shear induced aggregation[52]. Results from previous studies have established that P-

selectin mediates thrombogenesis, atherogenesis and contributes to cardiovascular disease progression[45;51].

Its involvement in pathological conditions like atherogenesis has made P-selectin a platelet marker of interest. Shear stress itself may cause platelet activation, which is evident from up-regulation of P-selectin expression[53]. Several groups have conducted *in vitro* studies to observe the expression of P-selectin on the platelet surface under different shear stress conditions. Goto *et al.* observed a significant increase in P-selectin expression after applying shear stress at the rate of 10,800 $s^{(-1)}$ on platelets[54]. Lu *et al.* applied 10, 15 and 20 Pa (1 Pa = 10 dyne/cm^2) shear stress on platelets in whole blood for 120 sec and found a significant increase in P-selectin expression with the increase in shear stress magnitude[47]. Zhang *et al.* used 100 dyne/cm^2 shear stress on platelets in platelet rich plasma and washed platelets in their study for short durations (10-20 sec), where minimal changes in P-selectin expression were observed. This study indicated that the duration of shear application, together with shear stress magnitude, are important for inducing changes in platelet P-selectin expression[55]. Rubenstein *et al.* applied several different shear conditions including constant shear of 4.75, 10, 40 dyne/cm^2 and pulsatile flow (varying from 4 to 40 dyne/cm^2) on washed platelets for 40 min, and observed an increase in the expression of P-selection with increased shear stress-exposure duration[13]. In another study conducted by Farzana *et al*., constant shear (1, 3, 10 and 30 dyne/cm^2) and dynamic shear stress (normal shear varying from 1 to 10 dyne/cm^2, recirculation shear varying from 0.6 to 4 dyne/cm^2, and elevated shear varying from 1 to 65 dyne/cm^2) was applied on washed platelets for 60 min. Results from this study indicated that there was no significant change in P-selectin expression under dynamic shear conditions.

However, under constant shear conditions, a significant increase in P-selectin expression was observed with increased shear stress magnitude[56]. Increased platelet activation due to the higher shear loading conditions with constant shear stress results in the fuse of more α-granules with platelet membrane resulting in an increased P-selectin expression on surface.

Along with shear stress, SHS is also considered to be a major risk factor for cardiovascular disease progression. Rubenstein *et al.* used SHS (at the concentration of smoke from 1 cigarette per/5 L) and found SHS to be a potent factor for platelet activation under normal flow conditions[12]. Though there have been many studies investigating the effect of shear stress and SHS on P-selectin expression separately, the combined effect of SHS and shear stress on P-selectin expression remains to be determined. Therefore the goal of this study was to investigate the separate effect of the two factors, as well as the combined effect of shear stress and SHS on P-selectin expression by platelets.

2.4.2 GPIbα Expression

Platelet surface receptor GPIb/V/IX is a complex composed of four transmembrane glycoprotein subunits, among which GPIbα plays a key role. GPIbα is non-covalently bound with other glycoprotein subunits (GPIbβ, GPV and GPIX) to form the complex[57]. GPIbα is the main binding site of the complex with an approximate molecular weight of 170kDa[58]. Approximately 25,000 GPIb/V/IX complexes are present on the resting platelet membrane[59].

GPIbα can initiate platelet adhesion through GPIbα interaction with plasma or subendothelial vWF during vascular injury. In the presence of high shear stress, GPIbα adheres to the subendothelial associated vWF, which triggers a conformational signal leading to platelet activation[60]. The GPIbα-vWF interaction simultaneously activates the platelet surface GPIIb/IIIa receptor, resulting in subsequent platelet aggregation[61].

In addition, the initial GPIbα-vWF adhesion facilitates platelets rolling on the subendothelium, slows down the platelet, and provides more time for platelet-subendothelium interaction resulting in stable adhesion[62]. GPIbα also contains binding sites for thrombin, which enables activation of platelets in the presence of thrombin[63].

Since shear stress can modulate platelet surface GPIbα expression, several studies have been conducted to observe the effect of shear stress on expression of this protein. Li *et al*. applied low level (100, 150, 1,000 s^{-1}) and high level (3000 s^{-1}) of shear stress for 7 min on whole blood to measure GPIb/V/IX complex expression on platelet surface[64]. Their results indicated that GPIb/V/IX expression was unchanged at low shear stress levels, while increased initially (for 1 min) and then decreased with time under high shear stress. In another study by Leytin *et al*., GPIbα expression was measured after shearing platelet rich plasma under physiological (10-44 dyne/cm^2) and pathological (117-388 dyne/cm^2) shear stress conditions for 90 sec[65]. GPIbα expression was found to be unchanged by physiological shear, whereas a pathological level of shear stresses decreased platelet surface GPIbα expression. White *et al*. applied shear stress (1000 s^{-1}) for longer duration (up to 20 min) on washed platelets and observed no change in GPIb/V/IX expression on the platelet surface[66]. Results from several studies have indicated that SHS can affect platelet activation and aggregation; however, platelet

surface GPIbα or GPIb/V/IX complex expression in the presence of shear stress and SHS has not been quantified in any studies[12;67]. Therefore, the goal of this study was to quantify the combined effect of SHS and shear stress on platelet surface GPIbα expression.

2.4.3 GPIIb Expression

The GPIIb/IIIa complex, also known as $\alpha_{IIb}\beta_3$, is a platelet surface glycoprotein that plays a major role in platelet aggregation. Approximately 80,000 GPIIb/IIIa receptors are presented on each platelet surface[68]. GPIIb/IIIa is a member of the integrin family, which contains two transmembrane glycoprotein subunits (GPIIb and GPIIIa) associated by non-covalent bonding[69]. Both subunits are equally important for binding and function of the GPIIb/IIIa complex[70]. Glycoprotein GPIIb has a molecular weight of about 136 kDa and is directly involved in ligand binding[71]. GPIIb/IIIa can bind several soluble ligands including fibrinogen, fibronectin, thrombospondin, and vWF[72]. On resting platelets, this integrin remains in an inactive (low affinity) state and cannot bind with the ligands. However, during platelet activation, degranulation of alpha and dense granules causes an increase in platelet surface GPIIb/IIIa receptor[73] and transduces a conformational change to activate (high affinity ligand binding state) the receptor that enables ligand binding[74].

Following platelet activation, GPIIb/IIIa mainly binds to fibrinogen facilitating firm adhesion[61]. Dimeric fibrinogen also enables binding of two adjacent platelets through GPIIb/IIIa-fibrinogen-GPIIb/IIIa cross-linking leading to platelet aggregation. Under high shear stress conditions, GPIb/V/IX-vWF interaction mediates shear induced

platelet activation resulting in GPIIb/IIIa receptor activation and stable aggregate formation mediated by GPIIb/IIIa-vWF interaction[75].

Due to its importance in platelet adhesion and aggregation, many studies have investigated the effects of mechanical and biochemical agonists on platelet surface GPIIb/IIIa. Li *et al*. applied low level (100, 150, 1,000 s-1) and high level (3000 s-1) shear stress for 7 min on whole blood to measure GPIIb/IIIa receptor expression on the platelet surface[64]. Their results indicated that GPIIb/IIIa expression increased at low shear stresses, but decreased at higher shear stress. Zhang *et al*. applied 100 dyne/cm^2 shear stress on washed platelets and platelet rich plasma for 10-20 sec and observed minimal change in GPIIb/IIIa expression[55]. Though several studies have examined the effect of shear stress on GPIIb expression, the effect of SHS on platelet GPIIb receptor expression under different shear stress conditions has yet to be elucidated. Therefore, this study aimed to quantify platelet surface GPIIb expression in the presence of shear stress and SHS, and to compare the results with the corresponding expression of normal resting platelets.

2.5 Platelet Aggregation

Platelet aggregation is another process by which platelets contribute to hemostasis. The aggregation mechanism of platelets has been discussed in section 2.1. Enhanced platelet aggregation or unregulated thrombus formation can be an indication of cardiovascular disease progression, as these processes can result in vascular occlusion [76;77].

Platelet aggregation can be measured *in vitro* using several methods including turbidimetric aggregometry, impedance aggregometry, flow cytometry, and lumiaggregometry[78-82]. Turbidity aggregometry measures optical transmittance in platelet rich plasma, whereas impedance aggregometry measures electrical impedance in whole blood. Flow cytometry is less common method. Lumiaggregometry can measure platelet aggregation and platelet dense granule secretion simultaneously in whole blood. In this study, we chose to use turbidity aggregometry to measure platelet aggregation.

Since notable changes of platelet aggregation are considered as a hallmark of cardiovascular disease, platelet aggregation has been studied extensively under different conditions. Moake *et al*. applied constant shear stress of 30-60 dyne/cm^2 for 30 sec on washed platelets and observed enhanced platelet aggregation[83]. In a similar study, Chow *et al* applied 15, 30, 60, 90, and 120 dyne/cm^2 shear stress on washed platelets for 100 sec and observed increased platelet aggregation with the increase in shear stress magnitude[84]. Rubenstein *et al*. found a significant increase in platelet aggregation at 10 and 40 dyne/cm^2 compared to 4.75 dyne/cm^2 shear stress. Davis *et al*., in a human study, observed increased platelet aggregation when human subjects were exposed to SHS for 20 min[85]. In another study by Rubenstein *et al*., an increased platelet aggregation was detected when washed platelets were exposed to SHS at the concentration of smoke of 1 cigarette per 5 L for 4 hr[67]. Since aggregation of platelets is an important indication of change in platelet function, our goal was to determine the combined effect of SHS and shear stress on platelet aggregation potential.

2.6 Platelet Thromboxane A_2/B_2

Thromboxane is a subfamily of lipids that are known as eicosanoids. Thromboxane A_2 (TXA_2) and thromboxane B_2 (TXB_2) are the two major thromboxanes produced by activated platelets. Upon activation by various agonists (including ADP, collagen, and thrombin), phospholipids of the platelet membrane are hydrolyzed into arachidonic acid. Oxygenation of arachidonic acid produces prostaglandin H_2, which in turn is converted into TXA_2 by thromboxane A_2 synthase[86]. TXA_2 induces a positive feedback mechanism, which enhances further platelet activation and promotes platelet aggregation by recruiting more platelets[87;88]. However, TXA_2 is chemically unstable, and has a short half-life (about 32 sec) at normal body temperature in biological fluids (plasma, urine)[89]. At the end of this short life, TXA_2 is metabolized into TXB_2 and excreted from human body via urine.

As only activated platelets synthesize thromboxane A_2/B_2, an increased level of plasma thromboxane indicates platelet activation. Both TXA_2 and TXB_2 can promote the onset and progression of atherosclerotic events in the vasculature[90]. An increased level of TXA_2 formation has been observed in patients with heart disease[91;92] and atherosclerosis[93]. Due to its pro-aggregatory activity, TXA_2 is considered to be an important factor for maintaining hemostasis and contributing to thrombosis. Studies have indicated that TXA_2 acts as a positive feedback element and can modulate platelet shape change and granular secretion[94]. In an animal study using stenotic canine coronary arteries, Aiken and Bush *et al*. reported prevention of arterial thrombosis by a thromboxane synthase inhibitor[95;96]. Findings from this study indicated that TXA_2 is

involved in thrombotic events. Fuse *et al*. observed a higher level of plasma TXB_2 in congenital heart disease patients[97].

In a human study conducted by Kritz *et al*., an increased level of plasma TXB_2 was detected in both smokers and non-smokers after they were exposed to passive smoke[98]. Passive smoking (SHS) enhances platelet activation and aggregation[34], suggesting that SHS could also enhance thromboxane release. Similarly, shear induced platelet activation could also enhance TXA_2 synthesis in platelets. Though several pharmacological studies have measured plasma thromboxane levels under various pathological conditions, not many studies have correlated platelet thromboxane release with risk factors such as altered shear stress and SHS. Since inhibition of the TXA_2 formation pathway and TXA_2 dependent platelet activation can be a potent tool to reduce CVD[99-102], investigating the level of TXA_2/TXB_2 generation by platelets exposed to altered shear stress and SHS is clinically important in developing new therapies for CVD.

CHAPTER III

MATERIALS AND METHODS

3.1 Platelets

Platelet pheresis bags were obtained from the Oklahoma Blood Institute. Platelets were separated from the plasma by centrifugation at 3000 rpm (1100×g) for 9 min. The supernatant (platelet poor plasma, PPP) was then discarded and platelet pellets were re-suspended in HEPES buffered modified Tyrode's solution (HBMT: 137 mM sodium chloride, 2.7 mM potassium chloride, 0.36 mM monobasic sodium phosphate, 12 mM sodium bicarbonate and 2 mM magnesium chloride, 0.2% BSA, 0.1% dextrose, and 0.01mL/mL of HEPES buffer (0.01 M – pH 7), pH 7.4). Platelet count was adjusted to 250,000/µL. Washed platelets were kept under mild agitation on a shaker at room temperature (RT), and used within 5 hour of preparation. Washed platelets were used to measure platelet surface protein expression using flow cytometry. Platelet rich plasma (PRP) was used for platelet aggregation and thromboxane B_2 generation experiments. PRP was diluted using the autologous platelet poor plasma (PPP), and the final platelet count was adjusted to 250,000/µL.

