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In vitro and *in vivo* assessment of platelet function in healthy dogs during low-dose aspirin therapy

By

Jillian Marie Haines

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

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2014

In vitro and *in vivo* assessment of platelet function in healthy dogs during low-dose aspirin therapy

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Low-dose aspirin therapy in dogs inconsistently inhibits platelet function, termed 'aspirin resistance'. There are no established diagnostic tests that can predict aspirin resistance in dogs prior to therapy. Platelet function was evaluated in healthy dogs prior to and during low-dose aspirin therapy using turbidimetric and impedance aggregometry, PFA-100, and urine 11-dehydro-thromboxane-B_2 concentration. Following a washout, platelet-rich plasma from the dogs was incubated with aspirin and evaluated via turbidimetric aggregometry. After aspirin, the majority of dogs were classified as 'aspirin responders' with 81% responding after 7 days. Platelet dysfunction was not consistent in all dogs at all times. Compared to turbidimetric, impedance and PFA-100 results were inconsistent when run concurrently, suggesting turbidimetric is the preferred technique. There was poor agreement between *in vitro* aspirin incubation and all other tests. Unlike in people, platelet function in dogs is consistently inhibited by aspirin incubation, making this a poor technique for predicting aspirin resistance.

DEDICATION

I would like to dedicate this research to my family who stood by me through the long process and of course to my two dogs, Riley and Cake, who always let me know that I am loved.

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INTRODUCTION

Platelet formation and function

Platelet formation (thrombopoiesis) occurs mainly in bone marrow within the megakaryocyte cell line. Megakaryocytes will mature and develop cytoplasmic processes (proplatelets), and both megakaryocytes and proplatelets will undergo a fragmentation process that results in shedding of platelets into the circulation.[1-3] After entering the circulation, the normal circulating lifespan of a platelet is about 5-7 days. [4]

Platelets play an essential role in blood hemostasis, a process involving interactions between vascular endothelium, coagulation factors, and other cell types. Hemostasis is divided into three phases: primary hemostasis, secondary hemostasis, and fibrinolysis. Primary hemostasis describes the interactions between platelets and vascular endothelium that lead to the formation of a platelet plug. This platelet plug acts as the first defense against hemorrhage, but is stable for only a short time and must be reinforced. Secondary hemostasis is characterized by the coagulation cascade and transformation of the platelet plug into a true blood clot through cross-linking of a fibrin meshwork. The third phase is fibrinolysis, in which plasmin is activated to break down fibrin and remove the blood clot. Alterations in the normal function of these phases can create ineffective hemostasis which can lead to inappropriate or excessive clot formation or bleeding.

Circulating platelets are inactive and travel along the periphery of blood vessels until they become exposed to damaged vascular endothelium.[5] Platelets will then become activated and adhere to the site of injury. Activation of platelets involves a process by which platelets change shape, release the contents of their granules, and express receptors on their surface that help to facilitate further aggregation. Once activated, platelets release vasoactive substances that lead to vasoconstriction, decreased blood flow, and blood stasis.[6] The decreased blood velocity and narrowed vascular lumen allow for increased contact time between platelets, damaged endothelium, and coagulation factors, and promote the generation of a platelet plug and then a blood clot. Adhesion and aggregation of platelets is further affected by shear conditions. Shear describes the frictional forces exerted on the endothelial layer of the vessel wall by blood flow.[7] Shear varies by size and type of vessel with high shear thought to occur mainly in small and medium sized arteries and low shear in larges arteries as well as veins.[8] In conditions of low shear, platelet adhesion to the endothelium occurs via collagen, fibronectin, and laminin.[5,9-11] Under high shear conditions, platelet adhesion is mediated by collagen and von Willebrand factor (vWF).[12] vWF is stored in the alpha granules of platelets and Weibel-Palade bodies in endothelial cells.[12] It helps platelets not only adhere to the endothelium, but also to other platelets, thus enhancing aggregation. vWF also acts to as a carrier protein and protector of factor VIII.

Platelets have two different receptors for vWF on their surface. The first is Integrin $\alpha_{IIb}\beta_3$ (previously GP IIb-IIIa complex) which after activation changes to allow binding of fibrinogen.[12,13] After endothelial injury, vWF adheres to exposed collagen and releases factor VIII. Platelets will then begin a process of rolling along the vessel wall

that is initiated by the second vWF receptor, GP Ibα in the GP Ib-IX-V complex. The loose attachments of vWF to the endothelium that occur during rolling are further mediated by P-selectin present on the activated endothelium.[14] After the activated platelet attaches to the endothelial collagen it undergoes a shape change that exposes the Integrin $\alpha_{IIb}\beta_3$ receptor.[15] The platelets produce membrane glycoproteins that further perpetuate the process of platelet recruitment and aggregation.[16]

Platelet aggregation occurs as two separate phases – primary and secondary aggregation. Primary aggregation describes a reversible process by which platelets develop direct interactions and undergo a shape change from a spherical to a stellate form consisting of multiple pseudopods. If no further stimulus is received, these platelet aggregates can potentially disperse. Once the stored contents of the alpha and dense granules of the platelet are released, aggregation becomes irreversible and the following wave of aggregation composes secondary aggregation.[17] In order for shape change to occur during primary aggregation, the agonist adenosine diphosphate (ADP) must be present. ADP is released during platelet degranulation and through its binding to the $P2Y_{12}$ receptor it creates the shape change by activating phospholipase C and causing calcium mobilization.[17,18] Binding of ADP to its receptors and associated calcium mobilization is a major mediator of full platelet aggregation. [18]

Platelets release many substances that are involved in creating the initial environment contributing to their activation, such as ADP, serotonin, and prostaglandins. Under high shear conditions, ADP is the main promoter of platelet activation.[19,20] One of the main mediators of platelet activation under low shear conditions is the prostaglandin thromboxane A_2 (TXA_2). TXA_2 is synthesized by platelets through conversion of

arachidonic acid by the cyclooxygenase (COX) enzyme. After its release, TXA_2 triggers vasoconstriction, leads to recruitment and subsequent activation of additional platelets, and enhances platelet aggregation. [21] Thromboxane A_2 works immediately but for only a short period of time (approximately 30 seconds). Thromboxane A_2 is rapidly metabolized into thromboxane B_2, and then 11-dehydro-thromboxane B_2 and 2,3-dinor-thromboxane B_2. These stable metabolites are ultimately eliminated in the urine. [22-24]

Platelets, like other cells, do not store thromboxane A_2, but instead synthesize it as needed from arachidonic acid. Arachidonic acid is released from phospholipid membranes via the enzymatic action of phospholipases. [25-27] Arachidonic acid is then converted into prostaglandin H_2 by COX-1 and 2, also known as prostaglandin H-synthase-1 and 2, respectively. COX-1 enzyme activity ultimately leads to the production of prostaglandins such as TXA_2 and prostacyclin (prostaglandin I_2).[26,28] Prostacyclin does not originate from platelets; instead it is released from vascular endothelium. Prostacyclin acts in opposition to thromboxane A_2 in that it causes vasodilation and inhibition of platelet aggregation.[25,26,29] By working together, these two prostaglandins keep hemostasis in a balanced state.

At least three isoforms of COX have been described in the dog. COX-1 is expressed constitutively in most body systems, and has the function of maintaining normal cell activities such as gastric mucosal health and protection and preservation of renal blood flow.[27,30] COX-3 is a splice variant of COX-1 and has similar functions to COX-1 but less activity (approximately 20% of the activity of COX-1).[31,32] COX-2 has a more limited distribution than COX-1, and a much lower level of expression. COX-2 is expressed in tissues such as the brain, thymus, kidneys and vascular endothelium, and

cell types such as circulating monocytes, fibroblasts, and tissue macrophages.[30,33,34] COX-2 is inducible, and levels can be increased through the action of inflammatory cytokines, growth factors, and endotoxins.[27,35,36]

Until recently, it was believed that COX-1 was the only isoform expressed by circulating platelets, therefore making COX-1 the primary mediator of platelet TXA_2 production. COX-2 was the isoform believed to be in control of prostacyclin production.[25,26,34,37,38] Recent research, however, has demonstrated that both COX-1 and 2 isoforms are expressed in human and dog platelets, with higher levels of COX-2 expression seen with increased platelet turnover and associated with higher levels of immature (reticulated) platelets.[28,33,37,39] These findings suggest a role for COX-2 in platelet function.[30,37]

The process of platelet activation involves complicated interactions between a variety of agonists including, but not limited to, thromboxane A_2. These agonists act to stimulate and activate platelets, cause vasoconstriction, and attract and then activate other platelets, with the ultimate goal of creating a platelet plug. Platelets are anucleate, and therefore rely on agonists and other stimuli to reach a state of activation. In order to facilitate activation, platelets contain stored granules ranging in size from 200-500 nm that contain a variety of proteins that aid in platelet adhesion, promote cell-to-cell interactions, and stimulate vascular repair.[40,41] These granules are referred to as dense (delta) granules and alpha-granules. Dense granules contain ADP, serotonin, and epinephrine.[41] Alpha-granules are the main secretory component of platelets, and contain adhesion molecules such as von Willebrand factor, P-selectin, and fibronectin. Alpha-granules also contain pro- and anti-angiogenic factors such as vascular endothelial

growth factor, as well as coagulation factors such as fibrinogen and factors V and VIII. Different molecules are selectively packaged within different alpha-granule subsets, both during platelet formation in the bone marrow and after uptake by circulating platelets.[40-44] During platelet activation, these selectively packaged molecules are released from alpha-granules depending on the specific receptor activated and the specific platelet agonist involved.[40,41,45]

Contained within alpha-granules is the molecule P-selectin, a cell adhesion molecule that mediates platelet and leukocyte aggregation. P-selectin also generates procoagulant microparticles that contain active tissue factor and enhance fibrin deposition.[46,47] Through these properties, P-selectin is thought to enhance both platelet aggregation and secondary hemostasis. Platelet P-selectin is found in higher concentrations in dogs suffering from primary immune mediated hemolytic anemia, and may contribute to the thromboembolic complications that develop in these patients.[48]

Platelets play a supporting role in the formation and stabilization of a true blood clot. Alpha-granules release pro-coagulant factors (factors V, VIII, and fibrinogen) that work with other coagulation factors to promote clot formation. Additionally, after activation, platelets undergo a shape change that creates a larger surface area for adherence to the endothelium as well as other platelets or cells. [49] Activated platelets may also express receptors and ligands on their surfaces that assist in thrombin and fibrin formation. In particular, aminophospholipid phosphatidylserine, which is found in the inner leaflet of inactivated platelets, will move to the platelet surface after activation and bind to prothrombin, accelerating the conversion of prothrombin to thrombin and the

formation of a blood clot.[50] The phosphatidylserine-rich platelet surface is able to synthesize thrombin at a rate 300,000 times faster than the fluid phase of plasma.[50]

Aspirin resistance

Unfortunately, treatment with low-dose aspirin does not consistently inhibit platelet function in all dogs and, despite preventative therapy, some dogs treated with aspirin will continue to develop thromboembolic complications. Previous research has demonstrated that only about one-third of normal dogs treated with low-dose aspirin respond to low-dose aspirin as expected, with prolongation in closure times as measured on an in-house platelet function analyzer (PFA-100®), while the majority of dogs either incompletely or inconsistently respond to the same dose of aspirin.[39] A similar phenomenon occurs in humans treated with low doses of aspirin, and people that are poorly responsive to the anti-platelet effects of aspirin are termed 'aspirin resistant'.[62]

The exact incidence of aspirin resistance in people is unknown, but the published incidence ranges from 8 to 45% of human patients.[56,62-64] Since canine clinic patients appear to inconsistently respond to low-dose aspirin in terms of prevention of thromboembolism, it is possible that, similar to humans, dogs also experience clinically relevant aspirin resistance. The exact incidence of aspirin resistance in canine clinic patients remains unknown.

