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Quantifying Nitric Oxide Production in Platelets using a Griess Reagent System

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ABSTRACT

Nitric oxide (NO) is a signaling molecule that regulates many physiological processes in the human body. Common examples of processes that NO is involved in range from immune responses to the regulation of blood pressure. In intact blood vessels, nitric oxide is constitutively released by vascular endothelial cells in order to prevent platelet adhesion and clot formation. Platelets have also been shown to release small amounts of NO as they aggregate on damaged blood vessels. Platelet-derived NO is hypothesized to play an important role in limiting the extent of clot formation. Although platelets produce nitric oxide during aggregation, it is difficult to accurately measure the concentration of nitric oxide that is released. The Griess reagent system is commonly used with other cell types and in other in vitro assays to measure NO production. Here, we used a commercially available Griess reagent kit (Cayman Chemical, Ann Arbor Michigan) to determine if the Griess reagent system could quantify nitric oxide production by aggregating platelets. Platelets were stimulated by common physiological agonists, including ADP, epinephrine, arachidonic acid, and collagen, and platelet NO production was quantified according to the manufacturer's instructions. We found that arachidonic acid was the strongest agonist and epinephrine to be the weakest. The Cayman Chemical kit we used did not quantify nitric oxide production as we had previously hypothesized and led us to conclude that the kit should not be used for clinical purposes.

INTRODUCTION

Hemostasis

Hemostasis is the process of platelets forming clots within blood vessels to stop blood loss or damage to the body. The process of hemostasis is simplified into two steps. Primary hemostasis is the process of platelet plug formation. Platelets perform five different functions during primary hemostasis: adhesion, activation and secretion, aggregation, clot retraction, and providing a procoagulant surface (Kickler, 2006). Secondary hemostasis is simply coagulation, (the process in which more and more platelets stick together) and helps to form the platelet plug is stabilized by a fibrin mesh. Multiple processes with specific enzymes occur within both steps of hemostasis and must precede one another to help facilitate the process of hemostasis. Figure 1 illustrates the formation of the platelet plug in primary hemostasis (Benz &Fleming, 2016).

Platelet plug formation begins with a disruption of the blood vessel from an injury. Within seconds, platelets begin to adhere to the subendothelium, the layer of skin and blood vessels found under the endothelium. Within the first minute of activation, fibrin strands are found interspersed among the platelets. Platelets also begin to release several factors such as ADP, epinephrine and arachidonic acid, all of which promote platelet plug formation. Several minutes after the process has started, a platelet plug is completely formed. The formation of a platelet plug occurs because additional platelets adhere to the surface of the subendothelium, specifically to collagen fibrils. Fibrin formation can then occur because the platelets have provided a surface for the assembly of coagulation factors (leading to the generation of thrombin

and fibrin) (Boon, 1993). This process is known as platelet aggregation and is part of the overarching process known as the coagulation cascade.

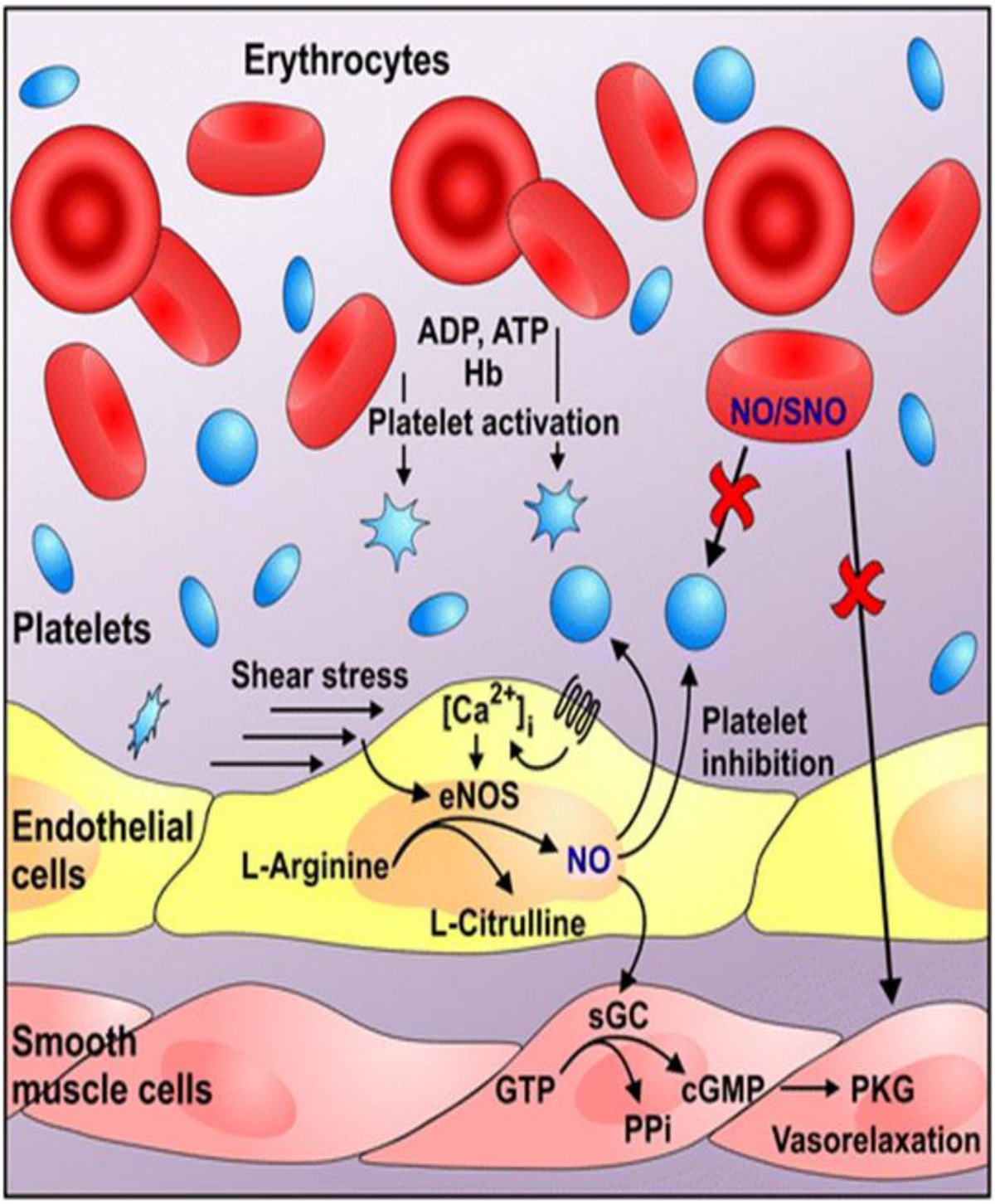

Figure 1: Platelet Plug Formation (Boon, 1993). The formation of a platelet plug in primary hemostasis is an intricate process that involves various players. As seen, NO is a major factor in platelet inhibition during an immune response to inflammation.

