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The Effects of Aspirin and Cyclosporine on Canine Platelet Function and Cyclooxygenase Expression

John Metcalfe Thomason

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By

John Metcalfe Thomason

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By

John Metcalfe Thomason

Approved:

Andrew Mackin Professor of Small Animal Internal Medicine (Director of Thesis)	Cory Langston Professor of Veterinary Pharmacology (Committee Member)
Robert Linford Professor of Equine Surgery (Committee Member)	Lesya Pinchuk Associate Professor of Immunology (Committee Member)
Stephen Pruett Department Head Department of Basic Sciences (Committee Member)	Mark Lawrence Associate Dean Director of Graduate Studies in the College of Veterinary Medicine

Kent Hoblet
Dean of the College of Veterinary Medicine

Name: John Metcalfe Thomason

Date of Degree: May 11, 2012

Institution: Mississippi State University

Major Field: Veterinary Medicine Science

Major Professor: Dr. Andrew Mackin

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Candidate for Degree of Master of Science

Immune-mediated hemolytic anemia (IMHA) is one of the most common causes of anemia in dogs. Despite aggressive therapy, there is a 50% mortality rate in IMHA patients, and the most common cause of death is thromboembolic disease, particularly pulmonary thromboembolism. With the high thromboembolism rate in dogs with IMHA, anti-platelet therapy with aspirin can be a life-saving preventative therapy. Along with anti-platelet therapy, immunosuppressive therapy is needed to decrease erythrocyte destruction. Cyclosporine has become a popular medication for immunosuppression in IMHA patients. Unfortunately, recent human reports have suggested that cyclosporine could activate platelets and contribute to a hypercoagulable state.

With the goal of improving therapy, these studies investigated the role aspirin plays in inhibiting platelet function and cyclooxygenase expression, an enzyme that enhances platelet reactivity. The effect of cyclosporine on platelet reactivity and hypercoagulablity was investigated to determine if this medication would create activated platelets and a prothrombotic state.

DEDICATION

I would like to dedicate this research to my fiancée, Emilia Morgan, our beloved basset hound, Coley, my parents, Claude and Becky Thomason, and my sister Elizabeth.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Immune-mediated hemolytic anemia (IMHA), also known as autoimmune hemolytic anemia, is one of the most common causes of red blood cell destruction and anemia in dogs, and is mediated by antibody and/or complement-mediated destruction of red blood cells.[1] In IMHA cases, the destruction of erythrocytes is usually associated with a type II hypersensitivity reaction that involves anti-erythrocyte antibodies, including IgG, IgM, and IgA, as well as complement components.[2,4] Extravascular hemolysis, or erythrophagocytosis, is typically associated with IgG antibodies and complement components C to C_3 stages (specifically C_3).[4] A majority of erythrophagocytosis occurs in tissues that contain a high population of macrophages, mainly the spleen, liver, and bone marrow.[5] Intravascular erythrocyte destruction is more commonly associated with hemolysis instead of erythrophagocytosis, and is due to the activation of the entire complement cascade, C_1 to C_9. Complement fixation on the erythrocyte membrane causes an influx of extracellular fluid, followed by red blood cell lysis.[6] Since IgM is more efficient at fixing complement proteins, intravascular IMHA is typically an IgM-mediated disease.[5,7,8]

Immune-mediated hemolytic anemia can be further classified as either primary or secondary, depending on the presence of an underlying disease. Patients diagnosed with primary, or idiopathic, IMHA do not have an identifiable underlying condition that

promotes erythrocyte destruction.[2] However, patients with secondary IMHA usually develop an immunologic response to foreign antigens that have either modified or become associated with erythrocyte membranes. Causes of secondary IMHA include infectious diseases (bacterial, viral, rickettsial), toxins, parasites, drugs (such as sulfa antibiotics, penicillins, and acetaminophen), vaccination, and neoplasia.[1,2] Primary IMHA is the most common form of IMHA, and 60 to 75% of all IMHA cases are considered to be idiopathic.[2]

The diagnosis of secondary IMHA requires the identification of an underlying cause; while treatment consists of the removal or treatment of the initiating cause, followed by temporary immunosuppressive therapy. On the other hand, since patients with primary IMHA do not have an identifiable underlying cause, the mainstay of therapy consists of immunosuppressive therapy (using drugs such as prednisone, cyclosporine, and azathioprine) and supportive care (for example, blood transfusion).

Unfortunately, despite the best treatment efforts, primary IMHA in dogs will often lead to death of the patient. The mean mortality rate for primary IMHA in dogs is approximately 50%,[3,8-10] but has been reported to be as high as 70%.[2] The most common cause of death in dogs with IMHA is thromboembolic disease, particularly pulmonary thromboembolism (PTE). Approximately 50% of dogs with IMHA have been shown to be in a hypercoagulable state that predisposes them to complications such as PTE.[2] The incidence of PTE in dogs with IMHA has been determined to be over 30%, and may be as high as 80%.[8] Since pulmonary thromboembolism is notoriously difficult to diagnose except at post mortem, it is even possible that the incidence of PTE in dogs with IMHA may be higher than 80%.[8]

Even though IMHA is a common condition in dogs, and almost all patients are predisposed to the development of thromboembolic complications, the exact mechanisms of excessive thrombus formation are not completely understood. However, it is hypothesized that a hypercoagulable state involving both blood platelets and clotting factors is implicated in the development of pathologic thromboembolic diseases.[2,11] Other possible factors contributing to the formation of PTE include vascular endothelial injury and blood stasis. Since a hypercoagulable state, vascular endothelial injury and blood stasis (so-called 'Virchow's triad') are all potentially present in IMHA patients,[7,8] it is hardly surprising that affected animals are prone to PTE and other thromboembolic complications. Dogs with IMHA have been shown to have hyperactive platelets,[11] suggesting that platelets may play an integral role in thrombus formation.

Platelet Formation

The formation of platelets, thrombopoiesis, occurs mainly in the bone marrow and involves the maturation of the megakaryocyte cell line (megakaryoblast, promegakaryocyte, and megakaryocyte).[12] The terminal differentiation phase of thrombopoiesis results in the shedding of platelets into circulation. Mature megakaryocytes develop multiple cytoplasmic processes, proplatelets, which penetrate into the venous sinusoids. Through a process of fragmentation, platelets are shed from both the megakaryoctye and proplatelet, and enter circulation.[12-14] Once in circulation, the normal circulating lifespan of canine platelets is approximately five to seven days.[15]

Newly formed platelets that have recently been released into circulation are known as reticulated platelets, because of the residual mRNA present within the cell.[16] In

the circulation, reticulated platelets will continue to mature and the residual mRNA will degrade within one day.[17] The percentage of circulating reticulated platelets can reflect megakaryocyte proliferation, and an elevated percentage of these new platelets usually indicates accelerated platelet production, while a lower percentage suggests poor megakaryocyte function.[16]

Mature platelets are anucleate cells and for this reason, it has often been stated that platelets are not capable of *de novo* changes in expression of proteins in response to endogenous or exogenous stimuli. However, since reticulated platelets contain residual mRNA and some rough endoplasmic reticulum derived from megakaryocytes,[18,19] the intracellular molecular mechanisms necessary for protein production remain functional.[17] The concentration and expression of proteins within newly formed platelets will depend on the protein expression within the megakaryocyte. Hence, the upregulation of a specific protein within the megakaryocyte will result in a similar increased expression in both the reticulated and mature platelet.[19] The platelet's ability to continue to synthesize and express proteins in a post-transcriptional level has previously been demonstrated in human platelets that contain active mRNA that continues *de novo* synthesis of plasminogen activator inhibitor 1.[19]

Platelet Function

Normal blood hemostasis is a complicated process that involves platelets, vascular endothelium, numerous coagulation factors, and several interacting cells. Hemostasis is typically broken down into three distinct phases: first, the formation of a platelet plug through the interactions of platelets and the vessel endothelium (primary

hemostasis), second, the interaction of coagulation factors to form a blood clot that strengthens the platelet plug with a cross-linked fibrin meshwork (secondary hemostasis), and finally, the activation of plasmin to break down fibrin and remove the blood clot (fibrinolysis). Defects in any of these phases can result in ineffective hemostasis, leading to either excessive hemorrhage or clot formation. The formation of a temporary platelet plug during primary hemostasis is the first step in coagulation, and plays a major role in preventing blood loss. Unfortunately, the platelet plug is only stable for a short period of time and, without coagulation factors for reinforcement, the plug will weaken and a blood clot may not form. On the other hand, continual platelet activation, especially in hypercoagulable states, will support excessive platelet plug and blood clot formation.

In circulation, platelets travel along the periphery of blood vessels in a non-activated state. When exposed to injured or damaged endothelium, either due to normal cell turnover or vessel trauma, platelets are activated and adhere to the damaged endothelium.[20] Activated platelets will release a multitude of agents such as serotonin, adenosine diphosphate (ADP), prostaglandins, and thromboxane A_2 (TXA_2), which have the combined effects of causing vasoconstriction, decreasing blood flow, and increasing blood stasis.[21] Disruption of the vessel endothelium and decreases in blood velocity due to vasoconstriction result in increased contact time between activated platelets, coagulation factors and damaged vascular subendothelium, a condition which promotes generation of the nidus needed to initiate clot formation.

One of the main mediators in platelet-induced vasoconstriction and platelet activation is the prostaglandin thromboxane A_2. Once synthesized and released by activated platelets, TXA_2 triggers vasoconstriction, recruits and activates additional

platelets, and enhances platelet aggregation.[22] Following synthesis by platelets, thromboxane A_2 has an immediate, but short lived effect (approximately 30 seconds). After release, thromboxane A_2 is metabolized to thromboxane B_2 and further metabolized into 11-dehydro-thromboxane B_2 and 2,3-dinor-thromboxane B_2 before being eliminated in the urine.[23-25]

Cells, including platelets, do not store thromboxane, but the prostaglandin is synthesized from arachidonic acid that has been released from phospholipid membranes through the actions of several phospholipases that include phospholipase A_2, prostaglandin H synthase, and thromboxane synthase.[26-28] Once mobilized, arachidonic acid is converted into prostaglandin H_2 by prostaglandin H-synthase-1 or -2, more commonly known as cyclooxygenase (COX)-1 and –2. Both COX enzymes play key roles in normal hemostasis, and lead to the production of several biologically active prostaglandins, mainly thromboxane A_2 and prostacyclin.[27,29] Unlike thromboxane A_2, which is mainly produced by platelets to enhance aggregation, prostacyclin (prostaglandin I_2) originates from the vascular endothelium and has the opposite physiologic effect on primary hemostasis (vasodilation and inhibiting platelet aggregation).[26,27,30] These two prostaglandins work in concert to maintain a balanced, normal hemostatic system.

