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Effects of aspirin dose escalation on platelet function and urinary thromboxane and prostacyclin levels in normal dogs

By

Natalie Marie McLewee

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 Research
in the College of Veterinary Medicine

Mississippi State, Mississippi

May 2017

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Effects of aspirin dose escalation on platelet function and urinary thromboxane and prostacyclin levels in normal dogs

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Pages in Study: 75

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Eight dogs were enrolled in a randomized, cross-over study that used optical aggregometry and a platelet function analyzer to evaluate platelet function before and after the administration of 5 aspirin dosages: 0.5 mg/kg q24h, 1 mg/kg q24h, 2 mg/kg q24h, 4 mg/kg q24h and 10 mg/kg q12h. Urine 11-dehydro-thromboxane-B_2 (11-dTXB_2) and 6-keto-prostaglandin-F_{1alpha} (6-keto-PGF_{1alpha}), were measured. Compared to pre-treatment, there were significant decreases in maximum aggregometry amplitude and increases in PFA-100 closure times for all doses except 0.5 mg/kg q24h. There was no difference in amplitude or closure time between the 2 mg/kg, 4 mg/kg, and 10 mg/kg q12h dosages. At 2 mg/kg q24h, 100 percent (aggregometry) of dogs were aspirin responders. There was a significant decrease in urinary 11-dTXB_2- and 6-keto-PGF_{1alpha}-to-creatinine ratios with aspirin administration. An aspirin dosage of 2 mg/kg q24h consistently inhibits platelet function in healthy dogs without decreasing prostacyclin synthesis significantly more than lower aspirin dosages.

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DEDICATION

I would like to dedicate this research to my parents, Joseph Donald Schroeter and Josephine Maria Assunta Schroeter, for their unwavering support and trust. Their constant motivation and enthusiasm has fueled me throughout this journey and will continue to do so for the rest of my life and career. I promise to "Never, never, never give up!"

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CHAPTER I

INTRODUCTION

Platelet Formation and Function

Platelets are formed in the bone marrow from megakaryocytes, descendants of large hematopoietic stem cells. As megakaryocytes mature, they will undergo fragmentation that results in the production of platelets. [1-3] Synthesis of platelets is controlled by a hormone, thrombopoietin, which is synthesized in the bone marrow and smooth muscle cells. [4] Canine platelets have an average lifespan of approximately 6 days [5], which is similar to that of humans, averaging 5-7 days. [6]

Platelets play an essential role in hemostasis, a process which causes bleeding to stop. Normal hemostasis occurs in 3 steps: platelet plug formation (primary hemostasis), the clotting cascade (secondary hemostasis), and clot removal (fibrinolysis). Endothelial damage and normal endothelial cell turnover is a daily occurrence. Disease processes including infection, acidosis, hypoxemia, inflammation, and hypotension can also cause endothelial damage.[7] After endothelial damage has occurred, the smooth muscle of the vessel will contract causing vasoconstriction and a reduction in blood flow. Platelets will adhere to the damaged or disrupted endothelium forming a loose platelet plug. This process is known as primary hemostasis. The platelet plug is unstable and without reinforcement from secondary hemostasis, it will weaken and fail to stop bleeding. Secondary hemostasis involves the clotting cascade and eventual stabilization of the

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blood clot with fibrin cross-linking. Fibrinolysis is ultimately needed to breakdown fibrin and dissolve the blood clots. This process is controlled mainly by plasmin.

Platelets travel inactivated within the bloodstream until called upon by damaged endothelium to help form a thrombus. Platelet adhesion, activation, and aggregation are the steps responsible for the initial platelet plug formation. Normal endothelial cells and circulating platelets avoid contact due to their electronegative repulsion. But once the vascular subendothelium is exposed, a complex process involving adhesive ligands and receptors ensues.[8] Platelet activation occurs both before and after adhesion has taken place. This process involves the platelet changing shape, releasing preformed granules, and expressing molecules for aggregation.[6] Platelets will swell, becoming larger in size and increasing their surface area, as well as forming pseudopodia. This facilitates adherence to the injured endothelium and to other platelets.[9] Although platelets lack a nucleus, there is still an abundance of substances contained within the platelet granules that aid in adhesion and aggregation and further activation.[6] Alpha granules contain platelet-derived growth factor B, fibronectin, p-selectin, transforming growth factor B, B-thromboglobulin, platelet factor 4, fibrinogen, factors V and VIII, and von Willebrand factor (vWF).[5] Dense granules contain adenosine diphosphate (ADP), adenosine triphosphate (ATP), histamine, epinephrine, serotonin, and calcium ions.[10] These granules aid in platelet adhesion, promote cell-to-cell interactions, and stimulate vascular repair.[11, 12] The alpha granules are the main secretory component within the platelet, and contain promoters of adhesion including p-selectin, fibronectin, and vWF. P-selectin is a cell adhesion molecule that mediates both leukocyte and platelet adhesion. P-selectin is also capable of producing procoagulant microparticles containing active tissue factor,

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and enhances deposition of fibrin. P-selectin is therefore involved in both primary and secondary hemostasis.[13, 14] Activated platelets will also express surface receptors and ligands, such as phosphatidylserine, that contribute to thrombin and fibrin formation. Aminophospholipid phosphatidylserine is found within the inner leaflet of resting platelets. Once the platelet is activated, phosphatidylserine will move to the platelet surface and bind prothrombin. This will precipitate the conversion of prothrombin to thrombin, thereby aiding in the formation of a blood clot.[15] The phosphatidylserine-rich platelet surface is able to synthesize thrombin at a rate 300,000 times faster than the fluid phase of plasma.[15] Thrombin is the most potent activator of platelets.[6]

Platelet adhesion involves binding of the platelet with the exposed subendothelium. Shear forces as well as frictional forces applied to the endothelial layer by the flow of blood influence these adhesive properties.[16] High shear conditions are thought to be present in small- and medium-sized arteries, and low shear conditions are present in large arteries and veins of any size.[17] Under high shear conditions, the primary mechanism of adhesion involves collagen and vWF. Under low shear conditions, platelet adhesion involves collagen, fibronectin, and laminin.[18-23] Shear forces also influence further activation of platelets. Under high shear forces, ADP promotes platelet activation, while under low shear forces, thromboxane A_2 (TXA_2) causes activation. [24-26] Thrombin is active as a platelet activator under all shear conditions.[8] vWF's involvement in primary hemostasis includes adhesion of platelets to the endothelial wall and conservation of factor VII.[27] vWF is synthesized by both megakaryocytes and endothelial cells.[28] Within the endothelial cells, vWF is stored in secretory vesicles called Weibel-Palade bodies, and within platelets and megakaryocytes it is stored within

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alpha granules.[27] Release of vWF can be stimulated by thrombin, fibrin, vasopressin, collagen, platelet-activating factor (PAF), epinephrine, and histamine.[28] The vWF receptors on platelets are the integrin αIIbB3 (formerly called GP IIb/IIIa) and GP Ibα in the GP Ib-IX-V complex.[27] Platelet collagen receptors include GP VI and the integrin α2B1.[29] During platelet adhesion, platelets will decelerate, marginate, and expose these receptors to the endothelium. Once bound to the endothelium by these receptors, platelets will undergo a conformational change that exposes the integrin αIIbB3 receptor.[30] This action is key as it provides a binding site for fibrinogen.[29, 31] The expression of the integrin αIIbB3 receptor can be achieved either by the direct binding of vWF to the receptor during adhesion or by amplification pathways initiated by agonist/receptor binding of additional platelet receptors, such as TXA_2 and ADP receptors.[29, 32]

The last step in platelet plug formation is the aggregation of platelets. 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 is termed secondary aggregation. ADP is responsible for primary platelet aggregation, although thrombin, fibrinogen, vWF, and TXA_2 are also important stimulators.[17]

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A special feature of platelets is their ability to synthesize eicosanoids, specifically thromboxane A_2. TXA_2 is a major platelet aggregator and vasoconstrictor.[33] When needed, TXA_2 can be synthesized within the platelet from arachidonic acid. Arachidonic acid (AA) is formed from the action of phospholipase A_2 on the lipid membrane.[34, 35] AA is then converted into prostaglandin H_2 by the cyclooxygenase enzymes COX-1 and COX-2, also known as prostaglandin H-synthase-1 and -2, respectively. From there, prostaglandin H_2 is then further converted into TXA_2 via thromboxane synthase. TXA_2 will then diffuse across membranes to exert its effects, although only for a short time, since its half-life is approximately 30 seconds.[36] 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 thromboxane metabolites are eliminated in the urine.[37-39] Prostacyclin (PGI_2) is another important prostaglandin produced from AA. Prostacyclin is produced constitutively from the vascular endothelium. In contrast to TXA_2, prostacyclin inhibits platelet activation and aggregation, and causes vasodilation.[35] Endothelial cells adjacent to those that are injured will release prostacyclin in order to stop the platelet plug from growing excessively. Thromboxane A_2 enhances platelet activation by binding with a G-protein-coupled thromboxane receptor, while prostacyclin inhibits platelet activation by binding to the prostacyclin receptor, activating adenylate cyclase and generating cyclic adenosine monophosphate.[9, 40] Working together, TXA_2 and prostacyclin help to balance and control hemostasis. The other prostaglandins produced by this pathway, such as prostaglandin E_2, prostaglandin D_2, and prostaglandin $F_{2\alpha}$, serve key roles in mediating inflammation, renal blood flow, and gastrointestinal wall protection.

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There have been three COX isoforms identified in dogs. COX-1 is constitutively expressed in most body systems, and is involved in maintenance of normal cellular activities, platelet production of thromboxane A_2, preservation and protection of gastric mucosa, and renal blood flow.[34, 41] The COX-3 enzyme has similar catalytic activities to COX-1, but only has about 20% of COX-1's activity level.[42, 43] COX-2 is more limited in distribution, being produced mainly in the brain, kidney, thymus, and vascular endothelium, and is expressed at much lower levels compared to the other COX enzymes.[41, 44-46] COX-2 has also been found in circulating monocytes, tissue macrophages, and fibroblasts.[41, 44] COX-2 enzymes are also inducible by inflammatory cytokines, macrophages, monocytes, growth factors, and endotoxin.[34, 41, 47, 48] Induction of the COX-2 enzymes occurs primarily at the post-transcriptional level.[49] Since platelets are devoid of a nucleus, it was wrongfully believed for a long time that only COX-1 was expressed in circulating platelets, and that COX-2 induction within platelets could only happen at the level of the bone marrow. This belief also made it seem more likely that the COX-1 isoform was mostly associated with thromboxane A2 production, while COX-2 was the isoform believed to be the primary stimulus for prostacyclin production in the endothelial cells. [35, 46, 50, 51] More recent research has shown that human and canine platelets express both COX-1 and COX-2 enzymes, with higher levels of COX-2 being produced by immature (reticulated) platelets.[45, 50, 52-54] Studies with human platelets have shown that platelets may also have the ability to regenerate COX-1 *de novo* in response to external stimuli.[55]

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Inhibitors of Primary Hemostasis

Understanding the steps of primary hemostasis is essential for treatment and prevention of thromboembolic disease. Translation of this knowledge to drug development is paramount. Inhibitors of primary hemostasis may involve inhibition of platelet activation, adhesion, or aggregation. Inhibitors of platelet activation include thromboxane inhibitors, ADP antagonists (thienopyridines, nonthienopyridines), and inhibitors of platelet signal transduction (phosphodiesterase inhibitors).[32] Although inhibitors of platelet adhesion are in the drug development phase, none are FDA-approved at this time. Inhibitors of platelet aggregation include GP IIb/IIIa (integrin αIIbβ3) receptor antagonists.

