Spring 2014

Interaction of blood cells with the vessel wall in thrombosis

Jessica Kay Hansen
University of Iowa

INTERACTION OF BLOOD CELLS WITH THE VESSEL WALL IN THROMBOSIS

by

Jessica Kay Hansen

A thesis submitted in partial fulfillment
of the requirements for the Master of
Science degree in Biomedical Engineering
in the Graduate College of
The University of Iowa

May 2014

Thesis Supervisor: Professor Steven R. Lentz

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

MASTER'S THESIS

This is to certify that the Master's thesis of

Jessica Kay Hansen

has been approved by the Examining Committee
for the thesis requirement for the Master of Science
degree in Biomedical Engineering at the May 2014 graduation.

Thesis Committee: _____
Steven R. Lentz, Thesis Supervisor

_____
Edward A. Sander

_____
Madhavan L. Raghavan

ACKNOWLEDGMENTS

First, I would like to thank Dr. Steven Lentz and Dr. David Motto, my two research advisors. Without their very generous support and that from the Department of Internal Medicine, I would not have been able to pursue the work that is presented here. Thank you, Dave, for not only hiring me as an undergraduate student, but for allowing me to continue on as a graduate student in your lab. And thank you, Steve, for allowing me to finish my graduate work in your lab and providing so much guidance over the last nine months.

I also need to thank Dr. Edward Sander, my academic advisor, who provided guidance and kept me connected to the Department of Biomedical Engineering. Thank you for including me in your lab meetings and allowing me to bounce ideas off of you when I needed the engineering input.

I would like to thank all of my co-workers from Motto Lab and Lentz Lab who taught me all of the lab techniques that I needed to complete this work and who provided me with good company on a daily basis. I would especially like to thank Dr. Sanjana Dayal and Van Schaffer, who both generously assisted me in completing many experiments and who taught me a lot along the way.

Finally, I would like to thank my friends and family for all of their support over the last two years, I couldn't have done this with you. Thank you, Mom and Dad, for pushing me to work hard and teaching me how important a good education is. Thank you, Grandpa Jerry, for being one of my biggest role models, my part-time tutor, and for inspiring me to continue my education as an engineer

ABSTRACT

Thrombotic events such as stroke, myocardial infarction, or deep vein thrombosis can be life-threatening; therefore it is important to understand the mechanisms of thrombosis and its correlations with clinical risk factors. It is well known that red blood cells (RBCs) can influence hemostasis and thrombosis by affecting the viscosity and rheological properties of blood. Recent evidence suggests that RBCs may play a more central and active role in some models of experimental thrombosis by interacting directly with the endothelium. It is still unclear, however, if the interaction of RBCs with the endothelial surface is facilitated by other blood components. In order to investigate the interaction of RBCs with endothelium in a defined system in vitro, human umbilical vein endothelial cells (HUVECs) were grown on coverslips and placed in a parallel-plate flow chamber system. Using this system, we tested the hypothesis that RBCs interact directly with ferric chloride-injured endothelium in the absence of other blood components. These experiments demonstrated that RBCs do interact with HUVECs in the flow chamber under both venous and arterial conditions. Using an anti-von Willebrand Factor (VWF) antibody, we demonstrated that the RBC-endothelium interaction is facilitated by VWF in vitro. These findings support a possible active role of RBCs in thrombosis and also demonstrate that RBCs interact with VWF, which was previously unrecognized.

Platelets are also known to play an important role in hemostasis and thrombosis, specifically through their interaction with exposed collagen located in the subendothelial matrix that becomes exposed to flowing blood after vascular injury. Platelets may become hyperactive in prothombotic conditions, leading to increased platelet aggregation and thrombotic events. Thrombotic events also increase in the presence of risk factors such as increased age or obesity. Previous data has shown that platelet hydrogen peroxide (H_2O_2) mediates platelet hyperactivity and that activated platelets from obese mice show increased H_2O_2 levels. In order to investigate the interactions of platelets with collagen in

vitro, the flow chamber system was used with collagen-coated coverslips to study platelet adhesion under arterial and venous conditions. We found that platelets from mice with increased levels of H_2O_2, due to the genetic deletion of glutathione peroxidase-1 formed larger aggregates than control mice. In preliminary experiments, we observed evidence for increased adhesion and aggregation of platelet from obese mice. These findings confirm the role of H_2O_2 in platelet hyperactivity and suggest that therapeutic strategies targeted toward lowering platelet H_2O_2 levels may have the potential to decrease thrombotic complications associated with certain prothrombotic disease states.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Hemostasis is a normal homeostatic process that maintains vascular integrity, prevents hemorrhage, and initiates wound healing after vascular injury. When pathological processes overwhelm the normal regulatory mechanisms of hemostasis, thrombosis is initiated by unregulated thrombin formation. Thrombotic events such as stroke, myocardial infarction, and deep vein thrombosis are all very serious health concerns and can be life threatening.[1] Therefore, studying the mechanisms of thrombosis is an important area of research with a large range of applications. Although there has been extensive research in the area of hemostasis and thrombosis, the pathological mechanisms of thrombosis are still incompletely understood.

It is well known that hemostatic and thrombotic processes involve endothelial cells, platelets, and several coagulation factors. It is also known that erythrocytes, or red blood cells (RBCs), can influence hemostasis and thrombosis by affecting blood's viscosity and rheological properties.[2] RBCs can also become trapped in developing thrombi, and recent evidence suggests that they may play more of a central and active role in thrombosis by a possible interaction directly with the endothelium.[3] Another important component in thrombotic processes is collagen located in the subendothelial matrix, which becomes exposed to flowing blood when the vessel wall is injured. This exposure leads to thrombus formation by allowing the interaction of the exposed collagen network with platelets via their glycoprotein VI and integrin $\alpha_2\beta_2$ receptors. In addition, platelets can interact with subendothelial collagen via the multimeric adhesive protein von Willebrand factor (VWF). Platelets may also become hyperactive in different conditions, leading to increased platelet aggregation and thrombotic events.[4]

There are several ways that mechanisms of thrombosis can be studied, including using animal models or in-vitro studies to isolate the specific interactions. Mice are often

used for in-vivo studies of thrombosis due to their short generation time, small size, and ease of manipulation of their genome.[5] Thrombosis in mouse models is induced in exposed vessels in various ways, including application of ferric chloride ($FeCl_3$), direct injury with a laser, or indirectly with a laser to excite photoreactive chemicals in the circulation (for example, rose bengal).[6] $FeCl_3$-induced injury has become the most common injury, specifically for use in the carotid artery, likely due to its low cost and ease of use.[7]

The use of flow chambers to study thrombus formation ex-vivo has become increasingly popular over the past few decades.[8] Flow chambers are a good option for studying the mechanisms of thrombosis because they allow isolation of the interactions occurring between different cell types and manipulation of physical conditions such as shear stress and imaging in real time. When studying these mechanisms outside of the body, it is necessary to mimic the physiological conditions that are found in the vessels as close as possible in order for the experiments to be mechanically relevant.

In this present study, two important interactions in thrombosis involving blood cells and the vessel wall were studied using a parallel-plate flow chamber. First, the interaction of RBCs with the endothelial surface, and second the interactions of platelets with collagen, as illustrated in Figure 1. One goal of this study was to test the hypothesis that RBCs are able to interact with the endothelium in the presence of $FeCl_3$ under venous and arterial conditions, and to determine whether this interaction is facilitated by other blood components. A second goal of this study was to define optimal conditions for examining the interaction of platelets with a collagen surface in the flow chamber and to use the system to test the hypothesis that platelet aggregation is mediated by hydrogen peroxide. The findings from these studies may help develop new therapies for those affected by thrombotic disorders.

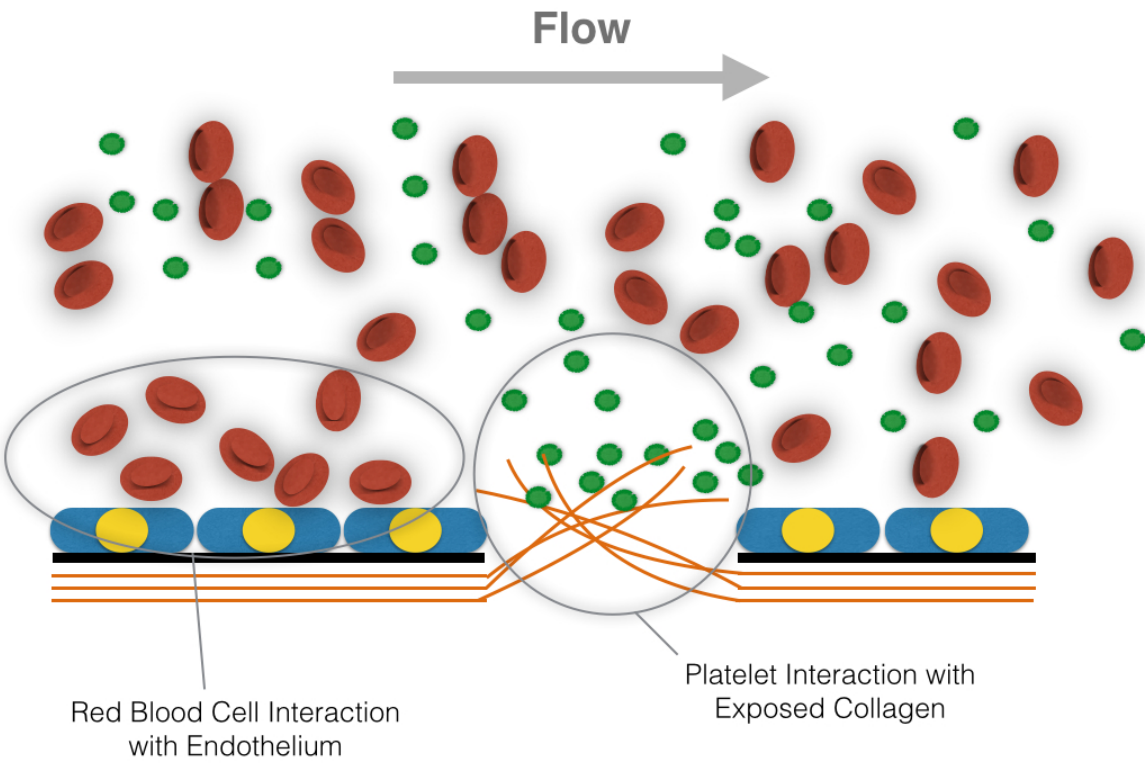

Figure 1 – Interactions in thrombosis between blood cells and the vessel wall. One interaction is that between RBCs (red) and stimulated endothelial cells (blue). If the endothelium is damaged, the subendothelial matrix, including collagen (orange), will be exposed to flowing blood and interact with platelets (green).

CHAPTER 2
INTERACTION OF RED BLOOD CELLS WITH THE ENDOTHELIUM

Background and Significance

Red blood cells (RBCs) make up nearly half of the whole blood volume and determine the majority of the viscous and elastic properties of flowing blood.[9] They have a unique biconcave shape, which allows RBCs to easily deform and roll over one another as they travel through the vasculature. Their shape and dense concentration allows them to move around and throughout whole blood, displacing smaller cells such as platelets. It has long been reported that RBCs are able to increase platelet and vessel-wall interactions by displacing them to the outer edges of the vessel.[2] In addition to influencing the rheological properties of blood, RBCs may also become trapped in developing thrombi and recent evidence suggests that in certain models of thrombosis they may have a more central role interacting directly with the endothelium at the site of injury.[3] Clinically, it is well known that RBCs are important in thrombosis. As early as 1910 it was noted that low hematocrits are associated with prolonged bleeding times.[10] Therefore, it is an important area of research to study role of RBCs in thrombosis.

As discussed in Chapter 1, the $FeCl_3$ model of thrombosis is one of the three main models used in research to study thrombosis in-vivo. $FeCl_3$ is thought to cause damage to the endothelial surface of the vessel, resulting in exposure of the subendothelial matrix.[11] In this model, filter paper saturated in $FeCl_3$ is applied directly to the adventitial surface of the vessel wall to initiate thrombosis. This exposure results in recruitment of platelets to the site of injury followed by activation of the coagulation cascade. Although there have been numerous studies that have used $FeCl_3$-induced injury, the mechanisms of $FeCl_3$ induced thrombosis are still not well understood.

Using scanning electron microscopy (SEM) and brightfield intravital microscopy, Dr. Motto and colleagues observed that RBCs are the first blood cells to adhere to the $FeCl_3$-treated endothelial surface in the carotid artery of mice. This interaction subsequently recruits platelets to the site of damage. They also observed that the $FeCl_3$ model does not result in endothelial denudation, as was previously believed. Further, they observed that genetic deletion of von Willebrand factor (VWF) or platelet glycoprotein Ib-α (GPIb-α) does not influence this interaction, suggesting that neither VWF or GPIb- α is required for the RBCs to adhere to the damaged endothelium.[3] These results suggest that RBCs interact primarily with the endothelial surface in $FeCl_3$-induced thrombosis. It remains unclear, however, whether interaction of RBCs to injured endothelial cells in the murine model is facilitated via other blood components.

In this in vitro study we sought to determine if RBCs adhere directly to injured endothelial cells independent of other blood components under venous and arterial shear stress. To address this question, washed RBCs were perfused over cultured endothelial cells in a parallel-plate flow chamber, and RBC-endothelial interactions were visualized by phase-contrast microscopy.

Materials and Methods

Endothelial Cells

Isolated human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (no. CC-2517). The HUVECs were maintained in an incubator at 37 degrees Celsius with a 5% CO_2 concentration. Endothelial growth medium (no. CC-4176, Lonza) was changed every third day. HUVECs were used from P2 to P6 when grown to confluency on glass coverslips.

Parallel-Plate Flow Chamber System

All of the in-vitro flow studies were carried out in a parallel-plate flow chamber (no. 64-1685, Warner Instruments) and connecting components as shown in Figure 2.