The precise mechanism for aspirin resistance in both humans and dogs is unknown, but several possible mechanisms have been proposed. These mechanisms can be divided into 'true' aspirin resistance and unproven aspirin resistance. Causes of true aspirin resistance include decreased bioavailability of aspirin, increased platelet turnover to expand the pool of non-aspirinated platelets,[65] competition with other nonsteroidal

anti-inflammatory drugs (NSAIDs), [66] alternative pathways for the formation of TXA_2,[37,67] the presence of variants of COX-1 which are less sensitive to aspirin inhibition[68], or poor compliance by the individual patient.[69] Unproven causes of aspirin resistance in people include increased sensitivity to ADP-induced activation of the platelet surface membrane glycoprotein (GP) IIb/IIIa, polymorphisms in GPIIb/IIIa, increased resistance to the platelet agonist collagen, high plasma von Willebrand factor concentrations, and the proaggregatory activities of isoprostanes, hyperlipidemia, smoking, and stress.[70-75]

One of the proposed mechanisms of aspirin resistance is the presence of an alternate source of thromboxane A_2.[28] As aspirin is typically highly effective at inhibiting COX-1 production of TXA_2, this mechanisms relies on the idea of a COX-1 independent pathway leading to ongoing platelet TXA_2 production. An alternate pathway for TXA_2 production could be production via the COX-2 isoform, either from circulating platelets of the vascular endothelium.[28,37] Genetic differences in the polymorphism of the COX-1 and/or COX-2 enzymes could affect individual sensitivities to aspirin. In order to inhibit COX-2 in people, a much higher dose of aspirin is needed than that needed to inhibit COX-1. COX-1 will be inhibited in people at doses of much less than 325 mg of aspirin per day (a typical human low dose of aspirin is 81 mg per day), but doses of greater than 325 mg per day are needed to inhibit the COX-2 enzyme.[28] A human low dose of aspirin of 81 mg is comparable to the dose of aspirin that is likely sufficient to inhibit COX-1 synthesis of TXA_2 in dogs (0.5 to 1 mg/kg per day).[53] Low dose aspirin administration can lead to an increase in COX-2 expression in dogs possibly due to up regulation of the enzyme.[39] In dogs receiving anti-inflammatory or high dose aspirin (10

mg/kg twice daily), COX-2 was found to be more consistently decreased.[61] This suggests that a 0.5-1 mg/kg per day dose might not be adequate for inhibition of COX-2 mediated thromboxane production.

Another possible mechanism to explain aspirin resistant may be variation in platelet thromboxane receptors. Thromboxane production in dogs receiving low-dose aspirin has been reported to be consistently inhibited, despite the fact that platelet function is only inhibited in one third. [39] This phenomenon suggests that there was a high degree of variability in platelet responsiveness to thromboxane. Genetic differences in thromboxane receptor structure might explain the different responses seen in individual. Genetic polymorphisms in other receptors that are involved in platelet function may also contribute to aspirin resistance. Polymorphism of PLA2, the gene coding for platelet GP IIIa, for example, has been associated with aspirin resistance in people.[72,76]

The term 'aspirin resistance' may actually encompass multiple mechanisms and phenomena. Three types of aspirin resistance have been identified in humans using a combination of *in vivo* and *in vitro* techniques utilizing platelet aggregometry. Individuals with type I aspirin resistance showed no affect on platelet aggregation or thromboxane synthesis with oral aspirin treatment, but marked suppression of aggregation and thromboxane synthesis with *in vitro* aspirin incubation. Type I aspirin resistance therefore has the potential to be due to variability in aspirin pharmacokinetics.[77] Type II aspirin resistance is characterized by patients that had no inhibition of platelet aggregation or thromboxane synthesis with oral aspirin therapy, and only partial inhibition of thromboxane synthesis and no significant inhibition of aggregation with *in vitro* aspirin incubation. The mechanisms behind type II aspirin

resistance are unknown, but the phenomenon may arguably be due to increased COX-2 expression or polymorphisms in the COX-1 gene.[78,79] Finally, type III aspirin resistance is characterized by patients that show inhibition of thromboxane synthesis with both oral aspirin and *in vitro* aspirin incubation, but no inhibition of platelet aggregation with either oral treatment or the *in vitro* additional of aspirin. Type III aspirin resistance has also been termed 'pseudo-resistance' as the aspirin appears to be exerting the expected pharmacodynamic affect on platelets, but platelet function is not affected, allowing for normal aggregation.[80] One potential explanation for type III aspirin resistance would be genetic polymorphisms in thromboxane receptor responsiveness to TXA_2. Interestingly, variable thromboxane receptor responsiveness to TXA_2 has been documented in dogs.[81]

Assessment of platelet function

There are currently a variety of options available for evaluating platelet function. Each method comes with its own set of pros and cons. and no single method provides all the information needed to fully assess a patient's platelet function. Determining which test or instrument should be used can be challenging, and is based on an understanding of the individual patient and clinical suspicion of the type of dysfunction suspected. Ultimately, it may require the use of multiple techniques or tests to fully elucidate cause or extent of changes in platelet function.

Multiple factors can affect which method for testing platelet function is chosen. Some tests are better able to evaluate drug effects on platelets, while others may better clarify hereditary or acquired function defects. Cost can have a large impact on not only the willingness of an owner to agree to testing, but also the availability of expensive instruments needed to run these tests. Advanced training may be needed in order to

operate specialty instruments and, due to special handling and time constraints, certain tests may be impractical for clinical use. There is a need for the development of patient side tests that deliver rapid and cost-effective results. However, these more user friendly options must first be proven to offer the same consistent and reliable results that we expect from the established laboratory methods already in place.

Platelet Aggregometry

Platelet aggregometry has been used for over 50 years, and is considered the gold standard for evaluation of platelet function.[59,82,83] Aggregometry uses either whole blood or platelet rich plasma in combination with an agonist that activates platelets. Aggregometry is then used to determine the ability of platelets to aggregate and form a platelet plug.[82,83] Aggregometry evaluates platelet function under low shear stress. The use of platelet aggregometry does have a number of drawbacks: it is time consuming, requires the use of expensive equipment and specialized training and, since samples must be processed shortly after collection, is difficult to utilize in a routine clinical setting.[82] There are two forms of platelet aggregometry available: turbidimetric aggregometry and impedance aggregometry.

Turbidimetric aggregometry

Turbidimetric (light transmission) aggregometry is considered more sensitive to subtle changes in aggregation compared to impedance aggregometry, and is therefore considered the preferred standard. Turbidimetric aggregometry utilizes sodium-citrated platelet rich plasma (PRP) and works by measuring the amount of light that is transmitted through the PRP after activation with a platelet agonist. A variety of agonists are

available for use with selection based on desired assessment and include collagen, ADP, thrombin, arachidonic acid, epinephrine, and others. A spectrophotometer records the light transmitted through the PRP as it is continually stirred over a set time period. The basis of the process is that as more aggregation occurs, more light will be transmitted through the PRP. A platelet poor plasma (PPP) sample is used from the same animal to act as a control. The aggregometer is adjusted such that the PPP sample represents 100% light transmission. The starting point of the curve is set to 0%. The results are displayed dynamically as a graphed curve, and parameters measured include maximal amplitude of aggregation (percentage) and a slope/rate (percentage/minute). The graph often shows an initial decrease in light transmission as activated platelets undergo shape change from discoid to spiny spheres, followed by a progressive increase in light transmission that will plateau as permanent and irreversible aggregation occurs. With weaker agonists, it is possible to see a biphasic aggregation with an early plateau signifying primary aggregation, followed by a secondary wave of irreversible secondary aggregation.[84,85]

Turbidimetric aggregometry is useful for the evaluation of both hereditary and acquired platelet dysfunction, as well as for monitoring of drug-induced platelet dysfunction. However, the results can be affected by multiple factors such as agonist type and concentration, stir speed, sample handling protocols, time elapsed from collection, and arguably platelet count.[85] Current recommendations are against adjusting PRP in order to have a standardized platelet count before the PRP is placed in the aggregometer, since this process appears to be unnecessary and may result in altered platelet function due to increased sample handling. Additionally, unadjusted samples may be a better indicator of *in vivo* hemostasis.[86-89]

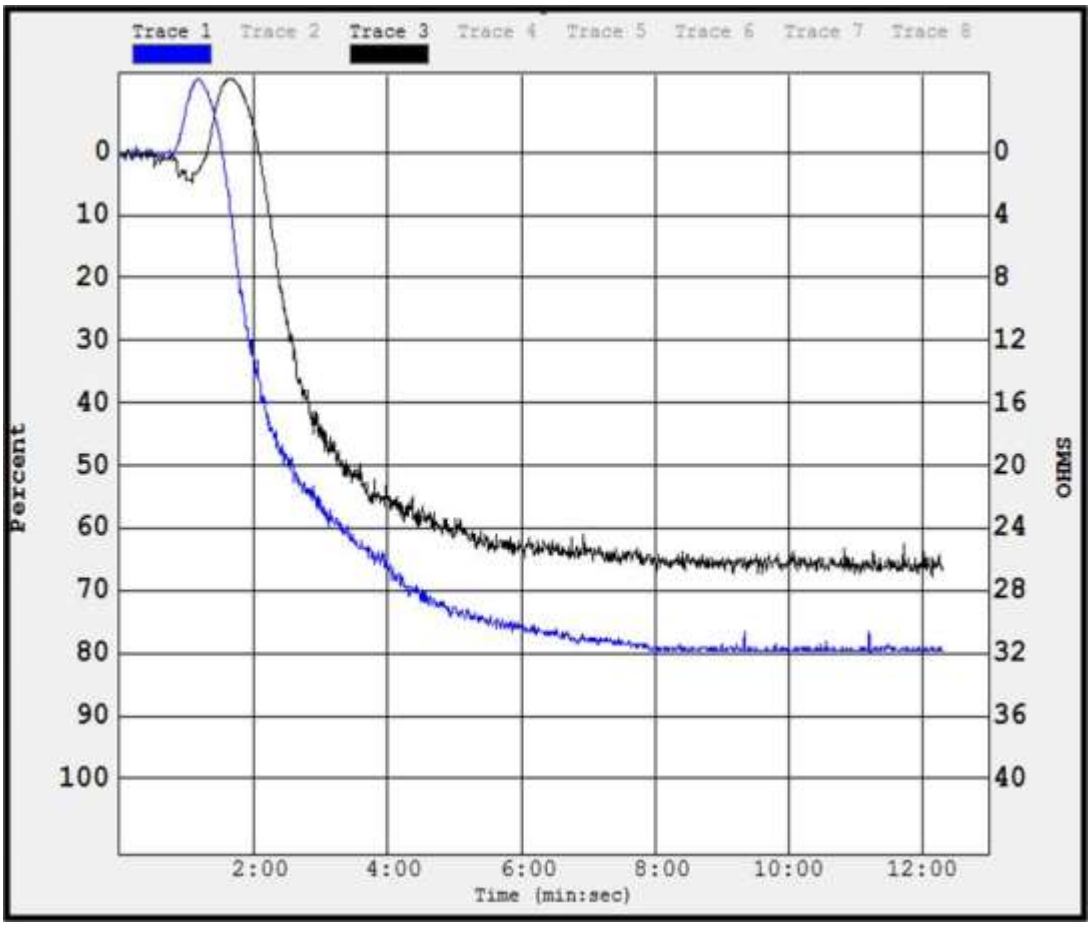

Figure 1.1 Turbidimetric aggregometry tracing showing normal platelet aggregation in a dog that has not received aspirin