Washed platelets or platelet rich plasma were treated with shear stress and/or second hand smoke to investigate the combined or separate effect of shear stress and SHS on platelet responses.

3.2 Shear Stress Treatment

3.2.1 Cone-Plate Shearing Devices

Different constant and dynamic shear stress was applied to platelets in a cone-and-plate shearing device. The cone-plate shearing device is a standard device to apply shear stress to cells[103]. Figure 3.1 represents the schematic view of the cone-plate shearing device. Shear stress generated in the cone and plate shearing device can be calculated using Equation (1).

$$\tau = \frac{\mu\omega}{\alpha} \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where, τ is the generated shear stress, μ is the dynamic viscosity of the fluid, ω is angular velocity and α is the cone angle.

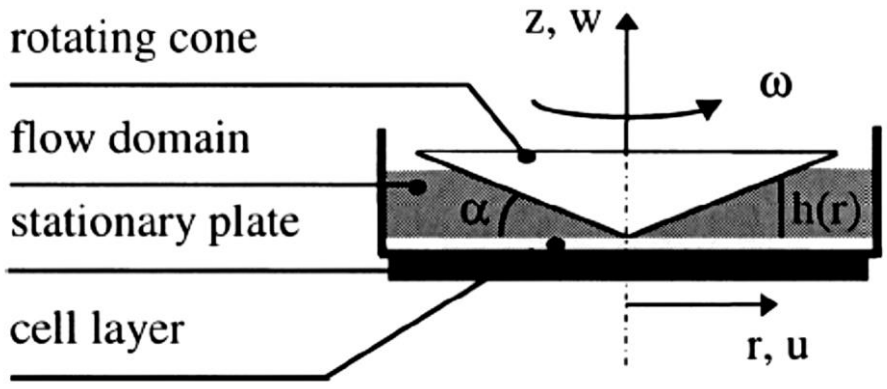

Figure 3.1: Schematic diagram of the basic cone-plate shearing device applying uniform shear stress on the fluid flow domain[104].

Since the dynamic viscosity of the fluid μ and the cone angle α are constant, shear stress in the flow field can be precisely controlled by adjusting the angular velocity of the cone. Under laminar flow conditions, shear stress is uniformly distributed throughout the flow domain. Two types of cone and plate shearing devices (single cone-plate and 6 well cone-plate) were used in this study.

A. Single Cone-Plate Shearing Device

The single cone-plate shearing device is shown in Figure 3.2. The diameter of the cone was 49.18 mm, and the angle of the cone was 2°. The maximum shear stress that can be generated by this device is 7.2 Pa (for laminar flow). Experiments were conducted in a 60-mm petri-dish containing approximately 3 ml of sample.

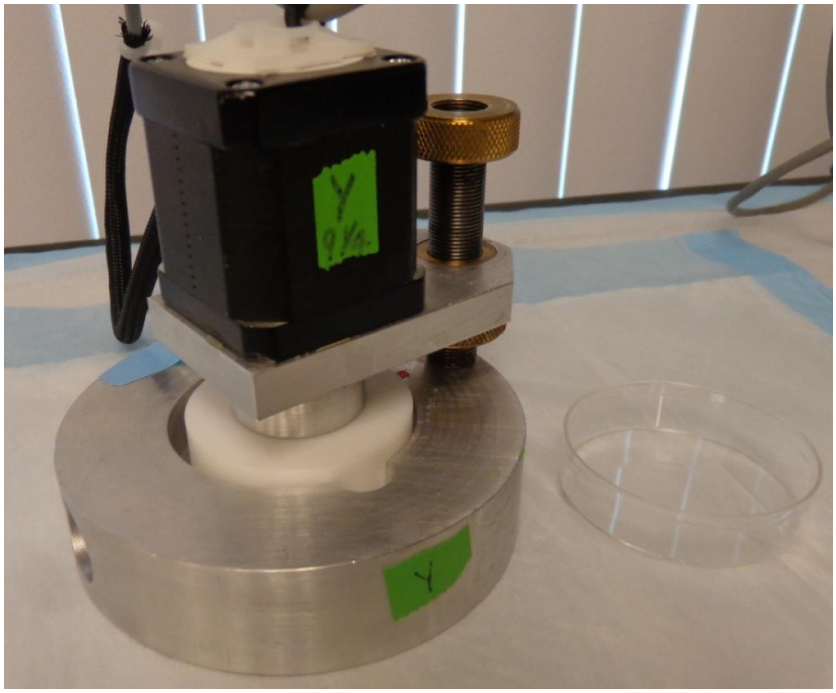

Figure 3.2: Hemodynamic Single Cone-plate shearing device used in this study for platelet shearing.

B. 6-well Cone-Plate Shearing Device

The design of the 6-well cone-plate device is similar to that of the single cone-plate dev1ice. This device (shown in Figure 3.3) had four cones, which can fit in the wells of a 6-well cell culture plate. The diameter of each cone was 34.66 mm, and the cone angle was 0.5°. The maximum shear stress generated by this device is 12 Pa (for laminar flow). Approximately 0.85 ml of sample was added to each well of the 6-well plate for shearing.

Figure 3.3: Hemodynamic 6-well cone-plate shearing device used in this study for platelet shearing.

Both shearing devices were used to shear platelets in different experiments depending on the volume of sample required for the downstream analysis. Angular velocity for both devices was adjusted accurately to keep the generated shear stresses identical. No variation was observed, using these two shearing devices to produce the same shear stress. Washed platelets and PRP were exposed to various shear stress

waveforms in the two shearing devices for 60 min (at RT).

3.2.2 Shear Stress Profiles

Two different types of shear stress were used in this study: 1) Constant shear stress at 1 and 3 Pa, and 2) Physiologically relevant dynamic (normal and elevated) shear stresses. 1 Pa was chosen to mimic the physiologically relevant normal mean shear stress[105;106], and 3 Pa to simulate the patho-physiological elevated shear stress condition in a diseased blood vessel. The pulsatile normal shear stress varied between 0.1 Pa and 1 Pa, mimicking shear stress conditions in a normal coronary artery. The pulsatile elevated shear stress was used to mimic shear stress experienced by platelets as they passed a stenosis. While the cells were passing through the simulated stenosis throat, they were exposed to high shear stress at 6.5 Pa for a short duration of time (~ 0.1 sec), and as they exited the diseased region shear stress applied to them returned to the normal level. The dynamic shear stress waveforms that were utilized in this study to stimulate platelets are shown in Figure 3.4.

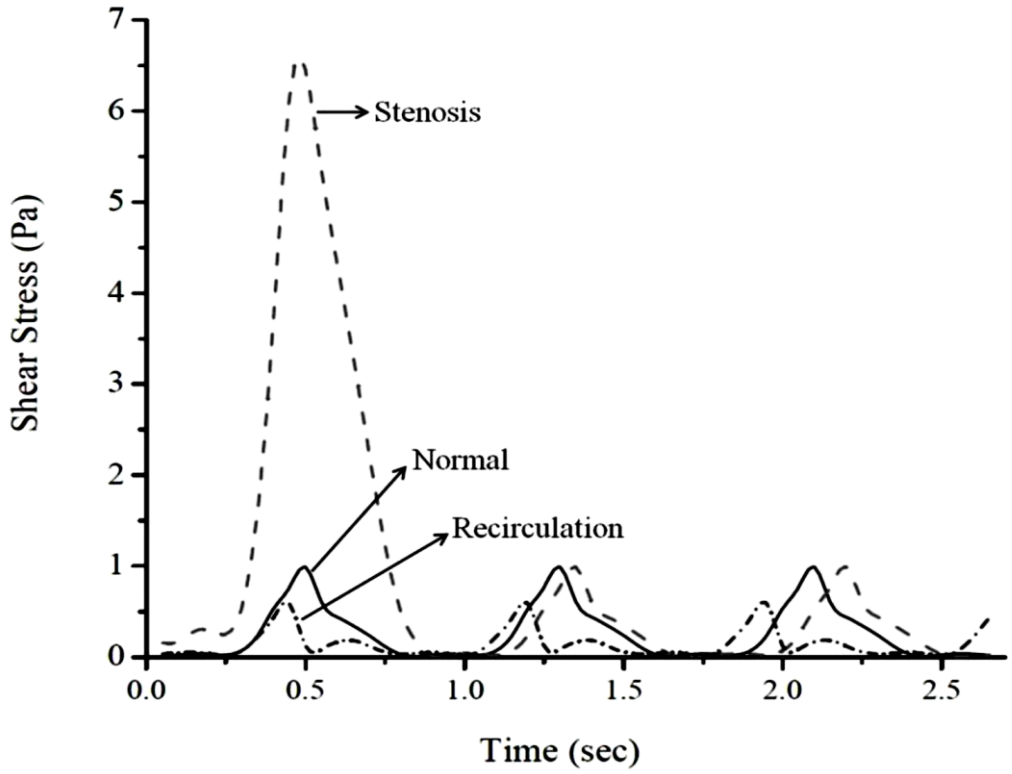

Figure 3.4: Shear stress profile of platelets passing through the left coronary artery under physiological and pathological conditions[107].

3.3 Secondhand Smoke Treatment

Side stream smoke, or secondhand smoke (SHS), was extracted from Marlboro cigarettes in HBS buffer (pH 7.4). Smoke extract was collected from the smoldering end and did not pass through any filters. The extraction procedure reported by Yin et al. was followed for SHS extraction[108]. Smoke extract was collected in the buffer at the concentration of smoke of 1 cigarette/50 mL. For all experiments SHS was used at a final concentration of smoke of 1 cigarette/5L, mimicking exposure to secondhand smoke from one cigarette. Platelets (both washed platelets and platelet rich plasma) were treated with SHS by mixing SHS extract at the specified concentration, followed by 60 min incubation (RT). Combined treatment with SHS and shear stress was attained by addition

of SHS extract (at the specified concentration) and then application of the desired shear stress on platelets for 60 min (RT).

3.4 Platelet Response Quantification

Platelet responses to SHS and/or shear stress were quantified through the measurement of platelet surface protein expression, platelet aggregation, and platelet thromboxane B_2 release as described below.

3.4.1 Platelet Surface P-selectin, GPIbα and GPIIb Expression

Washed platelets were treated with SHS and/or shear stress for 60 min. Untreated washed platelets were used as a negative control, and thrombin receptor activator peptide (TRAP at 20μM, 5 min, RT) activated platelets were used as a positive control. TRAP activated platelet represents complete platelet activation.

Post treatment platelet surface P-selectin expression was measured by incubating platelets with FITC conjugated monoclonal mouse anti-human P-selectin antibody (CD62P, Ancell Corporation, Bayport, MN) at 1:50 dilution (30 min, RT, in the dark). Samples were then diluted in HBMT (1:10) and read immediately using a flow cytometer (Accuri C6, Ann Arbor, MI).

Platelet surface GPIbα and GPIIb expression were also measured following the same procedure as for P-selectin.expression. FITC conjugated monoclonal mouse anti-human GPIbα anti-body (CD42b, Abcam, Cambridge, MA) at 1:10 dilution for (30 min, RT, in the dark) and FITC conjugated monoclonal mouse anti-human GPIIb antibody (CD41a, Ancell Corporation, Bayport, MN) at 1:50 dilution (30 min, RT, in the dark)

were used respectively for platelet surface GPIbα and GPIIb protein expression measurement. FITC conjugated MOPC antibody (MOPC, Ancell Corporation) was used at 1:100 dilution (30 min, RT, in the dark) to detect non-specific binding.

Platelet surface protein expression measurements were recorded by the cflow (Accuri C6) computer interface. The mean fluorescence value of specific platelet surface proteins was obtained from flow cytometry and was used for further data analysis. Data were normalized to the respective negative control of the same day to account for the variation in platelet donor. Therefore, there was no variations due to different platelet donors in the final data analysis.

3.4.2 Platelet Aggregation

Normalized PRP samples were treated with SHS and/or shear stress for 60 min and untreated PRP samples served as control. Aggregation in response to thrombin receptor activator peptide (TRAP, 20μM) was measured at 37°C, using an optical aggregometer (Chrono-Log Corporation, Model 490-2D, Havertown, PA). All the points for the aggregation curve were recorded using a computer interface program (Aggrolink). Using these saved points, the slope of the aggregation curve, percent aggregation, and maximum percent of aggregation were calculated. Slope and percent aggregation were calculated using a MATLAB program (which is included in the appendix) and maximum percent aggregation was obtained directly from the recorded points of aggregation. The slope for the first 60 points represents the rate at which platelets participate in TRAP induced aggregation. Percent aggregation quantifies the percentage of platelets participating in aggregation, whereas the maximum percent aggregation means the

maximum percentage of platelets participating in aggregation induced by TRAP. All the data were normalized to the respective control, to address variation from different platelet bags at different days.