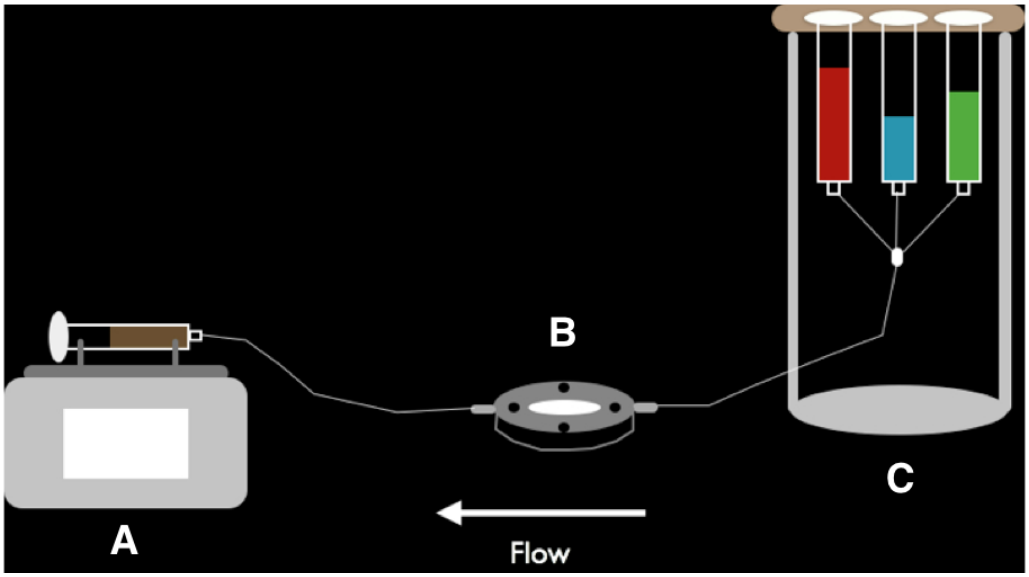

Figure 2 – Schematic diagram of parallel-plate flow chamber and connecting components used for in-vitro experiments. Syringe pump (A) withdraws solutions and blood components contained in syringes (C) through the parallel-plate flow chamber (B) at various flow rates.

To perfuse solutions through the chamber, a syringe pump (no. 703009, Harvard Apparatus) was connected to the downstream end of the chamber to perfuse solutions from the upstream syringe by negative pressure. The solutions were contained in 20 mL syringes with 2-way stopcocks so that the composition of the perfused solution could be varied throughout the experiment. For imaging purposes, the parallel-plate flow chamber was placed under a phase-contrast microscope (no. BX51, Olympus) and still images were captured as indicated using the DP2-BSW Olympus software.

In order to create a more physiological in-vitro environment, the shear stress in the flow chamber was an important component. In order to calculate the shear stress

based on the geometry of the chamber and the characteristics of the fluids being used, the following equation for wall shear stress (τ) on the cell monolayer in the chamber was used:

$$\tau = \frac{6Q\mu}{bh^2}$$

where Q is the flow rate, μ is the viscosity of the solution, b is the width of the chamber (shown between the arrows in Figure 3) and h is the height of the chamber. This equation assumes parallel plate geometry and Newtonian fluid behavior.[12] The parameters of the chosen gaskets and flow conditions for each experiment can be seen in Table 1. The chamber space was set with a silicone gasket placed between the top and bottom coverslip in the chamber.

Table 1. Flow Chamber Conditions for RBC-Endothelium Experiments

	Low Venous	**High Venous**	**Arterial**
Q	0.2 mL/min	0.096 mL/min	0.32 mL/min
μ	0.01 dyne-s/cm^2	0.01 dyne-s/cm^2	0.01 dyne-s/cm^2
b	3.2 mm	3.2 mm	3.2 mm
h	250 μm	100 μm	100 μm
τ	0.9 dynes/cm^2	3 dynes/cm^2	10 dynes/cm^2

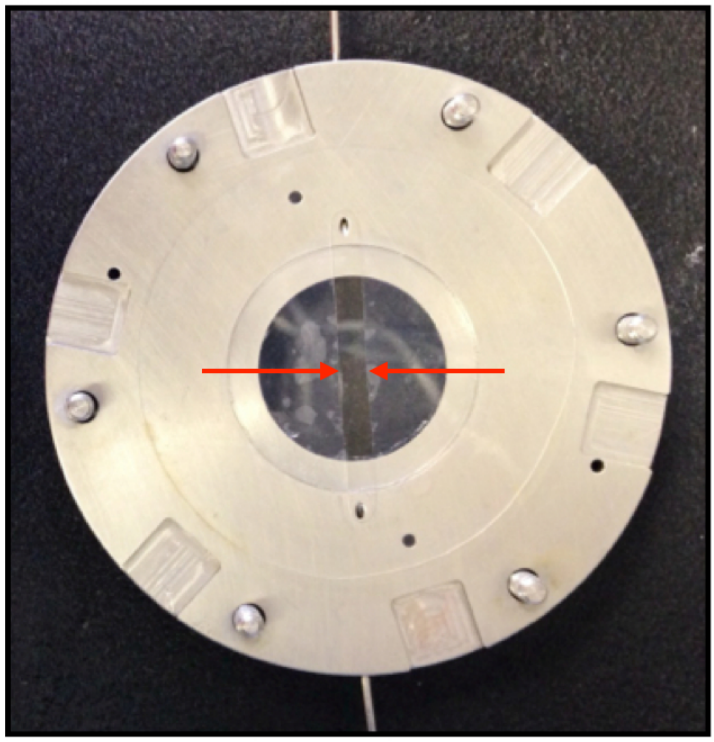

Figure 3 – Photograph of the top plate of a parallel-plate flow chamber. Arrows indicate the margins of the gasket.

Perfusion of RBC's over HUVECs

HUVECs were grown on 40 mm glass coverslips coated in 2% gelatin in 50 mm round petri dishes. Once they were grown to confluency, the coverslips were assembled into the parallel-plate flow chamber and perfused with washed human RBCs (de-identified samples from the University of Iowa Blood Bank) suspended at a hematocrit (volume/volume percent solution) of 12.5% in either phosphate buffered saline (PBS) (pH 6.5) or PBS plus 250 μM $FeCl_3$ (pH 6.5). The RBCs were perfused at a shear stress varying from 0.9 dynes/cm^2 (venous) to 10 dynes/cm^2 (arterial), depending on the gasket thickness and flow rate used for the specific experiment. Following perfusion of the RBC solutions, the chamber was then perfused with PBS to wash out any cells that had not

adhered to the surface. In some experiments anti-VWF antibody (peroxidase-conjugated rabbit anti-human VWF, Dako) or a negative control rabbit immunoglobulin fraction (Dako*)* was added to the RBC solution at a concentration of 100 µg/mL and perfused over the HUVECs. Images were collected using the phase-contrast microscope previously described and analyses were performed in Image J as indicated.

Image Analysis and Statistics

The images collected for each experiment were analyzed in Image J using the cell counting function. Individually adhered RBCs and RBC clusters were manually counted in all collected images. An RBC cluster was defined as three or more RBCs adhered to the endothelial surface together. The paired two-sided Student's t-test was performed to compare RBC and RBC cluster quantifications in the presence of ferric chloride to that in the absence of ferric chloride. Statistical significance was defined as a value of $P<0.05$.

Results

Adhesion of RBCs at Venous Shear Stress

To determine whether RBCs interact directly with endothelial cells in the absence of any other blood components, in-vitro experiments were designed to isolate the interaction by perfusing washed human RBCs over cultured HUVECs. The RBCs were first perfused at a low venous shear stress of 0.9 dynes/cm^2 in the presence or absence of $FeCl_3$. Results from these experiments can be seen in Figure 4, along with the quantification of these results in Figure 5. These experiments suggest that $FeCl_3$ is able to induce the adhesion of washed human RBCs to cultured human endothelial cells under conditions mimicking venous blood flow.

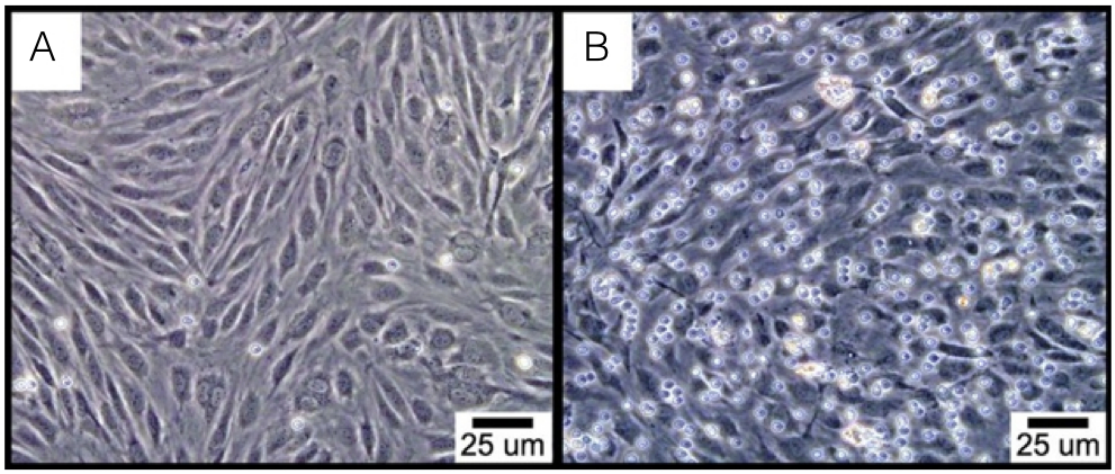

Figure 4 - Ferric chloride-induced adhesion of washed human RBCs to cultured HUVECs under low venous shear stress. A 12.5% HCT solution suspended in PBS (A) or PBS + 250 μM $FeCl_3$ (B) was perfused over confluent HUVECs for 5 minutes at a shear stress of 0.9 dynes/cm^2.

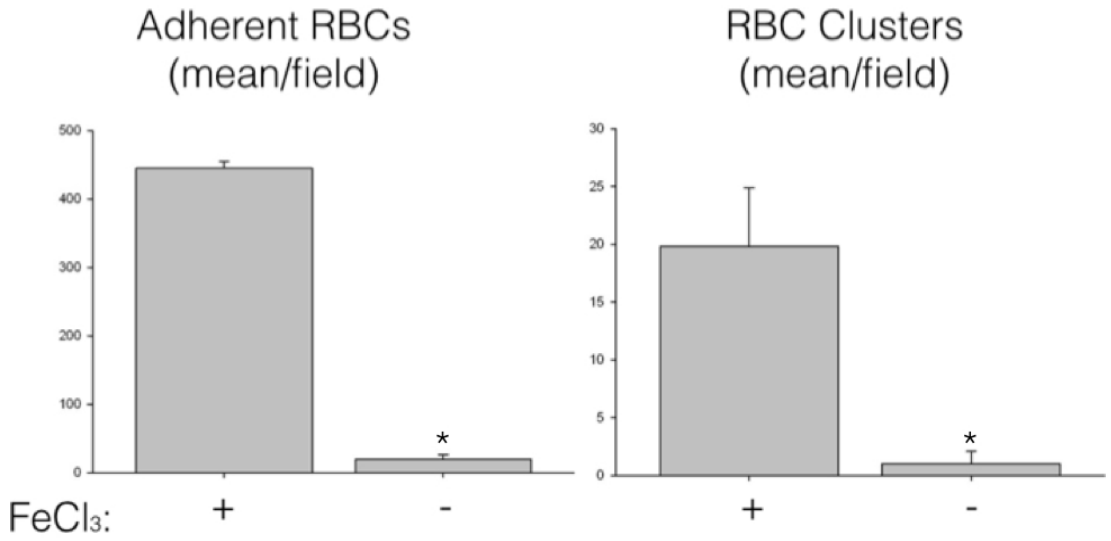

Figure 5 - Quantification of adherent RBCs and RBC clusters at a shear stress of 0.9 dynes/cm^2 with a maximum of 5 random 20x fields per experiment. Three or more RBCs together were considered a cluster. The error bars represent the standard error of the mean. Quantification was done in Image J. N=4. *P<0.05 compared to experiments with $FeCl_3$.

Typical venous shear stress in mammals ranges from 1 to 6 dynes/cm.[2,13] To examine RBC-endothelial cell interactions under more physiological venous conditions, the same protocol was repeated under a shear stress of 3 dynes/cm^2. As shown in Figure 6 and Figure 7, $FeCl_3$ drastically increased the number of adherent RBCs on the endothelial surface compared to the control experiment without $FeCl_3$. These results were similar to what was found at the lower venous shear stress. These findings suggest that $FeCl_3$ is able to induce the adhesion of washed RBCs to cultured human endothelial cells within the range of physiological venous shear stress.

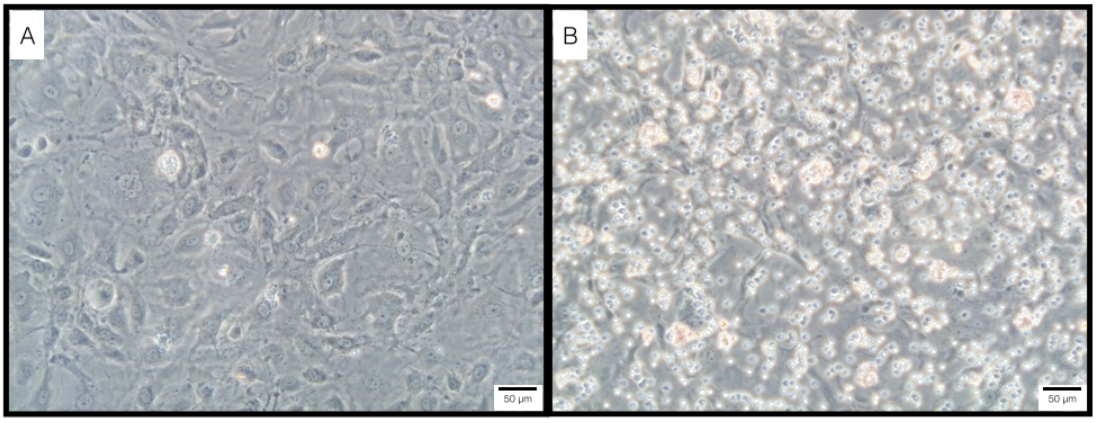

Figure 6 - Ferric chloride induced adhesion of washed human RBCs to cultured HUVECs under venous shear stress. A12.5% HCT solution suspended in PBS (A) or PBS + 250 μM $FeCl_3$ (B) was perfused over confluent HUVECs for 5 minutes at a shear stress of 3 dynes/cm^2.