One of the most commonly used anti-platelet medications is aspirin (acetylsalicyclic acid). Aspirin has been shown to be highly effective at inhibiting platelets, at high enough doses, in both humans and dogs.[33, 56-58] Aspirin irreversibly acetylates the hydroxyl group on a serine residue, position 529 and 516, located on the COX-1 and COX-2 enzyme isoforms.[34, 53, 59, 60] This acetylation blocks the active site of the COX enzyme, and prevents the conversion of AA to prostaglandin H_2, thus preventing the platelet from being able to synthesize thromboxane A_2.[33, 34, 53, 61] Since this action is irreversible, the inability to produce thromboxane A_2 will persist for the life-span of the platelet.[62] While thromboxane A_2 production appears to be quite sensitive to the inhibition of COX-1 isoforms, prostacyclin derived from the vascular endothelium utilizes both COX-1 and COX-2 enzyme isoforms, and has been shown to be less sensitive to the inhibitory actions of aspirin at anti-platelet doses. [33, 63, 64] Despite an early suppression of COX-1 mediated production of prostacyclin due to the

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effects of aspirin, COX-2 dependent prostacyclin synthesis can persist.[33]

Aspirin-induced COX enzyme inhibition also affects prostaglandin E_2, prostaglandin D_2, and prostaglandin $F_{2\alpha}$ production, making aspirin an effective anti-inflammatory medication, but also potentially causing side effects such as gastrointestinal hemorrhage and renal failure.[39, 65-67] Furthermore, gastrointestinal damage can occur from direct aspirin-induced mucosal damage, leading to ulceration.[66] When the ingested aspirin enters the stomach, the acidic environment causes the aspirin to diffuse into the mucosal cells. Within the mucosal cell, the aspirin becomes ionized and increases the amount of back diffusion of hydrochloric acid and pepsinogen, causing mucosal edema, inflammation, erosions, and ulceration.[66] In order to minimize this complication, buffered and enteric-coated aspirin was designed, with hopes of inhibiting dissolution within the stomach. This approach has been supported by studies showing that plain aspirin is more irritating to the stomach than the buffered and enteric-coated formulations.[68] The risk of ulceration is not entirely removed, however, as significant gastrointestinal hemorrhage can still be see with standard anti-inflammatory doses of enteric-coated aspirin.[66] These effects may be influenced by accumulation of enteric-coated tablets within the stomach.[69] Powdered aspirin formulations at high doses (greater than 25 mg/kg twice daily) have been administered to dogs for over a year without any significant gastrointestinal side effects.[70] A recent human study showed that, in contrast to the persistently high drug blood concentrations seen following overdose with aspirin tablets, drug concentrations decline more rapidly following ingestion of powder formulations, suggesting that prolonged absorption is unlikely with the powder formulation of aspirin.[71] Liquid formulations of aspirin are unstable and

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become inactivated within minutes of suspension, and their systemic effects are therefore uncertain.[72]

Another commonly used anti-platelet medication used in veterinary and human medicine is clopidogrel, which is an ADP receptor antagonist. ADP is released from platelet granules, and facilitates platelet aggregation. Once released, ADP interacts with receptors $P2Y_1$ and $P2Y_{12}$, both of which are platelet G-protein-linked purinergic receptors. Inhibition of either of these receptors will inhibit platelet function.[73, 74] Clopidogrel has become the most commonly used ADP receptor antagonist in human medicine.[75, 76] Clopidogrel is an irreversible inhibitor of $P2Y_{12}$, which causes permanent inhibition of circulating platelet function. Clopidogrel is classified as a thienopyridine, and requires hepatic metabolism into its active metabolite. In the majority of dogs, platelet function returns within 4 days of drug discontinuation, although some individuals may take longer.[77] Clopidogrel is available in tablet form for oral once daily administration. While other ADP receptor antagonists exist, there have not been any veterinary studies to evaluate the use of these medications in clinical patients.

Aspirin Resistance

Anti-inflammatory or “high” dosages of aspirin (10 mg/kg twice daily) reliably inhibit COX function and prostaglandin production in all cells that express the COX enzyme, including platelets and vascular endothelial cells.[78] Studies have shown that low doses of aspirin (0.5-1.0 mg/kg once daily) may inhibit platelet thromboxane synthesis without permanently inhibiting COX function within endothelium, thus allowing prostacyclin synthesis to persist.[37, 52, 56] These lower doses are also believed to decrease the risk of gastrointestinal and renal side effects. These attributes are ideal

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when considering the use of aspirin for its anti-platelet properties. Unfortunately, however, platelet inhibition does not appear consistent in all patients when lower doses are used.[37, 52, 79] Patients that are poorly responsive to the anti-platelet effects of low-dose aspirin are termed "aspirin resistant".[80] Aspirin resistance can be divided into two classifications: "clinical" and "laboratory". Clinical aspirin resistance is when thromboembolic disease occurs in the face of low dose aspirin. Laboratory aspirin resistance occurs when *in vitro* platelet function testing reveals inadequate suppression of platelets with low doses of aspirin. Ideally, the results of these classification schemes would agree, however discordance between classifications has been reported. The incidence of aspirin resistance in humans ranges from 8% to 45%[33, 80] and, in healthy dogs, the incidence is approximately 30%.[52, 79] The incidence of aspirin resistance in canine clinic patients remains unknown.

In human medicine, a new classification scale for aspirin resistance was developed in order to help determine the incidence of resistance, mechanisms involved, and clinical impact. For platelet responders, collagen-induced platelet aggregation and thromboxane formation are completely inhibited with aspirin. Type I resistance is characterized by ineffective inhibition of platelet function and thromboxane levels with aspirin administration, with platelet exposure to additional aspirin *in vitro* causing complete inhibition of function ("pharmacokinetic resistance"). In Type II resistance, platelets demonstrate minimal inhibition of platelet aggregation and thromboxane levels with aspirin administration, with no further inhibition of platelet aggregation following *in vitro* exposure, and only partial inhibition of thromboxane ("pharmacodynamic resistance"). In Type III resistance, there is persistent platelet aggregation despite

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complete thromboxane inhibition (“pseudo-resistance”). [81] Although these classification scales have not yet been specifically applied to the dog, studies have looked at platelet function and thromboxane levels in dogs receiving low doses of aspirin, and reveal complex results. One study showed that only 33% of dogs treated with low dose aspirin (1 mg/kg once daily) had impaired platelet function, with no significant differences between responders and non-responders with regards to concurrent thromboxane inhibition.[52] Another study showed 80% of dogs to have platelet function inhibition with low dose aspirin, and again results were independent of thromboxane inhibition. [79] Given these results, it is suspected that, similar to humans, dogs may experience aspirin resistance as well.

Currently the cause of aspirin resistance remains unknown in humans and dogs, and appears to be complex and multifactorial. Some of the mechanisms hypothesized in human medicine include single nucleotide polymorphisms, inflammation, metabolic syndrome, and patient compliance.[82]

Single nucleotide polymorphisms thought to be responsible for aspirin resistance include a wide variety of genetic mutations leading to changes in biological function and residual platelet reactivity. Proteins in human medicine that have been investigated include integrin αIIbβ3, GP Ia/IIa and GP VI (collagen receptors), vWF, $P2Y_1$, $P2Y1_2$, thromboxane A_2 receptor, and COX enzymes. Innate or acquired alterations in the genes that encode these proteins may be involved in aspirin resistance. For example, previous studies have shown that only 70% of canine platelets are responsive to thromboxane stimulation.[83-85] A possible explanation for this finding is impaired thromboxane A2 receptor linked G proteins.[83] For these patients, other agonists may be more important

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for platelet activation, which could explain the decrease responsiveness to aspirin.[32] Genetic polymorphisms may also cause enzymes, such as the COX enzymes, to respond variably to aspirin.[86]

Platelets play a crucial role in the regulation of neutrophils, and they may also be involved with amplification of inflammation. Once activated, platelets will release a variety of inflammatory mediators, and induce expression of inflammatory mediators in monocytes, macrophages, and granulocytes.[87] Recent studies have even suggested that platelets can trigger a thrombotic event through their inflammatory effects, which thereby reduces the anti-platelet effects of low dose aspirin.[88] COX-2 is inducible by inflammation and is reportedly sensitive to the effects of low dose aspirin in humans.[89] However, macrophages and monocytes are also important sources of thromboxane A2 during inflammation, and the COX-2 enzyme isoform within these cells appears to have a higher threshold for aspirin inhibition compared to COX-1 isoforms.[48] Human studies have shown that inhibition of COX-2 enzymes requires a much higher dose of aspirin compared to COX-1 inhibition.[53] Furthermore, inflammatory cells and newly generated platelets can synthesize increased amounts of COX-1 enzymes in an attempt to compensate for the COX-1 that has been irreversibly inhibited by aspirin.[48] Studies with human platelets have shown that platelets may also have the ability to regenerate COX-1 *de novo* in response to external stimuli.[55] Inflammatory conditions appear to have a significant effect on platelets and COX enzymes, and therefore may contribute to situations in which thromboxane A2 levels are inconsistently suppressed.

Metabolic syndrome, found in human patients with diabetes mellitus, hypertension, obesity, hypertriglyceridemia, and hypercholesterolemia, causes an

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increased risk of vascular events.[48] Human patients with diabetes mellitus have a high prevalence of resistance to aspirin and other anti-platelet drugs, along with persistent thromboxane-dependent platelet activation.[90-92] Interestingly, human studies have also found that obese populations have increased platelet activation and adhesiveness, higher concentrations of fibrinogen, vWF, and factor VII, and reduced sensitivity to anti-platelet drugs.[93, 94]

A few human studies have also shown that patient compliance is a cause of aspirin resistance.[95] This could potentially translate into poor owner compliance being a risk factor for aspirin resistance in canine patients. Additionally, enteric-coated aspirin tablets have been shown to accumulate in the stomach of dogs, and have been deemed not suitable for use in this species.[69, 96] Serum aspirin levels are also strongly influenced by feeding patterns in both humans and dogs, with the presence of food significantly decreasing the absorption of enteric-coated tablets.[96, 97]

Despite the years of experience and research with aspirin, still more research is needed, and the exact causes of aspirin resistance remain unknown.