3.4.3 Thromboxane B_2 (TXB_2) Assay

Activated platelets can release thromboxane A_2 which rapidly transforms into thromboxane B_2. Using a Thromboxane B_2 Express EIA kit (monoclonal, Cayman Chemical Company) the thromboxane B_2 (TXB_2) level was measured in plasma. After SHS and/ or shear stress treatment, PRP samples were centrifuged at 3000 rpm for 6 min. Platelet poor plasma (PPP) was collected and applied to a microtiter plate pre-coated with goat polyclonal anti-human antibody. Untreated PPP was used as control. Next TXB_2 tracer and TXB_2 monoclonal antibody were added (2 hr, RT, on orbital shaker) for binding with the polyclonal antibody in the wells. The Amount of TXB_2 antibody tracer complex bounded to the goat polyclonal antibody was detected by using Ellman's reagent (1hr, RT, on orbital shaker, in the dark) through color change. Color development was measured using a microplate reader (BioTek Instruments, ELx800) at 405nm. Color intensity was inversely proportional to the amount of TXB_2 present in the wells. TXB_2 express EIA standard (supplied with the assay kit) was used at different dilutions to obtain a linear relationship between color intensity and TXB_2 concentration. The concentration of TXB_2 in the plasma was calculated by comparing results with the linear relation obtained from the TXB_2 standard. Data normalization with respect to the control sample was conducted to avoid variations from different platelet bags at different days.

3.5 Statistical Analysis

For each platelet surface protein, expression (all mean fluorescence, AMF) values were obtained using flow cytometry and the values were normalized to negative control.

In platelet aggregation experiments from the collected aggregation points (obtained using aggregometer), slope of the aggregation curve, percent aggregation, and maximum percent of aggregation were calculated. Following normalization with respect to control all the three parameters were used for data analysis.

The concentration of thromboxane B_2, which was released by the platelets in plasma, was obtained from the optical density (OD) values obtained from the thromboxane B_2 assay. Thromboxane B_2 concentrations were normalized to the control sample prior to further analysis.

All data obtained from these experiments were normalized to the respective resting platelet control data for statistical data analysis. Single factor ANOVA was conducted to determine if there was any significant effect from SHS and shear stress treatment on platelet responses. If a significant difference was detected, Student-Newman-Keuls *post hoc* test was performed for multiple comparisons. Microsoft Excel and Statistical Analysis System (SAS 9.3) software was used for all statistical analysis with a significance level of $\alpha = 0.05$.

CHAPTER IV

RESULTS

4.1 Platelet Surface Protein Expressions

Platelet surface protein expression was measured after exposing washed platelets to second hand smoke (SHS) and/or shear stress for 60 min. Constant shear stress of 1 or 3 Pa or dynamic shear stress of pulsatile normal (varying from 0.1 to 1 Pa) or pulsatile elevated (varying from 0.1 to 6.5 Pa) was applied to washed platelets using a hemodynamic cone-plate shearing device. SHS treatment, was applied by adding SHS extract to washed platelets. Flow cytometry was used to quantify platelet surface proteins, utilizing fluorescent antibody binding with individual surface proteins. All mean fluorescence (AMF) values were obtained for each platelet protein. AMF values were used to compare the platelet surface protein expressions among different experimental conditions to investigate the effect of SHS and shear stress. At the first step, platelets treated with shear stress and SHS separately were compared with control platelets (resting platelets without any treatment) to look at the individual effect of these two treatments. Then platelets treated with both shear stress and SHS were compared with shear stress treated platelets to determine whether the effect of two factors are significantly different compared to the shear effect. AMF values for all experimental

conditions were normalized to the respective control value (resting platelets without SHS and shear stress) before all the comparisons to avoid variation from different platelet donors and experiments at different days.

4.1.1 P-selectin Expression

Treated washed platelets (exposed to SHS and/or shear stresses for 60 min) were incubated with FITC conjugated P-selectin antibody for 30 min and analyzed using a flow cytometer. Figure 4.1 shows histograms for P-selectin fluorescence intensity of platelet population. The positive control TRAP induced highest shift in fluorescence intensity for platelet population which is consistent with maximum activation. Higher shear stress loading showed shift in fluorescence intensity for increased platelet population.

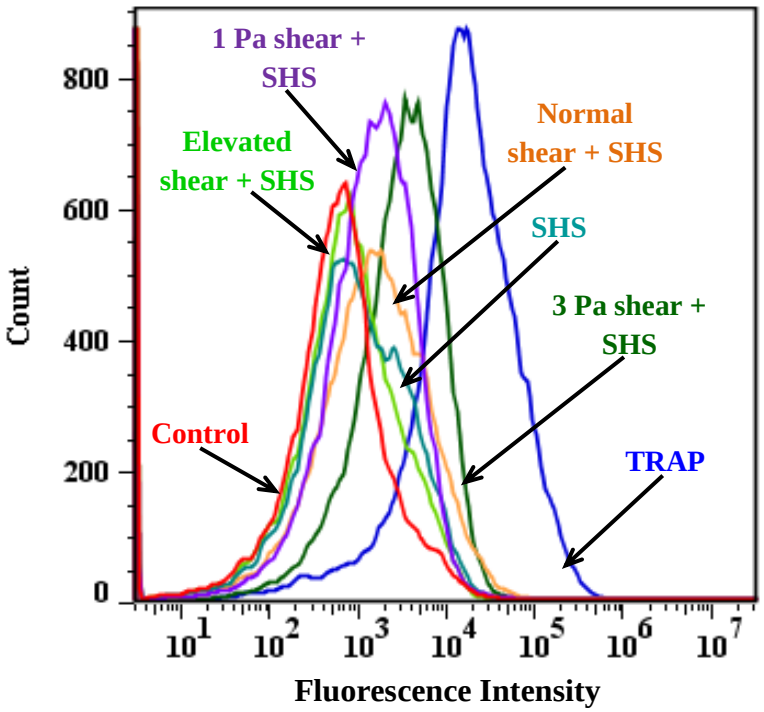

Figure 4.1: Representative P-selectin expression plots under combined effect of shear stress and SHS from flow cytomerty. Histograms showing activated platelet population shift of different combined treatment.

Normalized AMF values of platelet P-selectin expression for different conditions are shown in Figure 4.2. Statistical analysis using one way ANOVA showed a significant differences (P-value < 0.0001) in platelet P-selectin expression under the different conditions. Table 4.1 summarizes the P-values for the comparison of P-selectin expression among different conditions using Student-Newman-Keuls multiple comparison.

Platelets exposed to SHS, 1 Pa and 3 Pa have significantly (P-value < 0.05) increased P-selectin expression compared to that of resting platelets. SHS, 1 Pa and 3 Pa shear stress increased the average P-selectin expression by about 24%, 22% and 34% respectively compared to control. Therefore, both SHS and constant shear stress can induce platelet activation and increase P-selectin expression. Dynamic shear stresses (normal and elevated shear stress) did not show any effect on platelet P-selectin expression. Comparison between the two constant shear stress conditions shows that the effect of 1 Pa (1.22 ± 0.295, n=9) and 3 Pa (1.34 ± 0.27, n=9) shear stress was not significantly different. Similarly, no significant difference was observed in the effect of both dynamic shear stress of pulsatile normal (1.01 ± 0.1, n=7) and pulsatile elevated (0.98 ± 0.08, n=6). The combination of SHS and 3 Pa shear stress induced a significant increase in P-selectin expression (1.74 ± 0.25, n=9) compared to shear stress exposure at 3 Pa (1.34 ± 0.27, n=9) and SHS exposure (1.24 ± 0.35, n=32). No interaction between dynamic shear stress and SHS was observed, and P-selectin expression was unaffected by their combined exposure.

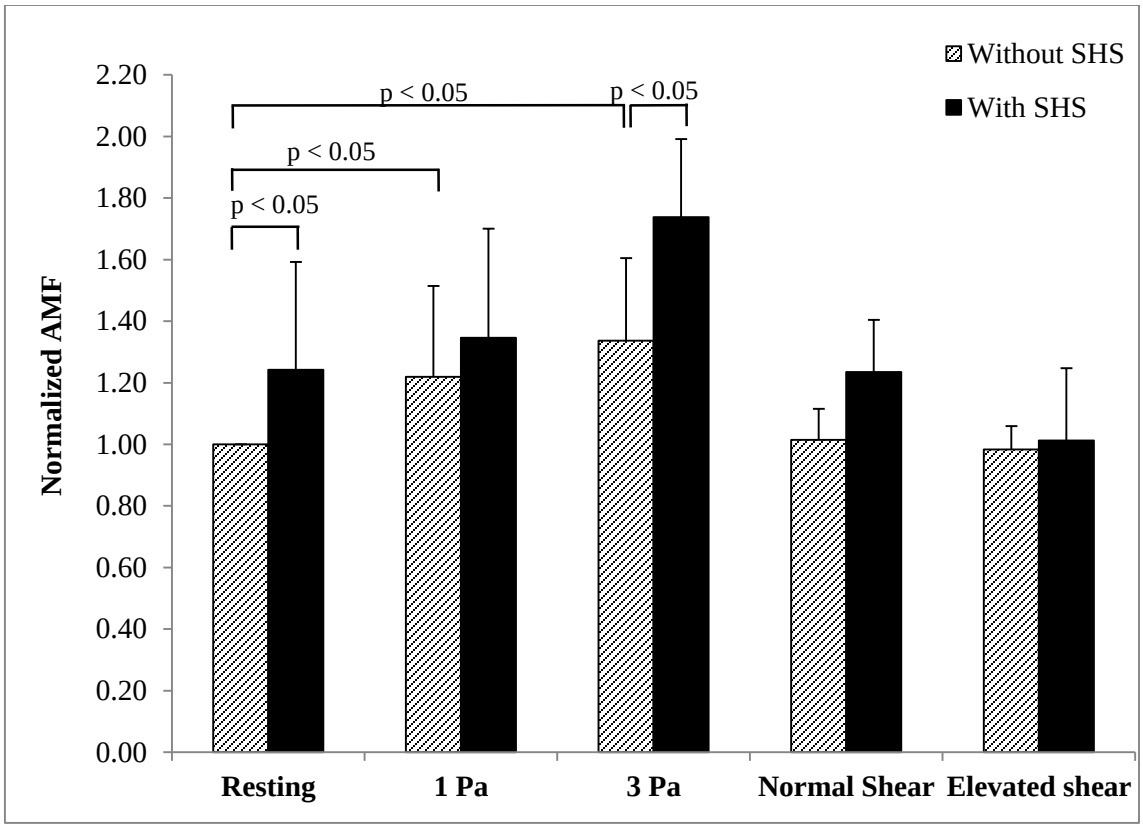

Figure 4.2: P-selectin expression on platelet surface after applying different shear stress and SHS. All mean fluorescence (AMF) values were normalized to CTR (resting platelets without SHS). All data (sample size, n = 6 - 9) are presented as mean + SD (standard deviation).

The results demonstrated that both constant shear stress and SHS exposure independently had a significant effect on platelet P-selectin expression, while dynamic shear stress showed no significant effect. In addition, higher magnitude of shear stress and SHS had significant effect on P-selectin expression.

Table 4.1: Summary of P-values obtained from multiple comparisons of P-selectin expression under different conditions. One way ANOVA p-value < 0.0001. N.S. indicates not significant.

Sample size, n=	**32**	**32**	**9**	**9**	**9**	**9**	**6**	**6**	**7**	**6**
	CTR	**SHS**	**Shear 10**	**SHS + Shear 10**	**Shear 30**	**SHS + Shear 30**	**Elevated**	**SHS + Elevated**	**Normal**	**SHS + Normal**
SHS	0.0002									
Shear 10	0.0205	(N.S) 0.8054								
SHS + Shear 10	0.0003	(N.S) 0.2661	(N.S) 0.2771							
Shear 30	0.0005	(N.S) 0.32	(N.S) 0.3208	——						
SHS + Shear 30	<.0001	<.0001	——	0.0012	0.0008					
Elevated	(N.S) 0.8803	0.0203	——	——	——	——				
SHS + Elevated	(N.S) 0.916	0.0383	——	——	——	——	(N.S) 0.8436			
Normal	(N.S) 0.8797	0.0301	——	——	——	——	(N.S) 0.815	——		
SHS + Normal	0.0355	(N.S) 0.9415	——	——	——	——	——	(N.S) 0.1219	(N.S) 0.115	

4.1.2 GPIbα Expression

Platelet surface GPIbα Expression was measured by flow cytometric analysis in a similar manner as for measuring P-selectin expression. After flow cytometric analysis, normalized fluorescence intensity values were compared among different experimental conditions. Normalized AMF values of platelet GPIbα expression for different conditions

are shown in Figure 4.3. Statistical analysis using one way ANOVA shows significant differences (P-value < 0.0001) among different conditions. Table 4.2 condenses the P-values for comparisons among different conditions.