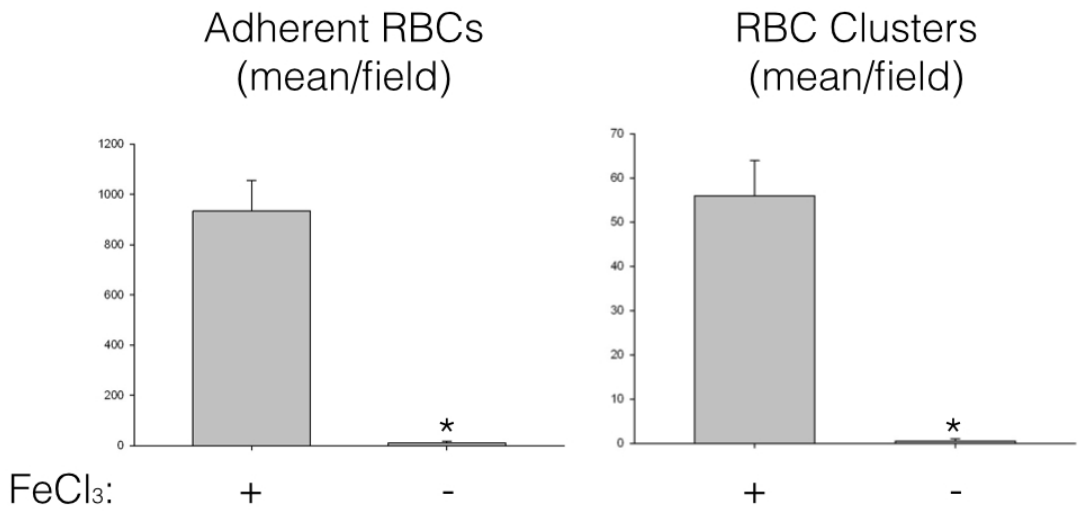

Figure 7 - Quantification of adherent RBCs and RBC clusters at venous shear stress of 3 dynes/cm^2 with a maximum of 5 random 20x fields per experiment. Three or more RBCs together were considered a cluster. The error bars represent the standard error of the mean. Quantification was done in Image J. N=3. *P<0.05 compared to experiments with $FeCl_3$.

Adhesion of RBCs at Arterial Shear Stress

In order to determine if RBC-endothelial interactions are also induced by $FeCl_3$ under arterial shear stress, the experiments were repeated with smaller gaskets that produced a shear stress of 10 dynes/cm^2. The results from these experiments can be seen in Figure 8 along with the quantified results in Figure 9. Similar to what was seen under venous shear stress, $FeCl_3$ was able to induce RBC adhesion to the endothelium under arterial shear stress. However, the number of RBCs and RBC aggregates decreased slightly compared to what was seen under venous shear stress. These results suggest that $FeCl_3$ can also induce RBC adhesion to the endothelium under arterial flow conditions, although the higher shear decreases some RBC adhesion.

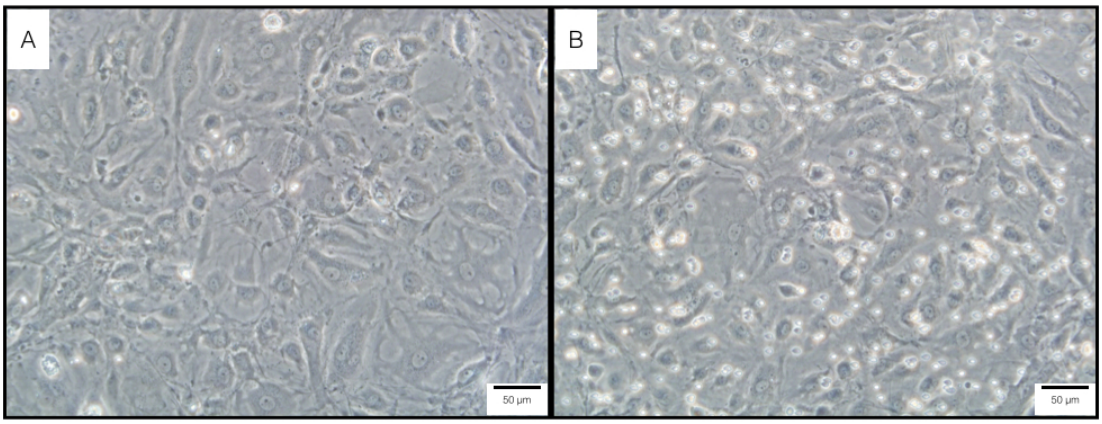

Figure 8 - Ferric chloride induced adhesion of washed human RBCs to cultured HUVECs under arterial shear stress. A12.5% HCT solution suspended in PBS (A) or PBS + 250 μM $FeCl_3$ (B) was perfused over confluent HUVECs for 5 minutes at a shear stress of 10 dynes/cm^2.

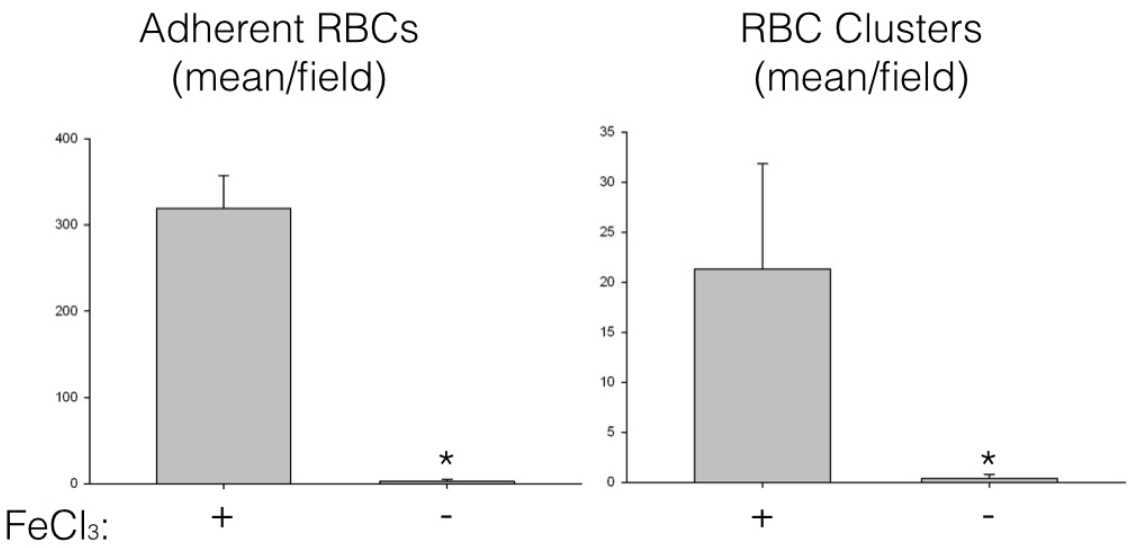

Figure 9 - Quantification of adherent RBCs and RBC clusters at venous shear stress of 10 dynes/cm^2 with a maximum of 5 random 20x fields per experiment. Three or more RBCs together were considered a cluster. The error bars represent the standard error of the mean. Quantification was done in Image J. N=3. *P<0.05 compared to experiments with $FeCl_3$.

Role of VWF in Interaction of RBCs with Endothelial Surface in Ferric Chloride Model

Although the in-vitro parallel-plate flow chamber experiments shown in Figures 4-9 were performed with washed RBCs in the absence of any other circulating molecules, it is possible that VWF secreted from the $FeCl_3$-injured HUVECs facilitates the adhesion of the RBCs to the endothelium. In order to investigate whether the RBCs were interacting with the endothelium surface in a VWF-dependent process, the venous experiments at a shear stress of 3 dynes/cm^2 were repeated with the addition of an anti-VWF antibody. The results from this experiment can be seen in Figure 10 along with the quantified results in Figure 11. The addition of the anti-VWF antibody drastically decreased the number of RBCs and RBC aggregates adhering to the surface. In order to provide a negative control for the addition of the antibody, the same experiments were also completed with the addition of a control antibody at the same concentration. As shown in Figure 11, the addition of the negative control antibody did not significantly decrease the adhesion of RBCs as seen with the anti-VWF antibody. The results suggested that VWF might be playing a role in RBCs adhering to the endothelium in the ferric chloride model of thrombosis. These results contradict what was seen in the in-vivo experiments with VWF knockout mice where SEM images showed RBCs adhering directly to the endothelium after the addition of ferric chloride to the surface of the vessel.[3]

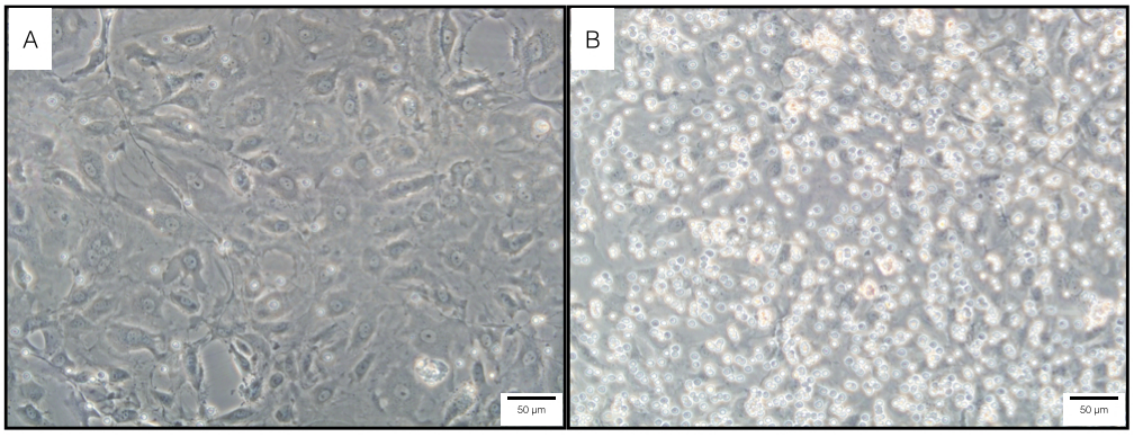

Figure 10 – Ferric chloride induced adhesion of washed human RBCs to cultured HUVECs under venous shear stress. A12.5% HCT solution suspended in PBS + 250 μM $FeCl_3$ + 100μg/mL anti-VWF antibody (A) or PBS + 250 μM $FeCl_3$ (B) was perfused over confluent HUVECs for 5 minutes at a shear stress of 3 dynes/cm^2.

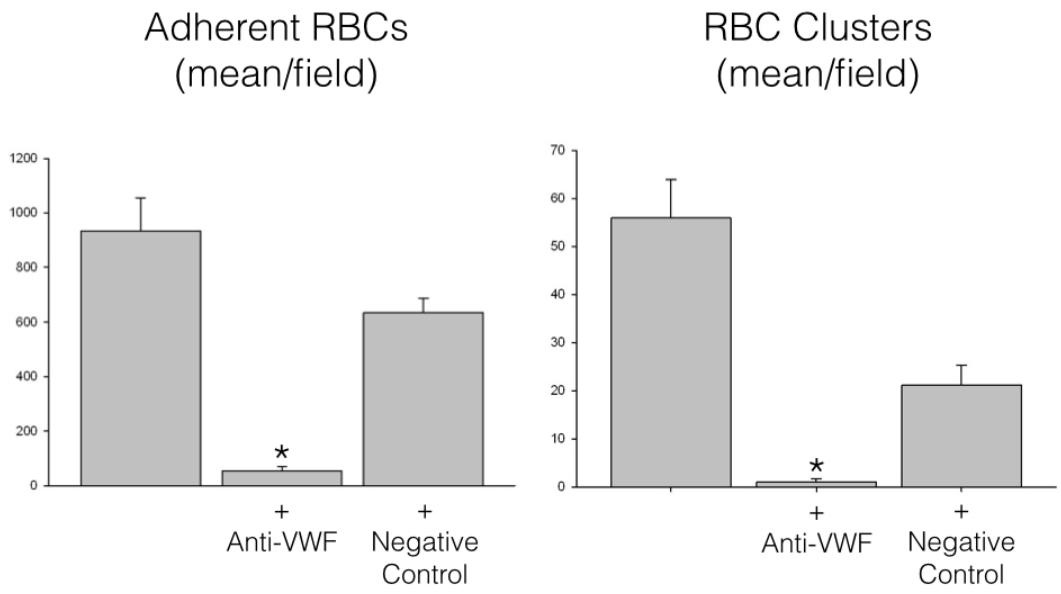

Figure 11- Quantification of adherent RBCs and RBC clusters at venous shear stress of 3 dynes/cm^2 and a comparison with the addition of anti-VWF antibody (100µg/mL) or negative control (100µg/mL) with a maximum of 5 random 20x fields per experiment. Three or more RBCs together were considered a cluster. The error bars represent the standard error of the mean. Quantification was done in Image J. N=3. *P<0.05 compared to experiments without anti-VWF antibody and compared to those with the negative control antibody.

Discussion

In this present study we investigated the interaction of RBCs with the injured endothelial surface in-vitro using a parallel-plate flow chamber system. We observed that RBCs adhere to $FeCl_3$-injured endothelium under both venous and arterial shear stress. Furthermore, we observed that the addition of an anti-VWF antibody interferes with this RBC-endothelium interaction. These observations suggest that the interaction of RBCs with the $FeCl_3$-injured endothelium does not require any other components that were not in the flow chamber, such as platelets, white blood cells, clotting factors, etc.

Additionally, we found that the interaction of RBCs with the $FeCl_3$-injured endothelial surface is facilitated via VWF, which is presumably expressed by the endothelial cells.