Impedance Aggregometry

The second type of platelet aggregometry commonly used to evaluate platelet function is impedance aggregometry. Since, unlike turbidimetric aggregometry, impedance aggregometry does not require light transmission, whole blood can be used instead of PRP. Impedance aggregometry employs the use of a current that passes between two metal wires. After the addition of an agonist, platelet aggregation will begin with the immediate adherence of platelets to the electrode wires. As the platelet plug

develops, the flow of current across the wires will be impaired and conduction will diminish. Impedance aggregometry graphs aggregation in a manner similar to turbidimetric aggregometry. Results are measured as maximal amplitude of aggregation, but the unit of measurement is expressed in ohms.[85]

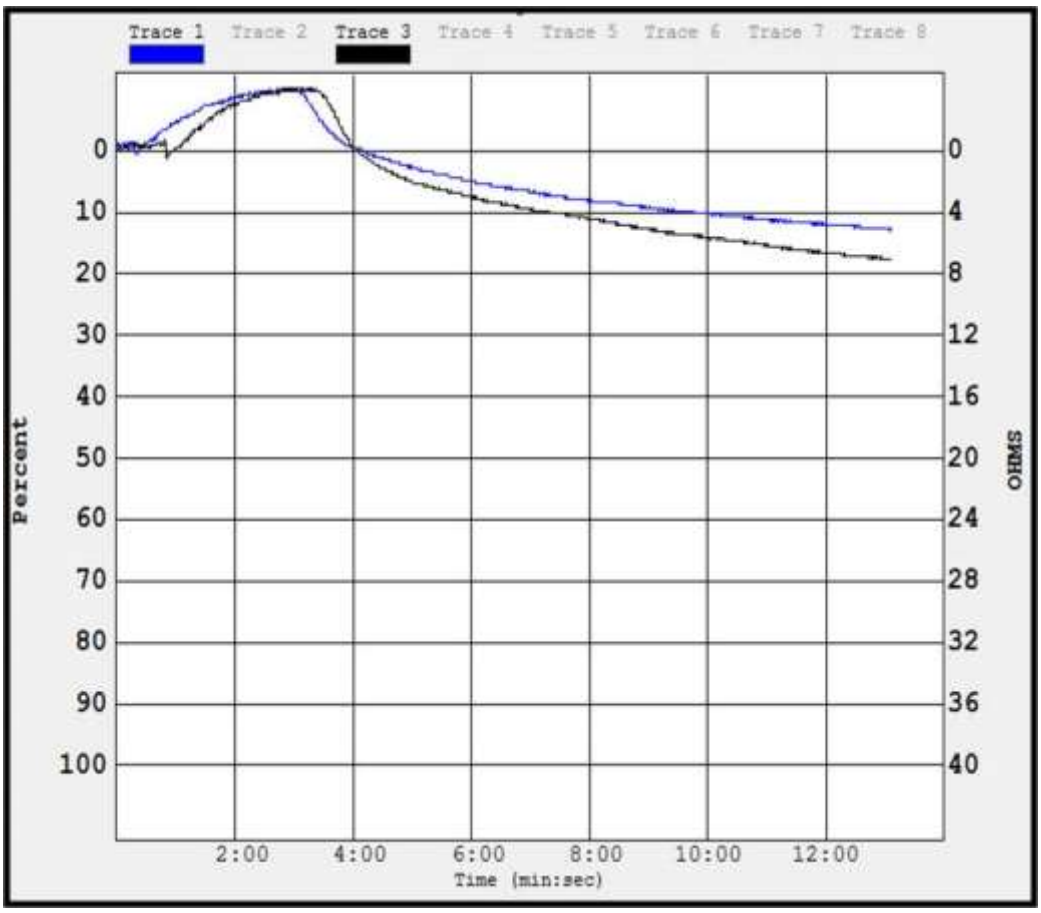

Figure 1.2 Impedance aggregometry tracing showing normal platelet aggregation in a dog that has not received aspirin

Advantages of impedance aggregometry compared to turbidimetric aggregometry include simplification of sample handling as centrifugation to create PRP is not required, less risk of platelet function disturbance due to the extra handling needed to create PRP, a

shortened preparation time, need for smaller sample volumes, and effectiveness with lipemic blood. As with turbidimetric aggregometry, various agonists are available for use, and impedance aggregometry can also be used to evaluate acquired and congenital platelet disorders as well as drug-induced platelet dysfunction.[85]

One specific impedance-type analyzer that has recently become available for use is the Multiplate® platelet function analyzer. The Multiplate® analyzer uses impedance aggregometry to evaluate platelet function under low, non-laminar shear conditions. The Multiplate® analyzer works in the same manner as other impedance analyzers in that it uses whole blood and measures conduction across an electrode. The analyzer utilizes five channels and disposable test cells, and is a rapid and user friendly instrument option because a computer both guides the user and controls volumes and order of procedures. These features potentially make the Multiplate® analyzer an attractive option for clinical use. Each test channel produces two aggregation curves and can be used separately, thus allowing for simultaneous evaluation of platelet function under the influence of different agonists.

The Multiplate® analyzer generates an aggregation curve and produces results such as area under the curve, maximal aggregation, and rate of reaction. Area under the curve is considered the best measurement of platelet function and overall aggregation response as it is dependent on both the height and slope of aggregation.[90] The manufacturer recommends the use of non-calcium chelating anticoagulants such as heparin or hirudin instead of the citrate used by traditional aggregometry instruments. Thrombin inhibitors such as heparin allow maintenance of physiologic concentrations of calcium to better represent *in vivo* hemostasis. However, as the samples are diluted with

0.9% sodium chloride during analysis, calcium levels are still likely affected to some degree.[83]

Recent studies using the Multiplate® analyzer in dogs have sought to validate its use and to determine the most appropriate combinations of anticoagulant, agonist, and test duration. Study results to date have not as yet provided reliable recommendations for use in dogs. While some results support its use in dogs, there remains disagreement on the best anticoagulant option. Hirudin was recommended in one study,[83] while another study recommended that shorter run times and the use of heparin anticoagulant were needed to optimize results[91] The agonists ADP, arachidonic acid, and collagen are effective at consistently inducing platelet aggregation with the Multiplate® analyzer, but ristocetin and thrombin receptor activating peptide (TRAP) are not.[83]

PFA-100

The Siemens Platelet Function Analyzer-100® (PFA-100®) is a bench top instrument used to evaluate platelet function. The PFA-100® has been previously evaluated for use as a commercial point-of-care platelet function analyzer in dogs.[58,59,92] The PFA-100® provides an *in vitro* evaluation of the time needed for formation of a platelet plug under high shear conditions. A variety of platelet agonists can be used, including as epinephrine, ADP and collagen.

The instrument contains an aperture that simulates the presence of damaged vascular endothelium. Results are recorded as 'closure time', or the time needed for a platelet plug to fully occlude the aperture. Use of this instrument requires sodium-citrated whole blood, and utilizes two different types of disposable cartridges. The collagen/epinephrine cartridge is best for evaluating drug-induced platelet dysfunction,

and the collagen/ADP cartridge is best for evaluating congenital or acquires platelet function defects.[93]

A prolongation of the PFA-100® closure time, when measured with the collagen/epinephrine cartridge, has been established as a sensitive indicator of aspirin-induced platelet function in both humans and dogs.[59] Both the collagen/ADP and collage/epinephrine cartridges have been used as an effective test for diagnosis of von Willebrand's disease in dogs.[94] The PFA-100® is insensitive at detecting the inhibitory effects of ADP antagonists.[95] Although the PFA-100® could potentially be used to detect increased platelet function, it is unclear if a shortened closure time is truly representative of platelet hyperactivity and thromboembolic risk.

The PFA-100® measures up to a maximum closure time of only 300 seconds. Closure times beyond 300 seconds strongly suggest platelet dysfunction. Interpretation of closure times can be difficult, however, especially when results are in the upper ranges of normal, and marginal values require repeated testing in order to verify results. Other factors such as even mild thrombocytopenia (150,000/µl), decreased hematocrit, sodium citrate anticoagulant concentration[92], dog breed[59], and the presence of underlying cardiac disease[96] can affect results.

Urine thromboxane

Measurement of thromboxane synthesis provides is a reliable indicator of COX function. After thromboxane A_2 undergoes metabolism, it is excreted into urine as stable metabolites. The most abundant urine metabolite, 11-dehydro-thromboxane B_2, as well as the metabolite 2,3-dinor thromboxane B_2, have been found in increased concentrations in people in association with hypercoagulable conditions.[97,98] In dogs, urinary

concentrations of 11-dehydro-thromboxane B_2 have been found to be elevated with gastric dilatation-volvulus.[97] Previous studies in humans have used urinary concentrations of 11-dehydro-thromboxane B_2 to assess aspirin-induced COX inhibition.[23,98]

Urine concentrations of thromboxane metabolites are preferred to plasma or serum levels as the mere act of blood collection and processing can lead to platelet activation and thromboxane synthesis. Platelet activation during blood sample collection, storage, and processing can lead to artifactually increased thromboxane concentrations that not indicative of true *in vivo* activity. For this reason, urine thromboxane metabolite levels are considered a more reliable measure of TXA_2 synthesis.[23,24] The urinary concentration of the metabolites 11-dehydro-thromboxane B_2 and/or 2,3-dinor thromboxane B_2 are preferred indicators of platelet thromboxane production compared to urinary thromboxane B_2, because thromboxane B_2 can be generated by the kidneys and excreted without undergoing metabolism.[97]

It has not yet been shown which of the two urinary metabolites, 11-dehydro-thromboxane B_2 or 2,3-dinor thromboxane B_2, best indicates in vivo platelet response to aspirin in dogs.[22] Disadvantages of using 11-dehydro-thromboxane B_2 include its low concentration in urine thus requiring minimal dilution during measurement that could allow for more interference as well as concerns about the metabolites relevance in indicating TXA_2 release from platelets. [22,99,100] However, the metabolite is used commonly in human medicine allowing extensive opportunity for comparison and thus extrapolation, even though possible differences between the affects of aspirin on venous versus arterial thrombosis remains unclear.[22]

The measurement of 2,3-dinor thromboxane B_2 offers the advantages of high urine concentrations in dogs making it easily detectible however it it has the potential to cross react with TXB_2 concentrations when measured using the ELISA method. This may be more of a concern in dogs with renal disease producing altered concentrations of renal TXB_2.[22] 2,3-dinor thromboxane B_2 has a shorter half life than 11-dehydro-thromboxane B_2 which has the potential to create more fluctuation in urine levels. Assays for this metabolite are less readily available and as it is not frequently measured in human medicine, there is little literature available for comparison or extrapolation.[22]

Additional methods for evaluating platelet function

Several other methods exist for evaluating platelet function. The buccal mucosal bleeding time (BMBT) is the most frequently used method in dogs for assessing platelet function *in vivo*. This test involves creating a small incision on the inside of the lip and measuring the time it takes for bleeding to be halted by the formation of a platelet plug. This same concept can also be assessed using a toe nail clip, however, the nail cuts may be less consistent and lead to more variable results. Both methods are affected by thrombocytopenia and are insensitive measures of drug induced platelet dysfunction.

The VerifyNow® platelet function analyzer is a point of care instrument that uses a technique similar to light aggregometry but in the form of disposable cartridges already preloaded with a platelet agonist. The different cartridges available were designed to evaluate the anti-platelet effects of drugs such as aspirin (arachidonic acid), $P2Y_{12}$ inhibitors (ADP and prostaglandin $E_{1)}$, and GP IIb/IIIa antagonists (iso-thrombin receptor-activating peptide). This instrument was designed for use in people, and has limits when applied to canine use, especially when evaluating the effects of aspirin.[95]

Canine platelets show variable response to arachidonic acid as well as iso-thrombin receptor-activating peptide (iso-TRAP) thus making this instrument an unreliable measure of the affects of aspirin or GPIIb/IIIa antagonists on canine platelet function.[101] As this method has so far seen limited use in the veterinary field, further research is needed using the VerifyNow® in veterinary patients to truly understand its capabilities and limitations.