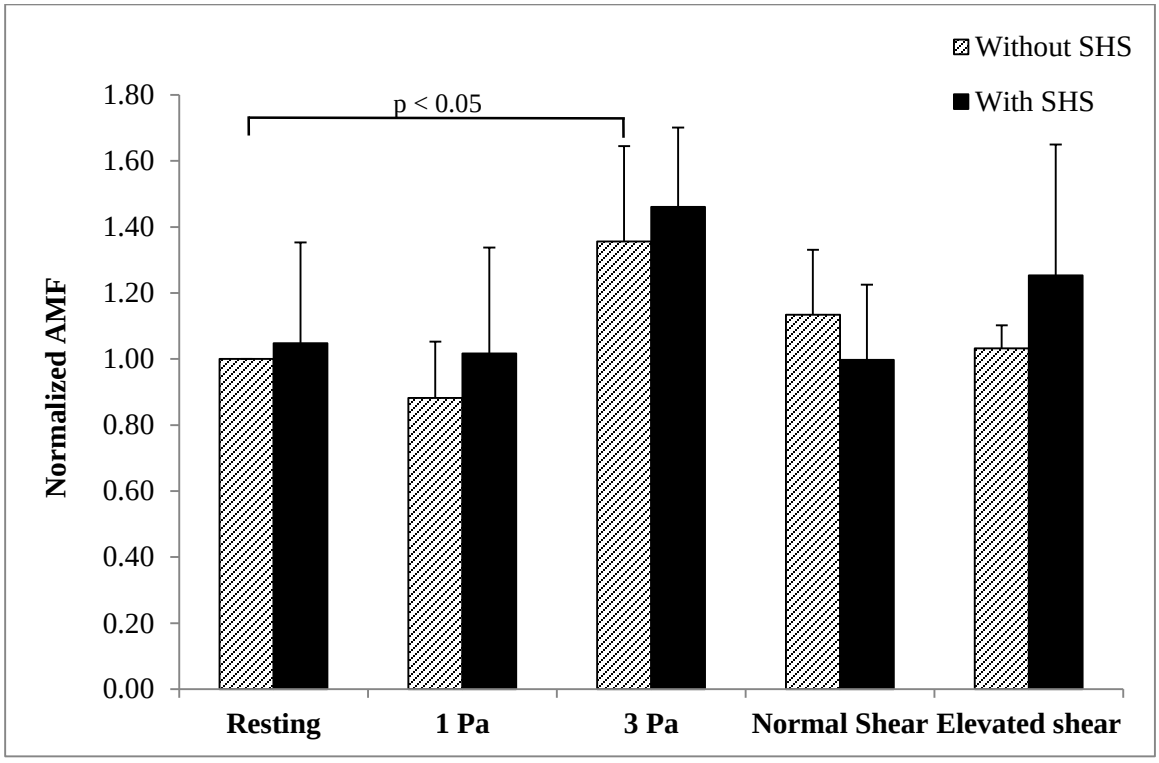

Figure 4.3: GPIbα expression on platelet surface after applying different shear stress and SHS. All mean fluorescence (AMF) values were normalized to CTR (resting platelets without SHS). All data (sample size, n = 6 - 8) are presented as mean + SD (standard deviation).

Table 4.2: Summary of P-values obtained from multiple comparisons of GPIbα expression under different conditions. One way ANOVA p-value < 0.0001. N.S. indicates not significant.

Sample size, n=	**29**	**29**	**8**	**8**	**7**	**7**	**6**	**6**	**6**	**7**
	CTR	**SHS**	**Shear 10**	**SHS + Shear 10**	**Shear 30**	**SHS + Shear 30**	**Elevated**	**SHS + Elevated**	**Normal**	**SHS + Normal**
SHS	(N.S) 0.4504									
Shear 10	(N.S) 0.2148	(N.S) 0.0837								
SHS + Shear 10	(N.S) 0.8529	(N.S) 0.7554	(N.S) 0.2547							
Shear 30	0.0005	0.0024	0.0002	____						
SHS + Shear 30	<0.0001	<0.0001	____	0.0004	(N.S) 0.4034					
Elevated	(N.S) 0.7651	(N.S) 0.8857	____	____	____	____				
SHS + Elevated	0.0191	(N.S) 0.0555	____	____	____	____	(N.S) 0.109			
Normal	(N.S) 0.2101	(N.S) 0.4154	____	____	____	____	(N.S) 0.4567	____		
SHS + Normal	(N.S) 0.9656	(N.S) 0.6072	____	____	____	____	____	(N.S) 0.0537	(N.S) 0.2964	

Enhanced GPIbα expression was observed with the condition of shear stress level of 3 Pa (1.36 ± 0.29, n=7) compared to control, which shows an average increase of GPIbα expression by 36%. SHS and other shear loading conditions (1 Pa, pulsatile normal, pulsatile elevated) did not show any significant changes of platelet GPIbα expression compared to control platelets. The effect of constant shear stress of 3 Pa was also elevated compared to 1 Pa (0.88 ± 0.17, n=8) shear stress, whereas both normal (1.13

± 0.2, n=6) and elevated (1.03 ± 0.07, n=6) pulsatile shear stress did not show significant difference in their effects. Combined effect of SHS and shear stresses (for both constant and dynamic shear) was also insignificant compared to the effect of shear stress and SHS separately. The results indicated that only constant high shear stress had a significant effect on GPIbα expression of platelets and no significant effect of combined treatments of shear stress and SHS was observed.

4.1.3 GPIIb Expression

Flow cytometric analysis was conducted for GPIIb expression as for P-selectin expression as described earlier. AMF values obtained from flow cytometry were normalized to control (resting platelets without SHS). Figure 4.4 shows all normalized AMF values of platelet GPIIb expression for different conditions represented as mean and standard deviation. Statistical analysis using one way ANOVA shows significant differences (P-value = 0.0023) in platelet GPIIb expression under different conditions. Table 4.3 summarizes all P-values for the comparisons of GPIIb expression among different conditions.

From figure 4.4 we observed that in SHS treated platelets (0.93 ± 0.12, n=24) GPIIb expression was down regulated from control (resting platelets without SHS). Platelets exposed to different shear stress loading conditions did not display significant differences in GPIIb expression in contrast to control. Not only were shear stress effects insignificant compared to control, but there were no significant differences in response between constant shear of 1 Pa (0.98 ± 0.04, n=7) and 3 Pa (0.95 ± 0.13, n=5). Similarly, the effect of dynamic shear of normal intensity (0.96 ± 0.07, n=7) was insignificant

compared to elevated (0.97 ± 0.07, n=6) shear stress. The combined effect of SHS and shear stress on platelets demonstrated down regulation of GPIIb expression compared to shear treated platelets, but this change was observed for two specific shear stresses (1 Pa and pulsatile elevated). The results here indicate that SHS had a significant effect on platelet GPIIb expression, while shear stress had no effect. SHS combined with 1 Pa and pulsatile evevated shear caused decreased GPIIb expression.

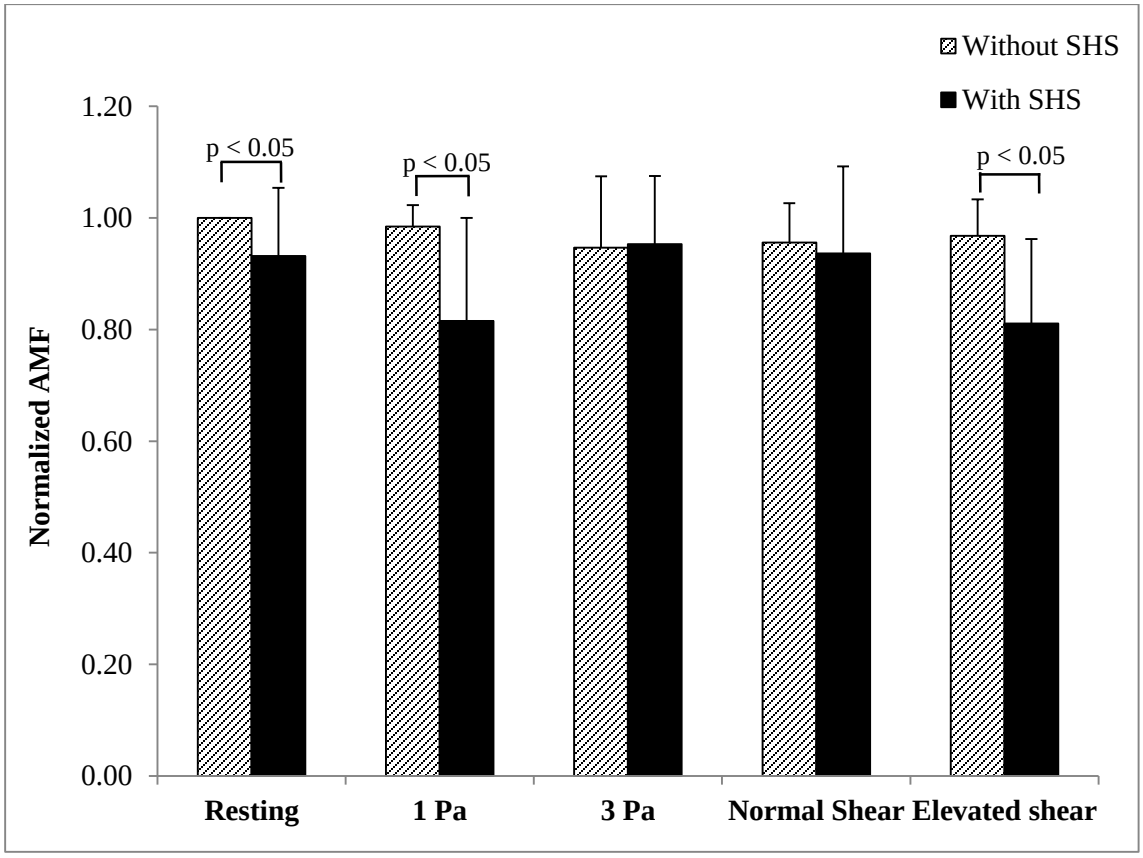

Figure 4.4: GPIIb expression on platelet surface after applying different shear stress and SHS. All mean fluorescence (AMF) values were normalized to CTR (resting platelets without SHS). All data (sample size, n = 5 - 7) are presented as mean + SD (standard deviation).

Table 4.3: Summary of P-values obtained from multiple comparisons of GPIIb expression under different conditions. One way ANOVA p-value = 0.0023. N.S. indicates not significant.

Sample size, n=	**24**	**24**	**7**	**7**	**5**	**5**	**6**	**6**	**7**	**6**
	CTR	**SHS**	**Shear 10**	**SHS + Shear 10**	**Shear 30**	**SHS + Shear 30**	**Elevated**	**SHS + Elevated**	**Normal**	**SHS + Normal**
SHS	0.0246									
Shear 10	(N.S) 0.7059	(N.S) 0.2499								
SHS + Shear 10	<.0001	0.012	0.0036							
Shear 30	(N.S) 0.2828	(N.S) 0.7938	(N.S) 0.5306	——						
SHS + Shear 30	(N.S) 0.3771	(N.S) 0.6501	——	0.0261	(N.S) 0.8811					
Elevated	(N.S) 0.5121	(N.S) 0.4327	——	——	——	——				
SHS + Elevated	0.0002	0.0155	——	——	——	——	0.0117			
Normal	(N.S) 0.3465	(N.S) 0.5565	——	——	——	——	(N.S) 0.8491	——		
SHS + Normal	(N.S) 0.2033	(N.S) 0.8697	——	——	——	——	——	0.0403	(N.S) 0.7491	

4.2 Platelet Aggregation

Platelet rich plasma (PRP at 250,000 platelets per µL) prepared from the platelet bag was exposed to SHS (smoke of 1 cigarette/5L of PRP) and/ or shear stress (1 Pa, 3 Pa, pulsatile normal and pulsatile elevated) for 60 min. The aggregation test was performed in the presence of an external agonist (TRAP- thrombin receptor activator peptide, at 20 µM concentration) using a chronolog turbidity aggregometer. Optical

transmittance was measured in the aggregometer through PRP in comparison with autologous platelet poor plasma (PPP). Light transmittance of PPP was set to 100 percent by setting it as reference and transmittance of the PRP sample to be measured was set as baseline with zero percent light transmittance initially. Figure 4.5 shows plots from aggregation test performed using the aggregometer. The aggregation plots show that the initial light transmittance was zero percent, and started increasing after the addition of the external agonist at 30 sec time. During aggregation light transmittance continued inreasing, until aggregation stabilizes (at around 5 min). The difference of light transmittance between the stabilized state and the initial state is termed as percent change of aggregation. Here plot A shows about 58 percent aggregation and B shows about 59 percent aggregation of PRP samples.