At least three COX isoforms have been described in dogs. COX-1 is constitutively expressed in most body systems, and functions to maintain normal cellular activities including platelet thromboxane A_2 production, maintenance of gastric mucosal protection, and preservation of renal blood flow.[28,31] The COX-3 isoform is transcribed from the COX-1 gene as a splice variant and has similar catalytic features, but only about

20% of the activity of COX-1.[32,33] Relative to COX-1, COX-2 has a far more limited tissue distribution, and expression is often at much lower levels. COX-2 expression occurs in the brain, kidney, thymus and the vascular endothelium.[31,34,35] COX-2 is also present in circulating monocytes, tissue macrophages and fibroblasts.[31] COX-2 expression can be induced by various endogenous and exogenous mediators including inflammatory cytokines, growth factors, and endotoxins.[28,36,37] Induction of the COX-2 enzyme occurs primarily at the post-transcriptional level.[38]

Since mature platelets do not contain a nucleus and are therefore incapable of mRNA synthesis, and because COX-1 is usually constitutively expressed by cells whereas COX-2 expression is more typically induced, it was long believed that COX-1 was the only COX isoform expressed in circulating platelets, and that COX induction could happen only at the level of the bone marrow. As a result, it was originally believed that COX-1 was the primary mediator of platelet thromboxane A_2 production, while COX-2 was the main isoform that controlled endothelial prostacyclin production.[26,27,35,39-41] However, a flow cytometry technique has been described that has identified and quantified the expression of both COX-1 and COX-2 in circulating human platelets.[39] In humans, platelet COX-2 expression is greater during times of increased platelet turnover and higher levels of reticulated platelets.[29,34,42] These recent discoveries suggest that platelet COX-2 could in fact play a role in platelet function.[31,39] Nucleated marrow platelet precursor cells, megakaryocytes, contain COX-1 and COX-2,[31,42] and changes in COX expression that are induced at the level of these platelet precursors would be expected to result in similar changes in platelets derived from affected megakaryocytes.

Platelet activation is a complicated process that involves much more than interaction with thromboxane, and also includes a variety of agonists that stimulate and activate platelets. Just as activated platelets release substances that cause vasoconstriction, platelets also release agonists that attract and activate other platelets to help form a platelet plug. Since platelets do not contain a nucleus, they are dependent on agonists and stimuli to reach an activated state. Stored throughout the platelet are granules, approximately 200 – 500 nm in size, that contain multiple proteins that enhance platelet adhesion, promote cell-to-cell interactions, and stimulate vascular repair.[43,44] Alpha-granules are the main secretory component of platelets, but dense (delta) granules and lysosomes are also present in platelets.[44] Substances found in α-granules include adhesion molecules (von Willebrand factor, P-selectin, and fibronectin), pro and anti-angiogenic factors (Vascular Endothelial Growth Factor) and coagulation factors (fibrinogen, factors V and VIII), while dense granules contain adenosine diphosphate, epinephrine, and serotonin.[44] It is well established that, both during platelet formation in the bone marrow and after being taken up by circulating platelets, molecules can be selectively packaged within different α-granule subsets.[43-47] During activation, the release of selectively packaged proteins from platelet α-granules is dependent on specific receptor activation and different platelet agonists.[43,44,48]

One of the molecules released from platelet α-granules during activation is P-selectin. Platelet P-selectin is a cell adhesion molecule that mediates platelet and leukocyte aggregation and generates procoagulant microparticles that contain active tissue factor and enhance fibrin deposition.[49,50] Therefore, platelet P-selectin is believed to have procoagulant effects that enhance both platelet aggregation and secondary

hemostasis. Platelet P-selectin expression has been shown to be expressed in higher concentrations in canine patients with primary IMHA, and could contribute to the high rate of thromboembolic complications seen in IMHA patients.[11]

Platelets play an important role in assisting the coagulation factors needed to stabilize the platelet plug and form a blood clot. First, platelet α-granules release pro-coagulant factors such as factors V and VIII and fibrinogen, which enhance the clot strength. Secondly, activated platelets will undergo a shape change, creating a greater surface area for adherence to both the endothelium and other platelets or cells.[51] Finally, platelets will also express additional receptors and ligands on the surface to facilitate thrombin generation and fibrin formation. In inactivated platelets, the aminophospholipid phosphatidylserine is located in the inner leaflet of the platelet plasma membrane but, upon activation, platelet phosphatidylserine is exposed on platelet surface membranes and binds to prothrombin, accelerating the conversion of prothrombin to thrombin, and expediting the formation of a blood clot.[52] It is estimated that a phosphatidylserine-rich platelet surface is capable of synthesizing thrombin 300,000 times faster than the fluid phase of plasma.[52] Phosphatidylserine expression during platelet activation occurs primarily through the opening of the mitochondrial permeability transition pore (MPTP).[53,54] During activation, the MPTP transiently opens and releases several activating mitochondrial contents into the cell cytoplasm, mediating caspase-3 activation and ultimately leading to increased phosphatidylserine expression on the cell surface.[55,56]

Aspirin Therapy

Normal platelet function and activation is necessary for the prevention of hemorrhage, but excessive platelet activation can lead to the development of large blood clots and thromboembolic complications, especially pulmonary thromboembolism. The risk of thromboembolism has been identified as a significant complication in many commonly encountered diseases, and can be a devastating complication of otherwise treatable conditions.[57] Besides IMHA, there are many diseases/conditions in veterinary medicine that are associated with an increased risk of thromboembolic complications, including protein-losing nephropathies, hyperadrenocorticism, sepsis, disseminated intravascular coagulation, and systemic inflammatory response syndrome.[58]

Diagnosing thromboembolic diseases can be very challenging. In human medicine, the diagnosis of a thromboembolism typically involves advanced imaging techniques that include venous compression ultrasonography and multidetector computed tomography and magnetic resonance angiography.[59] However, in veterinary medicine, some of these diagnostic techniques are not readily available. Because of the difficulty in establishing a definitive diagnosis in dogs, a combination of clinical signs, blood tests (decrease in platelet count, increase in D-Dimer and fibrin degradation products concentrations), and diagnostic imaging are needed to determine a presumptive diagnosis of a thromboembolism. Establishing a diagnosis of thromboembolism will also depend on the presence of an underlying disease that is associated with a hypercoagulable state.

Even with a definitive diagnosis, and the localization of a thrombus, treatment can be difficult and have devastating, life-threatening complications. If possible, either removal of the clot or administration of thrombolytic medications directly to the clot can

provide some relief and restore blood flow to the affected tissue. However, identifying and then isolating the thrombus can be very difficult, limiting the usefulness of these techniques. The use of systemic thrombolytic agents could be beneficial at dissolving a thrombus and restoring blood flow to the affected tissue. Unfortunately, severe complications can arise from systemic thrombolytic therapies which include hemorrhage, allergic reactions, and ischemia-reperfusion injuries after thrombus dissolution.[60]

Because of the devastating complications associated with both a thromboembolic event and thrombolytic treatment, preventative therapy is an essential component of treatment of patients in a hypercoagulable state. Inhibition of both primary and secondary hemostasis will provide the most complete preventative therapy. Unfortunately, many of the medications used in veterinary medicine that inhibit coagulation factors, such as heparins, can only be given by injection or are cost prohibitive for long term administration, making anti-platelet therapy the most commonly administered preventative therapy. The use of a safe, long term, affordable oral anti-platelet medication for the prevention of thromboembolism becomes a critical component of therapy in patients with in a hypercoagulable state. One of the most commonly used anti-platelet medications, aspirin, is highly effective at inhibiting both human and canine platelet function.[22,61-64]

Aspirin (acetylsalicylic acid) inhibits platelet function by irreversibly acetylating the hydroxyl group on a serine residue (position 530) located on the COX-1 enzyme.[28,29,65] This acetylation will permanently block arachidonic acid from reaching the active site of the COX enzyme, which is located between arginine 120 and tyrosine 385.[65] By acetylating the COX enzyme, aspirin prevents platelet thromboxane A_2

synthesis, and inhibits platelet activation and function.[28,29,61,65] Since platelets do not contain a nucleus, and are unable to synthesize new and functioning COX enzymes, aspirin-induced platelet dysfunction will persist for the entire lifespan of the platelet, and function will not return until the drug is discontinued and new platelets, not exposed to aspirin, are released from the bone marrow.

The aspirin-induced inhibition of the COX enzyme, not only inhibits the prostaglandins needed for platelet function, but also inhibits all prostaglandins generated through the arachidonic acid cascade. Other than thromboxane and prostacyclin, the COX enzyme is responsible for the conversion of prostaglandin H_2 to several other biologically active mediators, which include prostaglandin E_2, prostaglandin D_2, and prostaglandin $F_{2\alpha}$. These prostaglandins play key roles in mediating inflammation, tissue homeostasis, renal blood flow, and gastrointestinal wall protection. Besides inhibiting thromboxane production, aspirin's ability to inhibit the COX enzyme will also inhibit pro-inflammatory prostaglandin synthesis, making this medication an effective anti-inflammatory therapy. Unfortunately, by inhibiting the COX enzyme and decreasing prostaglandin synthesis, especially synthesis of the protective prostaglandins E_2 and D_2, aspirin can cause several life-threatening side effects that include gastrointestinal hemorrhage and renal failure.[23] An anti-inflammatory dose, or high dose, of aspirin (10 mg/kg twice daily), is very effective at consistently inhibiting platelet function, but can lead to multiple gastrointestinal and renal side effects, which makes this aspirin dose an undesirable preventative therapy.