Assessment of Platelet Function

There are a variety of options available for testing platelet function, each with its own unique set of features and limitations. Testing of platelet function is performed when trying to uncover the cause of a primary hemostatic disorder, evaluate for hypercoagulability and hypocoagulability, and evaluate the effects of anti-platelet medications. For any of the methods involved, sample collection must be carefully planned in advance to avoid interference with results. For example, excessively traumatic venipuncture commonly causes platelet activation and platelet clumping (especially in

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cats), leading to pseudothrombocytopenia. Pseudothrombocytopenia can also occur due to certain anticoagulants, such as EDTA.[98] Appropriate ratios of blood to anticoagulants should be used. Commercially available blood collection tubes come with a standard amount of anticoagulant and a vacuum that accepts the appropriate volume of blood. Handling of blood samples and the time between blood draw and testing should be carefully planned. Currently, the tests available to assess platelet function include: clot retraction, buccal mucosal bleeding time, Platelet Function Analyzer-100®, aggregometry, cone and plate(let) analyzers, viscoelastic coagulometers, Sonoclot®, thromboelastography, and flow cytometry.

Clot Retraction

Clot retraction is a crude and simple test influenced by the number and function of platelets, and the amount of fibrinogen. With this test, 5 mL of whole blood is placed into a sterile glass tube (without any anticoagulant), a wooden applicator is inserted into the sample, and the sample is sealed with plastic paraffin film, and incubated at 37C. The sample is visually analyzed over the next 8-24 hours. If clot retraction is complete, serum will be found surrounding a clot. If clot retraction does not occur, no serum will be found surrounding the clot. The percentage of clot retraction can also be estimated by using only 1 mL of whole blood, then 1 hour later, the amount of serum surrounding the clot is removed and measured. The volume is then multiplied by 50 to obtain the percent clot retraction. The normal range in animals is reported to be 25-60%.[99, 100] Clot retraction will be reduced with anemia and prolonged with polycythemia, and is impaired by thrombocytopenia, thrombocytopathia, and abnormalities or deficiencies with fibrinogen and some coagulation factors.[99]

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Buccal Mucosal Bleeding Time

The buccal mucosal bleeding time (BMBT) is the most common platelet function test available that is performed *in vivo*. Similar to clot retraction, a BMBT is a simple test that can be performed easily in a general practice setting. The BMBT evaluates the time needed to form a stable platelet plug following a standardized incision on the upper lip of the patient. The test uses a spring-loaded template device that creates a standardized incision. Blood is gently and carefully wicked away from the incision using filter paper. Once the incision is made, the time is recorded until no further bleeding is observed from the incision. Normal BMBT is less than 3 minutes in dogs.[101] Prolongation can occur with thrombocytopenia, and with thrombocytopathias such as von Willebrand's disease.[101-103]

Another *in vivo* test that has been described is the cuticle bleeding time (also called toenail bleeding time). This involves cutting the toenail quick to produce bleeding while the time to clot formation is recorded. The normal reference range are reported to be between 2 and 8 minutes.[104] The cuticle bleeding time, however, is not specific to abnormalities of platelet number and function, since it can also be prolonged in dogs with clotting factor deficiencies.

Platelet Function Analyzer®

The Platelet Function Analyzer® (PFA, Siemens HealthCare Diagnostics) is a point-of-care instrument that is able to evaluate platelet function using only a small amount of blood (800 μL), and is thought of as the *in vitro* equivalent to a BMBT.[105, 106] The PFA machine simulates primary hemostasis by aspirating a sample of citrate-anticoagulated whole blood under a high shear rate through a small aperture (150 μm) in

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a collagen membrane coated with platelet agonists (for example, ADP or epinephrine).[107, 108] This situation mimics the environment of a damaged vascular endothelium. Closure time (CT) is the time taken for a platelet plug to form and occlude blood flow. The maximum closure time is 300 seconds, therefore a CT of greater than 300 seconds strongly suggests platelet dysfunction. Results from a German study in dogs found a normal CT of 47-81 seconds using collagen/ADP cartridges and 67-210 seconds using collagen/epinephrine cartridges.[109]

The PFA can be used to evaluate inherited, acquired, or induced platelet dysfunction in patients with normal hematocrit and platelet counts. Studies in dogs have shown that a hematocrit (HCT) of less than 35% will significantly prolong CT. [110] CT may also be inaccurate in patients with polycythemia (HCT >60%) or thrombocytosis (>500 x 10^9 platelets/L). Dogs with heart murmurs may have a prolonged CT.[111] The PFA has been used in dogs to evaluate for inherited thrombocytopathies such as Scott syndrome and von Willebrand's disease.[112, 113] The PFA has also been shown as a sensitive indicator of aspirin-associated platelet dysfunction in both humans and dogs.[57, 58] The collagen/epinephrine cartridge is considered ideal for drug-induced platelet dysfunction, while the collagen/ADP cartridges are best for evaluating congenital or acquired platelet dysfunction.[108] The original model, PFA-100®, was insensitive for the detection of inhibitory effects of ADP antagonists.[114] However, there is now a newer version, the Innovance PFA-200® system, that offers a highly sensitive P2Y cartridge (Innovance PFA P2Y®) for the detection of $P2Y_{12}$ receptor inhibition.[115]

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Optical Aggregometry

Optical aggregometry has the ability to evaluate platelet function using various agonists at different concentrations using sodium-citrated platelet-rich plasma (PRP). Depending on the situation, agonists such as collagen, ADP, epinephrine, thrombin, arachidonic acid, and others can be used. The agonists cause activation of platelets and exposure of the GP IIb/IIIa receptor. Fibrinogen is able to bind to this receptor and aid in platelet aggregation. As platelet aggregation continues, the PRP suspension becomes clearer. This change is detected using a spectrophotometer to measure light transmission. A platelet-poor plasma (PPP) sample from the same patient is used as a control, such that this sample represents 100% light transmission. The results are displayed as a graphed curve, and the parameters recorded include maximal amplitude of aggregation (percentage) and slope/rate (percentage/minute). The graph will most often show an initial decrease in light transmission as activated platelets undergo their shape change (from discoid to spherical). This is followed by a gradual increase in light transmission that will eventually plateau as irreversible aggregation occurs. When weaker agonists are used, a biphasic aggregation can be seen with an early plateau, signifying primary aggregation, followed by a secondary curve and plateau, signifying irreversible secondary aggregation.

Disadvantages of optical aggegometry include that it evaluates platelet aggregation under low shear stress and does not mimic the physiologic environment in which platelet plug formation occurs, and that it requires large volumes of blood, technical expertise, and careful sample handling, and has limited availability. Despite these limitations, optical aggregometry is considered to be the gold standard method for

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evaluating aspirin-induced platelet dysfunction in humans.[57, 116, 117] It can be used to assess not only drug-induced platelet dysfunction, but also inherited and acquired causes of platelet dysfunction. Recently, it was also demonstrated that optical aggregometry was the most reliable test of platelet function that consistently identified dogs that responded to an anti-platelet dosage of aspirin.[79]

In human medicine, there is also a point-of-care, cartridge based system called VerifyNow®. This system uses citrated whole blood samples with light transmission technology. There are various cartridges available, depending on the drug-induced effects being evaluated. To date, there have been no studies using this method in domestic species.

Impedance Aggregometry

Impedance aggregometry is another method of testing platelet aggregation with the advantage of less sample preparation time than optical methods. Impedance aggregometry uses citrated whole blood instead of PRP. This technique relies on platelets forming a monolayer upon electrodes and changing the impedance of an electrical current, which is detected through wires.[118] Impedance aggregometry has been shown to correlate well with optical aggregometry in humans and, more recently, in dogs as well.[79, 119] Impendance aggregometers graph results in a manner similar to optical aggregometers. Aggregation is measured in maximal amplitude, but this is measured in units of ohms.[120] The Multiplate ® whole blood platelet function analyzer, also known as a multiple electrode impedance aggregometer (MEIA), is an impedance aggregometer that is available for clinical use and includes five channels with disposable test cells. Each test channel produces two aggregation curves and can be used separately, allowing

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simultaneous evaluation of platelet function using different agonists. When used to evaluate aspirin-induced platelet dysfunction, the conventional impedance aggregometer correlates reasonably well with optical aggregometer results, however there is poor correlation between the MEIA and turbidimetic aggregometry, therefore it was recommended to not use the MEIA method when evaluating aspirin responsiveness in dogs. [79]

Cone and Plate(let) Analyzers

The DiaMed Impact-R® is a cone and plate(let) analyzer (CPA) that evaluates platelet adhesion. Impact stands for the pneumonic "Image analysis, Monitoring, Platelet, Adhesion, Cone and plate Technology", with the "R" model representing its intended use in research. This technology aims to evaluate platelet function under near physiologic conditions, and uses image technology to measure platelets adhered and aggregated to the well surface. The software follows an algorithm to measure average size and surface area of platelet aggregates formed. There is limited research using this method in dogs.

Viscoelastic Coagulometers

Viscoelastic point-of-care coagulation instruments have become more popular in both human and veterinary medicine due to their unique ability to detect hypo- and hypercoagulability using a whole blood sample. [121] Viscoelastic analyzers measure changes in viscosity and elasticity of a blood sample as it turns from liquid to a fibrin clot. The most common machines used in veterinary medicine include: Sonoclot®, Thromboelastograph (TEG), rotational thromboelastometer (ROTEM), and Haemonetics (formly Haemoscope). These types of tests are useful for a broad understanding of the

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patient's coagulogram, but less sensitive and specific information is provided about platelet function. The Sonoclot® is a machine that measures changes in mechanical impedance exerted by a probe within a blood sample. The probe senses changes in blood viscosity or clot elasticity. The probe vibrates at 200 Hz and produces a continuous curve. As the blood sample clots, drag increases and the probe detects increased impedance. From this information, parameters such as activated clotting time (ACT), clot rate, and platelet function are reported out. This method is not indicated when assessing aspirin or clopidogrel drug-induced platelet dysfunction, however, because the methodology tests platelet function through thrombin activation. It could, however, be used to evaluate heparin therapy in dogs.[122]

Thromboelastography (TEG) uses a rotating plastic cylindrical cuvette (the cup) and a stationary suspended piston (the pin). In contrast to TEG, rotational thromboelastometry (ROTEM) uses a stationary cup and a rotary pin. TEG has a wider application and is used more frequently. As clot formation occurs, fibrin fibrils physically link the pin to the cup. The rotation of the cup is transmitted from the clot to the pin and is translated into a tracing by a torsion wire. Measurements from the graph provide a reaction time (R), K-time (K), alpha angle (α), maximum amplitude (MA), and clot stability (LY30/LY60). The reaction time is the time it takes to create a fibrin clot and reflects PT/PTT, but does not always correlate with these values. [123, 124] The K-time measures in minutes the time it takes from initiation of clotting for a TEG tracing to reach a predetermined level (20 mm) of clot strength. The α-angle measures in degrees the rate of fibrin buildup and crosslinking as a function of amplitude and time. Maximum amplitude (measured in mm) measures the widest part of the TEG tracing and represents

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the final clot strength. Elastic shear modulus (G, in dynes/second/cm^2) is calculated from the MA and serves as another measure of clot firmness. Clot stability (LY30/LY60) is a measurement of fibrinolysis, measuring the percent decrease in the amplitude tracing 30 and 60 minutes after the MA is determined. Results of TEG testing that could indicate hypercoaguloability include a shorter R, steeper α-angle, and increased MA.