Coagulation Cascade

The coagulation cascade is a process that occurs during secondary hemostasis. Simplified, coagulation involves activation, adhesion and aggregation of platelets which in turn produce a small ball of fibrin around the damaged area of the vessel. Adhesion is an important process as it allows platelets to undergo a morphological change and release the contents of their granules. The coagulation cascade is considered a series of sequential zymogen activation steps, where a factor is the substrate for the previous enzyme as well as the activator of the subsequent proenzyme (Boon, 1993). This cascade demonstrates the importance of timing and specific enzymes needed at every step. The cascade itself is detail-oriented as it has two pathways, both of which require specific cofactors and enzymes to help move the process along, that lead to fibrin formation.

The processes mentioned above illustrate the function of normal blood clotting, that is the normal process in which all factors and enzymes are functioning as they should after a traumatic injury. Virchow's triad is an idea that helps explain thrombosis as a pathologic process. Three factors that make up Virchow's triad (blood vessel injury, venous stasis, and clotting disorders) predispose one to blood clot formation. When everything is timed right, the body can take care of the injury in the span of a few days and the outside evidence is a wound that is covered with a band-aid to prevent infection. But in some cases, these factors and enzymes fail to do their job and thus lead to abnormal blood clotting.

If such factors are altered, the result can often lead to serious medical conditions such as deep vein thrombosis or stroke (Blann, 2015). Abnormal blood clotting and the medical conditions that it can lead to illustrate the need for identifying such clotting before it results in serious injury to the patient. Scientists in the past twenty years have set out to identify markers

that could help diagnose these problems before they get worse (Bryan & Grisham, 2007). Among the markers identified is nitric oxide, a signaling molecule that is produced by aggregating platelets and can potentially be measured as a marker for identifying blood clotting disorders (Freedman et al, 1997).

Nitric oxide- a signaling molecule

Nitric oxide has received more attention over the last twenty years for its ability to be quantified in diagnosing medical disorders. This research was preceded by studies in the 1940s and 1950s that focused on blood coagulation to determine regulation of dicoumarin drugs which eventually helped to establish the coagulation cascade we know today (Mannucci et al, 2013). Nitric oxide's functions as a signaling molecule make it one of the most important molecules produced during the process of hemostasis as it can inhibit platelet activation (Ku et al, 2007).

Nitric oxide is synthesized from L-arginine by the family of NO synthases. It is produced from L-arginine by the catalytic action of NOS isoforms in various types of cells (NOS) (Böhmer, Gambaryan & Tsikas, 2015). These synthases include endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) isozymes (Gambaryan & Tsikas, 2015). Nitric oxide can further be oxidized to nitrite and nitrate, two forms that can be measured by various chemical assays. Experiments showed the various rates of NO production as well as elimination of nitric oxide by products within the body (Himeno, 2004). This research is among the vast literature we have on NO.

Nitric oxide is produced at various locations in the body including major organ systems like the circulatory system. In the circulatory system, NO helps to regulate vascular tone and blood flow. When abnormalities occur in vascular NO production, endothelial dysfunction with

various cardiovascular pathologies such as cardiovascular disease result (Liuking, 2010). One medical disorder of interest is cardiovascular disease and the role of reduced NO signaling that can lead to serious damage. Reduced NO signaling is associated with several known risk factors for the most common cardiovascular diseases such as coronary artery disease (Lundberg, Gladwin & Weitzberg, 2015). In other parts of the body, excessive amounts of NO can signify an underlying health problem. Research has found NO activity in human platelets in patients who were in the severe stage of chronic kidney disease (Siqueira et al, 2011). While normal levels of NO throughout the body signify a healthy system, abnormal amounts of NO (either high or low) can point to a disorder within the body. Abnormal NO signaling is likely to contribute to a variety of neurodegenerative pathologies such as multiple sclerosis, Alzheimer's, and Parkinson's diseases (Forstermann & Sessa, 2012). Thus, the vast diseases that contain NO signaling identifies NO as an important molecule to study.

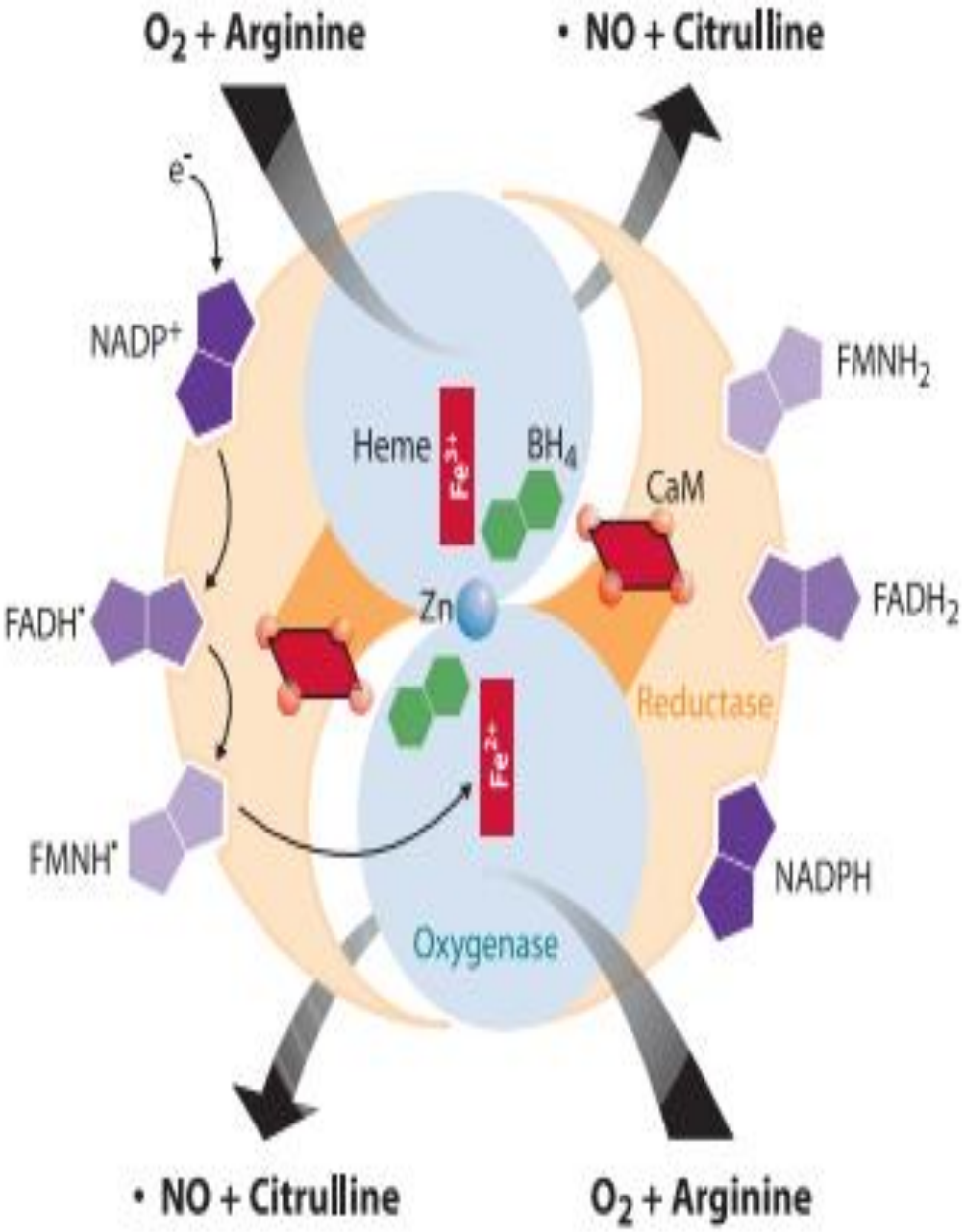

Figure 2: Nitric Oxide Synthesis; provided by Cayman Chemical. Nitric oxide synthesis involves various compounds that help produce NO and side products.