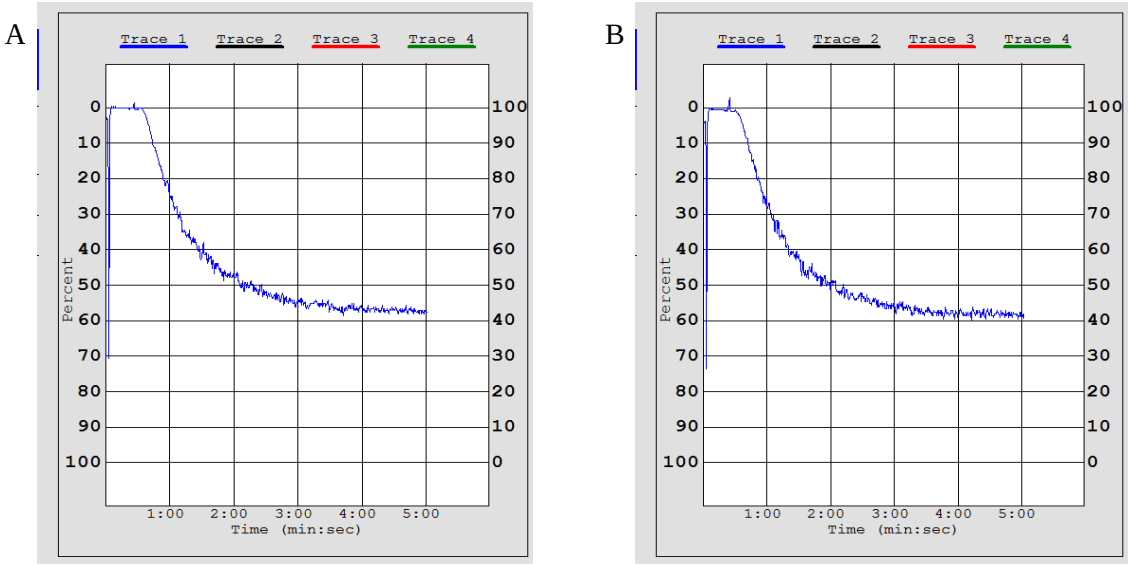

Figure 4.5: Aggregation plot obtained for PRP using the agonist TRAP (20 µM) in aggregometer. Initial light transmittance was adjusted to 0%, indicating no aggregation, and PPP was considered to have 100% transmittance. With initiation of aggregation the light transmittance increased gradually. A- Aggregation plot of control (resting PRP without SHS), B- aggregation plot of PRP treated with SHS.

For PRP under experimental conditions, all aggregation data points were recorded for about 5 min. From these data points three aggregation parameters were calculated, which were utilized to investigate the effects of SHS and shear stress on platelet aggregation. Those three parameters are aggregation slope for the first 30 sec, change of aggregation percentage, and maximum percentage of aggregation. For each experiment, obtained results were normalized to respective control (resting platelets without SHS) to account for the variation from different platelet donors. All results were presented as mean ± standard deviation. Finally using statistical analysis, results were compared to identify the effects of different experimental conditions. Aggregation of control platelets was first compared to SHS treated and then to shear stress treated platelets to observe if these two factors independently can cause any change in platelet aggregation. After that, aggregation of platelets treated with both SHS and shear stress was compared to that of shear exposed platelets. The last comparison was done to observe the significance of the combined effect of these two factors (SHS and shear stress) on platelet aggregation.

4.2.1 Aggregation Slope for first 30 sec

Platelet aggregation slope for the first 30 sec was calculated using the recorded aggregation data. Figure 4.6 shows the summarized results of aggregation slope. P-values for multiple comparisons among different conditions are presented in the table 4.4. From these comparisons we observed that SHS did not affect platelet aggregation slope significantly. Shear stress independently has a significant effect on platelet aggregation slope. Slope of aggregation decreased under the exposure to constant shear stress of 1 Pa (0.49 ± 0.11, n=7), 3 Pa (0.31 ± 0.18, n=6), and pulsatile shear stress of normal (0.64 ± 0.13, n=5), elevated (0.64 ± 0.14, n=6). Slope of aggregation for first 30 sec represents

the rate at which platelets are participating in aggregation. In essence, both constant and pulsatile shear stress was capable of inducing change in platelet aggregation rate. Besides that, the effect of constant shear at 3 Pa was significant over the effect of 1 Pa shear stress, while the effect of dynamic shear stress of pulsatile elevated and pulsatile normal was alike.

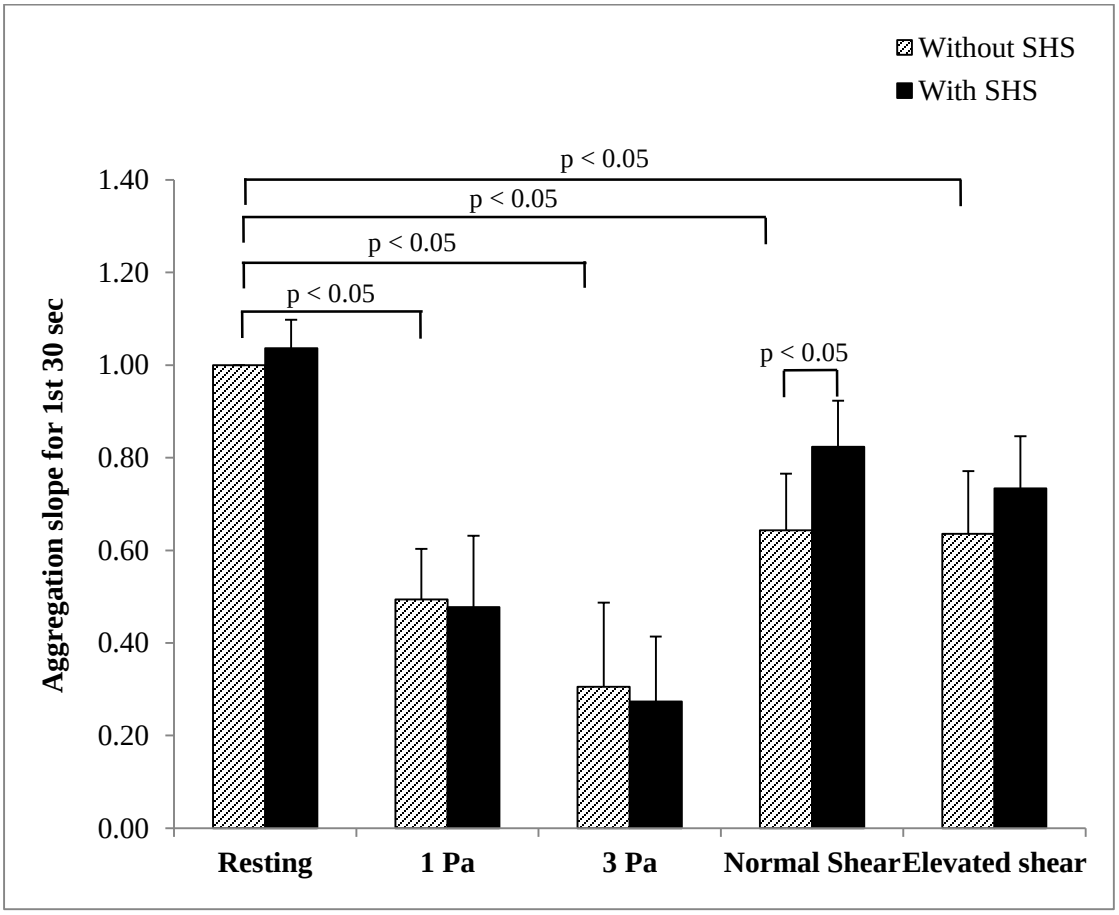

Figure 4.6: Slope of aggregation for first 30 sec after applying different shear stress and SHS on PRP. Aggregation slope values were normalized to CTR (resting platelets without SHS). All data (sample size, n = 5 - 7) are presented as mean + SD (standard deviation).

The combined effect of SHS and constant shear stress was also significant, but not different from shear stress effect. The combined effect of SHS and dynamic shear stress shows increase in aggregation slope, while normal pulsatile combined with SHS caused

significant increase. The results indicated that, only shear stress had significant effect, whereas SHS did not show any effect on aggregation slope under the experimental condition.

Table 4.4: Summary of P-values obtained from multiple comparisons of aggregation slope for first 30 sec under different conditions. One way ANOVA p-value < 0.0001. N.S. indicates not significant.

Sample Size, n=	**24**	**24**	**7**	**7**	**6**	**6**	**6**	**6**	**5**	**5**
	CTR	**SHS**	**Shear 10**	**SHS + Shear 10**	**Shear 30**	**SHS + Shear 30**	**Elevated**	**SHS + Elevated**	**Normal**	**SHS + Normal**
SHS	(N.S) 0.2003									
Shear 10	<.0001	<.0001								
SHS + Shear 10	<.0001	<.0001	(N.S) 0.7425							
Shear 30	<.0001	<.0001	0.0008	____						
SHS + Shear 30	<.0001	<.0001	____	0.0003	(N.S) 0.5744					
Elevated	<.0001	<.0001	____	____	____	____				
SHS + Elevated	<.0001	<.0001	____	____	____	____	(N.S) 0.0889			
Normal	<.0001	<.0001	____	____	____	____	(N.S) 0.9281	____		
SHS + Normal	0.0004	<.0001	____	____	____	____	____	(N.S) 0.1275	0.004	

4.2.2 Aggregation Percentage Change

Percentage change of aggregation was calculated from the difference of aggregation percentage of final state during aggregation testing and initial state for all experimental conditions. Summarized results of percentage change of aggregation are presented in figure 4.7. Table 4.5 contains the P-values for multiple comparisons among different experimental groups.

Percentage change of aggregation indicates the percentage of the platelet population participating in aggregate formation. Increased percentage of aggregation represents higher platelet numbers in aggregate. The effect of SHS on percentage change of platelet aggregation was insignificant compared to control platelets. All four shear stress profiles independently had a significant effect on percentage change of platelet aggregation. Percentage change of aggregation decreased under the exposure to constant shear stress of 1 Pa (0.09 ± 0.14, n=6), 3 Pa (0.07 ± 0.1, n=6) and pulsatile shear stress of normal (0.53 ± 0.24, n=5) or elevated levels (0.38 ± 0.29, n=6). So, all shear stress conditions had a significant effect on platelet numbers participating in aggregation. The difference between 1 Pa and 3 Pa was not significant. Likewise, for dynamic shear stress there was no significant between normal and elevated pulsatile shear. The combined effect of SHS and shear stress (0.78 ± 0.15, n=5) was significant for pulsatile normal shear stress waveform compared to the shear effect (0.53 ± 0.24, n=5), while both dynamic shear stress waveforms combined with SHS increased aggregation percent change. All the other conditions did not show a significant effect of combined treatments. To sum up, SHS had no effect on platelet aggregation percentage change, while shear

stress had significant effect, and interaction between these two factors (SHS and shear stress) was limited to dynamic shear stress waveforms.

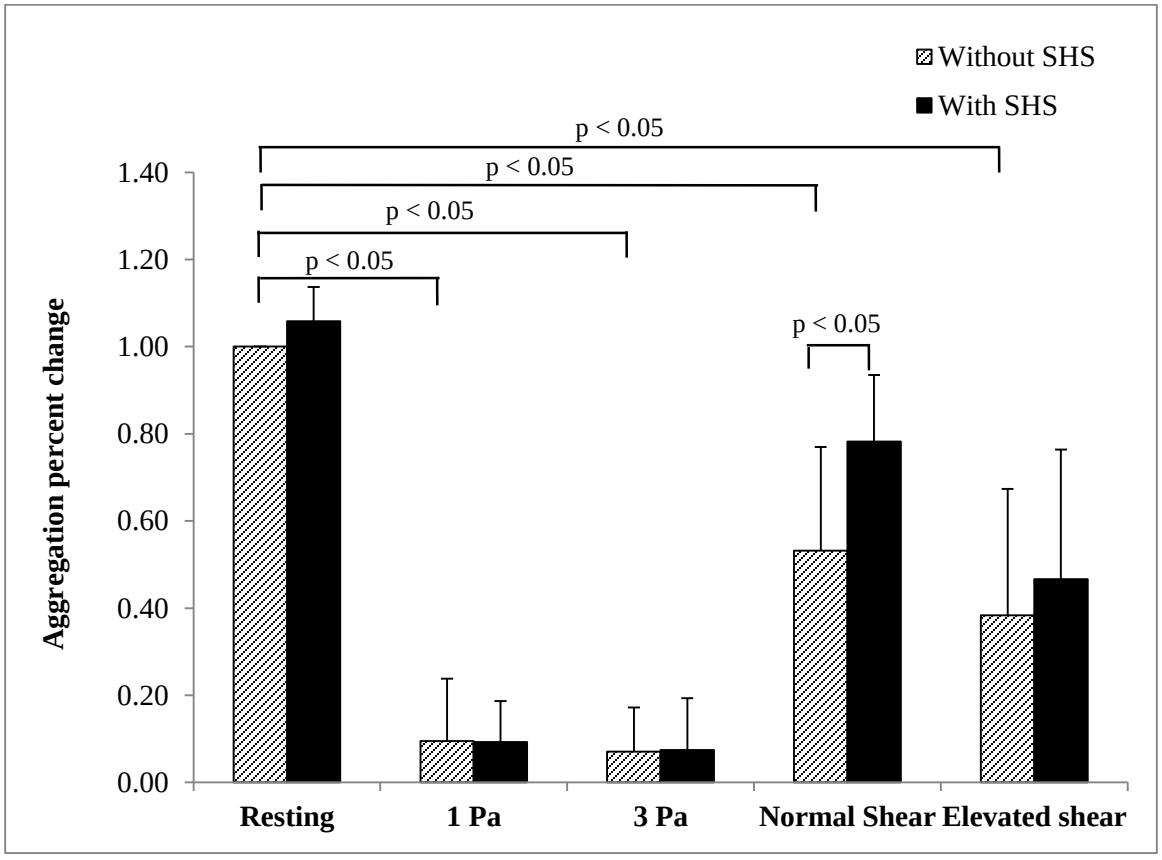

Figure 4.7: Percentage change of aggregation after applying different shear stress and SHS on PRP. Aggregation percentage values were normalized to CTR (resting platelets without SHS). All data (sample size, n = 5 - 6) are presented as mean + SD (standard deviation).