With thromboelastography, maximum amplitude, and, to a lesser degree, K-time and α-angle, are affected by platelet function. Multiple methods of platelet activation exist. Each sample should rest at room temperature for 30 minutes prior to analysis. [123, 125, 126] Use of whole blood is more physiologic and summates the contributions of clotting factors, platelets, and red blood cells. TEG typically uses citrated whole blood samples, which are re-calcified prior to activation. This method is not sensitive to platelet inhibition from drugs such as aspirin and clopidogrel. An alternative method uses heparinized whole blood samples. Modified TEG cups are made with a proprietary amount of heparinase, designed to negate the effects of unfractionated heparins or low-molecular weight heparin on tracing results. Reptilase and activated factor XIII (activator F) are added followed by the addition of ADP (to evaluate effect of clopidogrel) or AA (to evaluate effect of aspirin). The percentage difference between the MA with activator F compared to the MA with activator F combined with ADP or AA is calculated to show the reduction in platelet function. This method has been used in dogs and was shown to be sensitive, and could potentially work for aspirin as well.[77] Although thromboelastographic values and calculations are reportedly useful for the evaluation of platelet function, this technology was designed to focus on formation of a fibrin clot, and has not been well-established as a sensitive assessment of platelet function or drug-

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induced platelet function.

Flow Cytometry

The principle behind the use of flow cytometry for platelet function analysis is to test for deficiencies in platelet membrane glycoproteins and diseases of granular contents and storage. Flow cytometry is an analytical procedure used to evaluate cell suspensions with a laser- or impedance-based electronic detection apparatus. Using this technology, cell counting, cell sorting, and biomarker detection can be performed. This method is used to not only measure various cell types, but also chromosomes, proteins, or nucleic acids attached to particles within the fluid suspension.[127] Flow cytometry instruments have evolved to be able to measure up to 14 parameters simultaneously.[128] Platelets can be evaluated using flow cytometry at rest and after stimulation with various agonists.[129] This method has been used to evaluate p-selectin median florescence intensity (MFI) as an indicator of activated platelets in dogs with immune-mediated hemolytic anemia [130] Due to limited availability, the need for rapid sample handling, and specialized instrumentation and expertise, this technology is under-utilized, but could serve a vital role in future veterinary and human studies.

Future Directions

To achieve the ultimate goal of a reliable and effective prophylactic anti-platelet therapeutic protocol for use in hypercoagulable dogs, it is essential to have a thorough understanding of hemostasis, inhibitors of platelet function, and methods of measuring platelet function. Although there is a relative paucity of research evaluating the efficacy of aspirin in veterinary patients, aspirin is the most widely studied antiplatelet medication

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in humans.[131] Human studies have shown that aspirin reduces the rate of vascular death by approximately 15%, and of nonfatal vascular events by approximately 30%.[132, 133] In humans, aspirin has been shown to be an effective antithrombotic in conditions such as unstable and stable angina[134, 135], aortocoronary bypass surgery [136], patients with prosthetic heart valves[137], long-term hemodialysis patients[138], acute myocardial infarction[139], transient cerebral ischemia [140], and polycythemia vera[141].

In veterinary medicine, few studies exist that evaluate the clinical efficacy of aspirin. A retrospective study evaluating prognostic factors and survival rates of dogs with immune-mediated hemolytic anemia (IMHA) found improved survival rates when low-dose aspirin was used [142]. Although the use of aspirin in patients with IMHA is controversial, studies support that this disease process involves platelet activation[130]. While platelet activation may not be the sole mediator leading to thromboembolism in IMHA patients, evidence exists to support the use of antiplatelet therapies in these patients. Another veterinary study evaluating clinical response to antiplatelet therapy was a prospective comparison of aspirin and clopidogrel for the prevention of secondary cardiogenic arterial thromboembolism in cats. This study showed a significant reduction in the likelihood of secondary thromboembolism with clopidogrel administration compared to aspirin.[143]

Clearly, there is still much to learn about the role of aspirin in veterinary medicine, its pharmacokinetics and pharmacodynamics, the influence of comorbidities, optimal dosages, reliable platelet function testing, and mechanisms for aspirin resistance. Until further investigations are able to answer the multitude of questions that still exist,

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aspirin therapy will continue to be prescribed by many veterinarians. Given the low cost, ease of administration, and availability of aspirin, it serves as an important alternative therapy for many veterinary patients. Veterinary medicine would benefit from the determination of an ideal therapeutic dosage of aspirin in dogs that consistently inhibits platelet function and thromboxane A_2 synthesis but that does not inhibit prostacyclin biosynthesis. Alternatively, if there is no single dose that is ideal in all dogs, then an individualized approach to therapy would be needed. This leads to the necessity of a comprehensive understanding of the effects of a range of aspirin dosages in individual patients, which will enable a more effective use of aspirin in prophylactic anti-platelet therapeutic protocols.

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CHAPTER II

EFFECTS OF ASPIRIN DOSE ESCALATION ON PLATELET FUNCTION AND URINARY THROMBOXANE AND PROSTACYCLIN LEVELS IN NORMAL DOGS

Introduction

Thromboembolism is a significant complication of many commonly encountered medical conditions in veterinary medicine, including immune-mediated hemolytic anemia (IMHA), protein-losing nephropathies, and hyperadrenocorticism [1]. Anti-platelet therapy, including low-dose aspirin, is one of the most affordable and widely used options for prevention of thrombus formation (thromboprophylaxis). Compared to other thromboprophylactic drugs, dogs diagnosed with IMHA and treated with low-dose aspirin had improved short and long-term survival times [2]. Low-dose aspirin has also been recommended as thromboprophylaxis for dogs with glomerular disease [3, 4].

Aspirin is a cyclooxygenase (COX) inhibitor that irreversibly inhibits platelet function. The COX enzyme converts arachidonic acid to biologically active eicosanoids, including prostaglandins, which are necessary for normal homeostasis and hemostasis [5]. By inhibiting COX, aspirin prevents synthesis of thromboxane A_2 (TXA_2) and prostaglandins such as prostacyclin. TXA_2 is primarily produced by platelets, and triggers platelet activation and vasoconstriction [6]. Prostacyclin, in contrast, originates

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from the vascular endothelium, and inhibits platelet activation and causes vasodilation [5].

Based on our previous work, it was determined that anti-inflammatory or "high" dosages of aspirin (10 mg/kg twice daily) reliably inhibit COX function and prostaglandin production in all cells that express the COX enzyme, including platelets and vascular endothelial cells [7]. Unfortunately, high-dose aspirin may be associated with gastrointestinal and renal side effects [8-10], which make this dose less desirable for thromboprophylaxis [7]. In contrast to high-dose aspirin, a "low" dosage of aspirin inhibits platelet TXA_2 synthesis in humans, without permanently inhibiting COX function within the vascular endothelium and other cells, thereby allowing prostacyclin synthesis and vasodilation to continue, and reducing the likelihood of gastrointestinal and renal side effects [11, 12]. Unfortunately, low-dose aspirin (0.5 to 1 m/kg once daily), unlike high-dose aspirin, does not consistently inhibit platelet function in dogs [13-15]. Patients that are poorly responsive to the anti-platelet effects of aspirin are termed "aspirin resistant" [16]. The incidence of aspirin resistance ranges in humans from 5% to 57% [6, 16] and, in healthy dogs, our group has previously estimated that the incidence ranges from 19% to 56% depending on the technique used to assess inhibition of platelet function [14, 15].

Although there are many proposed mechanisms of aspirin resistance, one potential mechanism is aspirin underdosage. Our group has previously reported that aspirin-associated platelet dysfunction in dogs appears to be highly dose-dependent [7, 15]. While low-dose aspirin has inconsistent and variable effects in individual dogs, platelet function in dogs has been shown to be consistently inhibited by high doses of

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aspirin, regardless of the methods used to assess platelet function [7, 17]. Using both PFA-100® and optical aggregometry, previous studies have demonstrated that 28% to 57% of dogs treated with low-dose aspirin (1 mg/kg, q 24h) were considered non-responders, while all dogs were considered aspirin responders when treated with a much higher dose (10 mg/kg, q12h) [18]. Furthermore, when canine platelets are exposed to aspirin *in vitro*, at a concentration that consistently inhibits activation, platelet function is consistently markedly inhibited [15]. The pharmacokinetic profile of low-dose aspirin in dogs has not been evaluated, and a better understanding of drug pharmacokinetics would clearly be beneficial when exploring mechanisms for poor response to aspirin therapy. To date, only the anti-platelet effects of low and high doses of aspirin in dogs have been evaluated, and the wide range of potential doses between these two extremes have not been studied. Establishment of an optimal single "mid-range" aspirin dosage or a range of aspirin dosages which could reliably inhibit platelet function and TXA_2 synthesis without inhibiting prostacyclin synthesis has the potential to overcome the impact of the aspirin resistance associated with standard dosages of low-dose aspirin.

The purpose of this study was to use incremental increases in aspirin dosages to precisely determine the dosage or dosages of aspirin that consistently inhibited platelet function and TXA_2 synthesis without inhibiting prostacyclin synthesis in normal dogs. This study was designed to test the working hypotheses that small, incremental increases in aspirin dosages would lead to more consistent inhibition of platelet function and TXA_2 synthesis while having minimal effects on prostacyclin synthesis.

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Materials and Methods

Study Design, Animals

Eight healthy adult research Walker Hound dogs, four females and four males, were used in this study. The dogs were not exposed to any medications or vaccines for at least 2 weeks prior to the initiation of the study. Normal health status was established by detection of no abnormalities on physical examination, complete blood count (including manual platelet count), serum chemistry, urinalysis, and testing for heartworm and tick-borne disease (SNAP 4Dx Plus Test, Idexx Laboratories Inc, Westbrook, ME). The median age of the dogs was 1.5 years (range, 1 to 6.5 years), and their mean body weight was 27.4 kg (range, 25.6 to 30.5 kg). Body weight was obtained at the beginning of the study and used to calculate all subsequent drug doses. Animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee, and was in compliance with the requirements of a facility accredited by the American Association for Accreditation of Laboratory Animal Care.

In a five way, randomized, cross-over study, the dogs were separated into one of five groups, with each group given different oral dosages of aspirin (Aspirin, Major Pharmaceuticals, Livonia, MI): 0.5 mg/kg q24h, 1 mg/kg q24h, 2 mg/kg q24h, 4 mg/kg q24h or 10 mg/kg q12h. Aspirin doses were compounded into capsules by the Mississippi State University College of Veterinary Medicine Pharmacy. All drugs were administered orally for 7 days, followed by at least a 14 day washout period between dosing. After this washout period, the dogs switched groups, and the study was continued until all dogs had received each aspirin dosage.

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Prior to aspirin therapy (day 0) and after one week of drug administration (day 7), blood samples were collected for platelet function analysis (optical aggregometry and point-of-care platelet function analyzer), and urine samples were collected to measure urinary 11-dehydro-thromboxane B_2 (11-dTXB_2) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) concentrations (stable metabolites of TXA_2 and prostacyclin, respectively). Blood and urine samples collected on Day 7 were taken one hour after 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 anticoagulant. Urine was collected via cystocentesis using a 22-gauge 1.5-inch needle, and samples were stored at -80°C for later analysis.