Nitric Oxide Feedback Loop

In any case of blood clotting, activated platelets play an important role in further recruitment of platelets. Freedman and colleagues presented background on nitric oxide as well as present their findings. Their study was aimed at determining the extent to which nitric oxide helps regulate platelet recruitment. They established that platelet-derived NO was involved in the recruitment process and that adhesion of platelets to the vessel wall was partly prevented by endothelial cell production of nitric oxide (Freedman et al, 1997). Thus, nitric oxide is important as it inhibits adhesion as well as promoting platelet aggregation. Recent studies have also found that nitric oxide inhibits platelet adhesion by various agonists including collagen by cGMP dependent and independent mechanisms (Irwin, Roberts, & Naseem, 2009). This feedback loop as well as various mechanisms within the body help to regulate the amount of nitric oxide released in platelet aggregation. While these facts pertain to the function of the molecule itself, the physiology of nitric oxide is important as well.

Nitric Oxide Physiology

The physiology of nitric oxide within the human body varies from place to place as the nitric oxide itself is highly reactive. The half-life of NO in the blood has been found to be short due to oxidation by oxyhemoglobin to nitrite, thus NO in vivo is best measured as the concentration of nitrite (Liuking, 2010). This oxidized form remains stable for many hours and allows scientists to measure nitric oxide production (Bryan & Grisham, 2007). Our current and true understanding of NO in physiology is derived from in vivo experiments that sample multiple compartments simultaneously (Bryan & Grisham, 2007). Several methods for measuring NO and its products/metabolites in biological fluids currently exist and the method chosen for this project was the Griess Reaction

The Griess Reaction

The Griess reaction is an analytical chemistry tool that has been utilized to measure nitric oxide production. For nitric oxide to be measured, nitric oxide must be converted into nitrite. Figure 3 illustrates the process of dinitrogen trioxide reacting with sulfanilamide to produce a diazonium ion. This ion is then coupled with N- (1-Napthyl)ethylenediamine to form a chromopheoric azo product that can absorb light strongly at 540 nm. The nitrate and nitrite products are then detected as an azo dye product of the Griess reaction. In this present study, we examined the Griess reagent system and its ability to measure nitric oxide production efficiently in the presence of various agonists.

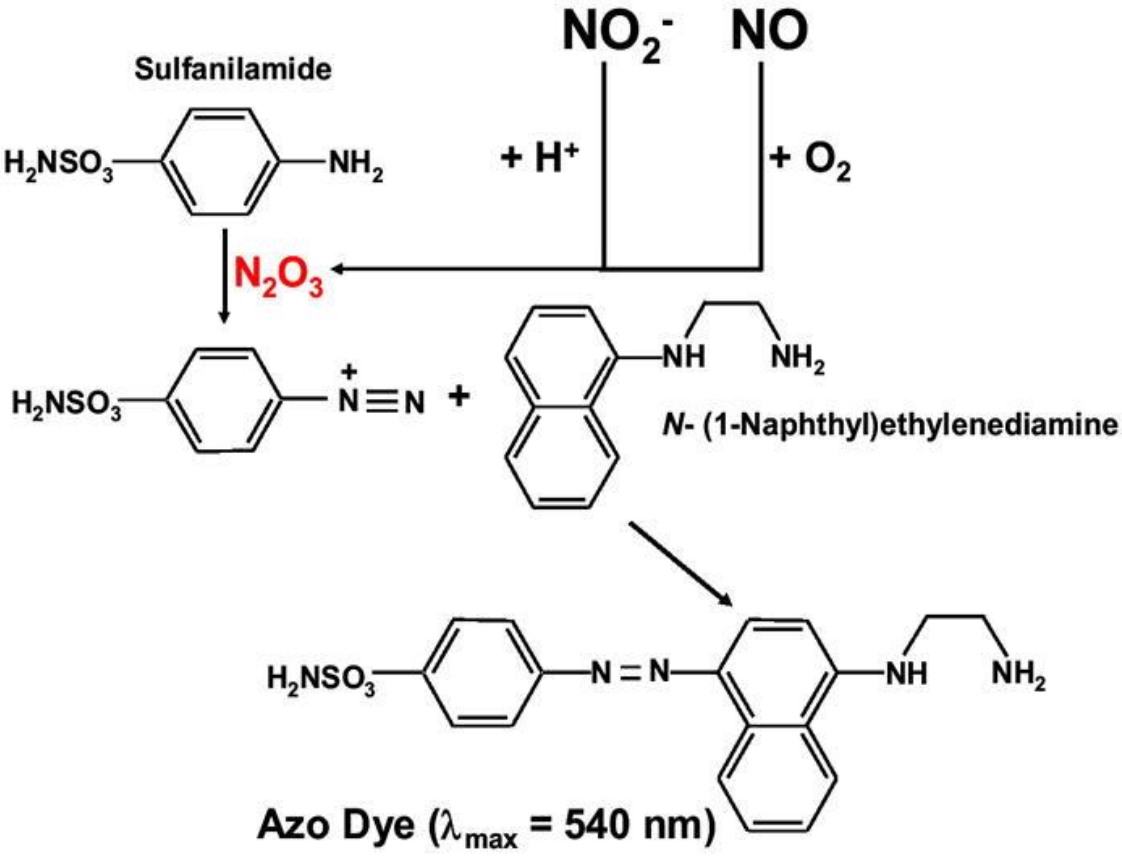

Figure 3: (Bryan & Grisham, 2007). The Griess Reaction is the process of nitric oxide being broken down into a form that can be easily read by fluorescence. This technique allows Nitric oxide to be measured indirectly.

Platelet Agonists

Platelet aggregometry is a series of tests performed on platelet-rich plasma using a variety of agonists. Gresele (2003) presented platelet history and identified various agonists that play an important role in aggregation. Banerjee and colleagues (2016) reported that ADP, epinephrine, and collagen were essential aggregating agonists that play a role in essential physiological phenomena such as life-saving blood coagulation. In this research project, four agonists were used to test platelet aggregation. The four agonists chosen are the most common platelet aggregation agonists, and they include ADP, collagen, epinephrine, and arachidonic acid.

Adenosine diphosphate (ADP) plays significant roles in various metabolic processes in the human body, including binding to specific platelet- membrane receptors causing platelet activation. When ADP is in the blood, it is often converted to adenosine, helping to inhibit any further platelet activation (Farabee, 2002). Like nitric oxide, ADP has its own feedback loop that keeps check on the amount of ADP present in the body.