Table 4.5: Summary of P-values obtained from multiple comparisons of aggregation percentage change under different conditions. One way ANOVA p-value < 0.0001. N.S. indicates not significant.

Sample Size, n=	**24**	**24**	**6**	**6**	**6**	**6**	**6**	**6**	**5**	**5**
	CTR	**SHS**	**Shear 10**	**SHS + Shear 10**	**Shear 30**	**SHS + Shear 30**	**Elevated**	**SHS + Elevated**	**Normal**	**SHS + Normal**
SHS	(N.S) 0.1467									
Shear 10	<.0001	<.0001								
SHS + Shear 10	<.0001	<.0001	(N.S) 0.9667							
Shear 30	<.0001	<.0001	(N.S) 0.7544	____						
SHS + Shear 30	<.0001	<.0001	____	(N.S) 0.8023	(N.S) 0.9834					
Elevated	<.0001	<.0001	____	____	____	____				
SHS + Elevated	<.0001	<.0001	____	____	____	____	(N.S) 0.3081			
Normal	<.0001	<.0001	____	____	____	____	(N.S) 0.0821	____		
SHS + Normal	0.0019	0.0001	____	____	____	____	____	0.0003	0.0053	

4.2.3 Maximum Percentage of Aggregation

From the obtained data of platelet aggregation by aggregometer, maximum percentage of aggregation values were calculated for all conditions. Figure 4.8 displays maximum aggregation percentages for platelets under all experimental conditions. Following the figure all P-values for designed multiple comparison among different conditions are shown in table 4.6.

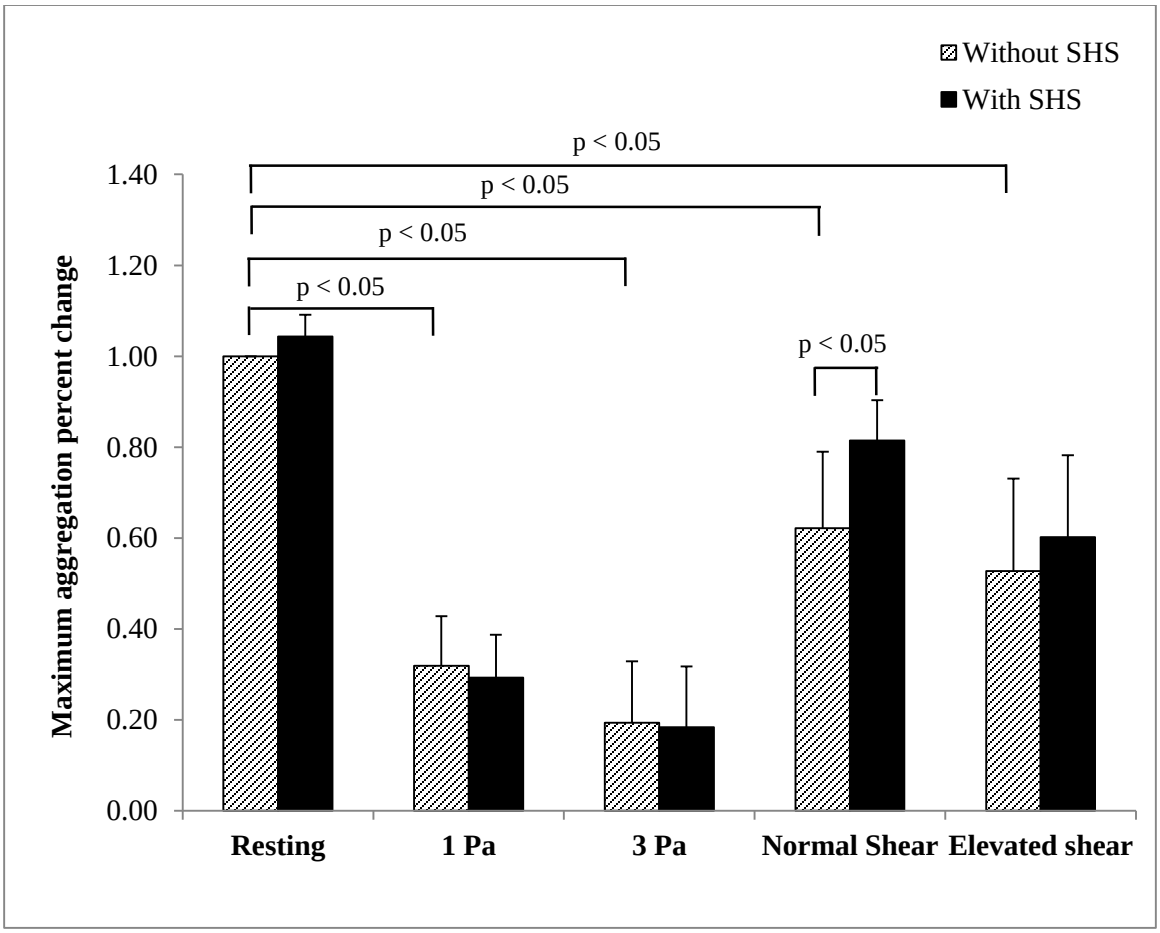

Figure 4.8: Maximum percentage of aggregation after applying different shear stress and SHS on PRP. Maximum aggregation percentage values were normalized to CTR (resting platelets without SHS). All data (sample size, n = 5 - 6) are presented as mean + SD (standard deviation).

From the results, a significant effect of shear stress (all shear loading conditions) was observed on the maximum percentage of platelet aggregation in comparison with resting platelets. Both constant shear stress of 1 Pa (0.32 ± 0.11, n=6), 3 Pa (0.19 ± 0.14, n=6) and pulsatile shear stress of normal (0.62 ± 0.17, n=5), and elevated levels (0.53 ± 0.2, n=6) resulted in decreased maximum percentage of aggregation. In contrast, SHS was not able to exert a significant effect on the maximum percentage of platelet aggregation under resting condition. Comparisons between the effect of shear stress conditions revealed that constant shear stress of 3 Pa was significantly different than 1 Pa

shear, whereas there was no difference between the effect of normal and elevated pulsatile shear stress.

Table 4.6: Summary of P-values obtained from multiple comparisons of maximum percentage of aggregation under different conditions. One way ANOVA p-value < 0.0001. N.S. indicates not significant.

Sample Size, n=	**24**	**24**	**6**	**6**	**6**	**6**	**6**	**6**	**5**	**5**
	CTR	**SHS**	**Shear 10**	**SHS + Shear 10**	**Shear 30**	**SHS + Shear 30**	**Elevated**	**SHS + Elevated**	**Normal**	**SHS + Normal**
SHS	(N.S) 0.1359									
Shear 10	<.0001	<.0001								
SHS + Shear 10	<.0001	<.0001	(N.S) 0.6475							
Shear 30	<.0001	<.0001	0.0344	____						
SHS + Shear 30	<.0001	<.0001	____	(N.S) 0.0581	(N.S) 0.8191					
Elevated	<.0001	<.0001	____	____	____	____				
SHS + Elevated	<.0001	<.0001	____	____	____	____	(N.S) 0.1907			
Normal	<.0001	<.0001	____	____	____	____	(N.S) 0.1216	____		
SHS + Normal	0.0003	<.0001	____	____	____	____	____	0.0009	0.0034	

Dynamic shear stress combined with SHS induced an increase in maximum aggregation percent, and combined effect of SHS and pulsatile normal pulsatile shear stress (0.81 ± 0.09, n=5) was significant over shear effect (0.62 ± 0.17, n=5) for maximum aggregation percentage. For constant shear stress, the combined effect of SHS

and shear stress was not significant for maximum percentage of platelet aggregation. Thus, shear stress had a significant effect on maximum aggregation percentage, whereas SHS had no effect. Interaction with SHS was observed for dynamic shear stress waveforms.

4.3 Platelet Thromboxane Release

Platelet rich plasma (PRP at 250,000 platelets per µL) prepared from the platelet bag was exposed to SHS (smoke of 1 cigarette/5L of PRP) and/ or shear stress (1 Pa, 3 Pa, pulsatile normal and pulsatile elevated) for 60 min. Thromboxane synthesized by platelets is released in the plasma, so PPP (platelet poor plasma) was separated from the PRP by centrifugation to perform the thromboxane B_2 (TXB_2) assay. The concentration of TXB_2 was obtained from the TXB_2 assay as described in the Materials and Methods section. For each experiment, obtained results were normalized to respective control (resting platelets without SHS) to account for the variation from different platelet donors. All results were presented with mean and standard deviation. Finally, using statistical analysis, results were compared to study the effects of different conditions. The control sample was first compared to SHS treated and then to shear stress treated sample to observe if these two factors independently cause any change in platelet thromboxane synthesis. After that, TXB_2 concentration for platelets treated with both SHS and shear stress was compared to shear exposed platelets. The last comparison was conducted to observe the effect of the combination of these two factors (SHS and shear stress) on platelet thromboxane release.

Figure 4.9 represents all the results in graphical form with mean and standard deviation. Significant differences for comparisons are also shown in the figure. Table 4.7 shows all P-values for conducted comparisons among experimental groups using Student-Newman-Keuls multiple comparison.

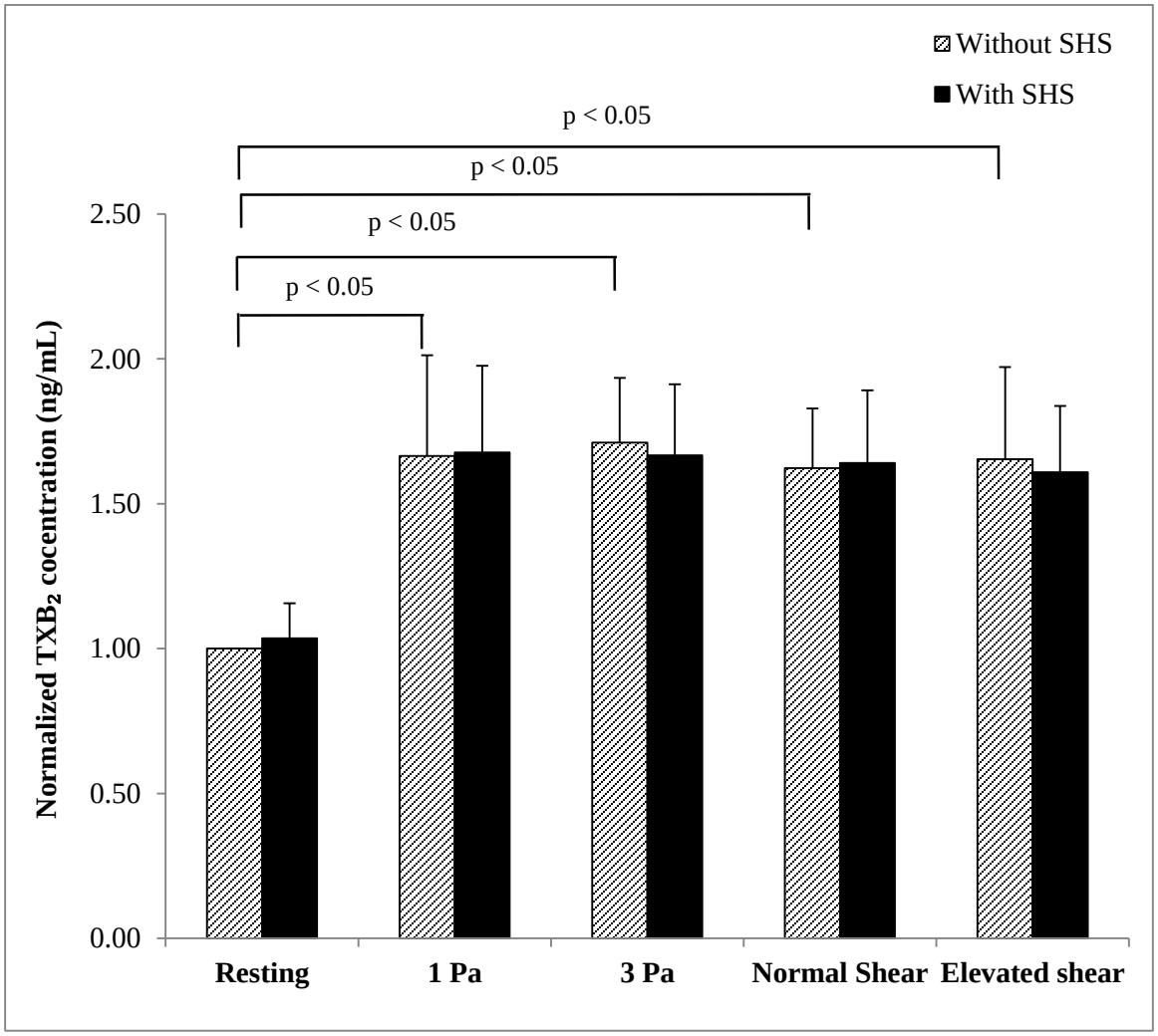

Figure 4.9: TXB_2 concentration in plasma after applying different shear stress and SHS on PRP. Concentration values were normalized to CTR (resting platelets without SHS). All data (sample size, n = 4) are presented as mean + SD (standard deviation).