The PlateletWorks® platelet function kit acts to indirectly evaluate platelet function by using a standard impedance cell counter to measure platelet counts before and after activation by several different agonists. Results are then reported as percent aggregation and percent inhibition. While this is a simple point-of-care test that offers rapid results, it is not only extremely time dependent but also strongly affected by individual user and, so far ,appears to lack correlation with other available tests of platelet function. Limited research with canine platelets is available for the PlateletWorks® platelet function kit.

Tests to evaluate platelet adhesion are also available. The DiaMed Impact-R® tests platelet function under close to physiological flow conditions by using imaging technology to measure platelets adhered and aggregated to well surface. However, limited research is available to assess use of the Impact-R® in veterinary patients. Various immunoassays, particularly enzyme-linked immunosorbent assays (ELISAs), and collagen binding assays have also been developed to measure the quantity and function of canine von Willebrand's factor, which plays a critical role in platelet adhesion.

Platelet role in clot stability can also be assessed using thromboelastography (TEG) or viscoelastometry, however this technology remains available in only limited

settings at this time, and its utility for evaluating this aspect of platelet function in dogs has not been established.

Primary hemostasis can also be assessed using a clot retraction test. This is a crude measure of clot formation that works by measuring the time it takes for a blood clot to form and then retract away from the walls of a glass container. Retraction normally takes 1-2 hours and is dependent upon a normal platelet count and normal platelet function.

Finally, flow cytometry can be used to evaluate platelet surface protein expression both before and after activation, and has a wide variety of applications to look at hereditary, acquired, immune, and drug induced platelet dysfunction. Unfortunately, limited availability, and the need for rapid sample handling and specialized instrumentation and expertise, create challenges for the routine clinical application of flow cytometry.

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CHAPTER II

EFFECTS OF LOW DOSE ASPIRIN ON PLATELETS

Introduction

Thromboembolism is an important complication of many conditions affecting dogs, including immune-mediated hemolytic anemia, hyperadrenocorticism, and protein losing nephropathy.[1,2] Low-dose aspirin is often recommended for the prevention of thromboembolic complications in humans and dogs. Unfortunately, despite low-dose aspirin therapy, a subset of patients still develop thromboembolic complications, a phenomenon known in human medicine as 'aspirin resistance'. The exact incidence of aspirin resistance in humans is unknown, but the phenomenon is estimated to affect up to 45% of human patients.[3-6]

Aspirin is a cyclooxygenase (COX) inhibitor that irreversibly inhibits platelet function. By inhibiting COX, aspirin blocks the arachidonic acid conversion pathway and prevents the synthesis of prostaglandins, particularly thromboxane A_2 (TXA_2) and prostacyclin (prostaglandin I_2), which work in concert to maintain a balanced, normal hemostatic system.[5] In contrast to TXA_2, which is produced by platelets to enhance local vasoconstriction, activate platelet adhesion, and promote platelet aggregation, prostacyclin originates from the vascular endothelium and has the opposite physiologic effect on primary hemostasis (vasodilation and inhibition of platelet aggregation).[7] After synthesis, TXA_2 has an immediate physiologic effect, and is then metabolized to

thromboxane B_2 and further metabolized into 11-dehydro-thromboxane B_2 (11-dTXB_2) and 2,3-dinor-thromboxane B_2 (2,3-dinorTXB_2) before being eliminated in the urine.[8-10] Measurement of urinary 11-dTXB_2 has been used as an indicator of aspirin-induced COX inhibition.[9,11-13]

Anti-inflammatory doses of aspirin (10 mg/kg orally twice daily) reliably inhibit platelet function in dogs by decreasing TXA_2 production, but can cause gastrointestinal and renal side effects.[11] Lower dosages (0.5-1 mg/kg orally once daily) of aspirin are used to maintain anti-platelet effects without significant adverse effects.[14] Low doses of aspirin, however, do not consistently inhibit platelet function in all dogs. Dudley et al. documented that low-dose aspirin therapy only completely and consistently inhibits platelet function in about one third of dogs, as measured by prolongation in closure times using a point-of-care platelet function analyzer (PFA), while remaining dogs either do not respond or inconsistently respond.[15] There is currently no pre-treatment diagnostic test that can reliably predict which dogs will respond to the anti-platelet effects of low-dose aspirin therapy.

In both human and veterinary medicine, platelet aggregometry is considered the gold standard method for evaluation of platelet function.[16-18] Platelet aggregometry analyzes platelet function by using specific agonists to activate platelets in either platelet rich plasma (PRP) (turbidimetric aggregometry) or whole blood (impedance aggregometry), and measuring the ability of platelets to aggregate.[17] Unfortunately, platelet aggregometry is expensive, labor intensive, and requires specialized equipment and training, thus making aggregometry difficult to utilize in a clinical setting.[11] Point-of-care PFAs have the potential to generate similar information to platelet aggregometers

about platelet function, and are more user-friendly instruments in a clinical setting. Correlating the results between platelet aggregometry and point-of-care PFAs, especially during low-dose aspirin therapy, may enable the establishment of point-of-care PFAs as user friendly instruments for identifying aspirin resistance in canine clinical patients during initial treatment.

In humans, a pre-treatment protocol has been developed for determining an individual patient's response to the anti-platelet effects of aspirin, as well as characterizing the nature of aspirin resistance when it is detected. This protocol involves *in vitro* exposure of platelets to acetylsalicylic acid followed by platelet aggregometry.[19] We hypothesized that this same *in vitro* protocol, performed on platelet samples collected prior to aspirin therapy, may similarly allow prediction of response or non-response to low-dose aspirin in dogs.

The objectives of our study were to develop and evaluate a protocol utilizing turbidimetric platelet aggregometry and *in vitro* platelet incubation in acetylsalicylic acid to determine the degree of aspirin-induced platelet inhibition in dogs, and to determine if this technique could reliably and accurately predict the degree of *in vivo* aspirin-induced platelet dysfunction. Additionally, in dogs receiving low-dose aspirin, an attempt was made correlate the results of turbidimetric aggregometry with point-of-care PFA results.

Materials and methods

Study Population:

Healthy client-owned adult dogs were recruited from students, staff, clients, and faculty associated with the Mississippi State University College of Veterinary Medicine. Informed owner consent was obtained before initiation of the study. The dogs did not

receive any medications or vaccines for at least two weeks prior to the initiation of the study, and no medications, other than aspirin, were administered during the testing period. Aspirin dosing was based on body weight obtained at the beginning of the study. Weight was rounded to the nearest whole kilogram. Normal health status was established via physical examination, complete blood count, serum chemistry, urinalysis, heartworm testing and platelet function analysis using a point-of-care platelet function analyzer[(a)] (collagen/ADP cartridge[(b)]). Dogs were excluded from the study if they were found to have abnormalities on physical examination, urinalysis, complete blood count or serum chemistry that could impact platelet function, heartworm or tick-borne disease, or platelet abnormalities based on either an abnormal platelet count or prolonged PFA closure time. Animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee.

Study Design

Aspirin[(c)] was administered orally to each dog at a dose of 1 mg/kg once daily in the morning for 7 days. Based on initial bodyweight, aspirin doses were compounded into capsules by the Mississippi State University College of Veterinary Medicine Pharmacy. Blood and urine samples were collected from all study participants for platelet function evaluation using platelet aggregometry and PFA[(a)] (collagen/epinephrine cartridge[(d)]) and urinary 11-dTXB$_2$ analysis on Day 0 (prior to initiating aspirin), and again on Days 3 and 7 during aspirin administration. Blood samples were collected via jugular venipuncture with a 20 gauge needle directly into a 4.5 ml vacutainer tube containing 3.2% sodium citrate[(e)] or hirudin [(f)] anticoagulant. Each sample was collected until the tube was completely filled to standardize the degree of anticoagulation. For

urine samples, an initial attempt was made to collect urine via free catch but, if unsuccessful, cystocentesis using a 22-gauge 1.5 inch needle with a 12 ml syringe was performed. The volume of urine collected varied between 3 and 10 mls. Blood and urine samples were collected between 2 and 5 hours, and 2 and 8 hours, after drug administration, respectively.

Platelet Aggregometry

Platelet aggregometry was performed using three different methods: turbidimetric and impedance aggregometry using a conventional aggregometer, and impedance aggregometry using a more recently developed multiple electrode aggregometer.

Conventional Aggregometer

A combined turbidimetric and impedance two channel platelet aggregometer[(g)] that allowed for two samples to be evaluated concurrently was used to analyze platelet aggregation in both platelet-rich plasma (turbidimetric) and whole blood (impedance). For each dog and time point, four total samples were analyzed, and the results were averaged to yield a single value at each time point. Aggregation was assessed using collagen as an agonist (final concentration 10 μg/mL[4-6)], a temperature of 37°C, and a stirring speed of 1,200 rpm. All samples were analyzed within four hours of collection. When compared to baseline (Day 0), a dog was considered to be an aspirin-responder if there was a greater than 25% reduction in maximal aggregation compared to pre-treatment values.[20]

Turbidimetric Aggregometry

To harvest platelet-rich plasma (PRP), whole blood collected into 3.2% sodium citrate was centrifuged at 1,200 rpm at room temperature for 3 minutes. Supernatant PRP was collected, and the remaining blood sample was centrifuged at 1,800 rpm at room temperature for 8 minutes to create platelet-poor plasma (PPP).

Samples were analyzed based on the manufacturer's standard guidelines[(h)]. Briefly, 450 µl of PRP was transferred into a glass cuvette containing a siliconized magnetic stir bar, and 500 µl of PPP was placed into a cuvette without a stir bar. Samples were incubated, at 37°C, for 5 minutes, and placed into the aggregometer, and stable baseline values for minimal (PRP) and maximal (PPP) aggregation were obtained (0% and 100%, respectively). Collagen was then added to the PRP sample, and aggregation was monitored for 12 minutes. The maximal percentage aggregation was calculated and recorded by using a program provided with the aggregometer[(i)].

Impedance Aggregometry

Samples were analyzed based on previously described protocols and the manufacturer's standard guidelines[(h)]. Briefly, 450 µl of saline and then 450 µl of citrated whole blood were transferred to a plastic cuvette containing a magnetic stir bar. Following incubation at 37°C for 5 minutes, samples were placed into the aggregometer, an impedance probe was inserted into each cuvette, and a stable baseline was obtained. Collagen was then added to the sample and aggregation was monitored for 12 minutes. The maximal amplitude, measured in ohms, was calculated and recorded as an indicator of maximal aggregation.

Multiple Electrode Aggregometer

Additional impedance analysis was performed using a recently available multiple electrode aggregometer[(j)], and analysis was based on the manufacturer's recommendations[(k)]. Aggregation was assessed using a collagen concentration of 10 μg/mL[4-6], a temperature of 37ºC, and a stirring speed of 1,200 rpm. 3 mls of blood was collected directly into a hirudin anticoagulant tube and transferred into a single-use test cell containing a dual sensor unit, a Teflon-coated magnetic stir bar, and warmed saline. Volumes for analysis were controlled by the automated pipette provided with the instrument. Within the dual sensor unit, the electrical resistance between the sensor wires was recorded. The change in impedance caused by platelet adhesion and aggregation on the electrodes was continuously detected. Collagen was added to the sample and aggregation was monitored for 12 minutes. Platelet aggregation was analyzed and recorded as area under the curve (AUC). For each dog and time point, four total samples were analyzed, and the results were averaged to yield a single value at each time point. All samples were analyzed within four hours of collection. When compared to baseline (Day 0), a dog was considered to be an aspirin-responder if there a greater than 25% reduction in maximal aggregation compared to pre-treatment values.[20]

Platelet Function Analysis

A commercial point-of-care platelet function analyzer[(a)] that has previously been evaluated for use in dogs[16,21-23] was used to analyze platelet function. This PFA is an *in vitro* platelet function analyzer that mimics a high shear force over an area of vascular damage, and stimulates platelet function with several platelet agonists to measure the closure time, in seconds, needed to form a platelet plug and inhibit blood flow. The cut-

off time for the instrument is >300 seconds. All cartridges were stored at 4°C and warmed to room temperature prior to analysis.