The role of collagen in hemostasis is very important as collagen makes up the subendothelial layer of the vessel wall, providing the surface for the attachment during aggregation. A barrier to platelet interaction is provided by various types of collagen that are present in the subendothelial matrix (Rumbaut, 2010). Although collagen plays a structural role, it does not show any primary wave of aggregation and is dependent on the membrane receptors that are present on the platelets for adhesion (Rumbaut, 2010). By depending on the membrane receptors, collagen relies on other molecules to help complete its task in coagulation.

Epinephrine is a hormone and is also a form of medication given in life-threatening situations; it is also commonly known as adrenaline. Adrenaline tends to cause the heart to race

and increase blood pressure in fight or flight situations. The focus of this agonist is related to its effects on platelets. Epinephrine causes ADP secretion and binds only to specific G-protein coupled receptors throughout the body. Epinephrine in the blood has also been related to an increase in the number of platelets.

The final agonist used in this project was arachidonic acid. Arachidonic acid is a polyunsaturated fatty acid that is present in phospholipids found in the human body's cells' membranes. Arachidonic acid is a key inflammatory intermediate as well as a vasodilator (Banerjee et al, 2016). In platelets, arachidonic acid is converted into thromboxane, a potent stimulator of platelet aggregation.

In the present study, we set out to test whether different platelet agonists would stimulate platelet NO production and whether that NO production could be measured by the Griess reagent system. We believe epinephrine to be the weakest agonist and ADP to be the strongest agonist (arachidonic acid and collagen were predicted to be somewhere in the middle). We predicted that arachidonic acid, ADP, and collagen stimulation would lead to measurable NO production while epinephrine would be too weak of an agonist to produce any measurable NO production. These predictions are based off previous research projects from Lynchburg College students (Keeney-Ritchie, 2016) as well as work by Ku and colleagues (Ku et al, 2007). The presence of these agonists in the blood may stimulate NO production and allow us to detect the presence of nitric oxide in the blood as well. By detecting nitric oxide in the blood, inflammation responses during critical health conditions such as cardiovascular diseases can be detected earlier and potentially limit the amount of damage one receives during events like heart attacks and strokes. Thus, the purpose of this project was to establish a test that would help detect nitric

oxide in the blood earlier. By detecting nitric oxide in the blood earlier, health professionals could receive results much quicker than conventional measures while also being cost effective.

A two-step research design was used for this research project. The first step was to test the ability of different agonists to stimulate platelet aggregation. The second step was to use these same platelet agonists to stimulate platelets and measure NO production via colorimetric assay. We predicted that arachidonic acid, ADP and collagen stimulation would produce measurable nitric oxide production in the blood as measured commercially available Griess reagent kit.

METHODS AND MATERALS

This study was approved by the Lynchburg College Institutional Review Board (LCHS1415094). Phlebotomy supplies were purchased from BD Medical (Franklin Lakes, NJ, USA). The process of drawing blood occurred at Lynchburg College's Graduate School of Health Sciences. The tubes were then transported to main campus of Lynchburg College and placed in a PDQ Platelet Function Centrifuge (Bio/Data Corporation, Horsham, PA, USA). Platelet rich plasma was extracted from the centrifuged blood samples. The PAP-8E Platelet Aggregation Profiler (Bio/Data Corporation, Horsham, PA, USA) runs samples and generates graphs of platelet aggregation over a specific period of time. Platelet aggregation supplies- including collagen, adenosine diphosphate (ADP) and arachidonic acid- were purchased from Bio/Data Corporation (Horsham, PA, USA).

The nitrate/nitrite fluorometric assay kit was purchased from Cayman Chemical (Ann Arbor, MI, USA). The chemical reactions facilitated by the kit are illustrated in Fig.4 where nitrite reacts with a dye that is used to measure enhanced fluorescence. The protocol for

measuring out the standards and wells as well as obtaining data for the standard curves was provided by the assay kit.

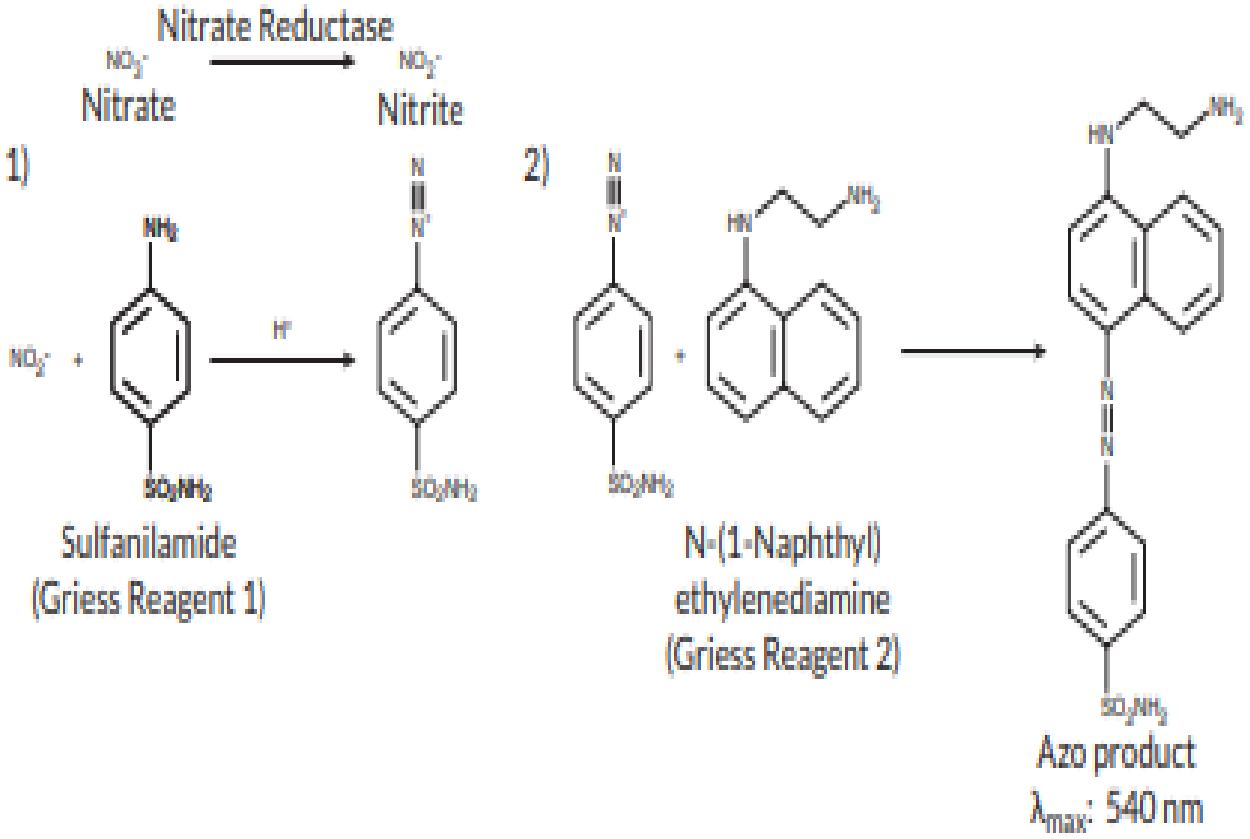

Figure 4: Process of nitrate being reduced to nitrite in the first step. The second step illustrates the addition of Griess reagents that convert nitrite into a deep purple azo compound (Cayman Chemical)