A high dose of aspirin permanently inhibits COX function in all cells that express this enzyme, including vascular endothelial cells. However, unlike the higher, anti-

inflammatory dose, a low dose of aspirin (0.5-1 mg/kg once daily) will inhibit platelet thromboxane synthesis and function without permanently inhibiting COX function of the vascular endothelium, allowing prostacyclin synthesis and vasodilation to continue.[22] Lower doses will also eliminate the potentially devastating side effects that occur with high dose aspirin, making low dose aspirin an appealing treatment for the prevention of thromboembolic complications. In fact, the administration of low doses of aspirin has been associated with an improved survival rate in dogs with IMHA.[66]

Unfortunately, despite preventative therapy, some dogs treated with aspirin will continue to develop thromboembolic complications. A similar phenomenon occurs in humans that are receiving low doses of aspirin, indicating that these patients are poorly responsive to the anti-platelet effects of aspirin ('aspirin resistant'). The true incidence of aspirin resistance in humans is unknown, but the published incidence ranges from 8-45% of human patients.[61,67,68] Since canine clinic patients appear to inconsistently respond to low dose aspirin, it is possible that, similar to humans, dogs also experience aspirin resistance. The true incidence of aspirin resistance in dogs is, however, unknown.

The exact mechanism for aspirin resistance in both humans and dogs is also unknown; however, there have been many proposed explanations. One proposed mechanism is the presence of an alternate source of thromboxane.[29] Since aspirin is highly effective at inhibiting COX-1 production of thromboxane, an additional thromboxane source would require a COX-1 independent pathway. One of the proposed alternate pathways of thromboxane production is the COX-2 isoform, either from the vascular endothelium or circulating platelets.[29,39] In humans, the inhibition of the COX-2 enzyme requires a much higher dose of aspirin than COX-1. For example, in humans, an

aspirin dose of 81 – 325 mg per day is adequate to inhibit COX-1 function, while >325 mg per day of aspirin is need to inhibit COX-2 function.[29] Low doses of aspirin, administered to dogs at a dose of 0.5 mg/kg per day,[66] may be sufficient to inhibit COX-1 synthesis of thromboxane, but insufficient to inhibit COX-2 production of thromboxane.

A second proposed mechanism of aspirin resistance includes genetic differences in the polymorphism of both COX-1 and COX-2, making these enzymes less sensitive to aspirin. In humans, polymorphism of the gene that codes for platelet glycoprotein IIIa, PLA2, has been associated with aspirin resistance.[69,70] Other proposed mechanisms of aspirin resistance include an increased reactivity to other platelet aggregating factors, increased production of new platelets that have not been exposed to aspirin, and increased production of isoprostanes, specifically 8-iso-$PGF_{2\alpha}$, which induce vasoconstriction and interact with TXA_2/PGH_2 receptors.[71] Finally, patient compliance and drug absorption play a significant role in assessing the anti-platelet effects of aspirin.

Evaluating Platelet Function

The use of platelet aggregometry has been utilized for over 50 years to evaluate platelet function, and has long been considered the gold standard for evaluating platelet function.[64,72,73] Aggregometry analyzes platelet function by using agonists to activate platelets in either platelet rich plasma or whole blood, and determines the ability of platelets to aggregate and form a platelet plug.[72,73] Platelet aggregometry is, however, expensive and requires specialized equipment and training, thus making it difficult to utilize in a clinical setting.[72]

Similar to aggregometry, the Siemens Platelet Function Analyzer-100® (PFA-100®) is an instrument that evaluates platelet function, but the PFA-100 is more useful in a clinical setting. The PFA-100® has previously been evaluated for use in dogs as a commercial point-of-care platelet function analyzer.[63,64,74] Briefly, the PFA-100® is an *in vitro* platelet function analyzer that measures the time needed to form a platelet plug in an environment similar to blood vessels. Platelet agonists such as collagen, epinephrine, and ADP are used to activate platelets under high shear conditions. The time taken for the activated platelets to form a plug and occlude an aperture designed to replicate damaged vascular endothelium is measured and reported as the closure time. The PFA-100® utilizes two types of cartridges with two platelet agonists per cartridge (collagen/epinephrine and collagen/ADP) to evaluate platelet function. The PFA-100® collagen/epinephrine cartridge detects drug-induced platelet dysfunction and the collagen/ADP cartridge evaluates congenital and acquired platelet function defects.[75] Prolongation of the PFA-100® closure time as measured with the collagen/epinephrine cartridge has become well established as a sensitive indicator of aspirin-induced platelet dysfunction in both humans and dogs.[64]

Thromboxane concentration is a reliable indicator of COX function, both in hypercoagulable states and during aspirin therapy. Urinary concentrations of 11-dehydro-thromboxane B_2 (the most abundant urinary metabolite of thromboxane) and 2,3-dinor thromboxane B_2 are increased in human diseases that are associated with a hypercoagulable state, including atherosclerosis, myocardial infarction, post-stroke dementia, and chronic obstructive pulmonary disease.[76,77] Urinary concentrations of 11-dehydro-thromboxane B_2 are elevated in dogs with gastric dilatation-volvulus.[76]

Measurement of urinary 11-dehydro-thromboxane B_2 has been utilized in previous studies as an indicator of aspirin-induced COX inhibition.[24,77,78]

Levels of thromboxane metabolites in plasma or serum can be artifactually increased due to platelet activation during sample collection and processing. The use of urine to measure thromboxane metabolites avoids the problem of *in vitro* platelet activation associated with blood collection, and is therefore considered to be a more reliable indicator of platelet thromboxane synthesis.[24,25] Urinary thromboxane B_2 can, however, be generated by the kidneys and excreted without metabolism; consequently, urinary thromboxane B_2 concentrations may provide an inaccurate representation *in vivo* platelet thromboxane A_2 synthesis.[76] Therefore, urinary assessment of 11-dehydro-thromboxane B_2 and/or 2,3-dinor thromboxane B_2 are useful indicators of *in vivo* platelet thromboxane production.[23,76,77]

Unlike instruments that evaluate platelet function, flow cytometry allows assessment of platelets with minimal manipulation of the sample. Flow cytometry detects fluorescence from labeled monoclonal antibodies that are bound to surface proteins to identify and quantitate the expression of a particular protein. Unlike aggregometry, where platelet activation is necessary, flow cytometry can evaluate surface protein expression before and after activation. Some of the surface markers that detect platelet activation include P-selectin, phosphatidylserine, and fibrinogen bound to glycoprotein IIb-IIIa.[79,80] Flow cytometry techniques have been developed to identify unactivated and activated platelets as well as microparticles released from activated platelets.[11,79] Even though flow cytometry does not evaluate the platelet's ability to form a platelet plug, understanding which proteins are expressed during clinical diseases helps

determines the activation status of platelets, and if there is an increased risk of thromboembolic complications.

Cyclosporine Therapy

Treatment of IMHA in dogs typically consists of immunosuppressive medications and supportive care. Although the cornerstone of immunosuppressive therapy for IMHA is treatment with glucocorticoids such as prednisone, additional immunosuppressive agents such as cyclophosphamide and azathioprine are often administered concurrently with glucocorticoids in order to achieve an effective level of immunosuppression. Prednisone, cyclophosphamide, and azathioprine all are commonly associated with side effects that can be dangerous for the patient and undesirable for the owners, such as myelosuppression with azathioprine and cyclophosphamide, hepatoxicity with azathioprine, sterile cystitis with cyclophosphamide, and polyuria, polydipsia, polyphagia and panting with prednisone. Side effects associated with standard immunosuppressive agents have in recent times led to the exploration of the use of alternative immunosuppressive agents such as cyclosporine.

Cyclosporine, a powerful immunosuppressive agent, was originally introduced into human medicine over 30 years ago for the use in organ transplantation. Cyclosporine is a promising drug for the treatment of immune-mediated diseases because it typically does not have many of the severe side effects associated with other immunosuppressive agents. Cyclosporine has slowly been adopted by the veterinary community over the past few decades and, more recently, since the development of a veterinary-approved cyclosporine product, has rapidly increased in popularity for the

treatment of a wide range of immune-mediated diseases in the dog.[81-84] Cyclosporine has been shown to be an effective, non-steroidal alternative in refractory allergic cases,[85] in immune-mediated dermatoses,[81] in inflammatory bowel disease,[81] and in perianal furunculous.[82-84,86] Although there have been few scientific studies that have evaluated the effectiveness of cyclosporine in the treatment of IMHA in dogs, the immunosuppressive agent has steadily increased in popularity for treating this particular condition because of limited associated side effects and the perceived safety of the drug.[86] Given the current high mortality rates associated with standard treatment of IMHA in the dog, veterinarians are hoping that cyclosporine will prove to be an effective new medication that improves survival rates and enhances safety and quality of life in patients with IMHA.

Recent human studies, however, have shown that the administration of cyclosporine increases the thrombogenic properties of platelets and accelerates thrombin generation.[52] In 2007, Tomasiak and others used platelet flow cytometry to determine that cyclosporine alters the platelet plasma membrane which results in increased expression of phosphatidylserine.[52] In another study comparing renal transplant patients to hypertensive control patients, P-selectin expression increased with all immunosuppressive agents, including cyclosporine, although platelet aggregation was not measurably affected.[87] Another study also demonstrated an increase in P-selectin expression in renal transplant recipients treated with cyclosporine, but similarly found no measurable increase in platelet aggregation.[88] The promotion of platelet phosphatidylserine and P-selectin will act as a catalysts for thrombin generation to expedite platelet procoagulant activity and thrombus formation.