Samples were collected directly into 4.5 ml blood collection tubes containing 3.2% sodium citrate, gently mixed, and kept at room temperature without agitation until analysis. The instrument was used according to manufacturer's instructions. For analysis, 800 µl of whole blood was transferred into either a collagen/ADP cartridge (performed initially to establish normal platelet function) or collagen/epinephrine cartridge (used throughout the study to assess aspirin-associated platelet dysfunction). Two collagen/epinephrine cartridges were analyzed at each time point for all dogs, and the closure times were averaged. All samples were analyzed within four hours of collection.

A dog was considered to be an aspirin-responder if the closure time on Day 7 was >300 seconds (ie. both cartridges were >300 seconds).[24] If, on Day 7, a dog had one cartridge <300 seconds and one cartridge >300 seconds, a third, 'tie-breaker' sample was analyzed.

In Vitro Aspirin Incubation

Following at least a two week washout period where no medications were administered, an additional 12 mls of blood was collected from each dog into 3.2% sodium citrate anticoagulant.[25] Acetylsalicylic acid was prepared via dissolution in 100% ethanol to create a 400 µM solution. *In vitro* incubation of platelets with acetylsalicylic acid was then performed, based on a previously described protocol.[19] Briefly, 100 µM of acetylsalicylic acid was added to 1 ml of PRP prepared by the previously described

technique, and incubated at room temperature for 15 minutes. Following this incubation, platelet aggregation was assessed via turbidimetric aggregometry, using the same technique that was previously described. All samples were analyzed in quadruplet and maximal percentage amplitude was averaged. To ensure that 100% ethanol alone would not influence platelet aggregation, PRP was also incubated for 15 minutes at room temperature with an equal volume of 100% ethanol for each dog. A dog was considered to be an aspirin-responder if there was greater than 25% reduction from Day 0 (baseline) results in the maximal percentage aggregation.[20]

Urine 11-dehydro-thromboxane B_2 Analysis

Urinary 11-dehydro-thromboxane B_2 (11-dTXB_2) concentration was analyzed using a commercially available enzyme-linked immunosorbent assay kit[(i)] that has been previously validated in the dog.[26] Urine samples was collected via free catch or cystocentesis and stored at -80°C for later analysis. Prior to analysis samples were thawed to room temperature and the assay buffer was used to dilute each sample. A correction factor was applied to account for these dilutions. Samples were analyzed in triplicate according to the manufacturer's instructions[(i)], and reported in picograms per milliliter of urine. Briefly, a 96-well plate was prepared by adding 50 µl of each dilute sample (vortexed thoroughly), 50 µl of 11-dTXB_2 monoclonal AChE tracer and 50 µl of 11-dTXB_2 monoclonal antibody to each well. Each plate was incubated at room temperature, in the dark and on an orbital shaker, for two hours, followed by the addition of Ellman's Reagent. Following a one hour incubation at room temperature, in the dark on an orbital shaker, each plate was analyzed a wavelength of 412 nm using a plate

reader[j] . Urine creatinine concentration was measured using a biochemistry analyzer[k] and used to calculate a urinary 11-TXB_2-to-creatinine ratio.[27,28]

Statistical Methods:

The means of the replications of measurement for each sample by each instrument were used in the statistical analyses. Histograms were used to visually assess if the turbidimetric, impedance, multiple electrode impedance, PFA and *in vitro* aspirin incubation aggregometry measurements were normally distributed using PROC UNIVARIATE in SAS for Windows 9.3[l]. The variables were found to not be normally distributed. Accordingly, a nonparametric analysis, Spearman Rank Correlation, using PROC CORR in SAS for Windows 9.3 was used to assess the correlation among the variables. A p value ≤ 0.05 was considered significant for all analyses.

Dogs were categorized as aspirin-responders or non-responders according to the results of each instrument for the Day 3 and Day 7 measurements. Agreement between the turbidimetric aggregometry response and each of the other instruments as well as between impedance and PFA results was assessed for Day 3 and Day 7 using PROC FREQ in SAS for Windows 9.3. Due to the sparse data in some cells, exact methods were used to determine the Kappa statistic. A scale proposed by Landis and Koch[29] (1977) was used to classify the strength of agreement. According to these guidelines, Kappa less than 0 equals "poor" agreement, 0.00 – 0.20 as "slight", 0.21 – 0.40 as "fair", 0.41-0.60 as "moderate", 0.61-0.80 as "substantial" and 0.81-1.00 as "almost perfect". An alpha level of 0.05 was used to determine statistical significance for all methods.

Results

Study Group Characteristics

A total of 36 dogs were initially screened for inclusion in the study, but 18 dogs were excluded due to problems with availability during the study period, positive results on infectious disease screening, pre-treatment thrombocytopenia, persistent lipemia, and the need for unexpected and unrelated surgery during the study period. Two further dogs were excluded from the study because an initial baseline PFA collagen/epinephrine cartridge closure time was greater than 300 seconds. A total of 16 dogs met the inclusion criteria and participated in the study. Mixed breed dogs were most common (n=4). There were 9 different breeds of purebred dogs, including Australian Shepherds (n = 2), Labrador Retrievers (n=2), Pit bull terriers (n=2), and one of each of the following: Alapaha Bulldog, German Shorthaired Pointer, Great Dane, Greyhound, Pembroke Welsh Corgi, and Standard Poodle. The study population consisted of 6 neutered males, 2 intact males, 7 spayed females, and 1 intact female. The mean age was 4.75 years (range, 1-9 years old) and the mean body weight was 29.07 kg (range, 14.2-50.5 kg). The 16 dogs received aspirin for 7 days with no reported adverse effects.

Conventional Turbidimetric Aggregometry

The mean turbidimetric aggregometry amplitude for all 16 dogs on Day 0 was 54.4% (range, 28.8-71.5%), while the mean amplitude for all dogs on Day 3 and Day 7 was 28.2% (range, 0.8-62.8%) and 20.6% (range, 0.8-55.8%), respectively. When compared to Day 0, there was a significant decrease in amplitude at both Day 3 ($p<0.001$) and Day 7 ($p<0.001$). There was no significant difference in amplitude between Days 3 and 7 ($p=0.414$). Mean turbidimetric amplitude was decreased by >25% (aspirin-

responsiveness) in 13 dogs (81.3%) at Day 3 and in 13 dogs (81.3%) at Day 7. Only two dogs were considered to be non-responders on both days. Two dogs inconsistently responded to low-dose aspirin between Day 3 and 7: one dog was classified as a responder on Day 3 and a non-responder on Day 7, and a second dog was classified as a non-responder on Day 3 and a responder on Day 7.

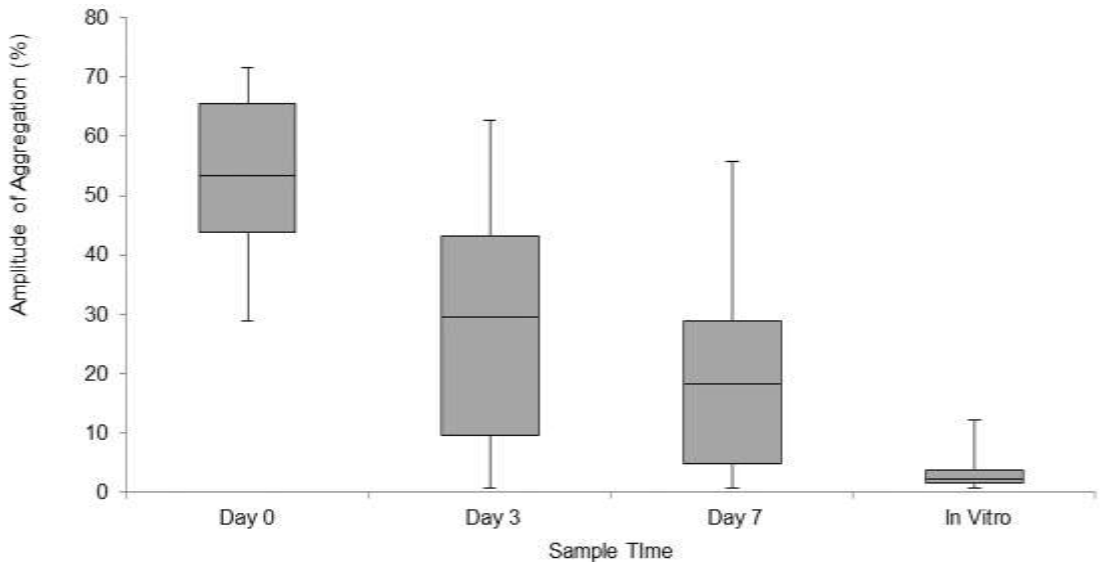

Figure 2.1 Amplitude (percentage) of aggregation of turbidimetric aggregometry

Turbidimetric aggregometry in 16 healthy dogs during treatment with low-dose aspirin (1 mg/kg PO every 24 hours for 7 days). There was a significant decrease in amplitude from Day 0 to Day 3 ($P<0.001$) (*) and from Day 0 to Day 7 ($P<0.001$) (**). There was no significant difference between Day 3 and Day 7. *In vitro* incubation with aspirin is shown for comparison. The box and whiskers plot demonstrates the median (line), interquartile range (box), and total range (whiskers).

Conventional Impedance Aggregometry

The mean impedance aggregometry amplitude for all dogs on Day 0 was 5.5 ohm (range, 0.8-13.8), while the mean amplitudes for all dogs on Day 3 and Day 7 were 2.4 ohm (range, 0-13.8 ohm) and 1.8 ohm (0-13 ohm), respectively. Due to unexpected machine error or insufficient sample volume, samples were only analyzed in triplicate (rather than quadruplicate) in five different dogs: 2 dogs on Day 0, 2 dogs on Day 3, and one dog on Day 7. When this occurred, the 3 results were averaged and incorporated into the analysis. When compared to Day 0, there was a significant decrease in amplitude at both Day 3 ($p<0.001$) and Day 7 ($p<0.001$). There was no significant difference in amplitude between Days 3 and 7 ($p=1.00$). Mean impedance amplitude was decreased by

>25% (aspirin-responsiveness) in 13 dogs (81.3%) at Day 3 and in 13 dogs (81.3%) at Day 7. One dog was considered to be a non-responder on both days. Four dogs inconsistently responded to low-dose aspirin between Day 3 and 7: two dogs were classified as responders on Day 3 and non-responders on Day 7, and two dogs were classified as non-responders on Day 3 and responders on Day 7.

When compared to aspirin responsiveness as determined by turbidimetric aggregometry, impedance aggregometry identified all of the same aspirin responders and non-responders on Day 3. However impedance only found 10 of the 16 dogs to have the same classification of aspirin response as turbidimetric on Day 7 (all were classified as aspirin responders). Impedance aggregometry results did not identify as non-responders any of the 3 dogs that were classified as aspirin non-responders via turbidimetric aggregometry on Day 7.