Preparation of Platelet-Rich Plasma

Blood was taken from healthy human individuals, and samples were obtained by the Lynchburg College Physician Assistant program. Blood was collected in vacutainer tubes with sodium citrate, which serves as a calcium chelator and helps act as an anticoagulant. The tubes were centrifuged for two min and were placed into a styrofoam container for holding. The platelet rich plasma (PRP) was then obtained from each tube using a Wheaton pipette (300-1000 microliters; Millville, NJ, USA). We obtained 7.5 mL of PRP and placed this into a BD falcon conical tube (Franklin Lakes, NJ, USA). The concentration of the agonists was measured at 20 µM per tube. In this investigation, platelet aggregation was stimulated by the addition of thrombin (20 µM), epinephrine (20 µM), ADP (20 µM), or collagen (20 µM). Six conical tubes were placed in the platelet function centrifuge and spun under the platelet poor plasma (PPP) option. The plasma from these tubes was then placed at room temperature into a second BD falcon conical tube for testing.

Platelet Aggregation Assays

Platelet aggregation assays were performed using PAP-8E platelet aggregation profiler (Bio/Data Corporation, Horsham, PA, USA). The PAP-8E is designed to support diagnoses of hemostasis disorders, chart important data during anti-platelet drug clinical trials, and measure the rate of aggregation by providing light transmission values. The reagents in the assay kit stimulate aggregation and these aggregation levels are evaluated by using the spectrophotometer to measure light transmission. Resting platelet-rich plasma allows 0% light transmission whereas fully clotted platelet-rich plasma (complete aggregation) will allow 100% light transmission. Testing with the PAP-9 incorporated using blanks and test samples of the

various agonists. The blank (well 1) was a vehicle containing water and resting platelets. Blanks were spun at 1200 RPMs in the PAP-8. Test samples consisted of 225 µL of PRP followed by 25 mL of the agonist for a total volume of 250 µL. The blank consisted of 250 µL of water only. Four wells were used in the PAP-8 for testing the agonists. The concentration of the agonists was measured at 20 µM per tube. In this investigation, platelet aggregation was stimulated by the addition of arachidonic acid (20 µM), epinephrine (20 µM), ADP (20 µM), or collagen (20 µM). This step allowed us to evaluate platelet aggregation in the presence of platelet agonists before attempting to test the Cayman Chemical assay kit. A bar graph depicting percent platelet aggregation can be found in the results section [Fig. 5].

Nitric Oxide Measurement

Table 1 depicts the plate setup that was used for the second step of the research project. In this step, we set up two columns of standards as well as four columns that had designated amounts of sample and buffer. The blank wells only received 200 µL of Assay Buffer. Columns 5 and 6 contained 80 µL of sample and columns 3 and 4 contained 40 µL of buffer. Enzyme cofactor mixture and nitrate reductase mixture (10µL each) were then added to each of the wells (both standards and unknowns). A plate cover was used to cover the plate, and the samples were incubated at room temperature for three hours.

Table 1:Plate setup. Adenosine Diphosphate (ADP), Epinephrine (EPI), Arachidonic Acid (AA), and Collagen (Coll) represent the four platelet agonists that were used to stimulate platelet aggregation.

	Standards		**40µL Sample** **40µL Buffer**		**80 µL Buffer**	
	1	**2**	**3**	**4**	**5**	**6**
A	**A1**	**A2**	**Rest**	**Rest**	**Rest**	**Rest**
B	**B1**	**B2**	**ADP**	**ADP**	**ADP**	**ADP**
C	**C1**	**C2**	**EPI**	**EPI**	**EPI**	**EPI**
D	**D1**	**D2**	**AA**	**AA**	**AA**	**AA**
E	**E1**	**E2**	**Coll**	**Coll**	**Coll**	**Coll**
F	**F1**	**F2**	**BL**	**BL**	**BL**	**BL**
G	**G1**	**G2**				
H	**H1**	**H2**				

After the required incubation time, 50 µL of Griess reagent R1 was added to each of the wells (standards and unknown). Griess reagent R1 was the sulfanilamide that helped reduce nitrate to nitrite as shown in Fig. 4. Immediately after, 50 µL of Griess reagent R2 was added. Griess reagent R2 was the N- (1-Napththyl) ethylenediamine that converted nitrite into a deep purple azo compound as shown in Fig. 4. Color was developed during a ten-minute waiting period at room temperature. The assay kit stated it was not necessary to cover the plate during this time. The absorbance was read at 540-550 nm using a µQuant spectrophotometer (BioTek Instruments, Inc. Winnoski, VT, USA). Corresponding absorbance values were displayed and used to make conclusions about the Griess reagent system's ability to measure nitric oxide production.

Data Analysis

The PAP-8E Platelet Aggregation Profiler reported percent aggregation. We used Microsoft Excel to calculate the mean percent aggregation, as well as the standard error of the mean.

RESULTS

Platelet Aggregation Assays

Platelet aggregation was stimulated in the presence of platelet agonists. ADP had the highest percentage aggregation while epinephrine had the lowest percentage aggregation out of the four agonists. We previously hypothesized that epinephrine would be the weakest agonist while ADP would be the strongest. Figures 5 through 8 are graphs of platelet agonists and each graph respectively shows a specific agonist and its ability to stimulate platelet aggregation compared to a vehicle.

After testing platelet aggregation in the presence of platelet agonists, we used the μQuant spectrophotometer to test the Griess reagent system and the kit's ability to quantify nitric oxide production. The results showed variation between the values of the standard as well as the sample wells. Results also showed low absorbance values from the spectrophotometer as it read the samples. Thus, we concluded that there were no significant results to indicate that the Cayman Chemical kit can quantify nitric oxide production.