Although it has long been reported that RBCs are indirectly involved in the formation of thrombi by: a) determining the rheological properties of blood and b) contributing to thrombus mass by getting trapped in a developing clot, they were not known to participate directly in hemostasis or thrombosis. However, recent evidence suggests that RBCs may be one of the first blood cells at the site of injury adhering to the damaged endothelial surface, at least in some models of experimental thrombosis. Recently, our group in Dr. Motto's lab demonstrated interactions of RBCs with the endothelium in-vivo using a $FeCl_3$ model of thrombosis. Dr. Motto and colleagues used SEM and brightfield intravital microscopy to visualize the interaction of RBCs with the endothelium in-vivo using mouse models.[3] Through these studies, they were able to demonstrate that RBCs are the first blood cells to adhere to the $FeCl_3$-treated endothelial surface in the carotid artery of mice. Thereafter, platelets were recruited to the site of injury. These novel observations contradicted the previously proposed model in which $FeCl_3$ induces thrombosis by causing endothelial denudation and subsequent exposure of the subendothelial surface to recruit platelets to the site of injury. In fact, they were able to demonstrate in their SEM images that $FeCl_3$ does not cause gross endothelial denudation, suggesting that thrombosis is initiated through a different mechanism, which they suggested must be through direct adhesion of RBCs to the endothelium. Their in-vivo models also suggested that VWF and GPIb-α are not required for the RBCs to adhere to the $FeCl_3$-injured endothelium. Since their studies were performed in-vivo, however, the RBC adhesion to the $FeCl_3$-injured vessel wall occurred in the presence of whole blood. Although some experiments were carried out in mice lacking VWF or GPIb-α, it was still unclear from their study whether or not this interaction involved any other vascular or blood components.

In the in-vitro studies presented here, we were able to study the interaction of RBCs with the $FeCl_3$-injured endothelium in the absence of other blood components (such as platelets, white blood cells, and clotting factors, etc.) by using washed human RBCs perfused over cultured HUVECs under physiological flow conditions. In agreement with the in-vivo studies of Motto and colleagues,[3] we observed adhesion of RBCs to $FeCl_3$-injured endothelium. Interestingly, however, we observed that this interaction was abolished in the presence of anti-VWF antibodies. These findings suggest that VWF is required for adhesion of RBC to $FeCl_3$-injured endothelium under the conditions of our in-vitro perfusion system. The findings from both the in-vivo and in-vitro studies together suggest that the interaction of RBCs with the injured endothelium has more than one mechanism, one of those being VWF-dependent. Dr. Motto and colleagues demonstrated that RBCs could still adhere to the $FeCl_3$-injured vessel in VWF-deficient or GPIb- α- deficient mice, but their results were not quantified for comparison to those in a wild type mouse. It may be that the amount of adhesion was decreased in the mice lacking VWF, leaving only the VWF-independent RBC adhesion that involves a different mechanism. An alternative explanation for the differences between the in-vivo and in-vitro studies is that there may be a difference in VWF-dependence between human and mice. Another possible explanation for the contradicting results is that in-vitro the anti-VWF antibody may interfere with another mechanism of RBC adhesion to the injured endothelial surface, giving the impression that the majority of RBC-endothelial interactions are VWF-dependent. Finally, it is possible that ADAMTS-13, a VWF-cleaving protease present in the circulating whole blood, may have eliminated the majority of VWF-dependent RBC adhesion in-vivo in the wild type mice. No ADAMTS13 was present in the in-vitro perfusion system, making the RBC-endothelium interaction more VWF-dependent. This scenario might explain why there was little difference in the amount of RBC adhesion to the $FeCl_3$-injured vessel in VWF-deficient mice compared to wild type mice.

The most novel finding from this present study is that RBCs interact with the $FeCl_3$-injured endothelium via VWF. This is a surprising finding since RBCs are not known to bind to VWF.[14] Although it is not clear from these studies how RBCs interact with VWF, future studies could examine this interaction in detail. To begin to pursue this question, pilot studies were performed to examine the RBC interaction with VWF strings secreted from HUVECs stimulated with histamine under venous flow in-vitro. These studies suggested that washed human RBCs were able to bind to the VWF strings, both in the presence and absence of platelets (see Figure A1). These results support our findings in the in-vitro $FeCl_3$ studies. In the future, additional studies could be pursued to further determine the mechanisms of the RBC-VWF interaction. To explore whether the anti-VWF antibody interferes with another mechanism of RBC adhesion to the $FeCl_3$-injured endothelium, other methods could be used to eliminate VWF in the system. Such methods include using endothelial cells from mice lacking VWF, addition of plasma to introduce ADAMTS13 into the system, or use of small interfering RNA (siRNA) to knockdown VWF. Additionally, future studies following up on Dr. Motto's work could be performed to examine other mechanisms of RBC-endothelial interactions in the mice lacking VWF, and whether these interactions are receptor mediated or nonreceptor mediated.

Although these in-vitro studies provided interesting results, there are some limitations of the results that are presented here. Since the experiments were carried out with washed human RBCs donated from the Blood Bank, they were not fresh samples. Research on RBC storage time has shown that over time RBCs in storage will undergo structural and function changes, making them undesirable for transfusion purposes.[15] Although there were more than likely some chemical and structural changes that had occurred in the RBCs used for these studies, it is unclear whether or not those would affect the RBC-endothelial surface interaction. In the future it would be advantageous to repeat the studies with fresh RBCs to determine if our in-vitro studies are clinically

relevant. Additionally, the experiments were completed at room temperature with no device to maintain the HUVECs and perfusing solutions at physiological temperatures. Although the experiments were performed over a limited time frame, lasting no longer than eight minutes each and the solutions were warmed to 37 degrees Celsius beforehand, the non-physiological temperatures during perfusion may have affected the properties of the blood cells, although RBCs are not as temperature sensitive as platelets. In the future it would be beneficial to complete the experiments using a temperature-controlling device on the parallel-plate flow chamber to confirm these results presented here. Furthermore, all conclusions from these studies and the studies that were done in-vivo using murine models[3], must be limited to the $FeCl_3$-induced model that was used to injure the endothelial surface and initiate thrombosis. As previously mentioned, this is only one model of thrombosis and the results that were found in this model may not be consistent with what is seen in other models or clinically. Future studies could be completed to test the other models of thrombosis to determine if this interaction observed here is dependent on $FeCl_3$. Also, to make the in-vitro experiments more physiological, other components of blood such as platelets, white blood cells, or other plasma components could be added to the washed RBCs to examine how these factors independently influence the interaction of RBCs with endothelium. Such a study may help determine other factors involved in facilitating the RBC interaction with injured endothelium. Finally, HUVECs were chosen to study the interaction of RBCs with $FeCl_3$-injured endothelium, however, the use of other cell types, such as human coronary artery endothelial cells, may provide more meaningful results and comparisons to the in-vivo studies.

In conclusion, these in-vitro studies revealed a previously unrecognized ability of RBCs to participate in thrombosis. We demonstrated that RBCs are able to adhere to the $FeCl_3$-injured endothelium under physiological conditions of blood flow and that VWF facilitates RBC adhesion. These novel findings demonstrate a previously unrecognized role of RBCs and, with future follow-up studies, may provide insight into the clinical role

of RBCs in thrombosis. One well-established clinical observation is that it is common for patients with low hematocrits to have longer bleeding times, regardless of their platelet counts, and that after RBC transfusion the bleeding times are improved.[16] These observations have been explained by the previously known rheological effects of RBCs in thrombosis, such as displacing platelets towards the endothelial surface in the vessel. However, the observations presented in this study, along with those presented by Dr. Motto's colleagues, suggest that exploring a more direct role of RBCs in hemostasis and thrombosis would be beneficial.

CHAPTER 3
INTERACTION OF PLATLETS WITH SUBENDOTHELIAL SURFACE

Background and Significance

Platelets are small cell fragments generated by megakaryocytes in the bone marrow. After they are released into circulation they play a critical role in vascular repair via their ability to quickly adhere to endothelial and subendothelial surfaces and aggregate at the site of injury.[17] One way platelets can adhere and aggregate at the vessel wall is through exposure of the subendothelial matrix, which is rich in collagen. In healthy vasculature, collagen lies underneath endothelial cell layers lining the vessel wall and is not exposed to blood flow However, when the endothelial surface is damaged, either by tissue injury or in certain disease states, the collagen matrix becomes exposed, as illustrated in Figure 1. When collagen comes into contact with platelets in the flowing blood, platelet glycoprotein VI interacts with collagen and platelet glycoprotein Ib-V-IX can interact with von Willebrand factor, leading to initial platelet adhesion at the site of injury. Further platelet-platelet aggregation is then mediated by binding of platelet surface $\alpha_{IIb}\beta_3$ integrin to the fibrinogen and von Willebrand factor.[18] Although these are important events in hemostasis, excessive platelet aggregation can also lead to thrombotic disorders, which may be life threatening.

Thrombotic events can be divided into two categories depending on whether they occur in the arteries or the veins. Traditionally, platelets have been thought to play a larger role in arterial thrombosis, which occurs at high shear stress, than in venous thrombosis, which occurs at lower shear stress.[19] Recent findings, however, have suggested that platelets contribute to both venous and arterial thrombosis in both mouse and human studies.[20, 21] Both arterial and venous thrombi formation can be life-threatening events as they can obstruct the blood flow to or from the major organs.

Depending on where the thrombotic events occur they can lead to myocardial infarction, stroke, deep vein thrombosis, and pulmonary embolism. These diseases account for a large portion of deaths worldwide and therefore thrombotic events are a serious healthcare problem.[22] Despite decades of research in developing new and more effective antithrombotic drugs to treat these diseases, their impact has been relatively small.[23] Susceptibility to both arterial and venous thrombosis increases in the presence of several risk factors including obesity, aging, diabetes, and smoking.[24] With the aging population and increasing number of obese people in the United States, the frequency of thrombotic events will continue to rise until we better understand the mechanisms behind these events and the correlation to their risk factors.

Several anti-platelet drugs are in use to limit thrombosis, but their beneficial effects are partial. One possible reason is that the mechanism of platelet activation may be different in different disease states. Studies from our group have demonstrated that platelet hydrogen peroxide (H_2O_2) plays an important role in platelet activation in aging.[4] Others have also demonstrated a role for platelet H_2O_2 in platelet hyperactivity in diabetes.[25] In this study we examined whether platelet H_2O_2 modulates aggregation on a collagen surface in the flow chamber. For this purpose, we first isolated the platelets from mice deficient in glutathione peroxidase-1 (Gpx1).[4] GPx1 is expressed in platelets and it reduces peroxides, such as H_2O_2, to water. We hypothesized that platelets from mice deficient in Gpx1 will form larger aggregates on the collagen-coated surface compared with platelets from wild type mice. We also examined whether catalase, which is another enzyme metabolizing H_2O_2, would limit the size of aggregates formed from platelets from wild type and Gpx1-deficinet mice. If we identify H_2O_2 as a possible mediator of platelet aggregation, studies can be directed to identify disease states with increased platelet H_2O_2. Further, clarification of the mechanisms of increased H_2O_2 in platelets in certain disease states would allow the development of targeted therapy.

As previously mentioned, obesity is one disease that is associated with thrombotic events, however the mechanism is not completely clear. Obesity, defined as a BMI of more than 30 kg/m^2, has become an epidemic in the United States, affecting nearly 1 in 3 adults.[26] The prevalence of the disease has steadily increased over the last decades, which is alarming considering the impact the disease has a person's health. It is estimated that every year 28 million people die as a result of consequences of obesity worldwide.[27] Obesity is associated with the development of cardiovascular risk factors leading to thrombotic disorders such as cardiovascular disease, stroke, and venous thromboembolism.[28] The correlation between obesity and thrombotic events has been recognized for some time, but the mechanisms behind this correlation are still poorly understood. It has been suggested that obesity promotes a state of chronic inflammation, activating prothrombotic signaling pathways in platelets and other vascular cells.[29] Studies have also indicated obesity is correlated with an increase in platelet reactive oxygen species (ROS).[30] Additionally, studies in our lab have demonstrated that mice fed on a high fat diet to induce obesity exhibit increased platelet H_2O_2, as shown in Figure A2. To pursue the hypothesis in-vitro, we sought to determine whether platelets from mice fed on an obesity diet form larger aggregates on collagen surface and whether the size of aggregates can be limited by addition of catalase. We hypothesized that platelets from obese mice will form larger aggregates on the collagen surface due to an increase in platelet H_2O_2, and therefore the size of the aggregates will decrease with the addition of catalase. If we are able to confirm this hypothesis, then this information can be used to develop targeted therapy to decrease the risk for cardiovascular events in obese individuals.

Methods

Mice

Wild type C57Bl6J mice were purchased from The Jackson Laboratory. Heterozygous mice deficient in glutathione peroxidase (Gpx*1*+/-), obtained from Dr. Yi Shi Ho[31], were bred to each other to generate littermates of wild type Gpx1+/+, heterozygous Gpx1+/-, and homozygous Gpx1-/- mice. Genotyping for the mutated Gpx1 was performed with real-time polymerase chain reaction. For diet induced obesity studies, C57BL/6 mice purchased from The Jackson Laboratory were placed on a high fat diet (60% calories from fat) at 5 weeks of age and maintained on this diet for 12 weeks before they were used for the study. All other mice were maintained on standard lab chow. The University of Iowa Animal Care and Use committee approved all animal protocols and all mice were maintained in the animal care facilities at the University of Iowa.

Preparation of Coverslips

To mimic the subendothelial surface, 40 mm round glass coverslips were coated in collagen. The glass coverslips were first soaked in a solution made of 50% 1M hydrochloric acid and 50% methanol for 1 hour. After drying overnight in a desiccator, the prepped coverslips were then coated with type I collagen from equine tendons (no. 385, CHRONO-LOG) diluted in a 5% glucose solution (pH 2.7) at a concentration of 100 µg/mL. All coverslips were then blocked in 1 mg/mL BSA in PBS for 1 hour, followed by rinsing with PBS. After a second period of drying in the desiccator, the collagen coverslips were ready for use in the flow aggregation studies.

Platelet Isolation and Preparation

Mice were anesthetized with CO_2 and blood was collected through heart puncture into acid citrate dextrose (9:1,v/v). Blood was diluted with tyrode buffer (1:1, v/v)(134

mmol/L NaCl, 2.9 mmol/L KCl, 2.9 mmol/L $CaCl_2$, 0.34 mmol/L Na_2HPO_4, 12 mmol/L $NaHCO_3$, 20 mmol/L HEPES, 1.0 mmol/L $MgCl_2$, 5.0 mmol/L glucose, 0.05% [wt/vol] fatty acid–free bovine serum albumin (BSA), pH 7.35) and centrifuged at 100G. Platelet rich plasma (PRP) was transferred to a fresh tube, mixed with prostaglandin E1 (PGE1) and re-centrifuged as above to get rid of contaminating RBCs and white blood cells (WBCs). PRP was collected, mixed with PGE1 and centrifuged at 1000G for 10 minutes to pellet platelets. Supernatant was removed and pellet was washed once with tyrode buffer. Hemavet (Drew Scientific) was used to determine platelet count and the suspension was diluted to adjust platelet counts to $3x10^5/\mu l$. The remaining RBCs from the first centrifuge were washed 3 times with RBC washing buffer (140mM NaCl, 10mM Hepes, 5mM glucose, pH 7.4) to mix with washed platelets during the assay.