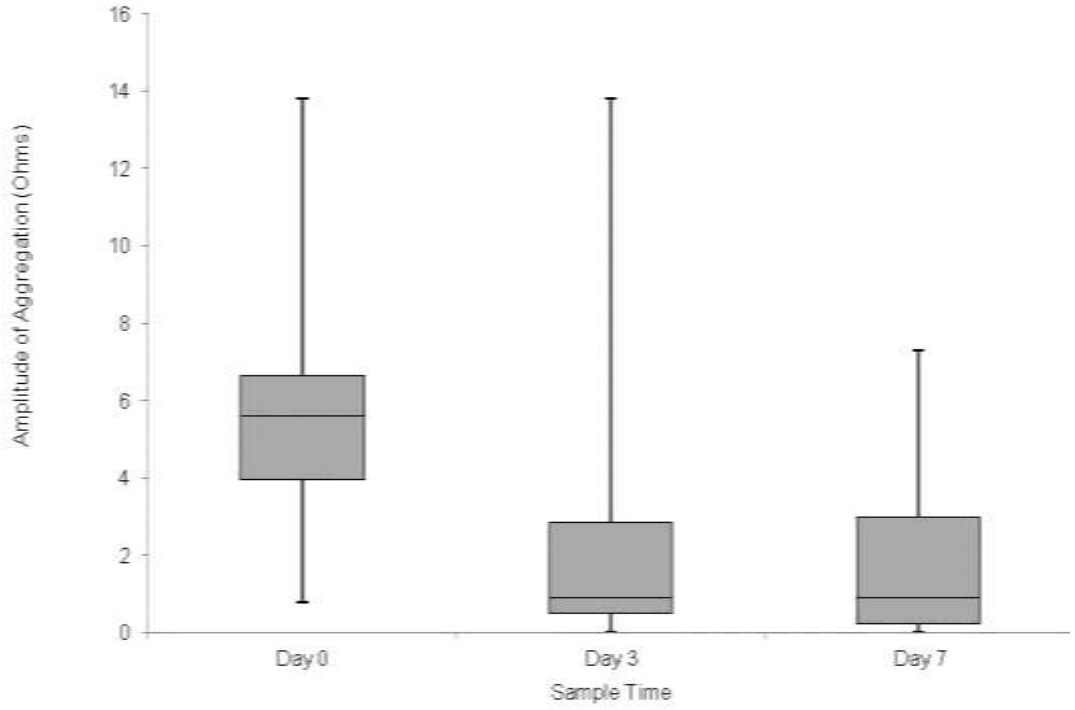

Figure 2.2 Amplitude of aggregation of impedance aggregometry in ohms

Impedance aggregometry in16 healthy dogs during treatment with low-dose aspirin (1 mg/kg PO every 24 hours for 7 days). There was a significant decrease in amplitude from Day 0 to Day 3 ($P<0.001$) (*) and from Day 0 to Day 7 ($P<0.001$) (**). There was no significant difference between Day 3 and Day 7 ($P=1.0$). The box and whiskers plot demonstrates the median (line), interquartile range (box), and total range (whiskers).

Multiple Electrode Aggregometer

Multiple electrode aggregometry results were only available for 9/16 dogs due to loss of instrument availability. Mean AUC for all 9 dogs on Day 0 was 1,681.7 U (range, 1,042.5-2,304.3 U). Mean AUC for Days 3 and 7 was 1,762.1 U (range, 1,280-2,944.8 U) and 1,960.9 U (range, 1,107.3-2,514.5 U) respectively. There were no significant differences in the AUC between Days 0 and 3, Days 0 and 7, and Days 3 and 7. Eight of the 9 dogs on Day 3 and all of the dogs on Day 7 were classified as aspirin non-responders.

When compared to aspirin responsiveness as determined by turbidimetric aggregometry, the multiple electrode aggregometer did not identify any of the same responders or non-responders on Day 3. In fact, the only aspirin responder identified via the multiple electrode aggregometer on Day 3 was classified as a non-responder with turbidimetric aggregometry. On Day 7, only one dog had the same classification (non-responder) with the multiple electrode aggregometer as with turbidimetric aggregometry.

Platelet Function Analysis

The mean point-of-care PFA collagen/epinephrine closure time for all 16 dogs on Day 0 was 119.7 seconds (range, 90-163 seconds), while the mean closure time of all dogs on Days 3 and 7 was 214 seconds (range, 112-300 seconds) and 232.7 seconds (range, 157.5-300 seconds), respectively. When compared to Day 0, there was a significant increase in closure times at both Day 3 ($p < 0.001$) and Day 7 ($p < 0.001$). There was no significant difference in closure times between Days 3 and 7 ($P=0.634$). The mean PFA closure time was greater than 300 seconds (aspirin-responsiveness) in 4 dogs (25%) on Day 3 and 7 dogs (43.8%) on Day 7. However, only 3 dogs were consistently greater than 300 seconds on both days. On Day 7, 5 dogs (31.3%) required a third sample to assist in the classification of aspirin responders or non-responders.

When compared to aspirin responsiveness as determined by turbidimetric aggregometry, PFA results only identified 4 of the same aspirin responders and 3 of the same non-responders on Day 3, and identified 5 of the same responders and only one non-responder on Day 7. PFA results identified the same categorization as turbidimetric aggregometry (responder versus non-responder) at Day 7 in 6/16 dogs. The PFA only

identified one of 3 dogs that were classified as non-responders by turbidimetric aggregometry on Day 7.

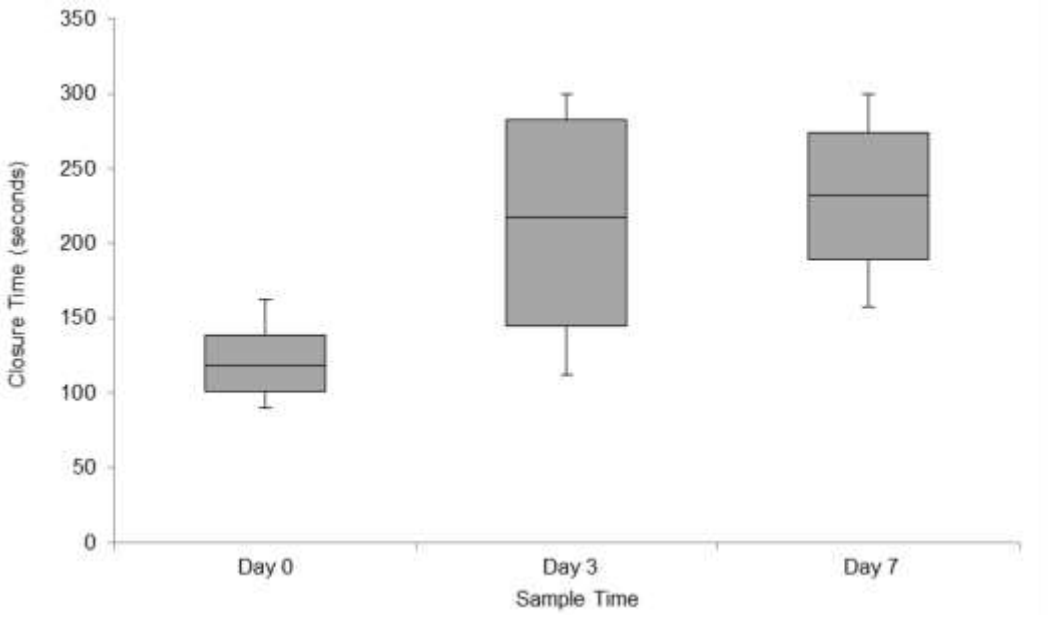

Figure 2.3 PFA closure times

PFA-100 closure times in 16 healthy dogs during treatment with low-dose aspirin (1 mg/kg PO every 24 hours for 7 days). There was a significant increase in closure time from Day 0 to Day 3 ($P<0.001$) (*) and from Day 0 to Day 7 ($P<0.001$) (**). There was no significant difference between Day 3 and Day 7 ($P=0.634$). The box and whiskers plot demonstrates the median (line), interquartile range (box), and total range (whiskers).

In Vitro Turbidimetric Aggregometry

The mean turbidimetric amplitude for all dogs following *in vitro* incubation with salicylic acid was 4% (range, 1-10%). The mean percent reduction from baseline for amplitude was 91.9% (range, 66.7-98%). All of the dogs demonstrated marked suppression in amplitude after *in vitro* incubation with salicylic acid, however, some residual platelet function did persist in three dogs as evidenced by weak aggregation.

Nevertheless, the reduction in amplitude from baseline was greater than 25% in all dogs, which categorized them all as aspirin-responders.

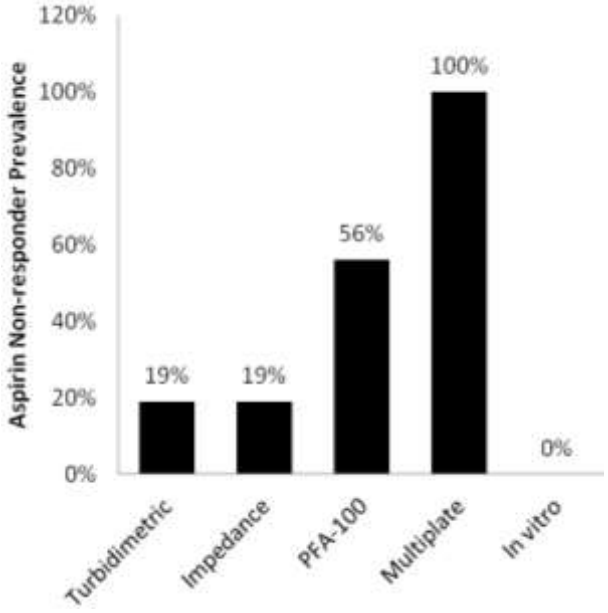

Figure 2.4 Prevalence of aspirin non-responders

Identified based on platelet function testing performed.

Urine 11-dehydro-thromboxane B_2 Assay

The mean urine 11-dTXB$_2$-to-creatinine ratio on Day 0 was 4.8 (range, 2.0-9.1), while the median ratios for Day 3 and Day 7 were 3.5 (range, 0.8-8.1) and 3.3 (range, 0.8-13.5), respectively. There was a 34.7% decrease in the urine 11-dTXB$_2$-to-creatinine ratio between Day 0 and 3, a 49% decrease between Day 0 and 7, and 21.9% decrease between Day 3 and 10. There was no significant change in the urinary 11-dTXB$_2$-to-creatinine ratio between Day 0 to 3 ($p=0.315$) and Day 3 and 7 ($p=0.664$). However, there was a significant decrease from Day 0 to 7 ($p=0.028$). Urine 11-dTXB$_2$ results

from 3 dogs on Day 0 and 2 dogs on Day 7 were unavailable because the concentrations were below the detection level of the assay.

Correlation

When the data from all three sample days were combined, the results from turbidimetric aggregometry were significantly correlated with impedance (r=0.63, p<0.001) and PFA (r=-0.56, p<0.001). In addition, impedance aggregometry was significantly correlated with PFA results (r=-0.55, p<0.001). When the data for each day were analyzed separately, no significant correlations were detected on Day 0 (p>0.099) or Day 7 (p>0.197). On Day 3, turbidimetric aggregometry was significantly correlated with impedance aggregometry (r=0.66, p=0.0052). Impedance aggregometry was correlated with PFA results (r=-0.49) with a p value approaching the level of significance (p=0.0532).

Agreement

The Kappa statistic was used as a measure of agreement for classification as aspirin-responder or non-responder between turbidimetric aggregometry and the other tests of platelet function, and between impedance aggregometry and PFA results. Agreement between aspirin responders or non-responders was determined for both Day 3 and Day 7. The only significant agreement occurred on Day 3 with perfect agreement (Kappa=1.00, p=0.002) between turbidimetric and impedance aggregometry.

Discussion

When discussing the concept of aspirin resistance it is important to differentiate between "clinical" aspirin resistance and "laboratory" aspirin resistance. Clinical aspirin

resistance occurs when low dose aspirin does not protect individuals from a thromboembolic event. Laboratory aspirin resistance is when aspirin does not show an antiplatelet effect when assessed with *in vitro* tests of platelet function. Ideally, these two concepts would be correlated allowing for the prediction of clinical aspirin resistance through the identification of laboratory aspirin resistance. In our study, we attempted to better evaluate multiple laboratory methods in order to work toward better prediction and therefore improved clinical handling of patients with possible aspirin resistance.