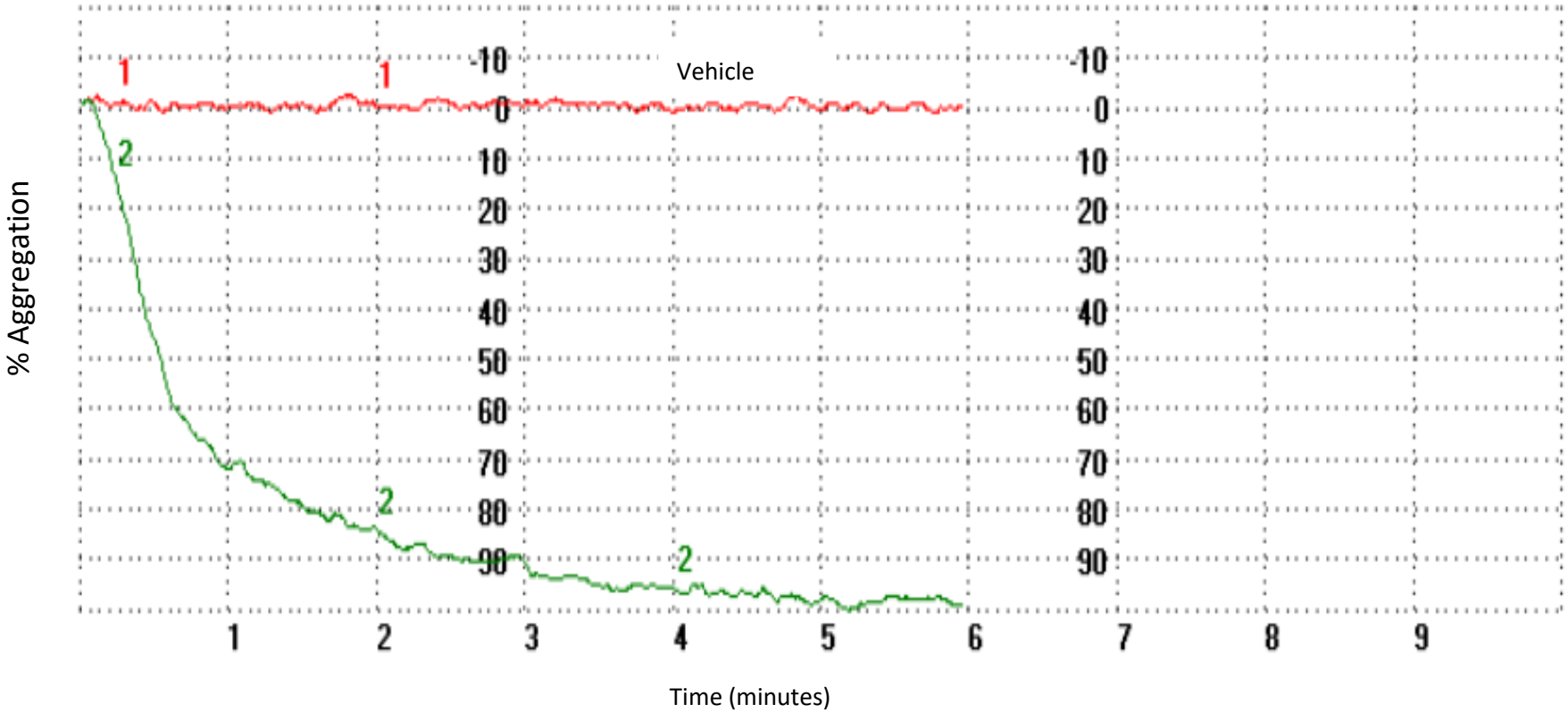

Figure 5: Platelet-rich plasma was incubated with vehicle (ddH_20) and then stimulated with collagen (20μM). Platelet aggregation was allowed to proceed for six minutes and representative tracings over this time are shown. The red line indicates the vehicle's inability to stimulate platelet aggregation. The green line indicates collagen's ability to stimulate platelet aggregation with over 90% aggregation occurring in six minutes.

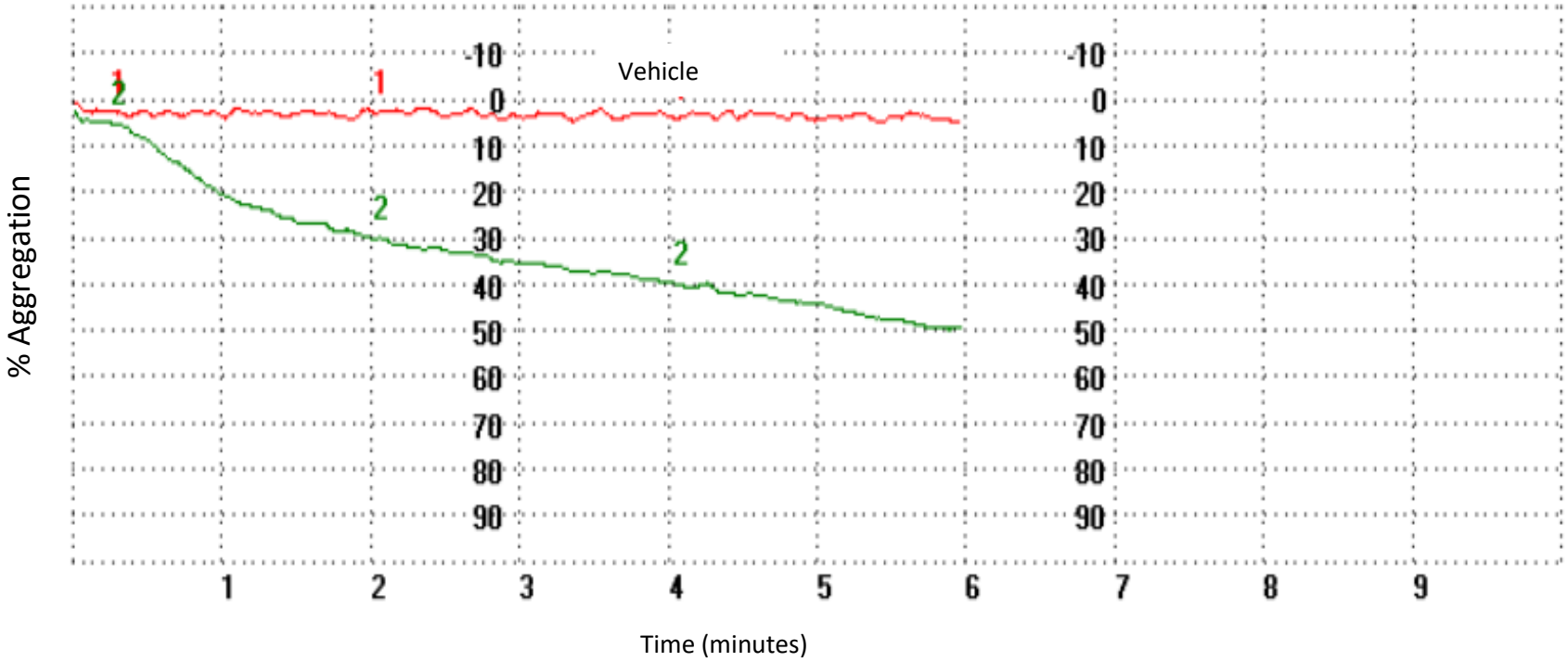

Figure 6: Platelet-rich plasma was incubated with vehicle (dd$H_2$0) and then stimulated with Epinephrine (20µM). Platelet aggregation was allowed to proceed for six minutes and representative tracings over this time are shown. The red line indicates the vehicle's inability to stimulate platelet aggregation. The green line indicates Epinephrine's ability to stimulate platelet aggregation with over 50% aggregation occurring in six minutes.