Cyclosporine has also been shown to increase platelet production of thromboxane A_2 and alter cyclooxygenase expression.[89-91] Several human studies have demonstrated an increase in thromboxane production during cyclosporine administration in renal transplant and coronary artery disease patients.[92-96] When compared to renal transplant recipients administered azathioprine, patients receiving cyclosporine had increased thromboxane production and increased rates of thrombus formation within the renal parenchyma.[93] Previous studies in rats have also demonstrated that cyclosporine suppresses COX-2 expression in a number of different tissues.[91,97]

The following manuscript consists of three separate studies that evaluated platelet function and cyclooxygenase expression during aspirin and cyclosporine administration. The initial study, using high or anti-inflammatory doses of aspirin, utilized flow cytometry to identify and quantify relative COX-1 and COX-2 expression in circulating canine platelets, and to investigate the effects of aspirin on platelet expression of both COX isoforms. The second study, using low doses of aspirin, utilized similar methodology, but tried to determine if low dose aspirin therapy would consistently affect platelet function and thromboxane production and, if not, whether pre-existing COX isoform expression or subsequent changes in isoform expression were associated with aspirin resistance. The final study was designed to thoroughly evaluate the effects of cyclosporine on hemostasis in normal dogs, concentrating on platelet function, thromboxane production and expression of P-selectin, phosphatidylserine, COX-1 and COX-2, and thereby ascertain whether treatment with cyclosporine could feasibly put canine IMHA patients at an increased risk of thromboembolic complications.

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CHAPTER II

PLATELET CYCLOOXYGENASE EXPRESSION IN NORMAL DOGS

Introduction

The cyclooxygenase (COX) enzyme plays a key role in normal platelet function. Platelet COX activity is required to convert arachidonic acid to prostaglandin H_2, leading to the production of several biologically active prostaglandins, including thromboxane A_2.[1,2] Thromboxane A_2 is a potent vasoconstrictor and platelet activator, and production of thromboxane A_2 by platelets is essential for normal platelet function.

At least three COX isoforms have been described in dogs. COX-1 is constitutively expressed in most body systems, and functions to maintain normal cellular activities including platelet thromboxane A_2 production, maintenance of gastric mucosal protection, and preservation of renal blood flow.[2,3] The COX-3 isoform is transcribed from the COX-1 gene as a splice variant and has similar catalytic features, but only about 20% of the activity of COX-1.[4,5] Relative to COX-1, COX-2 has a far more limited tissue distribution, and expression is often at much lower levels. COX-2 expression occurs in the brain, kidney, thymus and the vascular endothelium.[2,6-8] COX-2 is also

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present in circulating monocytes, tissue macrophages and fibroblasts.[2,8] COX-2 expression can be induced by various endogenous and exogenous mediators including inflammatory cytokines, growth factors, and endotoxins.[2,3,9] Induction of COX-2 occurs primarily at the post-transcriptional level.[10]

Because mature platelets do not contain a nucleus and are therefore incapable of mRNA synthesis, and because COX-1 is usually constitutively expressed by cells whereas COX-2 expression is more typically induced, it was long believed that COX-1 was the only COX isoform expressed in circulating platelets, and that COX induction could happen only at the level of the bone marrow. As a result, it was also believed that COX-1 was the primary mediator of platelet thromboxane A_2 production.[7,11-13] Recently, however, a flow cytometry technique has been described that has identified and quantified the expression of both COX-1 and COX-2 in some mature circulating human platelets.[11] The COX-2 isoform has also been identified in higher numbers of young (reticulated) platelets.[1,6,14] These recent discoveries suggest that COX-2 could in fact play a role in platelet function.[8,11] To our knowledge, COX-2 has not previously been identified in circulating canine platelets.

Aspirin (acetylsalicylic acid) is one of the most commonly administered anti-platelet medications in both human and veterinary medicine. Aspirin binds to and irreversibly acetylates serine residues in the active sites of the COX-1 and COX-2 enzymes, resulting in decreased platelet thromboxane A_2 production and inhibition of platelet function.[1,15-20]

The main objective of our study was to utilize flow cytometry to identify and quantify relative COX-1 and COX-2 expression in circulating canine platelets. To

further investigate the respective roles of the COX isoforms, our study also evaluated the effects of aspirin on platelet expression of both isoforms. Platelet function analysis and measurement of urinary 11-dehydro-thromboxane B_2 (11-dTXB_2) were also employed to explore the relationships between COX isoform expression, thromboxane production and platelet function in aspirin-treated dogs.

Material and Methods

Study Design

Eight healthy female intact Walker hound dogs were used in the study. The dogs were not exposed to any drugs or vaccines for at least two months prior to study commencement. Aspirin[a] was administered to each dog at a standard anti-inflammatory dose of 10 mg/kg PO every 12 hours for 10 days. The administered aspirin dose was 10.62 ± 0.610 (mean, SD) mg/kg, PO, every 12 hours. All dogs completed the study. Blood was collected for flow cytometric measurement of platelet COX expression prior to the first aspirin dose, 1 to 4 hours after aspirin administration on Day 10, and 14 days after the last dose of aspirin (washout). Blood and urine were collected for PFA-100® testing and urinary 11-dTXB_2 analysis at two time points, prior to the first aspirin dose and 1 to 4 hours after aspirin administration on Day 10.

Animals

The mean age of the dogs was 5 years (range, 1-6 years). Individual dog weights ranged from 20.5 to 24.2 kg (median, 22.3 kg). Body weight was obtained at the

beginning of the study and used to calculate all subsequent dosing. Normal health status was established based on the normal results of physical examination, buccal mucosal bleeding time, complete blood count (including manual platelet count), serum biochemistry, urinalysis, prothrombin time, partial thromboplastin time, von Willebrand factor antigen testing (ELISA method), heartworm testing, and rickettsial and *Babesia* serology. Animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee.

Flow Cytometric Analysis of Platelet COX-1 and COX-2 Expression

Blood was collected via jugular venipuncture with a 20 gauge needle into a glass vacutainer tube containing 3.8% sodium citrate.[b] Sample preparation for flow cytometry was initiated within 1 hour of collection.

COX-1 labeling*:* A modification of a previously described protocol for quantitating COX-1 expression on human platelets was used to label COX-1.[11] Briefly, 5 µL of citrated whole blood was added to 45 µL of FACS-PBS and incubated for 30 minutes with 10 µL of FITC-conjugated monoclonal mouse anti-ovine-COX-1 antibody.[c] Five microliters of IgG_2b-FITC isotype control[d] was run in parallel with each set of samples. The samples were then incubated for 30 minutes with 3 µL of monoclonal antibody mouse anti-human CD9:RPE antibody[e] to label platelets. Samples were fixed for 10 minutes at 4°C in the dark with 1% paraformaldehyde.[f]

COX-2 labeling*:* A modification of a previously described protocol for quantitating COX-2 expression in permeabilized human platelets was used to label COX-2.[11] Briefly, 5 µL of citrated whole blood was added to 45 µL of FACS-PBS and fixed at 4°C in the

dark for 10 minutes with 1% paraformaldehyde. Samples were washed with PBS and pelleted by centrifugation (400 g for 7 minutes). The supernatant was discarded, and the pellet was resuspended and incubated in 0.3% Triton X-100[g] for 10 minutes, and the wash step was repeated. The samples were then incubated at room temperature in the dark for 30 minutes with 15 µL of a monoclonal FITC-conjugated mouse anti-human-COX-2 antibody.[h] Platelets were labeled by adding 20 µL of monoclonal mouse anti-pig CD61-purified antibody[i] and incubated for 30 minutes, followed by the addition of 100 µL of goat anti-mouse IgG:RPE antibody[j] and incubated for 30 minutes. A FITC-IgG_1 isotype control[k] was run in parallel with all samples.

After labeling of platelets, samples were stored in the dark at 4°C until analysis. Flow cytometric analysis was performed within 2 hours of sample preparation. Platelets were analyzed using a flow cytometer[l] at a wavelength of 488 nanometers with CellQuest Pro software.[m] Platelet populations were displayed on log forward-scatter versus log side-scatter plots. Compensation was performed to compensate for spectral overlap of two different fluorochromes, FITC and PE. Gates were adjusted to baseline platelet populations. A total of 5,000 gated events were recorded for both COX isoforms. COX expression was quantified by the intensity of antibody staining and was expressed as mean fluorescence intensity (MFI). Fluorescence from the isotype control was subtracted from each sample. A histogram was created with MFI on the x-axis and number of events on the y-axis.

All of the antibodies used in this study were cross reactive with canine antigens, according to the manufacturers. Previous studies have demonstrated that all of the critical catalytic residues found in ovine COX-1 are also found in the canine COX-

1molecule, and that there is a greater than 90% sequence similarity between the human and canine COX-2 molecule.[21] Furthermore, anti-ovine and anti-human antibodies have been used previously for identification of canine COX-1 and COX-2, respectively.[21] In pilot studies in our laboratory prior to initiation of this study, the expression of both COX isoforms was analyzed in canine platelets via flow cytometry with both platelet rich plasma and whole blood samples, and samples were also analyzed with several antibodies (anti-CD9, anti-CD61, and anti-CD62P) known to be expressed on the surface of platelets. The sample type and antibody which provided the most consistent identification (isotype controls IgG2b:PE, IgG1:PE, IgG1:PE, respectively) of the platelet population for each isoform assay was then used in the study.

Platelet Function Analysis

Platelet function was analyzed using a commercial point-of-care platelet function analyzer (PFA-100®)[n]. The PFA-100® has been previously evaluated for use in dogs, and the instrument was used according to manufacturer's instructions.[22-24] Briefly, the PFA-100® is an *in vitro* platelet function analyzer that measures the time needed to form a platelet plug in an environment similar to blood vessels. Platelet agonists such as collagen and epinephrine are used to activate platelets under high shear conditions. The time taken for the activated platelets to form a plug and occlude an aperture designed to replicate damaged vascular endothelium is measured and reported as the closure time. The instrument cut-off time for non-closure of the aperture is greater than 300 seconds.

Blood samples were collected into 5 ml blood collection vacutainer tubes containing 3.8% sodium citrate, and samples were kept at room temperature until

analysis. An automated hematologic analyzer[o] was used to establish a platelet count and hematocrit for each sample prior to PFA-100® analysis. All samples were analyzed within two hours of collection. Samples were mixed well prior to analysis, and 800 µL of citrated whole blood sample were transferred into a PFA-100® cartridge containing the agonists collagen and epinephrine (collagen/EPI cartridge)[p], and analyzed. Cartridges were stored at 4°C and warmed to room temperature before analysis.