Optical Aggregometry

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

A two channel light transmission (optical) platelet aggregometer (Chronolog 700 Whole Blood/Optical Lumi-Aggregometer, Chronolog Corporation, Haverton, PA) that allowed for two samples to be evaluated concurrently was used to analyze platelet aggregation in PRP. Samples were analyzed based on the manufacturer's standard guidelines (Chrono-Log 700 manual. Havertown, PA: Chrono-Log Corp, 2006). 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

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incubated at 37°C for 5 minutes and then placed into the aggregometer, and stable baseline values for minimal (PRP) and maximal (PPP) aggregation were obtained (assigned values of 0% and 100% aggregation, respectively). Platelet numbers within the PRP were not adjusted to a standardized count by dilution with PPP prior to analysis, based on recommendations put out by the International Society of Thrombosis and Haemostasis Platelet Physiology and Scientific and Standardization Committee [19-22]. Collagen, 10 µg/mL, was then added to the PRP, and aggregation was monitored for 12 minutes. The maximal percentage aggregation was calculated and recorded using computer software (AGGRO/LINK 8, Chronolog Corporation, Haverton, PA). Platelet function analysis was performed within 4 hours of collection.

Platelet Function Analysis

A commercial point-of-care whole blood platelet function analyzer (PFA-100®, Siemens Healthcare Diagnostics, Deerfield, IL) that has previously been evaluated for use in dogs [23-26] was used to analyze platelet function. This platelet function analyzer stimulates platelet function with several agonists under high shear forces and measures the closure time, in seconds, needed to form a platelet plug and inhibit blood flow. The manufacturer's cut-off time for the instrument is 300 seconds.

The instrument was used according to manufacturer's instructions. Briefly, blood samples were gently mixed, and kept at room temperature without agitation until analysis. For analysis, 800 µl of whole blood was transferred into either a collagen/ADP cartridge (PFA Collagen/ADP Test Cartridge, Siemens Healthcare Diagnostics, Duluth, GA) (performed initially to establish normal platelet function) or collagen/epinephrine cartridge (PFA Collagen/EPI Test Cartridge, Siemens Healthcare Diagnostics, Duluth,

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GA) (used throughout the study to assess aspirin-associated platelet dysfunction) and analyzed. Two collagen/epinephrine cartridges were analyzed at each time point for all dogs, and the closure times were averaged. Platelet function analysis was performed within 2 hours of collection.

Aspirin Responsiveness

Based on our previously published criteria used for dogs [15], each dog was classified as either an aspirin responder or an aspirin non-responder. For optical aggregometry, a dog was considered to be an aspirin responder if there was a greater than 25% reduction in the percentage aggregation at maximum amplitude compared to day 0 values. For the whole blood platelet function analyzer, a dog was considered to be an aspirin responder if the closure time was >300 seconds.

Urine 11-dehydro-thromboxane B_2 Analysis

Urinary 11-dTXB_2 concentration was analyzed using an enzyme-linked immunosorbent assay kit (11-dehydro-thromboxane B_2 EIA kit-Monoclonal, Cayman Chemical Co, Ann Arbor, MI) that has been previously validated in the dog [13]. Prior to analysis, urine samples were thawed to room temperature, then handled according to the manufacturer's instructions. Samples were analyzed in triplicate on a plate reader (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA). Results were averaged, and reported as picograms per milliliter of urine. Urine creatinine concentration was measured using a biochemistry analyzer (ACE Alera® 201 Clinical Chemistry System, Alfa Wasserman, Inc.) in order to calculate a urinary 11-TXB_2-to-creatinine ratio.

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Urine 6-keto-prostaglandin $F_{1\alpha}$ Analysis

Urinary 6-keto-$PGF_{1\alpha}$ concentration was analyzed using an enzyme-linked immunosorbent assay kit (6-keto-prostaglandin-$F_{1\alpha}$ ELISA, Cayman Chemical Co, Ann Arbor, MI) that has undergone extensive analytic validation by the manufacturer, and has been used previously in dogs [27]. Using a similar technique as previously described with urinary 11-$dTXB_2$, urinary 6-keto-$PGF_{1\alpha}$ were analyzed according to the manufacturer's instructions. Samples were analyzed in triplicate, averaged, and reported in picograms per milliliter of urine. Urine creatinine concentration was measured to calculate a urinary 6-keto-$PGF_{1\alpha}$-to-creatinine ratio.

Statistical Methods

Mixed models using PROC MIXED in a computer software program (SAS for Windows 9.4, SAS Institute, Inc., Cary, NC) were fit for each outcome that included dose, sample and the dose*sample interaction as fixed effects. Dog identity within run and run were included as random effects with variance components covariance structure. An LSMEANS statement was used to make comparisons among levels of significant main effects. If the interaction term was significant, differences in least squares means between each of the levels of one variable were calculated for each level of the other variable in the interaction using an LSMESTIMATE statement. If there were significant main effects with more than two levels or significant interaction terms, the simulate option in the LSMEANS or LSMESTIMATE statements was used to adjust p-values to account for the effect of multiple comparisons. The distribution of the conditional residuals was evaluated for each outcome to ensure the assumptions of normality of the

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residuals and heteroscedasticity had been met. An alpha level of 0.05 was used to determine statistical significance for all methods.

Results

Optical Aggregometry

The mean optical aggregometry maximum amplitude for all aspirin dosages on days 0 and 7 are represented in Figure 2.1. There was no significant difference in the maximum amplitude for the 5 aspirin dosages on day 0 (pre-treatment). When compared to day 0, there was a significant decrease in maximum amplitude on day 7 for 1 mg/kg q24h ($p=0.0259$), 2 mg/kg q24h ($p<0.0001$), 4 mg/kg q24h ($p<0.0001$), and 10 mg/kg q12h ($p<0.0001$). On day 7, the maximum amplitude for 0.5 mg/kg q24h was significantly greater than for 2 mg/kg q24h ($p=0.0004$), 4 mg/kg q24h ($p=0.0007$), and 10 mg/kg q12h ($p=0.0003$). There was no difference in maximum amplitude among the 2 mg/kg q24h, 4 mg/kg q24h, and 10 mg/kg q12h dosages.

Two dogs were considered aspirin responders when treated with 0.5 mg/kg q24h. When treated with 1 mg/kg q24h and 4 mg/kg q24h, 6 dogs were considered to be aspirin responders. All dogs were considered to be aspirin responders when treated with 2 mg/kg q24h and 10 mg/kg q12h. One dog was classified as an aspirin responder with all five aspirin dosages.

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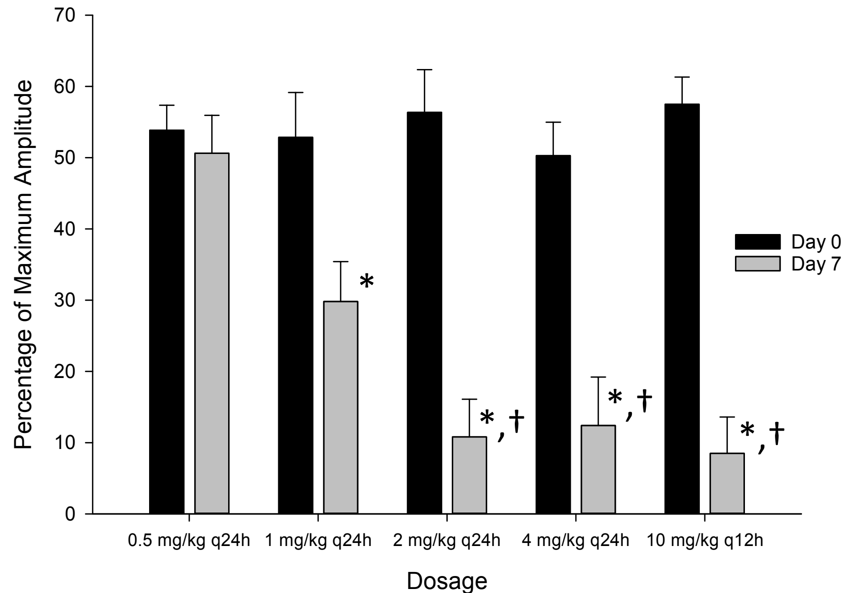

Figure 2.1 Maximum amplitude (percentage) of aggregation via optical aggregometry in 8 dogs treated with different oral dosages of aspirin: 0.5 mg/kg q24h, 1 mg/kg q24h, 2 mg/kg q24h, 4 mg/kg q24h, and 10 mg/kg q12h.

The graph demonstrates the mean +/- SEM. Sample points labeled * illustrate significant differences ($p<0.05$) from the corresponding dosage on day 0 values. Within day 7, samples labeled † were significantly different ($p<0.05$) from the maximum amplitude of 0.5 mg/kg q24h.

Platelet Function Analysis

The mean closure times for all aspirin dosages on days 0 and 7 are represented in Figure 2.2. There was no significant difference in the closure time for the 5 aspirin dosages on day 0 (pre-treatment). When compared to day 0, there was a significant increase in closure times on day 7 for 1 mg/kg q24h ($p=0.0002$), 2 mg/kg q24h ($p=0.0002$), 4 mg/kg q24h ($p<0.0001$), and 10 mg/kg q12h ($p<0.0001$). On day 7, the closure times for 0.5 mg/kg q24h were significantly greater than 2 mg/kg q24h ($p=0.0388$), 4 mg/kg q24h ($p=0.0002$), and 10 mg/kg q12h ($p=0.0006$). There was no difference in closure times among the 2 mg/kg q24h, 4 mg/kg q24h, and 10 mg/kg q12h dosages.

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None of the dogs treated with 0.5 mg/kg q24h were considered to be aspirin responders. Two dogs were considered to be aspirin responders when treated with 1 mg/kg q24h and 5 dogs were classified as aspirin responders when treated with 2 mg/kg q24h. When treated with 4 mg/kg q24h and 10 mg/kg q12h, 7 dogs were considered to be aspirin responders.

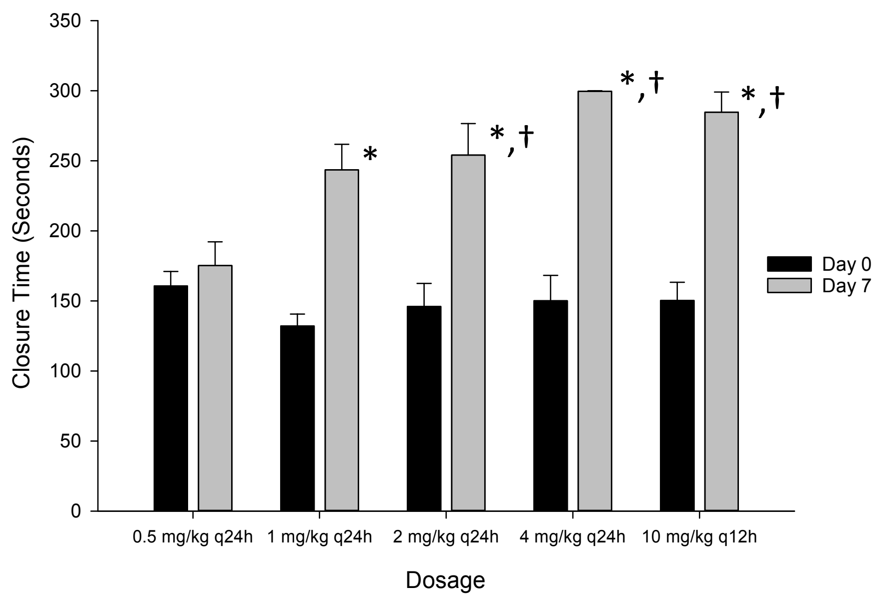

Figure 2.2 Whole blood point-of-care platelet function analyzer (PFA-100) closure times (collagen/epinephrine cartridge) in 8 dogs treated with different oral dosages of aspirin: 0.5 mg/kg q24h, 1 mg/kg q24h, 2 mg/kg q24h, 4 mg/kg q24h, and 10 mg/kg q12h.