Turbidimetric aggregometry is considered to be the gold standard method for assessing drug-induced platelet dysfunction in humans receiving aspirin. In dogs receiving aspirin, turbidimetric aggregometry was the most consistent of all of the tests of platelet function used in our study at identifying aspirin-responders or non-responders on both Days 3 and 7. With turbidimetric aggregometry, there were only two dogs that were inconsistently identified as either a responder or non-responder between Day 3 and Day 7. Impedance aggregometry was the second most consistent methodology at identifying aspirin responders or non-responders, with 4 dogs being inconsistently classified between Day 3 and Day 7, and the point-of-care PFA was the third most consistent instrument with 5 inconsistent classifications between Day 3 and Day 7. Our results are comparable to those of a previous study that evaluated the anti-platelet effects of low-dose aspirin in dogs [15], which also demonstrated inconsistencies in the aspirin-response classification of individual dogs on different days. Since, the anti-platelet effects of aspirin are typically expected to occur within minutes of ingestion of acetylsalicylate in people,[30] the platelet function results on Day 3 and Day 7 would be expected to be comparable, suggesting that the tests used in our study do not reliably detect aspirin-responsiveness in individual dogs

on a day-by-day basis. However, alternatively, the discrepancy between Day 3 and Day 7 results may not reflect inconsistent aspirin-responsiveness, but rather may reflect delayed drug effects that may not be fully evident until Day 7. In some dogs, the response to aspirin appears to change over time. One dog, based on turbidimetric results, and 2 dogs, based on impedance results, were classified as non-responders on Day 3 but as aspirin-responders on Day 7. These findings may suggest a progressive inhibition in platelet function during aspirin administration. However, in contrast, one dog, based on turbidimetric results, and 2 dogs, based on impedance results, were classified as aspirin-responders on Day 3 but as non-responders on Day 7. Possible explanations for variability in results include decreased drug bioavailability, poor owner compliance with drug administration, increased platelet turnover to release non-aspirinated platelets, or expression of a variant COX-I enzyme which is less sensitive to aspirin inhibition.

Since turbidimetric aggregometry is the historical gold standard for assessment of platelet function in patients receiving aspirin, in our study we chose to compare the results of all other diagnostic tests with this methodology. When comparing the response classifications between turbidimetric and impedance aggregometry, there was perfect agreement on Day 3, with 19% of dogs being classified as aspirin non-responders. However, on Day 7, there was not complete agreement between turbidimetric and impedance aggregometry. Although both techniques identified 3 dogs (19%) as aspirin non-responders, each technique identified a different 3 dogs as non-responders. In a comparable study performed in humans that were receiving low-dose aspirin, 6 different tests of platelet function were utilized to assess the anti-platelet effects of aspirin and the prevalence of aspirin resistance. Turbidimetric aggregometry was also used as the gold

standard in the comparable human study, which found that impedance aggregometry had the highest correlation and the second highest agreement with turbidimetric aggregometry when classifying patients as aspirin resistant. Interestingly, based on the results of impedance aggregometry, the prevalence of aspirin resistance in humans was 18%, similar to the 19% of dogs in our study classified as non-responsive to the anti-platelet effects of aspirin.[17] Based on our study, impedance aggregometry may provide useful data regarding aspirin-induced platelet dysfunction in dogs when turbidimetric aggregometry is not feasible or not available, although impedance results may vary from turbidimetric results in some dogs on some days.

Recently, an impedance-type multiple electrode aggregometer was validated for use in dogs, and we therefore chose to also utilize this aggregometer in our study.[18] In dogs receiving aspirin, we detected only one aspirin-responder on Day 3 using this aggregometer, and not a single aspirin-responder on Day 7. Based on visual assessment of the curves generated by the instrument, the multiple electrode aggregometer protocol used in our study detected little to no aspirin-associated platelet dysfunction, a finding that is very discrepant with the results obtained with other tests of platelet function. Platelet aggregation was inconsistent and often demonstrated an increase in aggregation during the course of aspirin administration. Even though the multiple electrode aggregometer is technically very user friendly compared to the other conventional aggregometer used in our study, our results suggest that this methodology does not provide an accurate assessment of aspirin-associated platelet dysfunction in dogs receiving aspirin, at lease using the protocol that we utilized. Our protocol used collagen as an agonist to closely match the protocols used in the conventional aggregometer, and

to match published protocols used to evaluate human aspirin responsiveness. The use of different agonists, such as arachidonic acid, may provide a better assessment of aspirin-induced platelet dysfunction using the multiple electrode aggregometer.[31] Additionally, because the multiple electrode aggregometer was only used to analyze 9 dogs, our results did not reach a high enough power for a true evaluation of correlation with other methods. However, based on an almost complete failure of the multiple electrode aggregometer to detect aspirin-associated platelet dysfunction, it is highly unlikely that the use of additional dogs would have significantly improved correlation or agreement.

Similar to previous studies in dogs[15,16,22], our study confirmed that aspirin-associated platelet function could be detected utilizing a user-friendly point-of-care PFA with a collagen/epinephrine cartridge, and that PFA closure times significantly correlated with both turbidimetric and impedance aggregometry results. However, when considering drug responsiveness in dogs receiving aspirin, there was poor agreement between the point-of-care PFA and turbidimetric aggregometry. On Day 7 of aspirin therapy, based on turbidimetric analysis, 19% of dogs were classified non-responsive while, based PFA results, 56% of dogs were classified as non-responsive to aspirin. The percentage of aspirin resistance in our study, based on PFA results, was somewhat greater than a previous study using the same PFA protocol in dogs, which demonstrated an aspirin resistance prevalence of about 33%.[15] In a comparable human study, the prevalence of aspirin resistance classified using the same point-of-care PFA and collagen/epinephrine cartridge used in our study was 59.5% of the study population.[17] In all of these studies, the point-of-care PFA, even though it is user-friendly and often used to clinically evaluate platelet function in human patients receiving aspirin, markedly

overestimates the degree of aspirin resistance compared to the gold standard turbidimetric aggregometry, and therefore may not be the ideal instrument to use when determining if a dog is truly aspirin resistant.

Weber *et al* previously described an *in vitro* technique that involves the incubation of PRP with acetylsalicylic acid and analysis via turbidimetric aggregometry to both diagnosis and categorize aspirin resistance in humans.[19] In humans, aspirin resistance can be categorized into different types based on the results of a panel of tests including evaluation of platelet function and measurement of degree of thromboxane suppression after patent treatment with aspirin, and evaluation of platelet function after *in vitro* incubation of patient platelets with acetylsalicylic acid.[19] Although, in people, the *in vitro* acetylsalicylic acid incubation technique is typically performed during a period of aspirin administration, the goal of our project was to see if this technique could also be used to predict aspirin responsiveness prior to drug administration. In our study, canine platelet function as measured by turbidometric aggregometry following *in vitro* incubation with acetylsalicylic acid was very consistently inhibited in all dogs. As a result, results of our *in vitro* acetylsalicylic acid incubation testing showed poor agreement with conventional turbidimetric and impedance aggregometry, with multiple electrode aggregometry, and with PFA closure times, suggesting that our particular *in vitro* technique is not effective at predicting aspirin resistance in dogs. The *in vitro* incubation technique used in our study was modeled on a comparable human study, and used the same acetylsalicylic acid concentration (10μM) as previously described.[19] However, since the pharmacokinetic profile of low-dose aspirin in dogs has not been established, it is unknown if the quantity of acetylsalicylic acid used in our incubation

study correlates with aspirin blood levels in dogs receiving low-dose therapy. One potential explanation for the high incidence of apparent 'aspirin-responders' as classified by our *in vitro* acetylsalicylic acid incubation study could be that the concentration of acetylsalicylic acid used was too high, potentially mimicking the effects of higher doses of aspirin that have been shown to consistently inhibit platelet function in most dogs.[11,16,22,26] Incubation studies using different concentrations and incubation times of acetylsalicylic acid may provide for a more realistic assessment of low-dose aspirin therapy.[32,33] However, the concentration of acetylsalicylic acid used in our incubation study was identical to that used in incubation studies in human patients receiving low-dose aspirin[19] and, at least at higher anti-inflammatory oral doses of aspirin, the pharmacokinetic profile of salicylate in dogs has been shown to approximate that of humans.[34-38] A pharmacokinetic study of salicylate levels in dogs receiving oral low-dose aspirin would be needed to facilitate accurate optimization of acetylsalicylic acid concentrations in a dog species-specific aspirin incubation study. In our laboratory, the only solution that allowed for proper dissolution of the acetylsalicylic acid was 100% ethanol. To ensure that the ethanol itself did not alter platelet function, samples were analyzed following incubation with pure ethanol. All dogs had strong, normal turbidimetric platelet aggregation, suggesting that the ethanol did not markedly influence platelet function.

Previous studies in humans have used the stable thromboxane A_2 metabolite 11-dehydro-thromboxane B_2 as an indicator of aspirin-induced COX inhibition.[9,28] Measurement of thromboxane metabolites in plasma or serum can cause artifactual increases because platelets can be activated during blood collection, storage, or

processing. Measurement of these metabolites in urine, in contrast, circumvents this complication, and is therefore considered a more reliable indicator of systemic thromboxane synthesis.[9,10] A previous study in dogs has demonstrated that, compared to the 11-dTXB$_2$ assay used in our study, urinary 2,3-dinorTXB$_2$ levels were a more sensitive indicator of aspirin-induced thromboxane inhibition.[26] However, although the urine 11-dTXB$_2$ assay used in our study is less sensitive than the 2,3-dinorTXB$_2$ assay, we have previously established that the 11-dTXB$_2$ assay is still sufficiently sensitive to detect aspirin-induced COX inhibition in dogs receiving low-dose aspirin.[15] One proposed mechanism of aspirin resistance in humans is a variable responsiveness of platelets to thromboxane and, in some dogs, it also appears as if thromboxane may not be a consistent or necessary platelet agonist. In fact, it has previously been shown that about 70% of canine platelets are insensitive to thromboxane stimulation due to impaired platelet thromboxane A$_2$ receptor-linked G proteins.[39] Based on our turbidimetric aggregometry results, 2 dogs were inconsistently classified as non-responders (platelet inhibition on only one of the 2 days that were evaluated), despite the fact that urinary 11-dTXB$_2$-to-creatinines ratio steadily declined from Day 0 to Day 7 in both dogs. Interestingly, 3 of the dogs that were classified as aspirin responders based on turbidimetric aggregometry actually had an increase in urinary 11-dTXB$_2$-to-creatinine ratios with aspirin therapy. Our results, particularly the findings that aspirin non-responsiveness was detected in several dogs despite documentation of suppressed thromboxane synthesis, and that there was no correlation between turbidimetric aggregometry and urine 11-dTXB$_2$-to-creatinine ratios, suggest that aspirin resistance in some dogs, as in humans, is due to more than a simple failure to suppress COX enzymes.

Further studies using the more sensitive urinary 2,3-dinorTXB_2 assay (unfortunately, currently not commercially available) would be needed to better define the role of thromboxane in aspirin resistance. Potential thromboxane-independent causes of aspirin resistance in dogs, as in humans, include decreased drug bioavailability, poor compliance, and platelet activation via alternative agonists such as ADP. Based on our results, aspirin resistance in dogs, as in people, appears to be multifactorial, and the precise cause may vary amongst patients.

Commonly published 'low doses' of aspirin for platelet inhibition in dogs range from 0.5 mg/kg once daily to 1 mg/kg once daily.[15,26,40-42] Interestingly, much higher doses of aspirin, such as 10 mg/kg orally twice daily, have been shown to reliably inhibit platelet function in all dogs, confirming that aspirin-associated platelet dysfunction is highly dose-dependent. It is therefore possible that one potential cause of 'aspirin resistance' in dogs receiving low (0.5 to 1 mg/kg once daily) doses of aspirin is simple drug under dosage, and that simply increasing the dose of aspirin that is considered to be 'low-dose' would eliminate aspirin non-responsiveness in some dog. Further studies would be needed to determine the dose (or dose range) of aspirin that maximizes inhibition of thromboxane synthesis and platelet function without the undesirable effect of also inhibiting prostacyclin synthesis.