Urinary 11-Dehydro-Thromboxane B_2 Analysis

Urinary 11-dTXB$_2$ concentration was measured using a multiplex analyzer[q] (which utilizes xMAP technology with fluorescently dyed microspheres to detect low concentrations of substrates) and a commercial competitive enzyme immunoassay kit[r] that has been previously validated in the dog.[25] Urine was collected via cystocentesis, batched, and stored at -80°C until analysis. Prior to analysis, the urine was thawed and the specific gravity was measured. The assay buffer was used to standardize the samples to a urine specific gravity range optimized for the analyzer working range (1.003 to 1.012). All samples were analyzed in duplicate according to the manufacturer's instructions, and reported in picograms per milliliter of urine. A correction factor was applied to account for the sample dilutions. Briefly, a 96-well plate was prepared by adding 100 µl of the diluted sample to the appropriate well, followed by the addition of 50 µl of both 11-dTXB$_2$ Phycoerythrin Tracer and 11-dTXB$_2$ beads to each well. The plate was placed on an orbital shaker and incubated in the dark at room temperature for 4 hours prior to analysis.

A biochemistry analyzer[s] was used to measure urine creatinine concentration by the Jaffe Reaction. Urine 11-dTXB$_2$ concentration was normalized to the individual's urine creatinine by determining the 11-dTXB$_2$ to creatinine ratio, as previously described.[25,26]

Statistical Analysis

A single population, repeated measures design was utilized in this study. After visual assessment of Q-Q plots, COX-1 and COX-2 expression, and 11-dTXB$_2$ concentration were deemed to be approximately normally distributed, although the maximal assay closure time was reached at some time points thus affecting normal distribution for the PFA-100 results. Results of platelet COX expression and urine 11-dTBX$_2$ concentration in dogs treated with aspirin were individually assessed by analysis of variance (ANOVA) using the MIXED procedure in SAS for Windows version 9.2[t]. Sample time was included in the model as a fixed effect. The repeated measures of dogs over time were accounted for by REPEATED statements assuming an unstructured covariance structure. Differences in least square means with Tukey-Kramer adjustment of p-values were used for multiple comparisons of the three time points for the COX-1 and COX-2 analyses. To accommodate the non-normal distribution of the PFA-100® results, differences in baseline and Day 10 levels were evaluated by a Wilcoxon Signed Rank Test using the UNIVARIATE procedure in SAS for Windows version 9.2. A p-value of less than 0.05 was considered to be significant for all analyses.

Results

Flow Cytometric Analysis of Platelet COX-1 and COX-2 Expression

Platelet COX-1 expression was measurable in all dogs prior to drug administration. Time was found to be a significant factor within the model ($p=0.003$). There was a significant increase ($p=0.003$) in COX-1 expression from baseline values by Day 10 of aspirin administration (Figure 2.1), on average by 250%, with a range of 63-476%. By the end of the washout period, there was a significant decrease ($p=0.002$) in COX-1 expression from Day 10 values (Figure 2.1). There was no significant difference between baseline and washout values ($p=0.140$). The aspirin-associated increase in COX-1 expression followed by the decrease after drug discontinuation was observed in all dogs.

Platelet COX-2 expression was also measurable in all dogs prior to drug administration. Time was found to be a significant factor within the model ($p=0.031$). There was no significant change in COX-2 expression from baseline values following administration of aspirin ($p=0.124$) (Figure 2.2). By the end of the washout period, however, there was a significant increase ($p=0.026$) in COX-2 expression from Day 10 values. There was no significant difference between baseline and washout values ($p=0.782$). Individual dog responses were variable: COX-2 expression decreased during aspirin administration in 6 dogs (average decrease 70%, range 56-80%), and increased in 2 dogs (by 8% and 116%).

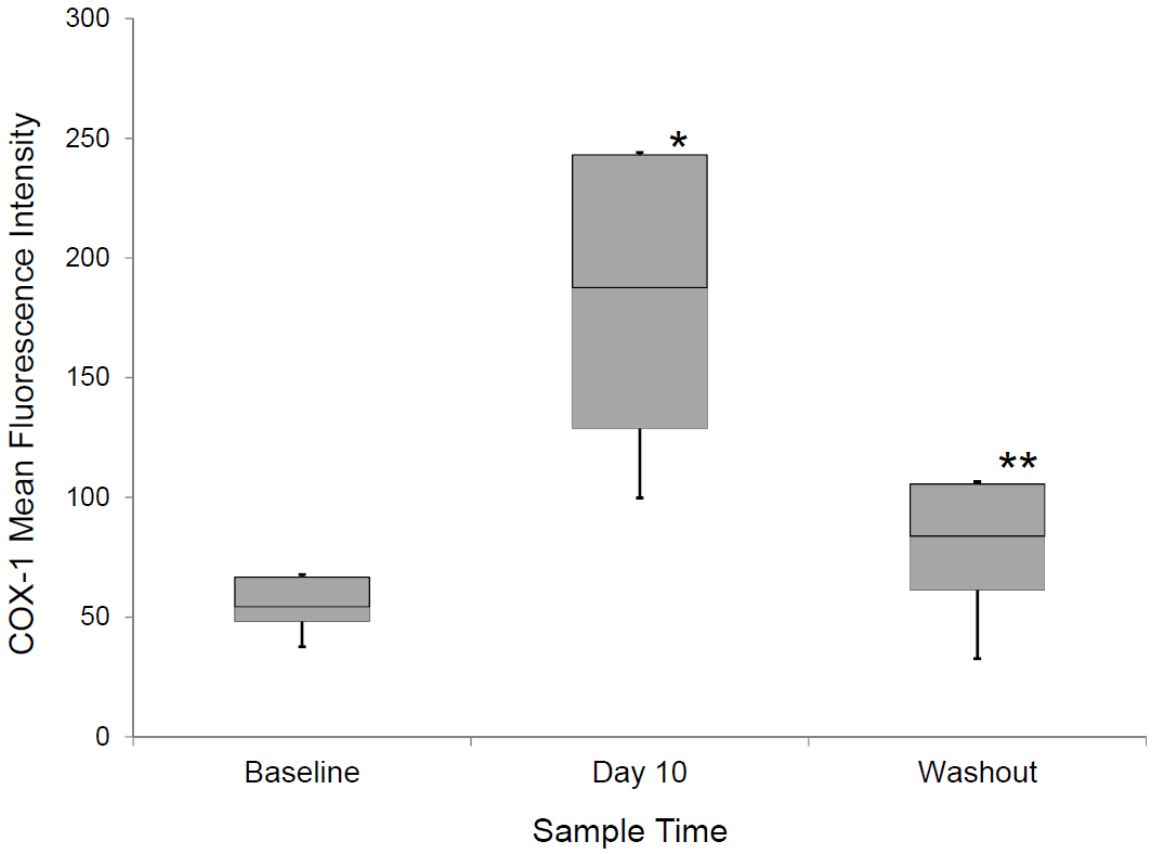

Figure 2.1 Mean COX-1 expression for 8 dogs after treatment with aspirin (10 mg/kg PO every 12 hours for 10 days). There was a significant (p=0.003) increase in COX-1 expression from baseline values after administration of aspirin (*), followed by a significant (p=0.002) decrease from Day 10 values after the discontinuation of aspirin (**). There was no difference (p=0.140) between baseline and washout time points.

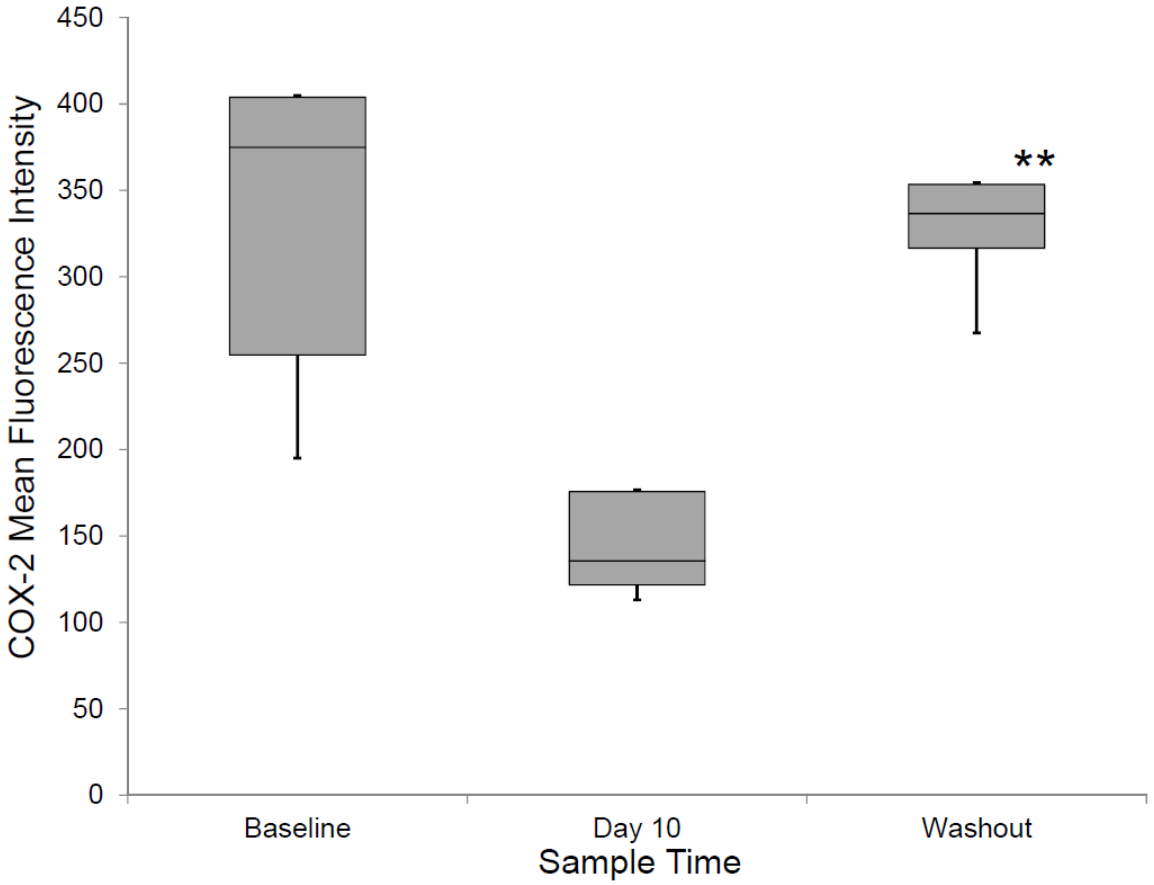

Figure 2.2 Mean COX-2 expression for 8 dogs after treatment with aspirin. Although there was a decrease in expression from baseline values after administration of aspirin, this decrease was not significant (p=0.124). There was a significant (p=0.026) increase from Day 10 values after the discontinuation of aspirin (**). There was no difference (p=0.782) between baseline and washout time points.