Experimental Protocol and Imaging

Washed platelets to be used in collagen-coated coverslip experiments were prepared by incubating for 2 minutes at 37°C with 3mM calcium chloride, and either 0.25% calcein green dissolved in DMSO (no. C34852, Life Technologies) or anti-mouse CD41 antibody (no. MHCD4104, Life Technologies).

The experiments were carried out in the in-vitro parallel-plate flow chamber described in Chapter 2. Platelets of interest for the individual experiments were perfused through the chamber containing the collagen-coated coverslips at varying shear stresses for 5 minutes. The chamber was perfused with PBS for 3 minutes to rinse out any platelets that had not adhered to the coverslip. Images were then taken at a minimum of 8 locations throughout the coverslip at 20x in phase contrast or fluorescence using the microscope that was described in Chapter 2. The percent surface coverage was estimated using the threshold function in Image J. Statistical analyses were performed using the paired Student's t-test to compare the percent surface coverage of wild-type Gpx1 mice

versus GPx1-/- mice and to compare that of obese versus control mice. Statistical significance was defined as a value of $P<0.05$.

Results

Optimizing Flow Chamber Conditions for Murine Platelets

When beginning the studies of the interaction of platelets with the sub-endothelium, it was first necessary to standardize our methods using the parallel-plate flow chamber. Since the availability of murine platelets is limited, the chamber size was optimized in order to reduce the total volume of sample required for each experiment. We used a hand cut gasket with a width of 1.5 mm that required approximately 60% less volume compared to using a gasket that was commercially available from Warner Instruments. A cutting guide with dimensions of 1.5 mm width by 37 mm length was developed at the engineering workshop at the University of Iowa Hospitals and Clinics. This tool was placed over a blank silicone gasket and a scalpel was used to cut the gasket to the precise dimensions required. The required flow rates and chamber conditions to simulate venous and arterial shear stress with this gasket, based on the previously mentioned equation for shear stress, can be seen in Table 2.

Table 2. Flow Chamber Conditions for Platelet-Collagen Experiments

	Venous	**Arterial**
Q	0.045 mL/min	0.225 mL/min
μ	0.01 dyne-s/cm^2	0.01 dyne-s/cm^2
b	1.5 mm	1.5 mm
h	100 μm	100 μm
τ	3 dynes/cm^2	15 dynes/cm^2

Optimizing Flow Conditions for Venous Shear Stress

Initial experiments with washed platelets alone did not show appreciable aggregation on the collagen surface using a concentration of $3x10^5$ platelets/mL, as seen in Figure 12-A. There was less than 1% surface coverage after 5 minutes of perfusion of the platelet solution and aggregates were very small in size. To increase platelet aggregation, washed RBCs were added to develop physiological rheological forces for platelets to adhere, as previously described.[32] In subsequent experiments several concentrations of platelets and RBCs were tested to obtain an optimal surface coverage using wild type murine platelets.

The first conditions examined were $3x10^5$ platelets/μL with 50% HCT (volume/volume percentage), as was used in previous studies.[32] These conditions provided approximately 24% surface coverage of platelet aggregates after 5 minutes of flow (Figure 12-B). In addition, there was also some RBC adhesion observed with the platelet aggregates. These results suggested that the addition of washed RBCs increased the amount of platelet aggregation on the collagen surface.

To reduce the amount of platelet aggregation on the surface as well as the number of RBC aggregates, the cell counts were reduced to $1.5x10^5$ platelets/μL and 25% HCT. This resulted in a decreased surface coverage of approximately 7% and a large decrease in RBC aggregation (Figure 12-C). These conditions provided a more optimal surface coverage of platelets for subsequent experiments with platelets from diseased mice.

To examine the effect of further reducing the amount of RBCs in solution, the RBC concentration was decreased to 12.5% HCT while maintaining the platelet concentration at $1.5x10^5$ platelets/μL. We were interested in lowering the cell counts as much as possible to minimize the amount of volume used per study. These cell concentrations provided similar surface coverage of approximately 9% and again no RBC aggregation (Figure 12-D). Although the surface coverage was comparable to what was seen with 25% HCT, the size of the individual aggregates were slightly smaller. These

results suggested that reducing the RBC count to 12.5% HCT would produce satisfactory results while using the smallest sample volume. For this reason, all subsequent experiments will be carried out at the optimized condition of $1.5x10^5$ platelets/μL and 12.5% HCT.

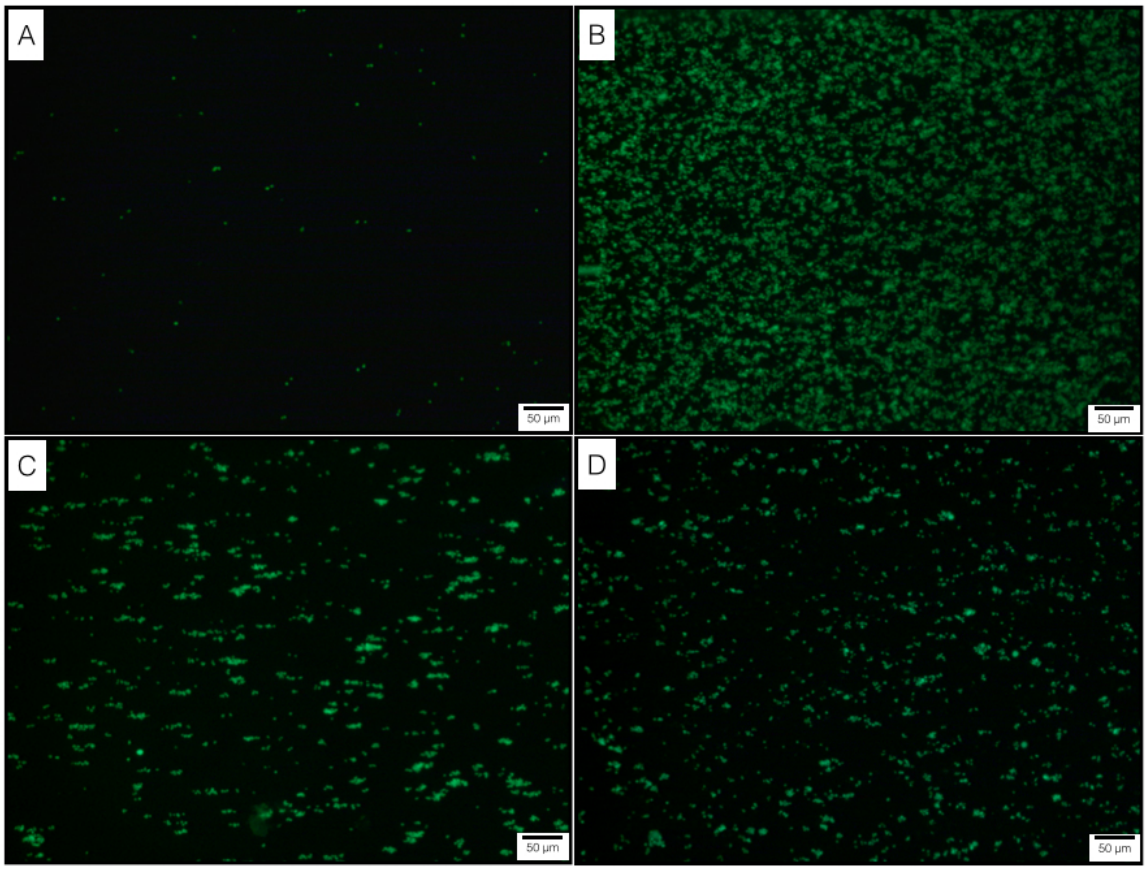

Figure 12 – Representative images of platelet adhesion on a collagen-coated surface from optimization experiments. Images show aggregation of platelets in the presence/absence of RBCs at cell counts of (A) $3x10^5$ platelets/μL with 0% HCT, (B) $3x10^5$ platelets/μL with 50% HCT, (C) $1.5x10^5$ platelets/μL) with 25% HCT, (D) $1.5x10^5$ platelets/μL with 12.5% HCT. All images were taken after 5 minutes of perfusion of the platelet and RBC solution at a shear stress of 3 dynes/cm^2 and after 3 minutes of rinsing with buffer.

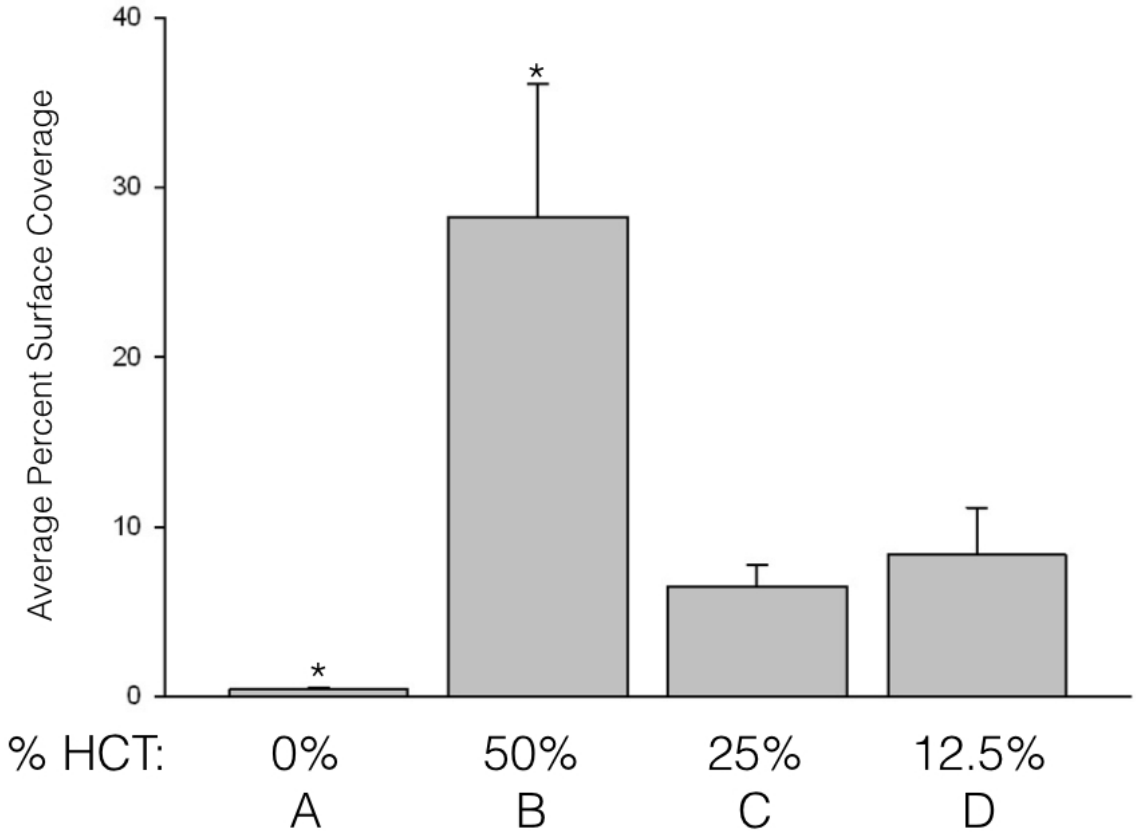

Figure 13 – Quantification of percent surface coverage by platelets on the collagen-coated surface during the optimization experiments, as pictured in Figure 13. The HCT (volume/volume percentage) was varied to determine the optimum cell counts to be used in future experiments. The percentages represent the mean surface coverage of 8 random 20x fields per experiment. The error bars represent the standard error of the mean. Quantification was done in Image J. *Platelet counts are also increased to $3x10^5$ platelets/μL.

Another parameter that we could adjust to further reduce the volume of the murine samples required for each experiment was the time the sample was flowed over the collagen-coated surface. In all of the previous experiments, samples were perfused for 5 minutes. To investigate whether or not this time could be reduced while still providing optimal surface coverage, the perfusion time was reduced to 2 minutes. This was done with the optimal cell count conditions determined from the previous optimization

experiments, $1.5x10^5$ platelets/μL and 12.5% HCT. The surface coverage after 2 minutes of flow was approximately 0.8% (Figure 14-B), which is significantly less than the 9% surface coverage seen in the 5-minute experiments (Figure 14-A). These results suggested that reducing the time of the experiments to 2 minutes at the optimized cell counts would not provide similar results. For that reason, in all subsequent experiments the platelet and RBC solutions were perfused for 5 minutes.