Table 4.7: Summary of P-values obtained from multiple comparisons of TXB_2 concentration under different conditions (sample size, n = 4). One way ANOVA p-value = 0.0004. N.S. indicates not significant.

	CTR	**SHS**	**Shear 10**	**SHS + Shear 10**	**Shear 30**	**SHS + Shear 30**	**Elevated**	**SHS + Elevated**	**Normal**	**SHS + Normal**
SHS	(N.S) 0.8404									
Shear 10	0.0006	0.001								
SHS + Shear 10	0.0005	0.0008	(N.S) 0.9541							
Shear 30	0.0003	0.0005	(N.S) 0.7957	____						
SHS + Shear 30	0.0005	0.0009	____	(N.S) 0.9656	(N.S) 0.8068					
Elevated	0.0007	0.0011	____	____	____	____				
SHS + Elevated	0.0014	0.0024	____	____	____	____	(N.S) 0.7847			
Normal	0.0011	0.0018	____	____	____	____	(N.S) 0.8629	____		
SHS + Normal	0.0008	0.0014	____	____	____	____	____	(N.S) 0.8516	(N.S) 0.9312	

SHS treatment here did not show any effect on TXB_2 concentration, whereas shear stress (all four shear stress conditions) significantly increased TXB_2 concentration. The average increase in TXB_2 concentration due to the exposure to shear stresses can be expressed as 66% under 1 Pa, 71% under 3 Pa, 62% under pulsatile normal, and 65% under pulsatile elevated shear stress condition. Comparison between shear stress conditions did not show any significant difference. As shown in the table 4.7, between 1 Pa (1.66 ± 0.35, n=4) and 3 Pa (1.71 ± 0.22, n=4) of constant shear stress, and also between dynamic normal (1.62 ± 0.21, n=4) and elevated (1.65 ± 0.32, n=4) pulsatile

shear stress there were no significant differences. The combination of SHS and shear stress exposure for 60 min also did not show any significant enhancement over shear stress alone. From the results, it is evident that SHS did not affect platelet thromboxane release and no added effect was observed when used in combination with shear stress. Shear stress itself significantly enhanced platelet thromboxane formation.

CHAPTER V

DISCUSSION

Cardiovascular disease (CVD) poses a major health risk in the United States. Circulating blood platelets are susceptible to various shear stress patterns generated in vasculature, especially under vascular disease conditions. Shear induced changes in platelet function contributes to initiation and progression of CVD. Recently, secondhand smoke (SHS) has been established as a potent risk factor for pathogenesis of CVD. However, the combined effect of various shear stress patterns and SHS on platelet functions has not been established yet, leaving a gap in understanding the combined effect. This study was aimed to investigate the combined effect of different shear stress profiles and SHS on platelet functions.

Numerous studies have investigated the effect of shear stress on platelets, although most of these studies are limited to the use of constant shear stress. This study investigated the effects on platelet functions using both constant and physiologically relevant dynamic shear stress using a feasible cone-plate hemodynamic shearing device[29;109;110]. The shear stress profiles utilized in this study were chosen to observe the change in platelet functions under both physiological and pathological level of shear stress. 1 Pa of constant shear stress represented physiological shear,

while 3 Pa was used for pathological level of shear. Similarly for dynamic shear, physiological and pathological level of shear stress was represented by normal and elevated pulsatile shear stress respectively. Both waveforms were obtained from a previous study conducted in our laboratory[111]. Normal shear stress waveform varies between 0.1 and 1 Pa over one cardiac cycle (~ 0.9 sec), while elevated shear stress waveform varies similar to the normal waveform with a very short exposure to high shear stress magnitude (6.5 Pa for 0.1 sec once every 90 sec).

Platelet functional changes measurement were done using standard methods. Platelet function as well as surface glycoproteins can be measured effectively using flow cytometry[112;113]. Platelet aggregation has been established as the 'gold standard' for platelet function measurement[114]. Agonist induced platelet aggregation evaluation using aggregometer and thromboxane B_2 measurement using immunoassay, both of these methods has been using for platelet function analysis[115-117].

5.1 Platelet Activation (Surface P-selectin Expression)

Constant and dynamic shear stress had a different kind of impact on surface P-selectin expression. Constant shear stress of 1 and 3 Pa significantly increased platelet surface P-selectin expression, while dynamic shear stress (normal and elevated) had no effect on P-selectin expression. Similar results were observed in a study conducted by Rubenstein *et al.*, where a significant increase in platelet surface P-selectin expression was observed under constant shear stress (1 Pa and 4 Pa) after 40 min of shear exposure[13]. Another study from our group by Yin *et al.* reported that dynamic (normal, low, and elevated) shear stress did not enhance P-selectin expression of platelets[118]. The

lack of effect of dynamic shear stress on platelet P-selectin expression could be attributed to the relatively low shear stress loading on platelets compared to constant shear stress.

The difference in shear loading on platelets is described by the shear stress-exposure time integral value as defined by Rubenstein *et al*.[13]. In their study, Rubenstein *et al*. found that the pulsatile shear waveform resulted in significantly reduced levels of platelet activation compared to constant shear waveforms of similar peak magnitude. In this present study, shear stress-exposure time integral values were approximately 1980 Pa·s for pulsatile normal and 2088 Pa·s for pulsatile elevated; while for constant shear stress of 1 and 3 Pa these values were 3,600 and 10,800 Pa·s respectively. Hence the results indicate that, P-selectin expression was highly sensitive to shear stress-exposure time integral value.

SHS also significantly increased platelet P-selectin expression. Similar to our current observation, enhanced platelet activation in the presence of SHS has been observed in previous studies[12;36]. Further, the combination of SHS and shear stress (both constant and dynamic shear stress) had increased platelet P-selectin expression compared to individual effect of shear stress for all shear conditions. Interestingly, the combined effect of high magnitude constant shear stress and SHS revealed a significant increase in P-selectin expression compared to all other treatment conditions. This could potentially be due to the higher shear stress-time integral value of the high magnitude constant shear waveform. Together the data indicate that these two biochemical and mechanical agonists could enhance platelet activation.

5.2 Platelet Surface Protein Expressions

Experimental results from this study indicated that constant shear stress of 3 Pa induced a significant increase in platelet surface GPIbα expression, while 1 Pa constant shear had no significant effect. Also, dynamic shear stress (pulsatile normal and pulsatile elevated) did not induce any change in GPIbα expression. This finding was in agreement with the observation of a study by White *et al*., where platelet surface activation and constant shear stress did not cause any reduction in platelet surface GPIb/V/IX receptor[66]. Constant shear stress of 3 Pa had increases GPIbα expression compared to other shear conditions which could be due to the variation in shear stress-time integral value. Higher constant shear (3 Pa shear) induced increase in GPIbα expression could possibly be mediated by a mechanism, which is different than that of other shear conditions. Another conclusion that can be drawn from this reasoning is GPIbα expression is more sensitive to shear stress-exposure time integral value.

On the other hand, SHS had no significant effect on platelet surface GPIbα expression. In addition, the combined effect of SHS and shear stress had no significant effect on platelet surface GPIbα expression compared to the effect of individual factors. Though, SHS (individually and combined with shear stress) induced elevated platelet activation as measured by increased P-selectin expression, no change in GPIbα expression was observed. This indicates that P-selectin and GPIbα are regulated by distinct pathways.

Further, it was observed from the results that platelet surface GPIIb expression was unaffected by shear stress (both constant and dynamic shear stress). This observation

implies that, changes in the platelet aggregation under varying shear stress conditions are not necessarily mediated by the effects of shear stresses on the platelet GPIIb expression. However, SHS individually caused a significant decrease in surface GPIIb expression. The down regulation of this surface protein could be attributed to receptor clustering, translocation and consumption by activated platelets[119;120].

SHS combined with shear stress of constant 1 Pa and pulsatile elevated induced a significant decrease of GPIIb expression compared to the individual effect of SHS or shear stress. Combined effect of SHS and shear stress (constant 1 Pa and pulsatile normal) did not display any change in GPIIb expression. Thus, when combined with the exposure of 1 Pa and elevated shear stress, the effect of SHS was intensified leading to significant decrease of GPIIb expression. This also indicates that different pathways or mechanisms were affected differently under various conditions which is demonstrated by the effect on GPIIb expression.

5.3 Platelet Aggregation

With platelet aggregation, slope for the first 30 sec of aggregation curve representing how fast platelets form aggregates when activated by external agonist was first investigated. Shear stress (both constant and dynamic shear stress) exposure caused a significant decrease of aggregation rate in agonist induced aggregation. Constant shear stress demonstrated more impact on aggregation rate than dynamic shear (both normal and elevated pulsatile shear), which could be attributed to the variation in shear time integral values as explained before.

The impact of SHS on aggregation rate was insignificant. Similarly, no enhancement of platelet aggregation was observed by Lardi *et al.*, when platelets were exposed to SHS (60 min exposure)[121]. Combined exposure of constant shear and SHS have resulted no change in aggregation rate, but dynamic shear stress combined with SHS increased aggregation rate. The results also revealed that combined effect of normal pulsatile shear and SHS enhanced aggregation rate significantly compared to normal shear effect. However, dynamic shear stress is interacting with SHS, showing opposite treatment effect as dynamic shear causing decrease in aggregation rate while SHS causing increase. Altogether, it indicates that combined exposure of shear stress (both constant and dynamic shear stress) and SHS have no intensified effect on platelet aggregation compared to shear stress effect.

To further investigate the effect of SHS and shear stress waveforms on aggregation, aggregation percent change and maximum percentage of aggregation was observed for agonist induced aggregation. Shear stress individually induced significant effect on both aggregation percent change and maximum percentage of aggregation. Dynamic shear stress had reduced impact than constant shear stress, which could be due to the variation in shear stress time integral.

SHS exposure did not induced significant change in aggregation percentage changes, which is in accordance with previous study[121]. Constant shear stress with SHS exposure induced no effect on platelet aggregation percent changes when applied together. Dynamic shear stress combined with SHS induced increase in platelet aggregation percent changes. The difference in induced effects by constant and dynamic shear stress when combined with SHS could be attributed to the variation in shear stress-

time integral. Dynamic shear stress and SHS shows opposite effect on platelet aggregation percent change similar to aggregation rate. It indicates that SHS is not inducing further effect on shear (both constant and dynamic shear stress) induced platelet aggregation, as expected compared to shear induced aggregation percentage.

In a study conducted by Ikeda *et al*., platelets were found to be aggregating even while they were stimulated in the shearing device[122]. The shear induced aggregation could potentially affect the post shearing agonist induced aggregation observed. Further, continuous platelet activation with shear stress exposure was observed in a parallel study conducted in our laboratory[56]. This transient platelet activation while being sheared could also potentially have affected platelet aggregation, which was not possible to quantify due to the experimental limitation of this study.

5.4 Platelet Thromboxane B_2 Release

Effect of shear stress and SHS on TXB_2 concentration was investigated in the present study to determine whether thromboxane synthesis is affected under the experimental conditions. All shear stress treatment conditions induced a significant increase in TXB_2 concentration at the end of 60 min exposure duration. Previous studies had also reported shear induced TXB_2 synthesis[123;124]. The TXB_2 concentrations observed under different shear stresses were not significantly different. However, transient changes in TXB_2 concentrations were not measured in this study.

SHS alone did not show any impact on TXB_2 at the end of 60 min exposure. A previous human study reported an increase in thromboxane generation caused by passive smoking[98], where neighboring cells of platelets could possibly influence the thromboxane

synthesis pathway. Another possible reason for the discrepancy between the current results and the human data is that the SHS extract used in this study may not be identical in composition to passive smoke exposure. Combined exposure of SHS and shear stress had no significant effect on accumulated TXB_2 at the end of exposure duration. Thus, SHS separately or along with the exposure of shear stress did not induce any change in thromboxane synthesis.

Previous studies have established that TXB_2 is able to induce pro-aggregatory activity. SHS alone did not affect platelet thromboxane synthesis or platelet aggregation. With all the shear stress waveforms platelet thromboxane release was enhanced, while decreased platelet aggregation was observed. These results are in contrary to our expectations. One possible explanation for the mismatch between thromboxane release and platelet aggregation is that local transient platelet aggregation may have been occurred while platelets were being sheared in the cone-plate shearing device[122] resulting in decreased platelet count, and consumption of required aggregation factors. Such a decrease in platelets and aggregation factors could have been possibly caused a decrease in post shearing aggregation at the end of 60 min shearing.

Besides that, platelet aggregates formed were exposed to continuous shearing (while were being sheared) which could potentially resulted in disaggregation of platelet clots and prevented platelets from further agonist induced aggregation. Thus, we cannot draw firm conclusions regarding the effect of shear stress on platelet aggregation in this model system. Further studies are needed to determine whether platelet aggregation during the shearing period confounded the post shear aggregation measurements. If so, the cone-plate system may need to be optimized in future experiments. Incorporating an

aggregation measurement device to measure the light transmittance in the cone-plate shearing device would facilitate understanding of the effect of shear stress on platelets.