The graph demonstrates the mean +/- SEM. Sample points labeled * illustrate significant differences ($p<0.05$) from the corresponding dosage on day 0 values. Within day 7, samples labeled † were significantly different ($p<0.05$) from the closure time of 0.5 mg/kg q24h.

Urine 11-dehydro-thromboxane B_2 Analysis

The urine 11-dTXB_2-to-creatinine ratio for all aspirin doses on days 0 and 7 are represented in Figure 2.3. Compared to day 0, there was a significant ($p=0.0003$)

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decrease in the 11-$dTXB_2$-to-creatinine ratio on day 7 for all aspirin dosages. However, there was no significant dose dependent decrease in the 11-$dTXB_2$-to-creatinine ratio for all doses.

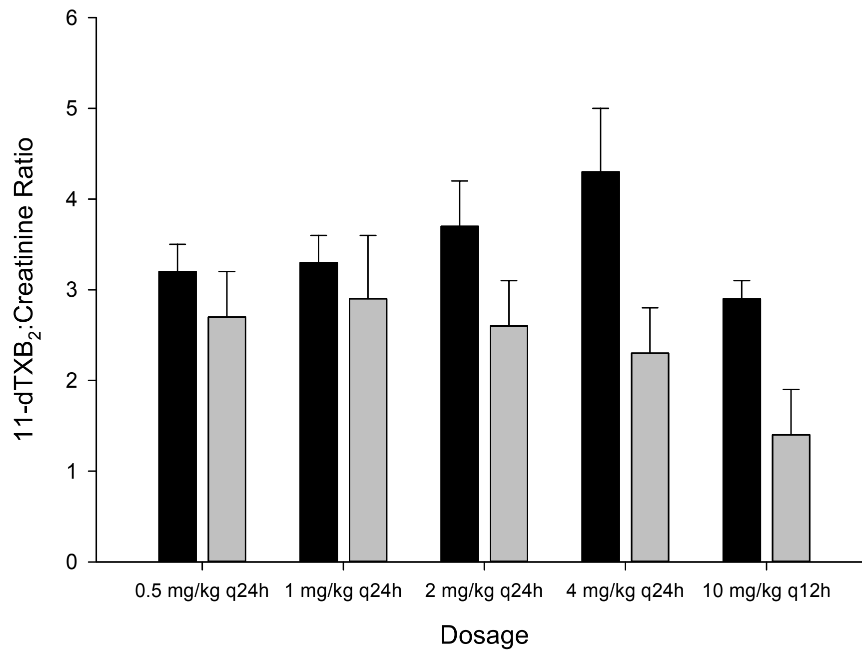

Figure 2.3 Urine 11-dehydro-TXB_2:creatinine ratio seen in 8 dogs treated with different oral dosages of aspirin: 0.5 mg/kg q24h, 1 mg/kg q24h, 2 mg/kg q24h, 4 mg/kg q24h, and 10 mg/kg q12h.

The graph demonstrates the mean +/- SEM. There was no dose effect on the urine 11-dehydro-TXB_2:creatinine ratio, and day 7 values were significantly different ($p<0.05$) than day 0 values.

Urine 6-keto-prostaglandin $F_{1\alpha}$ Analysis

The urine 6-keto-$PGF_{1\alpha}$-to-creatinine ratio for all aspirin doses on days 0 and 7 are represented in Figure 2.4. Compared to day 0, there was a significant ($p=0.0075$) decrease in the 11-$dTXB_2$-to-creatinine ratio on day 7 for all aspirin dosages. However,

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there was no significant dose dependent decrease in the 6-keto-PGF$_{1\alpha}$-to-creatinine ratio for all doses.

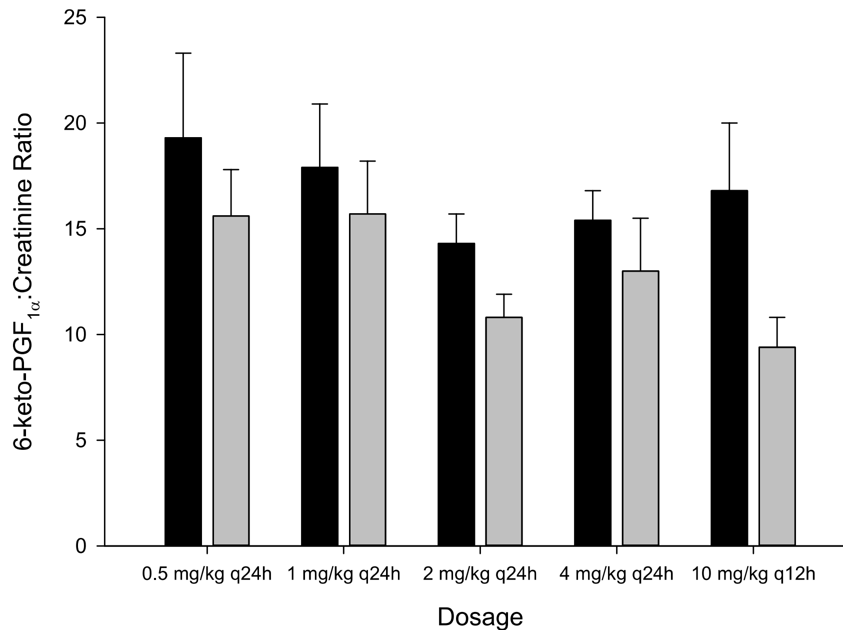

Figure 2.4 Urine 6-keto-prostaglandin F$_{1\alpha}$:creatinine ratio seen in 8 dogs treated with different oral dosages of aspirin: 0.5 mg/kg q24h, 1 mg/kg q24h, 2 mg/kg q24h, 4 mg/kg q24h, and 10 mg/kg q12h.

The graph demonstrates the mean +/- SEM. There was no dose effect on the urine 6-keto-prostaglandin F$_{1\alpha}$:creatinine ratio, and day 7 values were significantly different ($p<0.05$) than day 0 values.

Discussion

The ideal dose of aspirin that consistently inhibits platelet function in dogs, while minimizing any drug-associated side effects, is unknown. Currently used standard low or anti-platelet dosages (0.5-1 mg/kg/day) of aspirin, however, do not reliably inhibit platelet function [13-15]. Platelet function in dogs will, in contrast, be consistently inhibited by high dosages of aspirin [17], suggesting that, if dogs are treated with high

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enough doses of aspirin, there is an increased likelihood of inhibition of platelet function. Unfortunately, as the aspirin dosage increases, so does the risk of undesirable inhibition of prostacyclin, and of aspirin-induced side effects. The data reported in the current study suggest that a modified "low" dosage of aspirin (2 mg/kg q24h) will consistently inhibit canine platelet function in normal dogs, without the need to resort to much higher standard anti-inflammatory aspirin dosages of 10 mg/kg twice daily.

In humans, optical aggregometry is considered to be the gold standard method for evaluating aspirin-induced platelet dysfunction [23, 28, 29]. Recently, our group demonstrated that optical aggregometry was the most reliable test of platelet function that consistently identified dogs that responded to an anti-platelet dosage of aspirin [15]. In the current study, compared to pre-treatment values, all aspirin dosages except the very lowest dosage (0.5 mg/kg q24h) significantly decreased platelet aggregation after 7 days of drug administration. On day 7, there was no significant difference in the mean platelet amplitude among the aspirin dosages, except at the lowest dosage. Additionally, on day 7, compared to the 0.5 mg/kg q24h dosage, there was a significant decrease in platelet aggregation for the 2 mg/kg q24h, 4 mg/kg q24h, and 10 mg/kg q12h dosages, but not for the 1 mg/kg q24h dosage. These results are consistent with a dose-dependent decrease in platelet aggregometry that reaches maximal effect at an oral aspirin dosage of 2 mg/kg once daily.

Recently, using optical aggregometry, canine aspirin responsiveness was defined as a greater than 25% reduction in the percentage aggregation at maximum amplitude following aspirin treatment, compared to pre-aspirin values [15]. Using this definition, our group had demonstrated that, when aspirin was administered to dogs at an oral

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dosage of 1 mg/kg once daily for 7 days, approximately 80% of the dogs were classified as aspirin responders. These previous results were similar to the results reported here, in that when dogs were administered the same dose of aspirin for 7 days, 75% were considered to be aspirin responders. In contrast, in this current study, when treated with a 2 mg/kg once daily aspirin dosage, all dogs were considered to be aspirin responders, a rate of aspirin responsiveness that was identical to that seen with the high anti-inflammatory dosage of aspirin (10 mg/kg q12h). These results suggest that an aspirin dosage of 2 mg/kg once daily will provide consistent aspirin-induced platelet dysfunction in normal dogs, and that higher dosages of aspirin may not provide additional anti-platelet benefits. Although the 2 mg/kg q24h and 10 mg/kg q12h dosages achieved 100% aspirin responsiveness, only 6/8 (75%) of the dogs receiving the 4 mg/kg q12h dosage were considered aspirin responsive. In the two dogs considered non-responsive at this dose, platelet function was inhibited (by 7% and 20%) compared to pre-aspirin values, but did not achieve the 25% reduction required for classification as aspirin responders. When treated with the 10 mg/kg q12h dose, these same two dogs had the least reduction in aggregometry amplitude (73% and 43%) compared to an average of 98% reduction for the other six dogs. These findings suggest that additional pharmacodynamic effects, other than dosage, could influence the anti-platelet effects of aspirin.