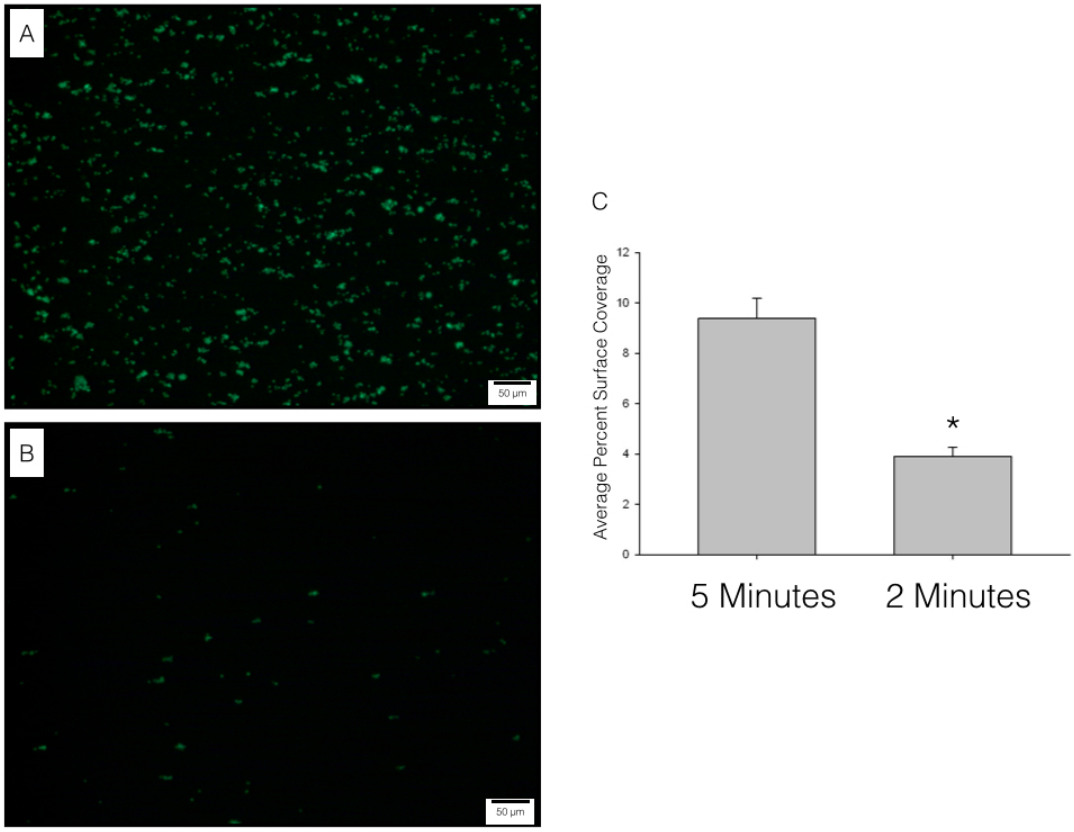

Figure 14 - Representative images of platelet adhesion on a collagen-coated surface at different time points. Images show aggregations after perfusion of solution with cell counts of $1.5x10^5$ platelets/μL and 25% HCT at 3 dynes/cm^2. Solutions were either perfused for 5 minutes (A) or 2 minutes (B) followed by 3 minutes of rinsing with buffer. (C) shows quantification of average percent surface coverage of a minimum of 8 random 20x fields per experiment. The error bars represent the standard error of the mean. Quantification was done in Image J. *P-value < 0.05 compared to 5 minute experiments.

Optimizing Flow Conditions for Arterial Shear Stress

In order to optimize the flow conditions for an arterial shear stress of 15 dynes/cm^2, we tested the conditions optimized for venous shear stress. From these experiments we found that after 5 minutes of perfusion of a 1.5x10^5/µl platelets and 12.5% HCT solution at 15 dynes/cm^2 gives slightly lower surface coverage compared to the venous shear stress experiments (Figure 15). These results may suggest that the shear stress is too high for adequate platelet aggregation on the collagen surface, but the decrease compared to venous conditions was not significant. Additionally, there was a large decrease in the amount of single cell adhesion with arterial shear stress although the size of the aggregates was much larger. These results suggest that although the surface coverage was lower, these conditions can be used at arterial flow rates in future studies of disease state platelets.

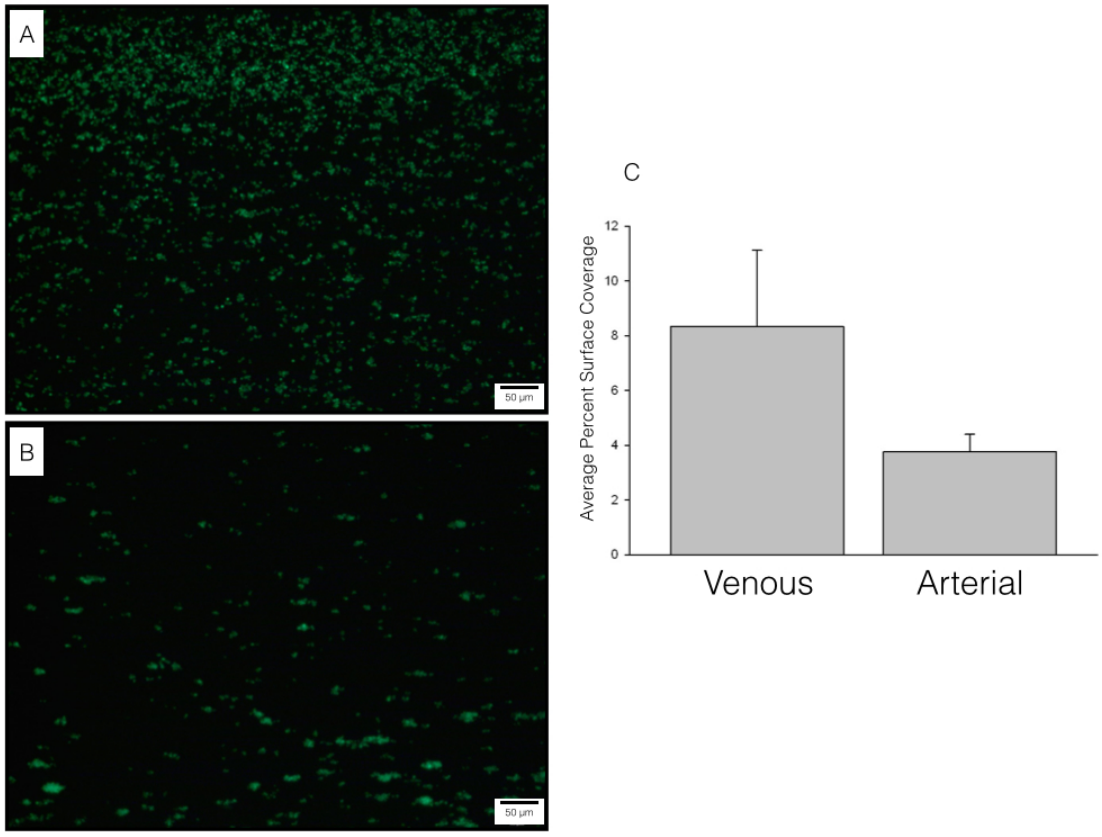

Figure 15 – Representative images of platelet aggregation from 5 minute perfusion of $1.5x10^5$ platelet/µL and 12.5% HCT under venous shear stress of 3 dynes/cm^2 (A), or arterial shear stress of 15 dynes/cm^2 (B). (C) shows quantification of percent surface coverage by platelets from the venous and arterial experiments. The percentages represent the mean surface coverage of 8 random 20x fields per experiment. The error bars represent the standard error of the mean. Quantification was done in Image J. N=2

Diseased State Experiments

H_2O_2 in Platelet Aggregation

In order to test the hypothesis that H_2O_2 mediates aggregation on a collagen surface, platelets from wild type (Gpx1 +/+) mice were compared to Gpx1-deficient (Gpx1 -/-) mice. Platelet aggregation on the collagen surface was first compared at a venous shear stress of 3 dynes/cm^2, using optimized cell counts of $1.5x10^5$ platelets/mL, and 12.5% HCT. As shown in Figures 16-A, B, and C, there was a significant increase in

the surface coverage when using platelets from the Gpx1 -/- mice compared to the wild type mice (p-value < 0.05). These results suggest that platelets from mice lacking Gpx1 are hyperactive compared to their wild type littermates. This finding supports our hypothesis that H_2O_2 modulates platelet aggregation on a collagen surface. This observation also agrees with in-vivo data from Dayal et. al.[4]

To further test the hypothesis that platelet hyperactivity in Gpx1 -/- mice is mediated by H_2O_2, as opposed to other peroxides reduced by Gpx1, the effect of catalase on platelet aggregation was examined. In these studies, platelets from wild type and Gpx1-deficient mice were both incubated with polyethylene glycol (PEG) catalase before being perfused at a venous shear stress of 3 dynes/cm^2. As seen in Figure 16-C, in preliminary studies, the addition of PEG catalase to platelets from Gpx1-deficient mice decreased platelet aggregation to levels that were seen in the wild type group without PEG catalase. Additionally, the addition of PEG catalase to platelets from wild type mice did not have an effect on platelet aggregation. Although the number of experiments with PEG catalase was low (n=2), this preliminary data supports the hypothesis that H_2O_2 does mediate platelet aggregation.

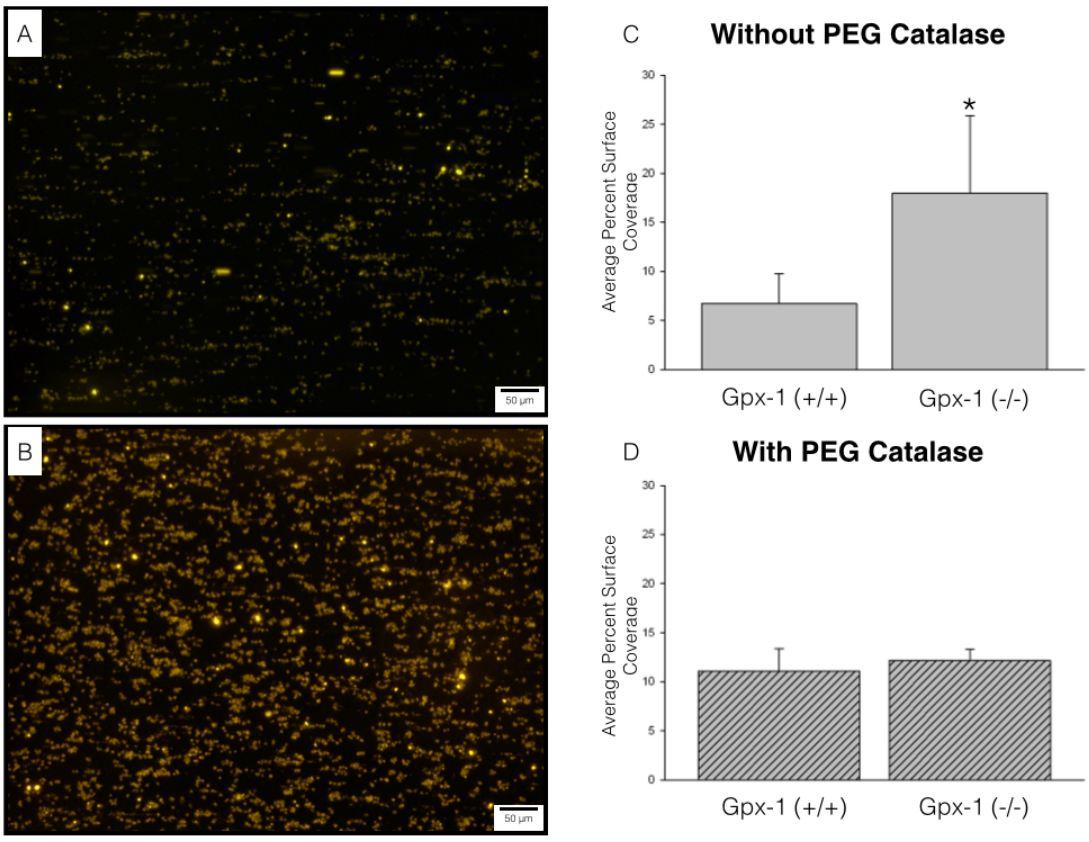

Figure 16 - Representative images of murine platelet aggregation on collagen surface from Gpx +/+ mice (A) and Gpx -/- mice (B) at a shear stress of 3 dynes/cm^2 and cell counts of 1.5x10^5 platelets/mL and 12.5% HCT. Images were taken after 5 minutes of perfusion and 3 minutes of rinsing with buffer. (C) and (D) show average percent surface coverage without and with added PEG catalase, respectively, of 8 random 20x fields per experiment. The error bars represent the standard error of the mean. Quantification was done in Image J. N=3 for experiments without PEG catalase and N=2 for experiments with PEG catalase. *P<0.05 vs Gpx-1 (+/+).

Platelet Aggregation in Obesity

The optimized flow chamber conditions were also used to study the hypothesis that obese mice show increased platelet aggregation on a collagen surface. After the mice had been on a high fat diet (consisting of 60% of calories from fat) for 12 weeks, their platelets could be compared to those of their control littermates in the flow chamber system. The experiments were carried out at the optimized conditions (1.5x10^5

platelets/mL and 12.5% HCT) for both venous and arterial shear stress. At venous shear stress, there was not a significant difference in average surface coverage between the control and obese mice (p-value = 0.628), as shown in Figure 17-A, B, and C. In a preliminary experiment at arterial shear stress, again there was not a significant difference between the two experimental groups (p-value = 0.126) but we did observe an increasing trend towards more platelet aggregation in the obese group, as shown in Figure 18-A, B, and C. These results suggest that platelets from obese mice may be hyperactive compared to their littermate controls, particularly under conditions of arterial shear stress, but the number of experiments needs to be increased to ensure that our trends are correct and to obtain significant results.

To examine the effect of PEG catalase on aggregation of platelets from control and obese mice, a solution consisting of the optimized cell counts ($1.5x10^5$ platelets/mL and 12.5% HCT) and added PEG catalase was perfused at venous and arterial shear conditions. At venous shear stress, the addition of PEG catalase significantly decreased platelet aggregation on the collagen surface for both control and obese mice, as illustrated in Figure 17-D. PEG catalase reduced platelet aggregation in the control and obese mice to similar levels, which is logical because we didn't observe a difference in platelet aggregation without PEG catalase between these two groups. In preliminary studies at arterial shear stress, the addition of PEG catalase increased platelet aggregation in both the control and obese groups, although the results were not significant (p=0.126 and p=0.087, respectively). In order to draw any conclusions from this data at arterial shear, the number of experiments needs to be increased to obtain significant data and to confirm the trends that we were seeing.