Platelet Function Analysis

The mean baseline PFA-100® closure time was 167 seconds (range 84-242 seconds). There was a significant ($p<0.008$) increase in closure time by Day 10 of aspirin administration on average by 75%, mean closure time was 294 seconds (range 274 – 300 seconds), although the increase was in all likelihood of a greater magnitude since 6 out of 8 dogs attained the maximum reportable closure time of 300 seconds. Increased closure times were seen in all dogs (Figure 2.3).

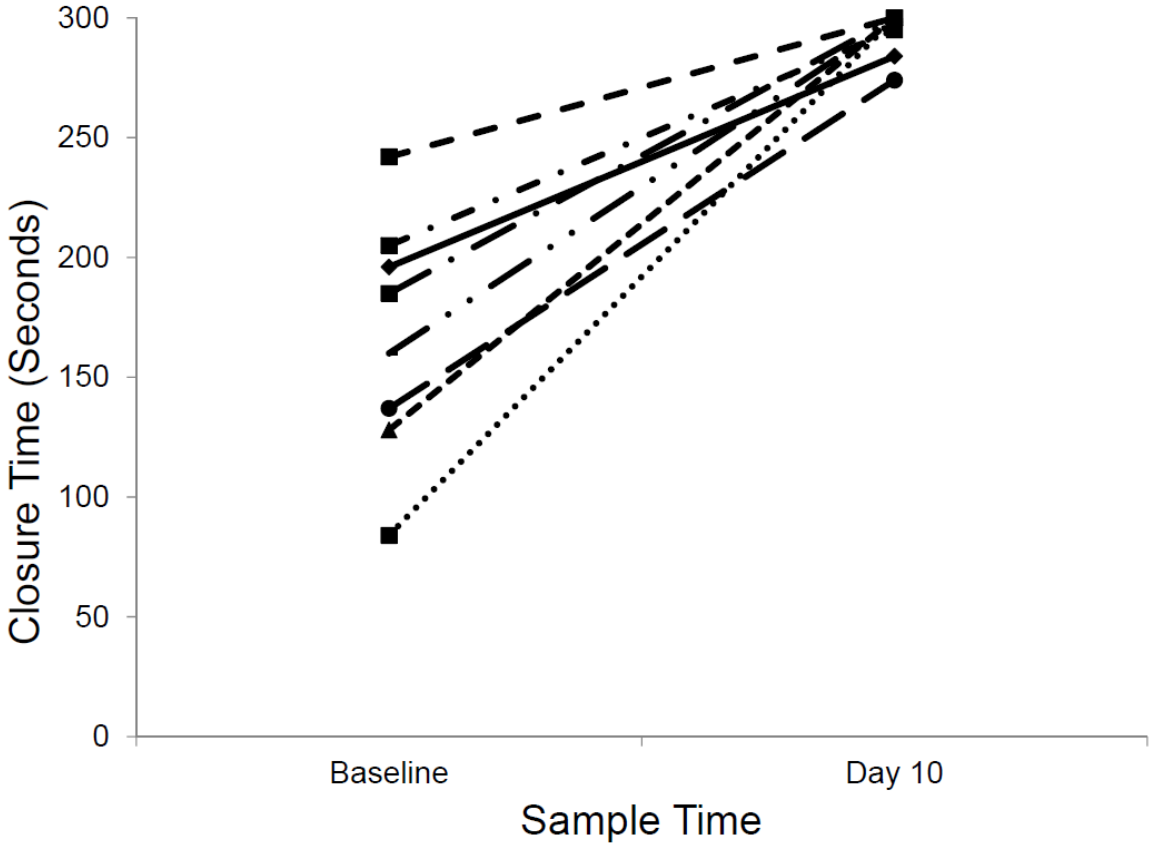

Figure 2.3 PFA-100® closure times in individual dogs after treatment with aspirin. Closure times were prolonged in all 8 dogs. Maximum reportable closure time is 300 seconds. Lines were added between data points to better demonstrate variation among dogs.

Urinary 11-Dehydro-Thromboxane B_2 Analysis

The mean baseline urinary 11-dTXB$_2$ to creatinine ratio was 23.7 (range 9.0-42.5). By Day 10 of aspirin administration, there was a significant (p=0.014) decrease in urinary 11-dTXB$_2$ to creatinine ratio to a mean ratio of 8.5 (range 4.7-12.7), an average decrease of 64%. A decrease in urinary 11-dTXB$_2$ to creatinine ratio was seen in all dogs (Figure 2.4).

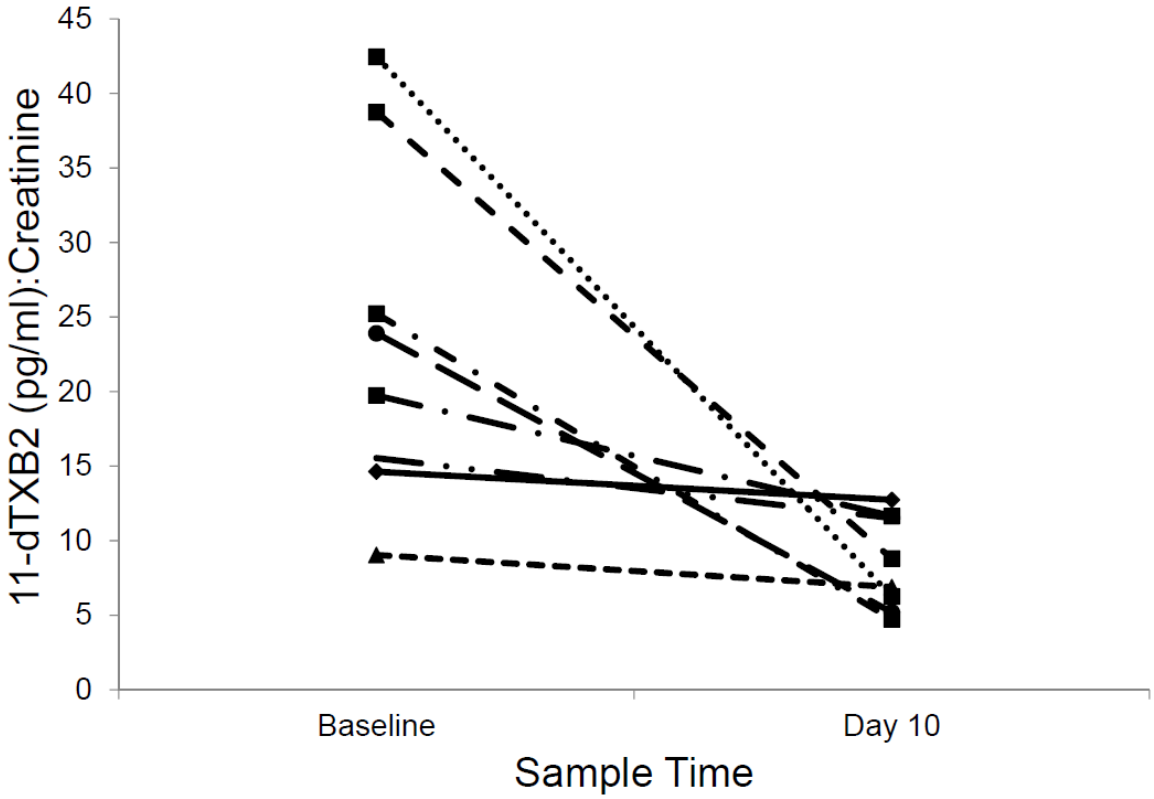

Figure 2.4 Urinary 11-dTXB$_2$ to creatinine ratios in individual dogs after treatment with aspirin. Urinary 11-dTXB$_2$ to creatinine ratios decreased in all 8 dogs. Lines were added between data points to better demonstrate variation among dogs.

Discussion

This study demonstrates COX-2 expression in circulating canine platelets. Previously published studies were often unable to detect COX-2 expression in circulating human and canine platelets.[7,11,13] As in recently published human platelet studies, addition of a permeabilization step prior to flow cytometry enabled us to detect COX-2 expression within the platelets of all of the dogs in our study. The functions of the COX-2 isoform in canine platelets are currently unknown.

Our study revealed that exposure to anti-inflammatory doses of aspirin altered canine platelet expression of both COX-1 and COX-2. Mature platelets are anucleate and would therefore not be expected to be capable of *de novo* changes in expression of proteins such as COX-1 or COX-2 in response to endogenous or exogenous stimuli. Nucleated marrow platelet precursor cells such as megakaryocytes contain COX-1 and COX-2,[8,14] and changes in COX expression that are induced at the level of these platelet precursors would be expected to result in similar changes in platelets derived from affected megakaryocytes. Young newly released platelets also contain residual mRNA derived from precursor megakaryocytes,[27,28] and retain the intracellular molecular mechanisms necessary for protein production. In fact, in many tissues, COX induction is thought to occur at the post-transcriptional level. Therefore, it is possible that some of the aspirin-induced changes in platelet COX expression that occurred in our study occurred in circulating platelets. Since our study measured COX expression only once during aspirin therapy, 10 days after the drug was commenced, we cannot determine at what time point altered COX expression became apparent in platelets, and thus cannot determine where COX induction occurred. Additional studies evaluating platelet COX

expression at several time points during the first week of aspirin therapy, and using flow cytometry to measure COX expression in immature 'reticulated' platelets, will help to more precisely determine the sequential effects of aspirin on COX expression at the level of the megakaryocyte, the immature platelet, and the mature circulating platelet.