Similar to previous studies in dogs [14, 23, 25], this study demonstrated that aspirin-induced platelet dysfunction could be determined by using a commercial whole blood point-of-care platelet function analyzer (PFA-100) with a collagen/epinephrine cartridge. Our group has previously reported that, compared to optical aggregometry, the PFA-100 is less reliable at determining drug responsiveness in dogs receiving aspirin

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[15]. In our previous studies using the PFA-100 in dogs treated with aspirin at a dosage of 1 mg/kg once daily for 7 days, only 33% to 44% of dogs were classified as aspirin responders [14, 15] and, when the same aspirin dosage was used in the current study, 25% of dogs were considered to be aspirin responders. Also in this study, at the same aspirin dosage of 1 mg/kg daily, 75% of the dogs were considered to be aspirin responders based on aggregometry results. This discrepancy was not unexpected because the PFA-100, although it is often used to clinically evaluate platelet function in dogs receiving aspirin, has been previously reported by this group to markedly overestimate the degree of aspirin resistance compared to optical aggregometry [15]. When receiving aspirin at a dosage of 2 mg/kg once daily, approximately 63% of the dogs in this study were classified as aspirin responders by the PFA-100, which is only slightly below the 88% of patients that were classified as aspirin responders when treated with the higher two aspirin dosages (4 mg/kg once daily, and 10 mg/kg twice daily). Based on PFA-100 results alone, an aspirin dosage of 4 mg/kg once daily would appear to be sufficient to maximize drug anti-platelet effects. However, when PFA results are considered concomitantly with aggregometry results, an aspirin dosage of 2 mg/kg may be sufficient overcome aspirin resistance, especially considering that aggregometry is more sensitive than the PFA-100 to the anti-platelet effects of aspirin.

During this study, mild transient gastrointestinal side effects were noted in some dogs with the administration of the anti-inflammatory high dosage of aspirin, 10 mg/kg twice daily, but no side effects were noticed with any of the lower aspirin dosages. This study was, however, not designed to specifically monitor for aspirin-induced gastrointestinal lesions such as gastric ulceration or erosions, and additional studies

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would be warranted to further explore the potential gastrointestinal side effects of using aspirin at dosages greater than 0.5-1 mg/kg daily. Furthermore, since low-dose aspirin is commonly administered concurrently with glucocorticoids to dogs with IMHA, additional studies would be necessary to determine the gastrointestinal side effects of a slightly increased "low" aspirin dosage, such as 2 mg/kg once daily, during concurrent glucocorticoid administration.

In previous studies in humans and dogs, the stable TXA_2 metabolite 11-dehydro-thromboxane B_2 has been used as an indicator of aspirin-induced inhibition of thromboxane synthesis [30, 31]. A second stable TXA_2 metabolite, 2,3-dinor-thromboxane-B_2 (2,3-dinor-TXB_2), is also present in the urine of dogs, and can also serve as a surrogate marker of thromboxane synthesis [13]. A previous study demonstrated that, compared to 11-dTXB_2, canine urinary 2,3-dinorTXB_2 concentrations were a more sensitive indicator of aspirin-induced thromboxane inhibition [13]. Unfortunately, an assay for 2,3-dinorTXB_2 in dogs was unavailable at the time of this study. However, although the urine TXA_2 metabolite assay used in this study has been published to not be the most sensitive indicator of aspirin-induced thromboxane inhibition, our previous work has shown that this assay is still sufficiently sensitive to detect a decrease in thromboxane synthesis during aspirin administration [14]. The results of the current study revealed that the administration of aspirin at any dosage caused a significant decrease in thromboxane synthesis, but there was no detectable dose-dependent relationship between the aspirin dose and thromboxane synthesis. The results reported here were similar to results from our previous work performed in dogs, in that aspirin-induced reductions in thromboxane synthesis were not dependent on drug dosage [7, 14,

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15, 17]. The current study suggests that the inhibition of thromboxane synthesis seen with an aspirin dosage of 2 mg/kg once daily is similar to that seen with the traditional lower anti-platelet dosages of 0.5 and 1 mg/kg once daily, and that an increase in aspirin dosages beyond 2 mg/kg once daily will not cause additional inhibition in thromboxane synthesis. However, it is conceivable that a more sensitive assay, such as the 2,3-dinorTXB_2 assay, might be able to detect more subtle variations in thromboxane synthesis, particularly considering that the current study detected a possible subtle (and not statistically significant) dose-dependent decrease in urine 11-dTXB_2 concentrations at higher aspirin dosages (Figure 3).

Prostacyclin (prostaglandin I_2), after being synthesized by the vascular endothelium, has a short half-life, and will quickly be converted into stable metabolites, including 6-keto $PGF_{1\alpha}$ and 2,3 dinor-6-keto $PGF_{1\alpha}$, which can be used as surrogate markers of prostacyclin synthesis [27, 32-34]. One proposed benefit of low-dose aspirin is that lower dosages will irreversibly inhibit platelet thromboxane synthesis without permanently inhibiting COX function in the vascular endothelium, allowing prostacyclin synthesis to continue. Anti-inflammatory high dosages of aspirin, in contrast, are purported to inhibit COX function and prostaglandin synthesis in both platelets and the vascular endothelium [11, 12]. The ideal anti-platelet dosage of aspirin would reliably inhibit platelet function, but would not inhibit prostacyclin synthesis. The results reported here, however, demonstrated that the administration of aspirin to dogs at any dosage leads to a decrease in prostacyclin synthesis. Similar to the findings with urinary thromboxane metabolites, there was no detectable dose-dependent relationship between aspirin dose and urinary prostacyclin metabolites. These results suggest that, although

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prostacyclin synthesis is reduced somewhat even at the lowest evaluated dosage of aspirin, the incremental increases in aspirin dosages needed to progressively inhibit platelet function will not further reduce the beneficial properties of residual prostacyclin synthesis. A slightly increased "low" aspirin dosage of 2 mg/kg once daily, therefore, would be expected to consistently inhibit platelet function while still maintaining prostacyclin concentrations comparable to those seen with traditional lower anti-platelet dosages of aspirin. Again, as with urine 11-dTXB_2, this study detected a possible subtle (and not statistically significant) dose-dependent decrease in urine 6-keto-$PGF_{1\alpha}$ concentrations at higher aspirin dosages. Therefore, the inclusion of a larger number of dogs in the study could conceivably have elucidated a statistically significant dose-dependent decrease in 6-keto-$PGF_{1\alpha}$ concentrations.

One limitation of the study is that there are no standard techniques that are consistently used to evaluate aspirin responsiveness. The platelet function assays and classification system used in this study are similar to previous studies that have evaluated aspirin-induced platelet dysfunction [15, 28]. However, other studies have used different methodologies and protocols to assess aspirin-induced platelet dysfunction [35], and this variability in methods does not allow for ready comparison between studies. Additionally, the dogs used in the study were healthy, and these results may not be directly applicable to prothrombotic patients. Finally, the dogs used in this study were only given aspirin for one week, whereas low-dose aspirin is commonly administered long term, and it is as yet unknown how canine platelet function and eicosanoid synthesis will respond to chronic aspirin administration.

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The role of aspirin therapy for thromboprophylaxis is controversial in veterinary medicine. The use of anti-coagulant therapy is commonly used for thromboprophylaxis and might be the more effective therapies for the prevention of venous thromboembolism because platelets are not the primary facilitator of thrombus formation in veins. However, platelets can still contribute to the formation and acceleration of venous thrombosis. For example, Weiss et al demonstrated that dogs with IMHA will have an increase in activated circulating platelets (Weiss et al., 2006). Additionally, dogs with IMHA treated with 0.5 mg/kg q24h of aspirin had a better short and long-term survival times compared to other anti-coagulant therapy. Recently, a study evaluated the safety of aspirin, clopidogrel, or aspirin and clopidogrel in dogs with IMHA. Although the study was underpowered to determine the most effective treatment option, when anti-platelet therapy was used as the sole thromboprophylaxis therapy, only 2 of 24 dogs developed a suspected thromboembolism (Mellett et al., 2011). Finally, aspirin and other anti-platelet therapies are readily available, inexpensive, easy to administer long term, and do not require routine assessment of coagulation to ensure appropriate inhibition. Therefore, aspirin or other anti-platelet therapies are commonly administered to patients at risk for formation of thromboembolism.

As discussed, low-dose aspirin is commonly used in an attempt to prevent thrombus formation in prothrombotic dogs but, at traditional anti-platelet dosages of 0.5 to 1 mg/kg once daily, platelet function appears to be inconsistently inhibited. Higher aspirin dosages may therefore be needed to provide appropriate platelet inhibition. As the aspirin dosage increases, however, so do the likely risks of aspirin-induced side effects and inhibition of prostacyclin. The results of this study suggests that, in healthy

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dogs, an oral aspirin dosage of 2 mg/kg once daily will consistently and adequately inhibit platelet function without decreasing the degree of prostacyclin synthesis significantly more than lower aspirin dosages. Additional studies will be required to determine if this higher aspirin dosage is associated with an increased risk of adverse drug effects.

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CHAPTER III

CONCLUSION

Using optical aggregometry, our study found that 100% of dogs were aspirin responders using a 2 mg/kg once daily aspirin dosage. For hypercoagulable conditions that include increased platelet activity, this newly recommended low dose rate of aspirin could improve overall efficacy and produce significantly improved survival times. Currently, there are no clear protocols for treatment or prevention of thromboembolism in dogs. Furthermore, despite treatment with various inhibitors of hemostasis, patients continue to experience thromboembolic events with life-threatening side effects. Lack of information regarding the causes for hypercoagulability, proper drug doses, and effective monitoring are undoubtedly related to the poor efficacy of these treatments. Depending on the specific disease pathophysiology, inhibitors of primary hemostasis, inhibitors of secondary hemostasis, or a combination of both, may be needed in order to prevent thrombosis and improve survival rates. While some studies report improved survival with certain hemostasis inhibitors [1-3], we are still lacking controlled studies with direct comparison between various treatment options.

Based on the results of our current study, we recommended a 2 mg/kg once daily dosage of aspirin for thromboprophylaxis. However, since only 6/8 dogs with a 4 mg/kg once daily dosage were considered aspirin responders, a superior approach to thromboprophylaxis may be to start at a 2 mg/kg/day dosage and use individualized dose

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adjustments based on serial platelet function testing, ideally platelet aggregometry. In clinical situations where platelet aggregometry cannot be performed, PFA-100® could be used as an alternative. Baseline and recheck evaluation could be recommended one week after starting aspirin therapy, and one week after any dose changes. If either test failed to show aspirin responsiveness, the aspirin dose could be increased incrementally until the desired affect is reached. Prior to initiating a treatment protocol of this nature, additional studies are recommended in order to understand the potential gastrointestinal side effects at higher aspirin doses.

One concern with using aspirin for thromboprophylaxis is the inability to standardize and optimize the methods for evaluating platelet function. Optical aggregometry is considered to be the gold standard method for evaluating aspirin-induced platelet dysfunction in humans.[4-6] It was also recently demonstrated that optical aggregometry was the most reliable test of platelet function that consistently identified dogs that responded to an anti-platelet dosage of aspirin.[7] While we currently accept optical aggregometry as the gold standard when evaluating aspirin-induced platelet dysfunction in dogs, there is uncertainty both in human and veterinary medicine as to whether or not this is actually the most appropriate methodology.