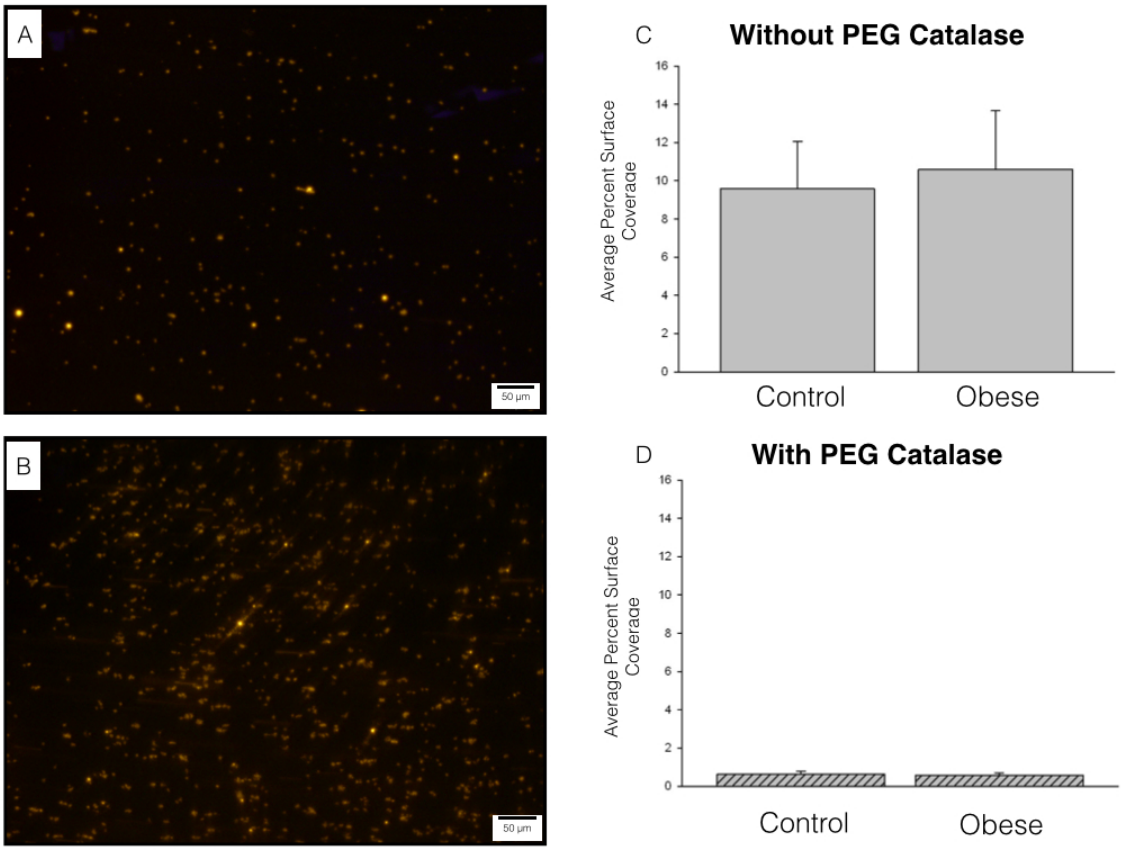

Figure 17 - Representative images of murine platelet aggregation on collagen surface from control (A) and obese (B) mice at a shear stress of 3 dynes/cm^2 and cell counts of 1.5x10^5 platelets/mL and 12.5% HCT. Images were taken after 5 minutes of perfusion and 3 minutes of rinsing with buffer. (C) and (D) show quantification of percent surface coverage without and with added PEG catalase, respectively. The percentages represent the mean surface coverage of 8 random 20x fields per experiment. The error bars represent the standard error of the mean. Quantification was done in Image J. N=4 for experiments without PEG catalase and N=2 for experiments with PEG catalase.

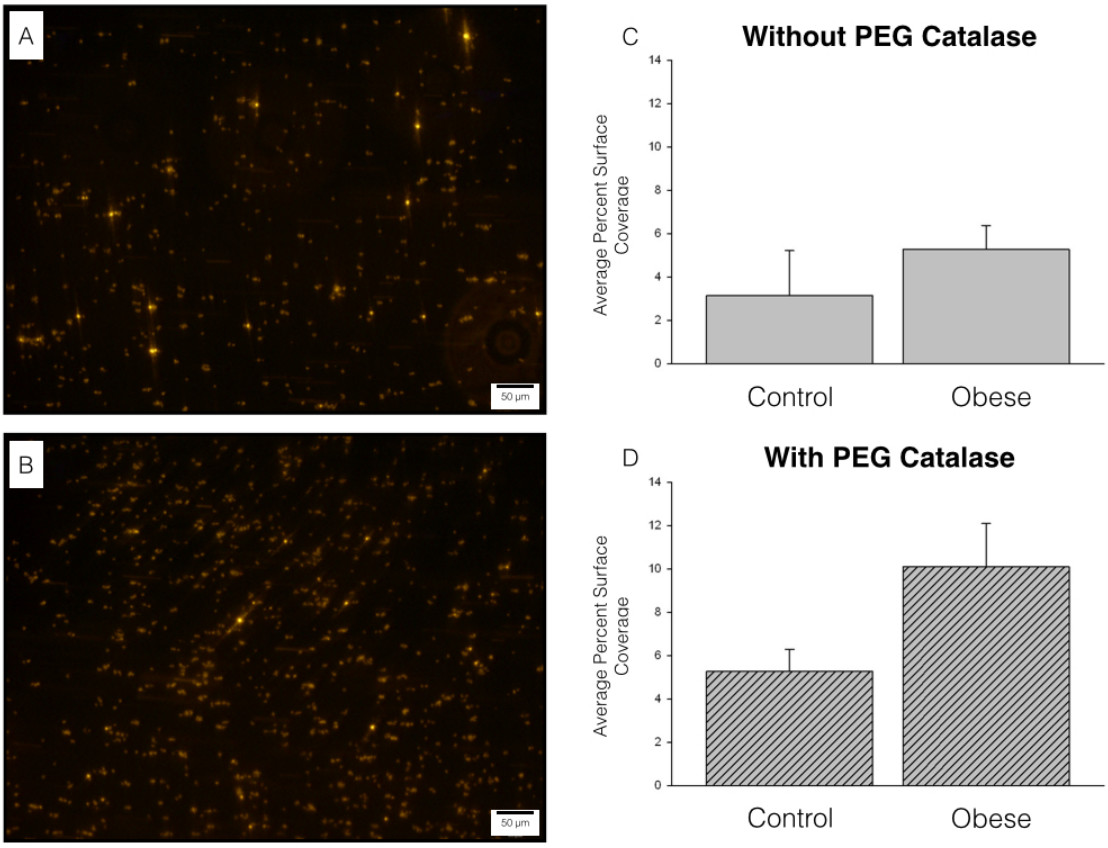

Figure 18 - Representative images of murine platelet aggregation on collagen surface from control (A) and obese (B) mice at a shear stress of 15 dynes/cm^2 and cell counts of 1.5x10^5 platelets/mL and 12.5% HCT. Images were taken after 5 minutes of perfusion and 3 minutes of rinsing with buffer. (C) and (D) show quantification of percent surface coverage without and with added PEG catalase, respectively. The percentages represent the mean surface coverage of 8 random 20x fields per experiment. The error bars represent the standard error of the mean. Quantification was done in Image J. N=2.

Discussion

In this present study we investigated the interaction of platelets with collagen using an in-vitro parallel plate flow chamber system. The initial studies regarded optimization of flow chamber conditions, where we were able to determine the optimal cell counts and shear stress conditions to provide the desired amount of platelet aggregation for future studies. From these experiments we determined that the optimal cell counts were 1.5x10^5 platelets/mL and 12.5% HCT and that this solution could be

perfused for 5 minutes at both venous and arterial shear stresses. With these conditions, the system could be used to study platelet aggregation from varying murine samples. Specifically, we were interested in the role of H_2O_2, as it has been shown to play a role in platelet activation in aging, diabetes, and possibly obesity.[4, 25]

To begin to study the role of H_2O_2 on platelet aggregation in-vitro, platelets isolated from mice deficient in Gpx1 were compared to their wild type littermates to determine if the modified mice would form larger aggregates when perfused over a collagen surface due to their increased levels of H_2O_2. In these studies we observed that mice deficient in Gpx1 show a significant increase in platelet aggregation compared to wild type mice. These observations suggest that H_2O_2 mediates platelet aggregation, supporting our hypothesis. However, we can only conclude that it is mediated by peroxide due to the fact that Gpx1 reduces not only H_2O_2, but also other peroxides, such as lipid peroxides.[33] To isolate H_2O_2 as the mediator of platelet aggregation, platelets were incubated with PEG catalase before perfusion over the collagen surface. PEG catalase reduced aggregation of Gpx1-deficient platelets to an average amount of surface coverage that was shown with wild type platelets that had not been treated with PEG catalase. Additionally, the aggregation of wild type platelets was not affected by treatment with PEG catalase. These observations further support our hypothesis that H_2O_2 mediates platelet aggregation on a collagen surface.

The identification of H_2O_2 as a mediator of platelet aggregation is consistent with what was demonstrated in-vivo by Dayal et. al.[4] In their study, they demonstrated that H_2O_2 plays an important role in platelet activation in aging, while others have demonstrated a role of H_2O_2 in platelet hyperactivity in diabetes. Since we have confirmed that H_2O_2 mediates platelet aggregation on a collagen surface in-vitro, future studies can be performed to identify other disease states that have increased platelet H_2O_2, and we have demonstrated that it would be possible to study these disease states in-vitro using the parallel-plate flow chamber system with the optimal conditions. Further studies

to provide clarification of the mechanism of increased H_2O_2 in these disease states would allow for the development of targeted therapy to reduce platelet aggregation leading to thrombotic events.

Since studies in our lab had demonstrated that obese mice exhibit increased platelet H_2O_2, we were interested in studying platelets from obese mice to determine whether or not they are more susceptible to platelet activation compared to control mice in-vitro. In these studies we observed that platelets from obese mice show an increasing trend in susceptibility to platelet aggregation on a collagen surface in the flow chamber system, particularly at arterial shear stress. To determine if H_2O_2 is mediating the increasing trend in platelet aggregation, we treated the platelets from control and obese mice with PEG catalase. At venous shear stress, aggregation was decreased in both groups to similar levels of surface coverage with the addition of PEG catalase. At arterial shear stress, aggregation in both groups increased with the addition of PEG catalase although the results were insignificant. The small number of experimental groups limited the studies with PEG catalase, making it difficult to interpret whether or not the increasing trend in platelet aggregation in obese mice is mediated by increased levels of H_2O_2. These results contradict what was observed by Dayal, et. al (Figure A2), where the levels of H_2O_2 were directly measured in control and obese mice, showing an increase in H_2O_2 in obese mice. In the future, these in-vitro studies can be continued to increase the sample size and confirm the trends that we observed. If we can confirm the data produced by Dayal et. al, it would be beneficial to investigate the potential sources of elevated H_2O_2 in obesity in order to develop therapies to reduce the occurrence of thrombotic events in obese individuals.

Although these studies provided interesting results, there are some limitations of the data that is presented here. First, although the flow chamber is an important tool for studying platelet aggregation under flowing conditions, it is still not equally physiological compared to studying these interactions in-vivo, which may lead to discrepancies

between in-vivo and in-vitro data as we saw. Such inaccuracies in the flow chamber include the concentrations of cells, as well as the composition and distribution of the collagen surface. The cell counts that we used were chosen because they provided the most optimal surface coverage under venous and arterial conditions in the flow chamber. However, these cell counts are not necessarily physiological and lack several blood components found in-vivo, which may contribute to platelet aggregation on exposed collagen. Additionally, the type of collagen that is used in platelet aggregation studies in the flow chamber varies from study to study, although it is not clear which provides the most physiological results in-vitro. In this study, type I collagen from equine tendons was used. Furthermore, it was not confirmed whether the collagen was evenly distributed on the glass coverslip. Other studies have used an airbrush technique to coat the coverslips with a collagen solution, while we simply spread the solution manually. In the future it would be beneficial to label the collagen surface for visual conformation that we are evenly coating the glass coverslip or invest in an airbrush, as this technique is much more consistent. Additionally, similar to the limitation presented in Chapter 2, the experiments were completed at room temperature with no device to maintain the system and perfusing solutions at physiological temperatures. Although the experiments were performed over a limited time frame, lasting no longer than ten to fifteen minutes each and the solutions were warmed to 37 degrees Celsius beforehand, the non-physiological temperatures during perfusion may have affected the properties of the blood cells. This is especially a concern with the platelets, which are known to be very temperature-sensitive. In the future, it would be beneficial to complete any additional studies using a device to maintain a physiological temperature to ensure that there are no adverse effects on the platelet aggregation being observed. Finally, the long-term goal of these platelet aggregation studies is to gain a better understanding of the mechanisms to help develop new therapies to decrease the occurrence of thrombotic events. Therefore, it would be most beneficial to use this system to study interactions of human platelets. Although it is

more difficult to obtain human platelets compared to those from a mouse, and even more so to obtain such to study different disease states, it would be more physiologically relevant to use human samples whenever possible.

In conclusion, these in-vitro studies presented here provided insight into the mechanisms behind platelet aggregation on a collagen surface using a parallel-plate flow chamber. We were able to determine the optimal cell counts and shear stress conditions to observe the desired amount of platelet aggregation on the collagen surface. These conditions were used to study the hypothesis that H_2O_2 mediates platelet aggregation, which was confirmed by our observations using mice deficient in Gpx1 and the addition of PEG catalase. The conditions were also used to study obese mice and determine whether or not platelets from these mice show increased platelet aggregation on a collagen surface in-vitro and if it is mediated by H_2O_2. Although we were unable to establish any significant differences between control and obese mice, the preliminary studies showed increasing trends in platelet aggregation in the obese mice. In the future these studies should be persued further to increase the sample size in all groups. Doing so will provide significant and meaningful results that can be used to better understand the mechanisms of platelet aggregation in thrombosis.

CHAPTER 4

CONCLUSION

The two separate studies presented here examined important interactions in thrombosis involving blood cells with the vessel wall. First, the interaction of RBCs with the endothelial surface, and second was platelet interactions with collagen. From these studies we made several important observations. We determined that $FeCl_3$ induces RBCs to adhere directly to the injured endothelium in the absence of any other blood components and this interaction is mediated by VWF that is presumably secreted from the $FeCl_3$-injured endothelial cells. Additionally, we demonstrated the optimal conditions to study platelet-collagen interactions in the parallel plate flow chamber and that platelet aggregation on a collagen surface is mediated by H_2O_2. We also demonstrated in preliminary studies that platelets from obese mice might show increased platelet aggregation on a collagen surface, although we could not show that this observation is due to increased H_2O_2 in obese mice.