Our study demonstrated a significant increase in platelet COX-1 expression after aspirin administration. While it is generally believed that COX-1 is constitutively expressed in most tissues, up-regulation of COX-1 expression has been demonstrated in human megakaryoblasts during thrombocytopoiesis, although the mechanism of enzyme up-regulation is not fully understood.[29,30] Up-regulation of COX-1 expression has also been identified in equine jejunal mucosa following periods of ischemia, in human gastric mucosa in association with gastric ulcers, and in humans experiencing rejection of a renal allograft.[31-35] Certainly it appears that in some circumstances, cellular COX-1 expression is induced rather than constitutive, and our study provides supportive evidence that suggests that exposure to aspirin induces COX-1 expression by platelets or platelet precursors in dogs, although the mechanism for this induction remains unknown. Although platelet COX-1 expression was increased, platelet function was concurrently decreased as evidenced by prolonged PFA-100® closure times and decreased urinary 11-dTXB$_2$ levels. Prolongation of PFA-100® closure time using the collagen/EPI cartridge is considered to be a sensitive indicator of aspirin-induced platelet dysfunction.[23] Thromboxane B$_2$ is a metabolite of platelet-derived thromboxane A$_2$, and measurement of urinary thromboxane B$_2$ metabolites such as 11-dTXB$_2$ is also considered to be a sensitive indicator of aspirin-induced inhibition of platelet cyclooxygenase activity and resultant decreased thromboxane A$_2$ production.[36-38] Taken together, the increased PFA-

100® closure times and decreased urinary 11-dTXB$_2$ levels seen in the dogs in our study provide strong supportive evidence of aspirin-induced inhibition of platelet COX activity, a finding that is to be expected given the relatively high doses of aspirin used. The increase in platelet COX-1 expression with a significant decrease in urinary 11-dTXB$_2$ concentrations could signify a negative feedback control to compensate for decreased COX-1 function.

Our study also revealed that, in contrast to COX-1, platelet COX-2 expression did not significantly change during the period of aspirin administration. By the end of the post-aspirin washout period, however, there was a significant increase in COX-2 expression compared to Day 10 values, suggesting that aspirin does have an effect on platelet COX-2 expression. Further studies with a larger number of dogs will be needed to determine the true nature of aspirin's effect on canine platelet COX-2 expression.

Previously described flow cytometry techniques for measuring platelet COX-2 have utilized platelet-rich plasma in order to minimize any potential interference associated with whole blood cellular components.[11] The extra platelet handling associated with the creation of platelet-rich plasma, however, has the potential to activate platelets and thereby artifactually alter test results. Prior to the initiation of this study, we conducted a preliminary experiment comparing the use of platelet-rich plasma and whole blood for analysis of platelet COX-1 and COX-2 expression via flow cytometry, and found similar results with both methodologies. We therefore elected to use whole blood for this study as the use of whole blood was less time-consuming and not as likely to lead to platelet activation artifacts.

Recently, a COX-2 splice variant was identified from platelet mRNA that appears to be induced by coronary artery bypass grafting in people, with a 200-fold increase in this variant after surgery. The role of this 'COX-2a' splice variant in platelet function is unclear, although it does not appear to be involved in thromboxane synthesis. In patients undergoing coronary artery bypass grafting, COX-1 mRNA is the predominant platelet COX isoform, with minimal COX-2 mRNA expression before surgery. Post-operatively, however, there is marked variability in COX expression, with induction of COX-2a mRNA accounting for more than half of the total COX mRNA in some patients.[39] Interestingly, those patients that expressed the COX-2a splice variant were also aspirin resistant following surgery. The human COX-2a splice variant is COX-2 immunoreactive, and it is possible that similar immunoreactive splice variants occur in the dog, and that at least some of the platelet COX-2 detected in our study consisted of splice variants. Our study, however, was performed in healthy dogs, therefore it is unlikely that induction of COX-2a splice variants would have occurred. Further studies are needed to identify possible splice variants in dogs and to determine the role of COX-2 and its variants in platelet function.

Some human patients do develop thromboembolic complications despite prophylactic treatment with standard anti-platelet 'low-dose' aspirin. This treatment failure has been termed 'aspirin resistance'. Although the exact incidence of aspirin resistance in people is undetermined, an estimated 8-45% of patients appear to be poorly responsive to aspirin therapy.[15,20,40,41] The mechanism of aspirin resistance is currently unknown, but it is likely that the etiology is multifactorial. Platelet COX-2, by providing an alternative pathway to COX-1 for platelet thromboxane A_2 synthesis, has been

proposed as one potentially important cause of human aspirin resistance.[20] Although low-dose aspirin has been shown to produce marked suppression of COX-1 activity in most people, clinical studies have revealed that approximately 20% of patients receiving aspirin have incomplete suppression of urine thromboxane metabolites, suggesting the presence of an alternative pathway (such as COX-2) for thromboxane A_2 synthesis.[42] In conditions associated with high rates of platelet turnover and increased thrombopoiesis such as immune-mediated thrombocytopenia and recovery from stem cell transplantation, younger platelets have greater COX-2 expression than mature platelets.[8,43] One recent study found that while only a small portion (about 10%) of circulating human platelets typically express the COX-2 isoform, up to 60% of all circulating platelets will express COX-2 during conditions of increased platelet turnover and regeneration.[1,8] Additional studies are required to determine whether COX-2 plays a direct role in resistance to low dose aspirin.

Since all of our dogs responded to relatively high anti-inflammatory doses of aspirin by exhibiting significant platelet dysfunction, as demonstrated by prolonged PFA-100® closure times and decreased urinary thromboxane B_2 concentrations, our study revealed no evidence of aspirin resistance. However, since aspirin resistance is typically only considered to be a problem in patients receiving aspirin at lower 'anti-platelet' doses, the findings of our study are not surprising. The dose used in this study was much greater than the dose typically used for thromboprophylaxis in dogs. Additional studies are required to determine whether dogs respond in the same manner to anti-platelet doses of aspirin using a typical low-dose regimen of 0.5 to 1.0 mg/kg PO Q24 hours,[44] or whether aspirin resistance will become apparent at lower drug doses. However, even at

the high aspirin doses used in our study, platelet COX-2 expression in response to drug treatment appeared to vary markedly between individual dogs. Studies designed to evaluate platelet COX-1 and COX-2 expression and concurrently measure platelet function and thromboxane A_2 synthesis in dogs receiving low-dose aspirin may enable us to determine whether the variable individual dog COX-2 responses to aspirin at high drug doses correlate with aspirin resistance at lower drug doses.

Aspirin is commonly administered to dogs, both for its anti-inflammatory properties and for its anti-platelet effects in patients at risk for thromboembolic complications. Our results demonstrate that circulating canine platelets express both COX-1 and COX-2. Additional research is required to better delineate the functions of platelet-derived COX-2 and its potential role in aspirin responsiveness in both normal and clinically ill dogs.

Materials, Instruments, and Supplies

a. Aspirin, Major Pharmaceuticals, Livonia, MI

b. 3.8% sodium citrate, Vacutainer tube, Becton Dickinson, Franklin Lakes, NJ

c. FITC-conjugated monoclonal COX-1, Clone CX111, Cayman Chemical Co, Ann Arbor, MI

d. FITC-conjugated IgG_{2b} isotype control, Santa Cruz Biotechnology, Inc, Santa Cruz,CA

e. Monoclonal anti-human CD9:RPE, Clone MM2/57, AbD Serotec, Raleigh, NC

f. Paraformaldehyde, Biolegend Inc., San Diego, CA

g. Triton X-100, Sigma-Aldrich, St. Louis, MO

h. FITC-conjugated monoclonal COX-2, Clone CX299, Cayman Chemical Co, Ann Arbor, MI

i. Monoclonal CD61-purified, Clone JM2E5, Accurate Chemical, Westbury, NY

j. Goat anti-mouse IgG:RPE, AbD Serotec, Raleigh, NC

k. FITC-conjugated IgG_1 isotype control, Santa Cruz Biotechnology, Inc., Santa Cruz,CA

l. FACSCalibur, Becton Dickinson, San Jose, CA

m. CellQuest software, Becton Dickinson, San Jose, CA

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

o. Abbott Cell-Dyn® 3700, Abbott Laboratories, Abbott Park, IL

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

q. Luminex® 200 System xMAP Technology, Luminex Corporation, Austin, TX

r. Luminex® 11-dehydro Thromboxane B_2 Kit, Cayman Chemical Co, Ann Arbor, MI

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

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

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CHAPTER III

CYCLOOXYGENASE EXPRESSION AND PLATELET FUNCTION IN NORMAL DOGS RECEIVING LOW DOSE ASPIRIN

Introduction

Thromboembolic complications in dogs are associated with multiple diseases, including immune-mediated hemolytic anemia (IMHA), protein-losing nephropathies, and hyperadrenocorticism.[1,2] Anti-platelet therapy plays an important role in the prevention of thromboembolic complications. One of the most commonly used anti-platelet medications, aspirin, is highly effective at inhibiting both human and canine platelet function.[3-7] Anti-inflammatory doses of aspirin will inhibit platelet function, decrease inflammation, and provide analgesia, but can cause significant side effects.[8] Lower doses of aspirin, in contrast, will maintain the anti-platelet properties of the medication without the side effects, making low dose aspirin an appealing treatment for the prevention of thromboembolic complications. The administration of low doses of aspirin has been associated with an improved survival rate in dogs with IMHA.[9]

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Unfortunately, despite preventative therapy, some dogs treated with aspirin will continue to develop thromboembolic complications. A similar phenomenon occurs in humans that are receiving low doses of aspirin, indicating that these patients are poorly responsive to the anti-platelet effects of aspirin ('aspirin resistant'). The true incidence of aspirin resistance in humans is unknown, but the published incidence ranges from 8-45% of human patients.[3,10-12] Since canine clinic patients appear to inconsistently respond to low dose aspirin, it is possible that, similar to humans, dogs also experience aspirin resistance. The true incidence of aspirin resistance in dogs is, however, unknown. The exact mechanism for aspirin resistance in both humans and dogs is also unknown, but possible explanations include genetic and acquired differences in cyclooxygenase (COX) enzyme activity or thromboxane receptors, increased platelet reactivity to other platelet agonists, and alternative pathways of thromboxane generation.[10]

Once synthesized and released by activated platelets, thromboxane A_2 (TXA_2) triggers vasoconstriction, enhances platelet aggregation, and induces further platelet activation.[5,13] The COX enzyme, which exists in two major isoforms, COX-1 and COX-2, is primarily responsible for platelet generation of thromboxane A_2.[14,15] Previously, circulating platelets were thought to contain only the COX-1 isoform,[16-18] but recent studies have identified the COX-2 isoform in both human and canine platelets.[15,17,19] The COX-1 isoform was originally believed to be the sole source of platelet TXA_2 production,[16] but platelet COX-2 could provide an alternate source of TXA_2 production, offering a possible mechanism for aspirin resistance.