Our study chose, as well as utilizing optical aggregometry, to include other methods of platelet function, such as the PFA-100®, because of the user-friendly technique and increased availability of these methods. At 2 mg/kg q24h, 100% of dogs were aspirin responders using optical aggregometry, while only 62.5% of dogs were aspirin responders using PFA-100®. Relying solely on PFA-100®, there would have been an additional 37.5% of dogs classified as non-responders. Utilizing the PFA-100® to

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assess platelet function is therefore questionable, and could lead to inappropriate doses being used in patients. Additionally, while the PFA-100® may potentially provide information about increased activity of platelets, it is uncertain whether or not shortened closure times represents increased platelet activity and hypercoagulability. One alternative method may be the VerifyNow® system, a point-of-care analyzer which has received approval by U.S. Federal Drug Administration for assessing platelet function in humans. The sensitivity of the VeryifyNow system for detecting aspirin-induced inhibition of platelet function in human medicine has been reported to vary widely, from 38% to 95%. [5, 8, 9] The correlation between VerifyNow and light transmission aggregometry with arachidonic acid as an agonist was found to be poor in human patients with stable coronary artery disease receiving aspirin [5], whereas in healthy patients it was shown to have good correlation.[10] In studies where high sensitivities of the VerifyNow system were reported for aspirin-induced platelet dysfunction, it was suggested that this method may optimal. Of course, additional studies are needed in order to better understand the utility of this method, particularly in dogs. Identifying a method that is sensitive and correlates well with light transmission aggregometry, but is more readily available and user friendly, would have the potential to provide an enormous amount of information in veterinary clinical patients receiving aspirin.

Evaluation of blood acetylsalicyclic acid and salicyclic acid levels may help to solve some of the mystery involved with finding the ideal dose of aspirin to inhibit platelets. Early human studies evaluating aspirin, however, showed an absence of a definable relationship between plasma acetylsalicyclic acid and its effects.[11] Canine studies attempting to correlate anti-inflammatory effects or analgesic properties of aspirin

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with blood acetylsalicyclic acid levels have also shown that onset and duration of analgesia with either oral or intravenous routes of administration could not be correlated to plasma levels of acetylsalicyclic acid.[12] This was likely because acetylsalicyclic acid is rapidly converted into salicyclic acid. Pharmacokinetic studies evaluating plasma salicyclate levels in dogs have shown marked individual variations in blood levels.[13, 14] A simple and sensitive analytical method using liquid chromatography-tandem mass spectrometry for determination of acetylsalicylic acid (aspirin) and its major metabolite, salicylic acid, in animal plasma has been developed and validated, and could be used in pharmacokinetic studies.[15] While many human studies incorporate acetylsalicyclic acid and salicyclic acid assays into their pharmacodynamic evaluations of platelet inhibition, similar studies in veterinary patients are lacking. A human study used high-performance liquid chromatography to compare plasma levels of acetylsalicyclic acid and salicyclic acid when two doses of aspirin were administered (160 mg/day vs 80 mg/day). [16] Combining this pharmacokinetic information with pharmacodynamic assays (inhibition of platelet thromboxane generation and urinary excretion of thromboxane and prostacyclin metabolites) allowed for a more complete evaluation of these two doses. Given the short half-life of acetylsalicyclic acid, and evidence that only platelets exposed to non-hydrolyzed acetylsalicyclic acid are inactivated, other delivery methods for acetylsalicyclic acid have been developed to provide coverage over a full 24 hour interval.[17-22] For example, one study compared the pharmacokinetics and pharmacodynamics of once daily immediate-release aspirin to an extended-release formulation of aspirin.[23] This study concluded that both aspirin formulations showed dose-dependent anti-platelet activity and that, compared to the immediate-release

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formulation, the extended-release formulation released active drug more slowly, resulting in prolonged absorption and lower systemic drug concentrations. The data from this study suggested a higher dose (162.5 mg) of extended-release aspirin is needed to achieve the same pharmacodynamic response as immediate-release aspirin 81 mg in steady state. Similar to human studies, investigating these factors in veterinary medicine could also help to better understand the pharmacokinetics and pharmacodynamics of aspirin in the dog, and could improve pharmacological and clinical success of this drug.

While it seems likely that the adjusted low-dose of aspirin (2mg/kg q24h) will improve inhibition of platelets in healthy dogs, it is unknown whether the same response will be obtained in patients with hypercoaguable states. Activation of platelets has been demonstrated in dogs with diseases such as IMHA, *Babesia rossi* infection, and endotoxemia, and in critically-ill dogs.[24-27] However, there have been no studies that have reported the impact of anti-platelet therapy on platelet function in dogs with diseases causing hypercoagulability associated with platelet hyperactivity.[28] This leaves some clinicians skeptical of the usefulness of aspirin in canine clinic patients, although the low-cost and ease of treatment continues to be a driving force for its use. The next step moving forward involves elucidating which hypercoagulable diseases involve platelet activation, studying platelet function in dogs with diseases known to have increased platelet activity contributing to thromboemboli while receiving anti-platelet therapy, and comparing outcome and survival in these patients compared to those without anti-platelet therapy.

Increasing the recommended low-dose of aspirin to 2 mg/kg q24h may increase the risk of gastrointestinal upset and hemorrhage. Significant gastrointestinal hemorrhage

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has been detected in dogs receiving the standard anti-inflammatory dose of aspirin (10-35 mg/kg q8-12h). [29-35] The risk of gastrointestinal damage with low-dose aspirin has not been thoroughly evaluated in dogs, but few studies have suggested that these doses (0.5-1 mg/kg/day) will decrease the likelihood of gastrointestinal side effects.[2, 36-38]. An increased risk of gastrointestinal ulceration or hemorrhage when aspirin is used in combination with prednisone or other glucocorticoid medications has also been theorized. Glucocorticoids are thought to induce gastrointestinal ulceration by the inhibition of phospholipase A.[39-41] In addition, glucocorticoids have been shown to decrease healing of gastrointestinal ulcers by altering the composition of gastric mucus, decreasing gastric mucosal cell turnover, inhibiting capillary and fibroblast proliferation, and enhancing collagen breakdown.[39, 40, 42-44]. While studies have shown that dexamethasone and extremely high doses of methylprednisolone cause gastrointestinal ulceration, few studies have evaluated the gastrointestinal effects of prednisone or immunosuppressive doses of prednisone in dogs.[45, 46]

Ultimately, the risk of gastrointestinal ulceration and hemorrhage associated with low-dose aspirin and immunosuppressive doses of prednisone has not been evaluated in canine clinic patients separately or in combination, or long-term. In healthy dogs, one study did evaluate the effects of prednisone (2 mg/kg/day) alone and in combination with low-dose aspirin (0.5 mg/kg/day) on the gastroduodenal mucosa.[47] This study found that the concurrent use of prednisone and low-dose aspirin did not increase the severity of gastroduodenal lesions compared to prednisone alone, or placebo. Some of the dogs in this study did experience mild, self-limiting diarrhea when given the combination of prednisone and low-dose aspirin. This study was performed in healthy beagle dogs, and

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the results could vary when these medications are given to clinically-ill patients. The other concern is whether or not gastrointestinal side effects are severe enough to be clinically relevant for the patient. The previously mentioned study did identify individual healthy dogs from each treatment group that developed gastric and duodenal mucosa lesions of varying severity.[47] While there was no significant difference in clinical signs compared to the placebo group, the authors suggested that the same may not be true for sick, geriatric, or debilitated patients.[31, 32, 48] Other studies have also shown the lack of clinical evidence of gastrointestinal side effects in dogs treated with prednisone and low-dose aspirin.[2]

In order to more thoroughly understand the gastrointestinal side effects of increased doses of aspirin, and aspirin combined with prednisone, and the clinical impact on sick or debilitated patients, additional studies are needed. First, a randomized, prospective study evaluating these effects in healthy dogs could be performed. Physical examination, complete blood counts, reticulocyte counts, serum iron level, total iron binding capacity, serum ferritin, serum hepcidin, serum biochemistry, upper gastrointestinal endoscopy with biopsies, gross fecal examination, fecal occult blood testing, and validated psychometric quality of life tools could be performed before, during, and after treatment. Ideally, these parameters would be evaluated in patients with aspirin alone, aspirin and prednisone combined, and a control group. After completing a study in healthy dogs and, if proven to be relatively safe, these methods could be used to evaluate sick, debilitated patients as well. While it is not practical to place sick, debilitated patients under anesthesia for elective procedures such as baseline upper

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gastrointestinal endoscopy, modifications to the prior protocol may be utilized to include these types of procedures once patients have been stabilized.

Another aspect of thromboprophylaxis requiring additional research is the role and efficacy of alternative anti-platelet medications to aspirin, such as clopidogrel. Clopidogrel has many similar appealing attributes that aspirin does, with the exception that the proprietary product is more expensive. While proprietary clopidogrel may cost more than aspirin, compounding either of these medications into specific and individualized capsules makes the cost of these two medications similar. Regardless, clopidogrel and aspirin both remain considerably less expensive than anticoagulant treatments, and do not require frequent injections. Furthermore, the combination of aspirin and clopidogrel has become popular in human and veterinary medicine. In fact, a recent human study investigated the pharmacokinetics and relative bioavailability of a fixed-dose combination capsule of clopidogrel and aspirin, and found this formulation to be equivalent to individual formulations, with the added benefit of improved compliance.[49, 50] In humans, using clopidogrel alone or concurrently with aspirin has been shown to significantly reduce the incidence of thrombotic events following myocardial infarction, ischemic stroke, vascular death, and restenosis of intracoronary stents. [51-53] Few studies have investigated the use of clopidogrel in veterinary patients, with only a single study showing the use of clopidogrel to be superior to aspirin. In this particular study, cats were given either aspirin (81mg/cat every 3 days) or clopidogrel (18.75 mg/cat once daily) for the prevention of cardiogenic arterial thromboembolism. Cats given clopidogrel had a significantly longer survival time compared to cats receiving aspirin.[54]

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Similar to aspirin, there have been recent human studies suggesting that 20-40% of patients receiving clopidogrel are resistant to the drug's anti-platelet effects, with a subset of clopidogrel non-responders and poor responders. [55] Additional studies are needed to determine the prevalence of clopidogrel resistance in veterinary patients, to establish whether dual anti-platelet therapy with aspirin and clopidogrel is superior to single drug therapy, and to determine whether the specific disease condition or species has any influence on treatment success. In the meantime, until the incidence of clopidogrel resistance is established in dogs, it may be wise to utilize a combination of both aspirin and clopidogrel in patients requiring anti-platelet therapy, in order to minimize the chances of ineffective therapy due to drug resistance.

In humans, genetic testing for sensitivity to anti-platelet medications such as aspirin and clopidogrel is also under current investigation. Genetic variables such as COX-1 haplotype polymorphism, $P1^{A1/A2}$ polymorphism to the beta (3) subunit (glycoprotein IIIa), polymorphism of P2Y12 receptor (H2 haplotype), and CYP2C19*2 polymorphisms have been identified in humans, and are believed to contribute to aspirin and clopidogrel resistance.[56-59] Similar genetic polymorphisms have yet to be investigated in veterinary medicine but, if such polymorphisms can be identified, genetic testing prior to therapy may have the potential to become a convenient method of recognizing potential aspirin and clopidogrel non-responders.

Ultimately, our hope with the information gained from this study is to optimize the platelet inhibition induced by aspirin, to overcome the limitations of aspirin resistance, and to decrease the risk of thromboembolic complications in at risk canine patients. Additional studies are needed in order to determine if a dose of aspirin of 2

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mg/kg/day will achieve these goals, and if this dose will do so without increasing the risk of significant complications in these patients.

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