Although the interactions of these two blood cells, platelets and RBCs, were studied separately, it became clear that physiologically the interactions of multiple cell types together play a very important role. In Chapter 3, we demonstrated that platelet-collagen interactions were best studied in the presence of RBCs to induce physiological rheological forces in the flow chamber system. Without RBCs in the solution, platelet aggregation on the collagen surface was limited. In Chapter 2, we made the novel finding that RBCs are able to interact with VWF, which is more commonly thought to interact with platelets during hemostasis and thrombosis. Additionally, in preliminary studies we were able to demonstrate that RBCs interact with VWF in the presence and absence of platelets. Future studies could be completed to determine if there is any competition between platelets and RBCs for binding sites on VWF.

Overall, the in-vitro studies presented here led to interesting and novel findings regarding interactions of blood cells with the vessel wall in thrombosis. These findings can continue to be persued in the future to help develop new therapies for those affected by thrombotic disorders.

APPENDIX

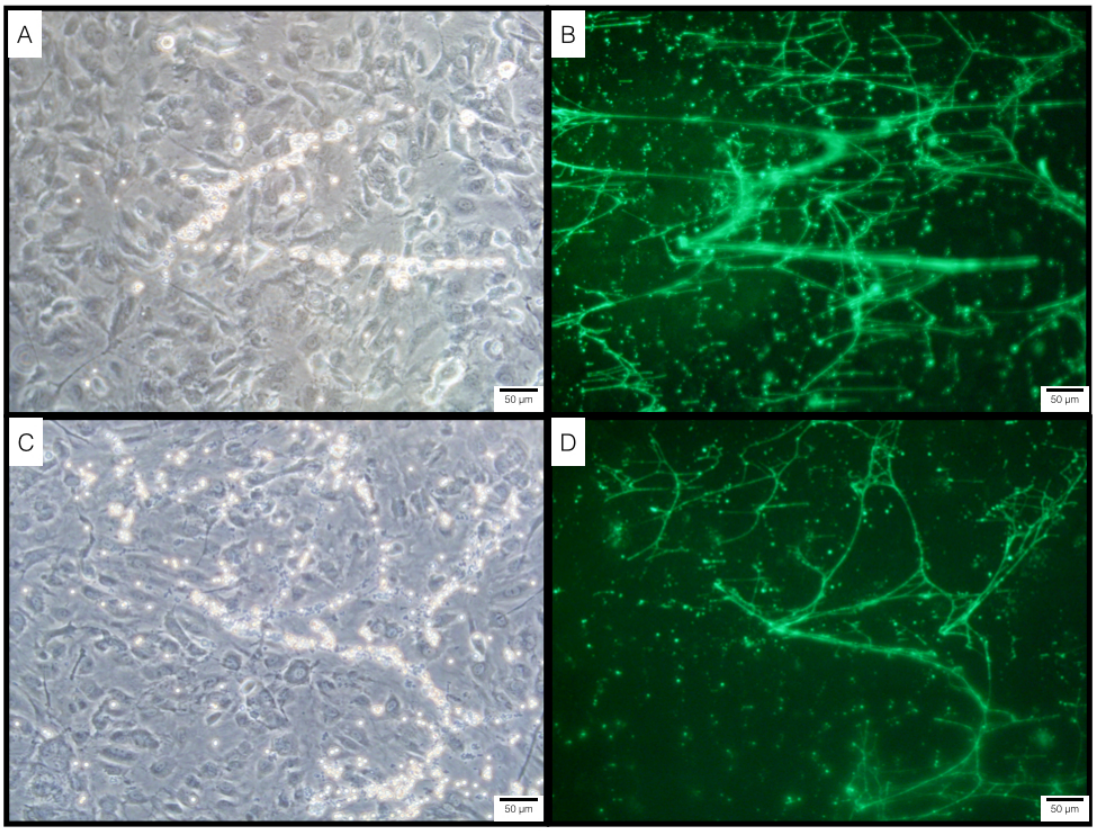

Figure A1 – Representative images from initial pilot studies investigating the RBC-VWF interaction. (A) shows the binding of RBCs to VWF strings secreted from histamine stimulated HUVECs in the absence of platelets. (B) shows the same field under fluorescence showing the labeled VWF strings. (C) shows the binding of RBCs to VWF strings in the presence of platelets, which also bind to the secreted VWF strings. (D) shows the fluorescently labeled VWF strings in the same location.

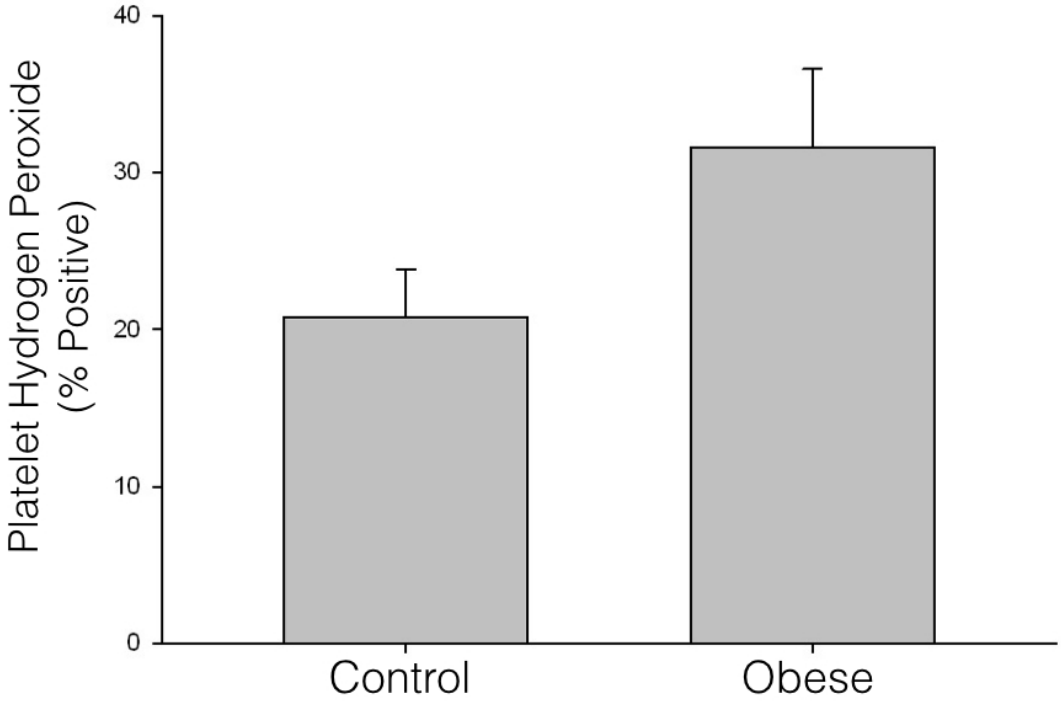

Figure A2 – Accumulation of hydrogen peroxide is increased in platelets from mice fed on a high fat diet consisting of 60% of calories from fat for 12 weeks. Platelets from 9-11 mice were studied in each group. Error bars represent the standard error of the mean. (Unpublished data from Dayal, et. al.)

REFERENCES

1. Furie B and Furie BC. Mechanisms of Thrombus Formation. *New England Journal of Medicine*. 2008;359:938-949.

2. Hathcock JJ. Flow Effects on Coagulation and Thrombosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2006;26:1729-1737.

3. Justin D. Barr AKC, Gilbert V. Schaeffer, Jessica K. Hansen, David G. Motto. Red blood cells mediate the onset of thrombosis in the ferric chloride murine model. *Blood*. 2013;121:3733-3741.

4. Sanjana Dayal KMW, David G. Motto, Francis J. Miller Jr, Anil K. Chauhan, Steven R. Lentz. Hydrogen peroxide promotes aging-related platelet hyperactivation and thrombosis. *Circulation*. 2013;127:1308-1316.

5. Cooley BC. Murine Models of Thrombosis. *Thrombosis Research*. 2012;129:S62-S64.

6. Sanjana Dayal AKC, Melissa Jensen, Lorie Leo, Cynthia M. Lynch, Frank M. Faraci, Warren D. Kruger, Steven R. Lentz. Paradoxical absence of a prothrombotic phenotype in a mouse model of severe hyperhomocysteinemia. *Blood*. 2012;119:3176-3183.

7. Owens III AP LY, Whinna HC, Gachet C, Fay WP, Mackman N. Towards a standardization of the murine ferric chloride- induced carotid arterial thrombosis model. *Journal of Thrombosis and Haemostasis*. 2011;9:1862-1863.

8. M. Roest AR, J. J. Zwaginga, M. R. King, J. W. M. Heemskerk, and The Biorheology Subcommittee of the SSC of the ISTH. Flow chamber-based assays to measure thrombus formation in vitro: requirements for standardization. *J Thromb Haemost*. 2011;9:2322-2324.

9. Hathcock JJ. Flow Effects on Coagulation and Thrombosis. *Arteriosclerosis , Thrombosis, and Vascular Biology*. 2006;26:1729-1737.

10. Duke W. The relation of blood platelets to hemorrhagic disease. *The Journal of the American Medical Assocaition*. 1910;60:1185.

11. Kurz KD MB, Sandusky GE. Rat model of arterial thrombosis induced by ferric chloride. *Thrombosis Research*. 1990;60:269-280.

12. *The Biomedical Engineering Handbook, Second Edition*. Boca Raton, FL: CRC Press; 1999.

13. Theodoros G. Papaioannou CS. Vascular Wall Shear Stress: Basic Principles and Methods. *The Hellenic Journal of Cardiology*. 2005;46:9-15.

14. PJ Lenting JP, E Groot, PG de Groot. Regulation of von Willebrand factor-platelet interactions. *Thrombosis and Haemostasis*. 2010;104:449-455.

15. CG Koch PF, L Li, JK Sabik 3rd, T Mihaljevic, EH Blackstone. Red blood cell storage: how long is too long? *The Annals of Thoracic Surgery*. 2013;96:1894-1899.

16. A. Anand SF. Hematocrit and bleeding time: and update. *Southern Medical Journal*. 1994;3:299-301.

17. Yiming Wang MA, Yan Yang, Sean Lang, Joseph W. Jin, Alison Cameron-Vendrig, Guangheng Zhu, Adili Reheman and Heyu Ni Platelets in thrombosis and hemostasis: old topic with new mechanisms. *Cardiovascular and Hematological Disorders-Drug Targets*. 2012;12:126-132.

18. Farndale RW. Collagen-induced platelet activation. *blood Cells, Molecules, and Diseases* 2005;36:162-165.

19. JM Cosemans AA-S, NJ Mattheij, JW Heemskerk. The effects of arterial flow on platelet activation, thrombus growth, and stabilization. *Cardiovascular Research*. 2013;99:Cardiovasc Res. .

20. von Brühl ML SK, Steinhart A, Chandraratne S, Konrad I, Lorenz M, Khandoga A, Tirniceriu A, Coletti R, Köllnberger M, Byrne RA, Laitinen I, Walch A, Brill A, Pfeiler S, Manukyan D, Braun S, Lange P, Riegger J, Ware J, Eckart A, Haidari S, Rudelius M, Schulz C, Echtler K, Brinkmann V, Schwaiger M, Preissner KT, Wagner DD, Mackman N, Engelmann B, Massberg S. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *The Journal of Experimental Medicine*. 2012;209:819-835.

21. Brighton TA EJ, Mann K, Mister R, Gallus A, Ockelford P, Gibbs H, Hague W, Xavier D, Diaz R, Kirby A, Simes J; ASPIRE Investigators. Low-dose aspirin for preventing recurrent venous thromboembolism. *New England Journal of Medicine*. 2012;367:1979-1987.

22. Jackson SP. Arterial thrombosis - insidious, unpredictable and deadly. *Nature Medicine*. 2011;17:1423-1436.

23. Collaboration AT. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *BMJ*. 2002;324:71-86.

24. Emanuele Previtali PB, Serena M. Passamonti, Ida Martinelli. Risk factors for venous and arterial thrombosis *Blood Transfusion*. 2010;9:120-138.

25. PC Redonodo IJ, JM Hernandez-Cruz, JA Pariente, GM Salido, JA Rosado. Hydrogen peroxide and peroxynitrite enhance Ca2+ mobilization and aggregation in platelets from type 2 diabetic patients. *Biochemical and Biophysical Research Communications*. 2005;333:794-802.

26. AS Go DM, VL Roger, et al. . Heart Disease and Stroke Statistics—2014 Update: a report from the American Heart Association. *Circulation*. 2014;129:e28-e292.

27. Marjorie Bastien PP, Isabelle Lemieux, Jean-Pierre Despres. Overview of Epidemiology and Contribution of Obesity to Cardiovascular Disease. *Progress in Cardivascular Diseases*. 2014;56:369-381.

28. D. Wormser SK, A.E. Di et al. Separate and combined associations of body-mass index and abdominal adiposity with cardiovascular disease: collaborative analysis of 58 prospective studies. *Lancet*. 2011;377:1085-1095.

29. Ilya O Blokhin SRL. Mechanisms of Thrombosis in Obesity. *Current Opinion in Hematology*. 2013;5:437-444.

30. Victor M. Victor MR, Eva Solá, Celia Bañuls, Katherine Garcia-Malpartida, Antonio Hernández- Mijares. Oxidative Stress, Endothelial Dysfunction and Atherosclerosis. *Current Pharmacutical Design*. 2009;14:2988-3002.

31. Cheng WH HY, Ross DA, Han Y, Combs GF Jr., Lei XG. Overexpression of cellular glutathione peroxidase does not affect expression of plasma glutathione peroxidase or phospholipid hydroperoxide glutathione peroxidase in mice offered diets adequate or deficient in selenium. *J Nutr*. 1997;127:675-680.

32. Tara C. White-Adams MAB, Erik I. Tucker, Jacqueline M. Gertz, David Gailani, Rolf T. Urbanus, Philip G. de Groot, András Gruber and Owen J.T. McCarty. Identification of Coagulation Factor XI as a Ligand for Platelet Apolipoprotein E Receptor 2 (ApoER2). *Arteriosclerosis , Thrombosis, and Vascular Biology*. 2009;29:1602-1607.

33. Xin Gen Lei W-HC, James P. McClung. Metabolic Regulation and Function of Glutathione Peroxidase-1. *Annual Review of Nutritrion*. 2007;27:41-61.