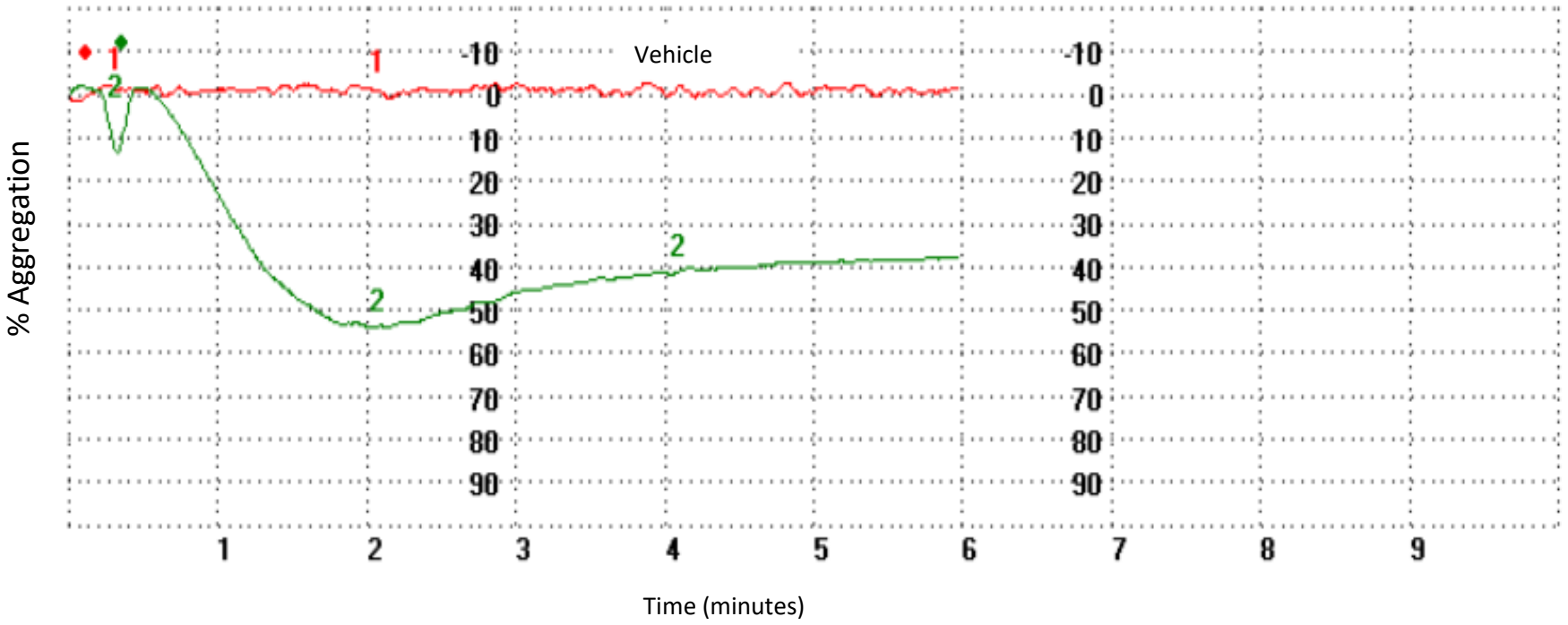

Figure 7: Platelet-rich plasma was incubated with vehicle (ddH_20) and then stimulated with Arachidonic Acid (20μM). Platelet aggregation was allowed to proceed for six minutes and representative tracings over this time are shown. The red line indicates the vehicle's inability to stimulate platelet aggregation. The green line indicates Arachidonic acid's ability to stimulate platelet aggregation with over 40% aggregation occurring in six minutes.

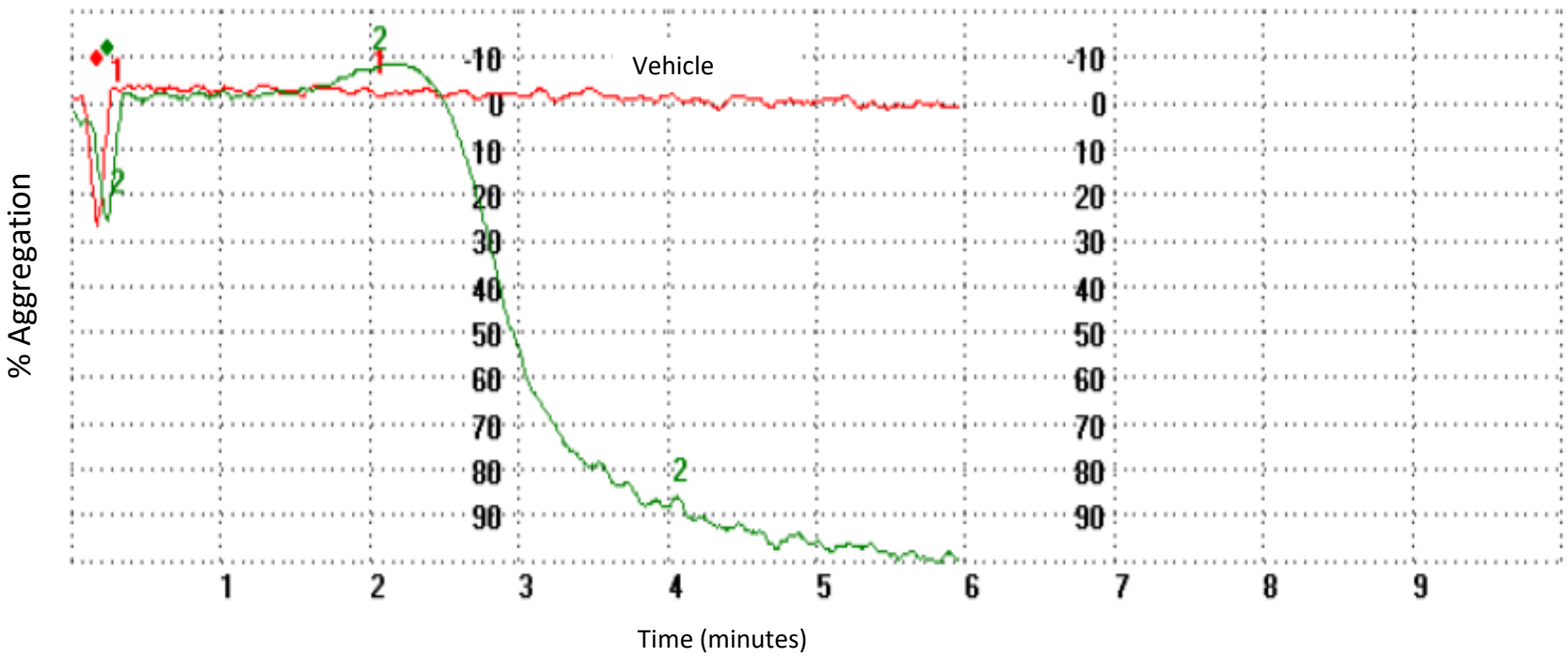

Figure 8: Platelet-rich plasma was incubated with vehicle (ddH_20) and then stimulated with ADP (20μM). Platelet aggregation was allowed to proceed for six minutes and representative tracings over this time are shown. The red line indicates the vehicle's inability to stimulate platelet aggregation. The green line indicates ADP's ability to stimulate platelet aggregation with over 95% aggregation occurring in six minutes.