One limitation of our study is that, by using dogs from a community-owned rather than research population, we were unable to precisely control all aspects of the dog's environment. Evaluation of a wide variety of dog breeds rather than a relatively homogenous research dog population allowed for a more clinically relevant assessment of aspirin resistance in the general canine population. However, although not reported by

the owners, some community-owned dogs could have potentially been exposed to non-aspirin medication. Additionally, even though it was also not reported by owners, poor compliance could have contributed to inconsistent aspirin responses. Another limitation of our study was that urine from each dog on each day was not collected at the same time. Although an attempt was made to collect urine at the same time each day, some dogs urinated prior to admission or had a minimal amount of urine in the bladder. Variation in time of collection after aspirin administration may potentially have affected urine thromboxane levels. Finally, our population of healthy dogs had no obvious health problems, and the results of our study therefore may not be directly applicable to the diseased clinic population. Further research would need to be done looking at populations of dogs with specific disorders.

In conclusion, our study demonstrated that most, but not all, dogs respond to the anti-platelet effects of low-dose aspirin as measured by conventional aggregometry. Conventional impedance aggregometry results correlate with turbidimetric aggregometry results with reasonable but not perfect agreement while, in contrast, multiple electrode impedance aggregometer results cannot be used to establish aspirin responsiveness. Platelet function as measured by a point-of-care PFA showed poor agreement with conventional aggregometry, and tended to overestimate the incidence of aspirin resistance, suggesting that this instrument may not provide a reliable measurement of aspirin-induced platelet dysfunction in dogs. Turbidimetric aggregometry after acetylsalicylic acid incubation using an *in vitro* methodology established in humans did not predict aspirin response in dogs.

Materials, Instruments, and Supplies

a. PFA-100®, Siemens Healthcare Diagnostics, Deerfield, IL

b. PFA Collagen/ADP Test Cartridge, Siemens Healthcare Diagnostics, Duluth, GA

c. Aspirin, Major Pharmaceuticals, Livonia, MI

d. PFA Collagen/EPI Test Cartridge, Siemens Healthcare Diagnostics, Duluth, GA

e. 3.2% sodium citrate, Vacutainer tube, Becton Dickinson, Franklin Lakes, NJ

f. Hirudin Vacutainer tube, Verum Diagnostica GmbH, Munich, Germany

g. Chronolog 700 Whole Blood/Optical Lumi-Aggregometer, Chronolog Corporation, Haverton, PA

h. Chronolog 700 Manual, Chronolog Corporation, Haverton, PA

i. AGGRO/LINK 8, Chronolog Corporation, Haverton, PA

j. Multiplate® Analyzer, Verum Diagnostica GmbH, Munich, Germany

k. Multiplate® Analyzer Manual, Verum Diagnostica GmbH, Munich, Germany

l. 11-dehydro-thromboxane B_2 EIA kit-Monoclonal, Cayman Chemical Co, Ann Arbor, MI

m. SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, California

n. ACE Alera® Clinical Chemistry System, Alfa Wasserman, Inc., West Caldwell, NJ

o. SAS for Windows version 9.2, SAS Institute, Cary, NC, 2008

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CHAPTER III

CONCLUSION

In this study a variable rate of canine aspirin responders was identified. Using the gold standard method of turbidimetric aggregometry, approximately 81% of dogs would be considered to show a normal inhibition of platelet function when exposed to aspirin. These dogs would therefore be "aspirin responders". That leaves an additional 19% of dogs that did not show a significant inhibition of platelet aggregation defined as >25% reduction in maximum amplitude of aggregation.

The definition of a gold standard is that which is the best available under reasonable conditions.[1] This does not mean that it is always the best possible test in absolute terms. Turbidimetric aggregometry has long been the gold standard in human medicine for evaluating platelet aggregation. However, skepticism remains as to whether this is an appropriate distinction and there remains no standardized methods for performing this technique.[2,3] The use of turbidimetric aggregometry to assess platelet function in veterinary medicine has been extrapolated from its use in human medicine. It remains unproven whether that this extrapolation can truly be made. When performing our research we accepted the concept of turbidimetric as the gold standard, but the possible exceptions to this concept cannot be completely forgotten or disregarded.

With this in mind, our research also found impedance aggregometry to have the same percent of dogs identified as "aspirin responders" as the gold standard

turbidimetric. The two aggregometry methods did not, however, have good agreement and only moderate correlation. It was also noted that the two methods both classified three dogs as non-responders, but each method identified three different dogs.

Turbidimetric aggregometry was compared with the more user friendly options available, the Multiplate® and PFA-100. Multiplate® found all dogs to have poor response to aspirin administration in that all dogs continued to have normal aggregation readings even after being administered aspirin. Therefore, all dogs would be classified as "aspirin non-responders". This method had neither agreement nor correlation with any of the other methods. The PFA-100 found 44% of dogs have appropriate response to aspirin administration showing a closure time of >300 seconds on day 7. This test had poor agreement but moderate correlation with both turbidimetric and impedance aggregometry.

The results found with the more user friendly modalities bring into question their ability to replace or act as a surrogate for the more traditional aggregometry methods for the purpose of studying aspirin response. The Multiplate® does not appear to function at all as an indicator of aspirin induced platelet dysfunction. The PFA-100 only found the same final result as turbidimetric in 10 of 16 dogs. That means inappropriate therapy could potentially be administered based on these results. In this setting, PFA-100 is an unreliable method for evaluating platelet function in dogs given aspirin. It is not known if better agreement or correlation would be seen with higher aspirin doses or when used with other medications or conditions affecting platelet function. Further studies in this area would be needed.

Given the findings in this study, with the acceptance of turbidimetric as the gold standard, there does not appear that either of the more user friendly options can replace turbidimetric in this capacity. Furthermore, while impedance findings were similar, discordant results also do not make it a strong option for the replacement of turbidimetric aggregometry.

Aspirin was given for a total of 7 days with evaluation of function performed on days 3 and 7. In multiple cases, the results on day 3 did not match those on day 7. Some dogs progressed from a non-responder to a responder while others actually became less aspirin responsive over time, switching from a responder to a non-responder. The reasons for these findings are not completely known. Some dogs may have required more time for aspirin to have complete effect such that a progressive inhibition of platelet function was occurring. Other dogs may have become aspirin resistant due to any of a variety of causes such as changes in thromboxane production or response, increased platelet turnover to produce more non-aspirinated platelets, administration complications such as poor compliance, bioavailability issues, or co-administration of other NSAIDs.

In most dogs, a final aspirin response was seen by 3 days. This may indicate that evaluation of platelet function could be performed early on in therapy. This would shorten the time period of inappropriate treatment if a dog was an aspirin non-responder. Unfortunately, testing at 3 days would not necessarily prove that a non-responder would not further progress into a responder or rule out that resistance would not develop. Our study did not look beyond 7 days to determine if further changes in aspirin response developed with longer term use. Further research would be needed looking at these effects. Our study results do not allow for a recommendation of testing of platelet

function on day 3 of aspirin administration. Testing would need to be performed after at least 7 days of consecutive low dose aspirin administration. It would be possible that response time would vary and perhaps be faster in dogs given a higher dose of aspirin.

Not all dogs in this study showed consistent results throughout the testing period. A fair amount of individual variation was seen especially on day 3 but also on day 7. Some dogs had markedly different results even between samples run simultaneously under all the same conditions with blood coming from the same tube. These dogs may have had one sample that showed complete suppression of aggregation and another sample that showed normal aggregation. These dogs seemed to demonstrate a threshold response in which some samples may have been on the verge of aggregation and were therefore more sensitive and could be more easily tipped over into aggregation. This finding could also suggest a dose dependant mechanism. It may be that by making a small dose increase that this threshold response could be overcome, leading to more consistent aggregation. Further research looking at gradual dose escalation would be warranted to determine the optimal dose of aspirin that would inhibit platelet function while still allowing for the production of prostacyclin. The ideal dose would still be a low dose that could be given once daily, but would not cause any of the side effects seen with higher anti-inflammatory aspirin doses.

One of the major goals of this study was to find a way to more easily predict aspirin response. Unfortunately, none of the more user friendly options were consistent enough to replace turbidimetric in standard testing. We next looked at an *in vitro* method of evaluating aspirin induced platelet dysfunction. When platelet rich plasma was incubated with 100 uM of acetylsalicylic acid we found that platelet function was

strongly inhibited in all the dogs. A few dogs showed a very weak aggregation but still greatly reduced from baseline. This method, therefore, did not allow for prediction of individual aspirin response. The choice of protocol for the *in vitro* portion of this study was taken from a similar study done by Weber et al, which used human patients.[4] The goal was to mimic the results of their study to see if comparable results would be obtained. Their study found variation in turbidimetric aggregometry results in patients given oral aspirin in combination with *in vitro* aspirin incubation. When also combined with thromboxane levels, three different groups of aspirin responders were identified. Our study did not find that differences among dogs could be found using these techniques. Following the previous classification system discussed *in vitro* incubation found all the dogs to be aspirin responders.

The findings of our study do not rule out the possibility of using *in vitro* aspirin incubation to predict aspirin response. The methods we used may not be ideal for the canine patient. The aspirin levels obtained in the *in vitro* testing may far exceed what is reached *in vivo*. The concentration of aspirin we used may be more comparable with a high dose aspirin administration, which could lead to more extensive platelet suppression. It is also possible that canine platelets are more sensitive to the effects of thromboxane such that lower doses of aspirin have more profound effects then what is seen in people. This theory seems less likely give the fact that human patients in the Weber et al study showed marked suppression (>95%) of thromboxane levels while the dogs in our study showed a mean suppression of urine thromboxane levels of 49%.[4] Finally, it may simply be that the causes for aspirin resistance in dogs are different then what are seen in people.

The ways in which aspirin resistance are evaluated and classified in people may not extrapolate well to canine patients.

Urine thromboxane levels decreased significantly in all the dogs as a group. When looking at the three dogs that were classified as aspirin non-responders via turbidimetric as individuals, only 2 of the 3 dogs showed a decreased thromboxane level. This could indicate different mechanisms were responsible for their aspirin resistance. Dogs that did show a decreased urine thromboxane level appeared to be reacting in a normal manner to aspirin. In other words, the aspirin was working properly but for some reason the dogs still were not showing the anti-platelet effects that would be expected. This could be due to variability in thromboxane receptors between individuals. One of the dogs showed what appeared to be thromboxane independent aspirin resistance. This would imply that aspirin did not have the expected affect on suppression of thromboxane. This could be due to an alternative pathway formation of thromboxane or other factors such as affects on bioavailability, compliance, increased platelet turnover, etc.

Interestingly, several dogs that were classified as aspirin responders based on turbidimetric aggregometry results did not have a significant decrease or had an increase in thromboxane levels during treatment. Some dogs even showed a rebound with a decrease at day 3 followed by an increase of thromboxane on day 7. A possible cause for this is inaccuracy with the measurement of thromboxane. It has been proposed that 2,3 dinor thromboxane B_2 is a better metabolite for evaluation of aspirin response in dogs.[5] However, these results could also suggest that there are different ways in which aspirin affects platelet function. Aspirin has been shown to affect several aspects of secondary hemostasis including thrombin formation and proposed to inhibit tissue factor synthesis.[6]

It is possible that aspirin may also have additional affects on primary hemostasis not yet determined that not only affects platelet response to aspirin but allows for different avenues of aspirin resistance.

Ultimately the causes for aspirin resistance in dogs remains unknown and is likely multifactorial. The causes may be similar to what is suspected in humans but other mechanisms and causes cannot be ruled out. It may remain difficult to elucidate these causes due to what may be extensive individual variation. With further research, it may become possible to at least predict aspirin resistance in a global sense, even if the exact cause remains unknown.

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