Platelet thromboxane release in different shear conditions were not different, even the pulsatile normal shear stress with lowest shear stress-exposure duration value produced about the maximum level of thromboxane similar to that of elevated constant shear stress. It could be possible that platelets exposed to shear stress for 60 min are producing thromboxane up to their maximum capacity that is why we could not observe any difference between shear stress conditions. Previous studies have reported basal plasma TXB_2 concentration as less than 2 pg/mL, which is subjected to transient ($t_{1/2}$ = 5-7 min) excretion to urinary metabolites in normal human circulation[125;126]. This in vitro study on the other hand shows higher concentration of plasma TXB_2 which could possibly indicate the saturation level of thromboxane generation by platelets under the experimental conditions. Study with varying shear exposure duration is required to investigate the effect on the change in platelet thromboxane synthesis.

Additionally, the shearing device used in this study has a limitation that there is some loss of sample volume due to evaporation during shearing[127;128] for longer exposure duration (60 min). Concentration of the samples may have thus caused artifactual platelet TXB_2 accumulation after shear exposure. Above all, the present study was not designed to capture the change in shear induced platelet aggregation and TXB_2 concentration during exposure to shear stress. Study is required to investigate platelet aggregation and TXB_2 concentration during shearing.

5.5 Overall Summary

To summarize, based on the background study and current hypothesis increased platelet activation and aggregation was expected from this study. Both SHS and altered constant shear stress have been found to have significant increase in platelet activation, aggregation and thromboxane generation from previous studies, which are described in the background section. The combined effect of SHS and altered constant shear stress was expected to further enhance platelet functional changes following the trend of individual effect.

The effect of dynamic shear stress on platelet activation is insignificant, whereas the effect is not determined on platelet aggregation for *in vitro* studies. Significant enhancement by the combined effect of SHS and altered dynamic shear stress was not expected to observe on platelet aggregation. Combined effect of SHS and dynamic shear stress on platelet thromboxane generation also was expected to increase further following the increased expression by dynamic shear stress and SHS. Table 5.1 shows a comparison between expected and observed results from this study for platelet activation and aggregation parameters under different experimental conditions.

Expected trend of combined effect was reflected in the observed results that can be seen in the table 5.1 for platelet activation. The combined effect of shear stress and SHS on platelet aggregation and thromboxane generation did not match the expected trend.

Table 5.1: Summary of hypothesized and observed effect of SHS and shear stress on platelet activation and aggregation. SS – shear stress, Const – constant shear stress, Dyn – dynamic shear stress, and N.S. – not significant.

End Point	Characteristics	Expected					Observed				
		SS		SHS	SS + SHS		SS		SHS	SS + SHS	
		Const	Dyn		Const	Dyn	Const	Dyn		Const	Dyn
Platelet Activation	P-selectin expression	↑	N.S.	↑	↑	?	↑	N.S.	↑	↑	N.S.
Platelet Aggregation	Aggregation rate	↑	?	↑	↑	?	↓	↓	N.S.	N.S.	N.S.
	Percentage of aggregation	↑	?	↑	↑	?	↓	↓	N.S.	NS	N.S.
	Maximum aggregation percentage	↑	?	↑	↑	?	↓	↓	N.S.	N.S.	N.S.
	Thromboxane B_2 generation	↑	↑	↑	↑	↑	↑	↑	N.S.	N.S.	N.S.

To sum up, this study investigated the interaction between SHS and altered shear stress and their effect on platelet functions (activation, aggregation). Alteration in normal platelet functions could be detrimental for the cardiovascular system. The findings from this study indicate that, exposure of platelets to side stream smoke and elevated constant shear stress under experimental conditions had increased platelet activation. However, platelet aggregation and thromboxane synthesis were not altered by the combination of SHS and shear stress. Hence, we conclude that exposure to SHS could potentially increases shear induced platelet activation (P-selectin expression), but has no significant effect on platelet aggregation.

5.6 Limitations

Under normal conditions, interaction between platelets and other cells in the vasculature (like endothelial cell, white blood cell, leukocyte etc.) can influence platelet functions. Such cellular interactions were not possible to incorporate in this study. This is another limitation of this study. Washed platelets and platelet rich plasma were used to investigate the change in platelet functions, which is a further limitation of this study since washed platelets and platelet rich plasma are not found *in vivo*. Additionally, some protective mechanisms may work in parallel to platelet functions in physiological system, which are absent in the *in vitro* study. Finally, although by using the hemodynamic cone-plate shearing device we tried to mimic the shear stress waveforms generated in physiological systems, the shearing was not completely physiological. Volume loss of the shearing sample is also a limitation of the cone-plate shearing device.

Transient response of platelet functional changes was not designed to capture in this study. Further study is required to investigate the effect of combined exposure of SHS and altered shear stress on platelet functional changes like aggregation and thromboxane generation. Besides that, post shearing agonist induced aggregation measurement using aggregometer shows the relative aggregation potential after treatments, so it was difficult to differentiate the effects of experimental conditions from the results. Alternative aggregation measurement methods like measuring change in light transmittance through PRP in the shearing device[122], or using flow cytometry before and after shearing could be more effective to study the combined effect on platelet aggregation[129;130].

CHAPTER VI

CONCLUSION

The global aim of this study was to investigate the effect of secondhand/ sidestream smoke and pathological shear stress on platelet activation and aggregation. Pathogenesis of cardiovascular disease (CVD) alters local blood flow conditions in a particular vasculature. Altered shear stress distribution causes enhanced platelet activation and aggregation. However, the role of secondhand smoke exposure on shear induced platelet functions has not been established so far. The results from this study revealed that secondhand smoke (SHS) exposure aggravated shear induced platelet responses under the specified conditions, and resulted in enhanced platelet activation as observed by enhanced expression of platelet surface P-selectin expression. In addition, SHS and shear stress together could alter the platelet aggregation response. However, the alteration of normal platelet aggregation was not accompanied by an increase in the expression of the platelet surface GPIbα or GPIIb receptor. Further study is required to investigate the mechanism behind the changes observed. The observations from this study indicate that, exposure to environmental SHS can potentially cause detrimental

effects on the cardiovascular system by enhancing platelet activation which poses the risk of CVD progression, especially in patients diagnosed with CVD

6.1 Recommendations

Future studies should incorporate endothelial cells with platelets to look at the combined effect of altered shear stress and SHS on platelets in the presence of adjacent cells. Transient platelet responses should be captured by varying treatment time duration to capture the changes of responses due to the treatment effect with time. In using cone-plate shearing device, future study should investigate if the shearing treatment is affecting the post shear aggregation measurements. The cone-plate system may need to be customized to avoid the interference with treatment effect. Besides that more effective method should be chosen for aggregation measurement to quantify the aggregation rather than measuring the relative post treatment aggregation. The aggregation measurement methods could be improved by measuring change in light transmittance through PRP in the shearing device or using flow cytometry before and after treatment for platelet aggregation detection.

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APPENDICES

A. BASIC PROGRAMS USED FOR CONE-PLATE SHEARING DEVICE

1. Program for Constant Shear Stress Experiments

rem 2667 is 2.4 dynes/cm2 and 10004 is for 9 dynes/cm2

```
joff
sposx 0
accx 0
B = 1111
velx B
jogx
0
end
$
```

2. Programs for Dynamic Shear Stress Experiments

Normal Shear Stress

```
joff
300
sposx 0
accx 0
A = 332
B = 308
C = 360
velx A
jogx
wait 250

100
A = A + B
velx A
```

```
wait 10
if A > 11108 then goto 200
goto 100
200
A = A - C
velx A
wait 10
if A > 340 then goto 200
goto 300
end
$
```

Stenosis Shear Stress

```
joff
N = 0
300
sposx 0
accx 0
N = N+1
A = 332
B = 308
C = 360
velx A
jogx
wait 250
100
A = A + B
velx A
wait 10
if A > 11108 then goto 200
goto 100
200
A = A - C
velx A
wait 10
if A > 340 then goto 200
if N < 70 then goto 300
velx 72000
jogx
wait 1000
N = 0
goto 300
end
$
```

B. MATLAB PROGRAM USED FOR CALCULATING AGGREGATION PARAMETERS

```
%|Output is the following (by column):
%|1) Slope between 0-60sec, 2) Slope between 61-120sec, 3) Slope between
%|121-180sec, 4)Slope between 181-240sec, 5) Slope between 241-300sec,
%|6)Slope from start to level off point, 7) Time at level off, 8)Value at
%|level off, 9) Avg value from level off to end, 10) Absolute value change
%|from begining to level off (avg), 11) Perecent change from beginning to level
%|off (avg)
%|Developed by D. Rubenstein January 2008
%|------------------------------------------------------------------


% Inputs for the program to work
A=input('What is the name of the text file that you want to load? (use single quotes and full file name test.txt) ');
B=load (A); % sets B as the geometry file, with all of the data
C=size(B);


data=zeros(C(2)-1,11);
% Loops to calculate the regression lines
for i=2:C(2)
   x=0;
   x_sq=0;
   y=0;
   xy=0;
   for ii=1:61 %calculates slope during first minute
      x=x+B(ii,1);
```

```
    x_sq=x_sq+B(ii,1).^2;
    y=y+B(ii,i);
    xy=xy+B(ii,1)*B(ii,i);
end
D=[61,x;x,x_sq]\[y;xy];
data(i-1,1)=D(2);
x=0;
x_sq=0;
y=0;
xy=0;
for ii=62:121 %calculates slope during second minute
    x=x+B(ii,1);
    x_sq=x_sq+B(ii,1).^2;
    y=y+B(ii,i);
    xy=xy+B(ii,1)*B(ii,i);
end
D2=[60,x;x,x_sq]\[y;xy];
data(i-1,2)=D2(2);
x=0;
x_sq=0;
y=0;
xy=0;
for ii=122:181 %calculates slope during third minute
    x=x+B(ii,1);
    x_sq=x_sq+B(ii,1).^2;
    y=y+B(ii,i);
    xy=xy+B(ii,1)*B(ii,i);
```

```
end
D3=[60,x;x,x_sq]\[y;xy];
data(i-1,3)=D3(2);
x=0;
x_sq=0;
y=0;
xy=0;
for ii=182:241 %calculates slope during fourth minute
   x=x+B(ii,1);
   x_sq=x_sq+B(ii,1).^2;
   y=y+B(ii,i);
   xy=xy+B(ii,1)*B(ii,i);
end
D4=[60,x;x,x_sq]\[y;xy];
data(i-1,4)=D4(2);
x=0;
x_sq=0;
y=0;
xy=0;
for ii=242:301 %calculates slope during fifth minute
   x=x+B(ii,1);
   x_sq=x_sq+B(ii,1).^2;
   y=y+B(ii,i);
   xy=xy+B(ii,1)*B(ii,i);
end
D5=[60,x;x,x_sq]\[y;xy];
data(i-1,5)=D5(2);
```

```
end

% this is code to find the spot where the slope levels off
for j=2:C(2)
   for jj=1:C(1)
      if jj == C(1)-29
         data(j-1,7)=B(jj,1);
         data(j-1,8)=B(jj,j);
         break
      else
         E=0.03*B(jj,j); % a five percent error rate is assumed exceptable
         E_min=B(jj,j)-E; % lower boundary for comparision
         E_max=B(jj,j)+E; % upper boudnary for comparison
         dummy=0;
         for jjj=jj+1:jj+30 % checks for the nearest 30 if it is within that range
            if B(jjj,j) >= E_min
               if B(jjj,j) <= E_max
               else
                  break
               end
            else
               break
            end
            dummy=dummy+1;
         end
         if dummy == 30
            data(j-1,7)=B(jj,1);
```

```
            data(j-1,8)=B(jj,j);
            if data(j-1,8) == 0
               data(j-1,7)=B(jj-30,1);
               data(j-1,8)=B(jj-30,j);
            end
            break
         end
      end
   end
end

for l=2:C(2)
   final=data(l-1,7);
   x=0;
   x_sq=0;
   y=0;
   xy=0;
   for ll=1:final+1 %calculates slope between first and point where the slope levels off
      x=x+B(ll,1);
      x_sq=x_sq+B(ll,1).^2;
      y=y+B(ll,l);
      xy=xy+B(ll,1)*B(ll,l);
   end
   D6=[ll,x;x,x_sq]\[y;xy];
   data(l-1,6)=D6(2);
end
```

```
for m=2:C(2)
   level_sum=0;
   count=0;
   final=data(m-1,7);
   for mm=final+1:C(1)
      if B(mm,m)==0
         break
      else
         level_sum=level_sum+B(mm,m);
         count=count+1;
      end
   end
   data(m-1,9)=level_sum/count;
   data(m-1,10)=B(1,m)-data(m-1,9);
   data(m-1,11)=(data(m-1,10)/B(1,m))*100;
end

save data.txt data -ASCII
clear A B C D D2 D3 D4 D5 D6 E E_max E_min count data dummy final
clear i ii j jj jjj l  level_sum ll m mm x x_sq xy y
```

VITA

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Master of Science

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