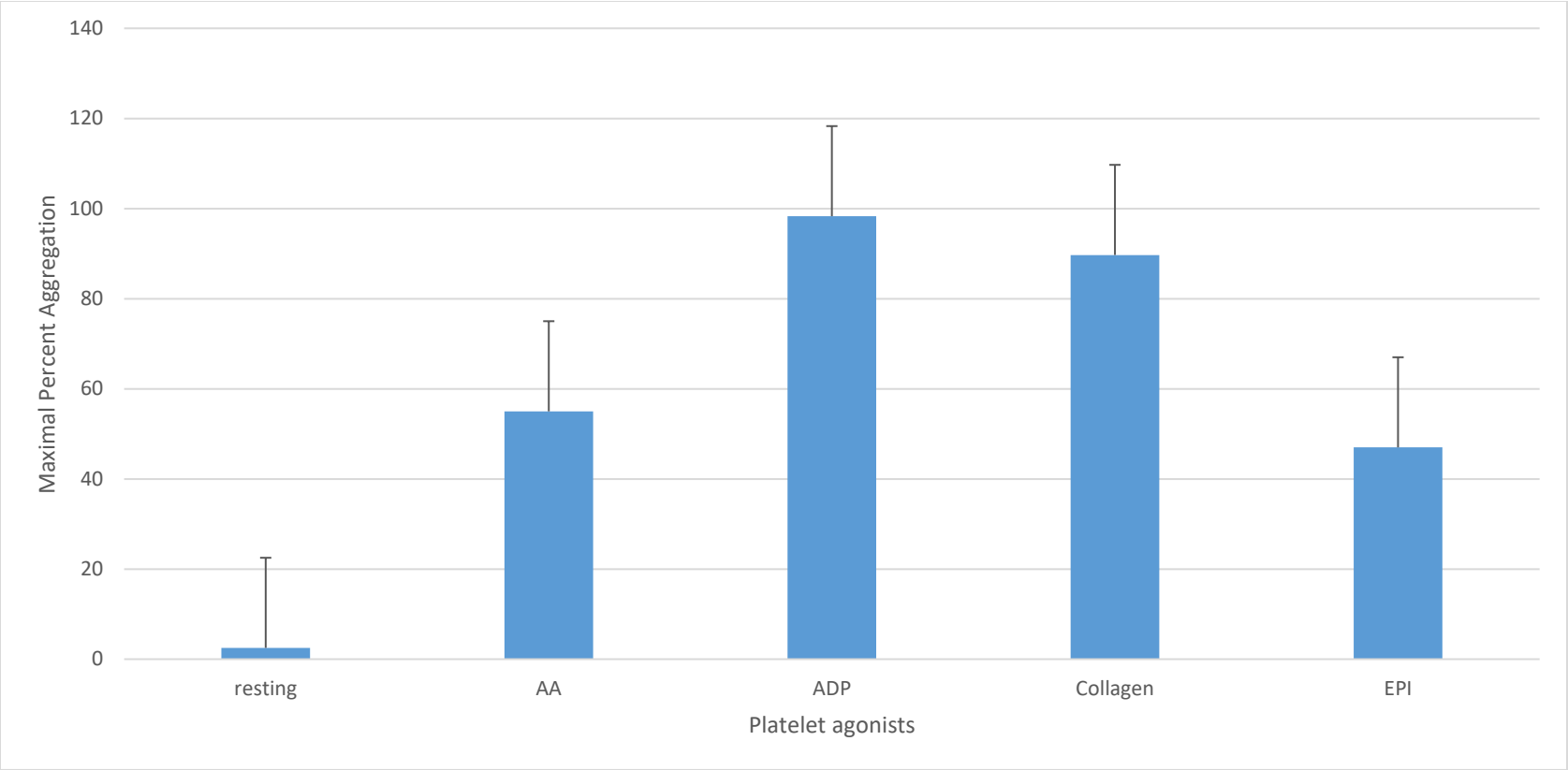

Figure 9: Platelet agonists stimulate platelet aggregation. Platelet-rich plasma was stimulated by the four platelet agonists arachidonic acid (AA, 20µM), ADP (20µM), Collagen (20µM), and Epinephrine (20µM). Platelet aggregation was allowed to proceed for six minutes. Mean percent maximal aggregation for each group is shown and error bars indicate standard error from the mean. The sample size for each agonist was the same.

Nitric Oxide Detection

After stimulation of the platelets by the platelet agonists, the Cayman Chemical kit was used to measure nitric oxide production. We believed that after stimulation, nitric oxide would be produced and be quantified by the kit. However, there was little NO detection.

DISCUSSION

The purpose of this study was to determine whether the Griess reagent kit can serve as a tool to quantify nitric oxide production. We proposed that arachidonic acid, ADP and collagen stimulation would produce measurable nitric oxide production in the blood as measured by the Cayman Chemical kit. Our data however suggests that the Cayman Chemical kit was unable to detect nitric oxide in aggregated platelets.

The Cayman Chemical kit should not move from a lab to clinical setting for diagnostic purposes as we believe low sensitivity of the system itself and perhaps contamination of nitrate in the chemicals during the reaction attributed to little NO detection. The low sensitivity of the Griess reaction would explain why variations were found between the various wells on the plate. If contamination (in the form of interfering reactions that produce artifacts), is not avoided it could skew data and alter conclusions about the concentration of NO (Giustarini et al, 2004). Risk of skewed data would most likely deter researchers from using this kit.

Other articles in the past have also discussed the pros and cons of the Griess reagent system. The Griess reaction is a two-step process in which numerous variations exist among the published assays. The most popular version is a sequential method where nitrite is mixed with sulfanilamide (SA) followed by N-naphthyl-ethylenediamine (NED). This version is found in most commercial kits (Fein et al., 2003). Pros of the kit include reasonable cost, easy to follow instructions, and the ability to modify the Griess reaction to eliminate interfering reactions that could ultimately lead to skewing data results. However, we suggest that one should evaluate both pros and cons before using the Cayman Chemical kit and avoid using the kit for diagnostic purposes.

As stated by Ku and colleagues (2007), measuring NO produced in platelets by using technologies like fluorescence spectrophotometry may allow healthcare professionals to efficiently monitor NO evolution. Measuring NO production would be important in cardiovascular conditions as well as conditions involving any nitric oxide signaling. The importance of platelets and the production of nitric oxide is reflected in the various thrombotic disorders that can arise if abnormal NO signaling occurs. For example, an imbalance between positive and negative platelet stimuli can contribute to thrombotic disorders, myocardial infarction being the most recognized example (Apostoli et al, 2014). The benefits of a system that can measure nitric oxide production is highly regarded by scientists and health professionals and it is our hope that future research and fixes to the kit will allow the kit to be used in a clinical setting.

Future research projects that could come from this project include modifying the Cayman Chemical assay kit, evaluating the use of two or more kits at a time within one project, and modifying the dilution within the kit during the preparation of the assay. As stated in various articles on the pros and cons of the kit, modifying the Griess reaction allows one to avoid any interfering reactions that might take place during the reaction of interest. It would also allow a researcher to modify the sensitivity of the system, hopefully allowing results that can be measured and analyzed. By evaluating two kits at a time within one project, one could compare a control (a methodology that is highly reliable and produces accurate results) with the Cayman Chemical kit and compare how close the kit can quantify nitric oxide production efficiently and accurately. Finally, a future research project could look at modifying the dilution within the kit during the preparation of the assay. In our experiment, we had two columns either 80µL buffer

or 40µL sample/ 40 µL buffer. Perhaps if different dilutions had been used, nitric oxide production might have been measurable and allowed us to confidently say that the Cayman Chemical kit can quantify nitric oxide.

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