Aspirin functions as a permanent inhibitor of the COX enzyme via acetylation of a serine residue.[10,20,21] Permanent inhibition of the COX enzyme prevents conversion of

arachidonic acid to prostaglandin H_2 and many subsequent prostaglandins, including TXA_2 and prostacyclin. While platelet-derived TXA_2 plays a critical role in vasoconstriction and promoting platelet aggregation, prostacyclin originating from the vascular endothelium has the opposite effect by causing vasodilation and inhibiting platelet aggregation. Low dose aspirin, unlike higher anti-inflammatory doses, will alter platelet function without permanently inhibiting COX function in the vascular endothelium, allowing prostacyclin production to continue and contribute to the prevention of thrombus formation.[5]

Previous studies in dogs have demonstrated that high aspirin doses consistently inhibit platelet function[6,7,19] and thromboxane production,[19,22] while concurrently altering platelet COX-1 and COX-2 expression.[19] Platelet COX enzyme expression in dogs receiving low dose aspirin has not been previously evaluated, but variations in COX isoform expression could provide an explanation for aspirin resistance. A better understanding of the relationship between platelet COX isoform expression and response to low doses of aspirin could potentially allow clinicians to predict which individuals will demonstrate aspirin resistance.

The objective of this study was to evaluate the effects of a recommended low dose of aspirin (1 mg/kg/day)[a,8] on platelet function and COX expression in healthy dogs. The goals of our study were to determine whether low dose aspirin therapy would consistently affect platelet function and thromboxane production, and if not, whether pre-existing COX isoform expression or subsequent changes in isoform expression were associated with aspirin resistance.

Material and Methods

Study Population

Healthy client owned adult dogs were recruited from students, staff and faculty associated with the Mississippi State University College of Veterinary Medicine. Owner consent was obtained before initiation of the study. The dogs were not exposed to any medications or vaccines for at least two weeks prior to initiation of the study, and no non-aspirin medications were administered during the testing period. All drug dosing was based on the body weight obtained at the beginning of the study. Normal health status was established via physical examination, complete blood count, serum chemistry, urinalysis, heartworm testing and platelet function analysis using a point-of-care platelet function analyzer, the PFA-100®[b] (collagen/ADP cartridge[c]). Dogs were excluded from the study if they were found to have abnormal physical examination findings, abnormal complete blood count or serum chemistry results, heartworm or tick-borne disease, or platelet abnormalities based on either an abnormal platelet count or prolonged PFA-100® (collagen/ADP) closure time. Animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee.

Study Design

Aspirin[d] was administered orally to each dog at a dose of 1 mg/kg once daily for 10 days. Based on individual body weight, the aspirin dose was compounded by the Mississippi State University College of Veterinary Medicine Pharmacy. Blood and urine samples were collected from all study participants for platelet function evaluation (PFA-

100® collagen/epinephrine cartridge[e]), measurement of platelet cyclooxygenase expression, and urinary thromboxane analysis on Day 0 (prior to initiating aspirin), and again on Days 3 and 10 during aspirin administration.

Platelet Function Analysis

The PFA-100® has previously been evaluated for use in dogs[6,7,23] as a commercial point-of-care platelet function analyzer. The PFA-100® is an *in vitro* platelet function analyzer that records the closure time, in seconds, needed to form a platelet plug after activation by platelet agonists. Blood was collected via jugular venipuncture with a 20 gauge needle directly into a 5 ml vacutainer tube containing 3.8% sodium citrate[f]. Samples were well mixed, and 800 µl of whole blood was transferred into either a collagen/ADP cartridge (establishment of normal platelet function prior to the study) or collagen/epinephrine cartridge (main study) for analysis. All samples were analyzed within four hours of collection and were kept at room temperature until analysis. The cut-off time for the instrument is greater than 300 seconds, and results were considered to be prolonged if the closure time was greater than 300 seconds. All cartridges were stored at 4°C and warmed to room temperature before analysis.

Based on PFA-100® collagen/epinephrine closure times following aspirin administration, all dogs were divided into one of three groups: aspirin responders (prolonged closure times at Day 3 and 10), aspirin non-responders (no prolongation of closure times at either time point), and inconsistent aspirin responders (prolonged closure times on either Day 3 or 10, but not both). The initial Day 0 blood samples for platelet function analysis (collagen/epinephrine cartridge) were discarded due to an unanticipated

instrument malfunction. Blood samples were obtained 4 weeks after completion of administration of aspirin to represent the baseline analysis for the PFA-100® collagen/epinephrine cartridge.

Flow Cytometry

Blood was collected via jugular venipuncture with a 20 gauge needle directly into a glass vacutainer tube containing 3.8% sodium citrate. Sample preparation was initiated within 1 hour of collection.

COX-1 labeling*:* Platelet COX-1 expression was quantified by utilizing a previously described protocol.[19] Briefly, 5 µL of whole blood was combined with 45 µL of FACS-PBS and incubated with a FITC-conjugated mouse anti-ovine-COX-1 monoclonal antibody[g]. For platelet identification, samples were incubated with a mouse anti-human CD9:RPE monoclonal antibody[h]. Samples were fixed for 10 minutes at 4°C in the dark with 1% paraformaldehyde[i].

COX-2 labeling*:* A previously reported protocol[19] was used to quantify platelet COX-2 expression. Briefly, 5 µL of whole blood was added to 45 uL of FACS-PBS and fixed with 1% paraformaldehyde at 4°C in the dark. Samples were washed and then incubated in 0.3% Triton X-100[j]. Following another wash, samples were incubated with a FITC-conjugated mouse anti-human-COX-2 monoclonal antibody[k]. Platelets were identified with a mouse anti-pig CD61-purified monoclonal antibody[l] and goat anti-mouse IgG:RPE antibody[m].

Samples were stored in the dark at 4°C prior to analysis. Isotype matched monoclonal antibodies were used for both COX antibodies. Analysis was performed

with a flow cytometer[n] with CellQuest Pro software[o]. Platelet populations were displayed on log forward-scatter versus log side-angle light scatter plots. Gates were adjusted to baseline platelet populations, and a total of 5,000 gated events were recorded for each labeling. Expression was quantified by the intensity of antibody fluorescence and expressed as mean fluorescence intensity (MFI). A histogram was created with MFI on the x-axis and events on the y-axis.

Urinary 11-Dehydro-Thromboxane B_2 Analysis

Urinary 11-dehydro-thomboxane B_2 (11-dTXB$_2$) was measured using a multiplex analyzer[p] and a commercial competitive enzyme immunoassay kit[q] that has been previously validated in the dog.[24] Urine was collected either by free catch or cystocentesis and stored at -80°C until analysis. Prior to analysis, urine samples were thawed and the specific gravity was measured. The assay buffer was used to standardize each sample to a urine specific gravity (range, 1.002 to 1.014), which was the optimal working range for the analyzer. Each urine sample was analyzed in duplicate and reported in picograms per milliliter of urine. A correction factor was applied to account for the sample dilutions. A 96-well plate was prepared by adding 100 μL of the urine sample and 50 μL of both the 11-dTXB$_2$ Phycoerythrin Tracer and the 11-dTXB$_2$ XMAPR® beads to the appropriate well. Each plate was incubated at room temperature, in the dark and on an orbital shaker prior to analysis. The urine creatinine concentration of each sample was determined by a biochemistry analyzer[r], and the urine 11-dTXB$_2$ concentration was normalized to create a urinary 11-dTXB$_2$ to creatinine ratio.[24,25]

Statistics

A power analysis was performed prior to study initiation, based on results from a previous study evaluating the effects of non-steroidal anti-inflammatory drugs on platelet function.[26] The results of this analysis indicated that a sample size of 27 dogs would be needed to achieve a power value of 0.80 for detection of post-aspirin changes in platelet function. A single population, repeated measures design was utilized in this study. After visual assessment of Q-Q plots, COX-2 expression appeared to be normally distributed; however, COX-1, 11-dTXB$_2$, and the PFA-100® (collagen/epinephrine) were not considered to be normally distributed. Consequently, nonparametric methods for analysis of repeated measures were utilized[27,28] for all results. For each outcome, the data were ranked and then analysis of variance type statistics were obtained through PROC MIXED (SAS)^s by using the ANOVAF option and the MIVQUE0 estimation method for the covariance parameters and a REPEATED statement specifying an unstructured covariance structure. Sample time was included in the model as a fixed effect. Differences in least square means were used for multiple comparisons of the three time points for the analyses; the simulation option within SAS was used for adjustment of p-values for the pair-wise comparisons. Following classification into response groups based on PFA-100® collagen/epinephrine closure times, the effect of responder classification was assessed by the addition of response group and the time by response group interaction into the COX-1, COX-2, and 11-dTXB$_2$ models. A p-value of less than 0.05 was considered to be significant for all analyses.

Results

Study Group Characteristics

A total of 25 dogs satisfied the inclusion criteria and participated in the study. There were 14 different breeds with Labrador Retrievers being the most commonly represented breed (n = 6). Other breeds included English Springer Spaniels (n = 2), Australian Shepherds (n = 2), Shar-Pei (n = 1), Pug (n = 1), Yorkshire Terrier (n = 1), Chesapeake Bay Retriever (n = 1), Chihuahua (n = 1), Boxer (n = 1), Border Collie (n = 1), German Shepherd (n = 1) and mixed breed dogs (n = 7). The study population consisted of 8 neutered males, 12 spayed females, and 5 intact females. The mean age was 3.7 years (range, 1-9 years old) and the mean body weight was 23.2 kg (range, 5.4-48.9 kg). One dog, a female intact Labrador Retriever, was excluded from the study after detection of a prolonged baseline PFA-100® collagen/epinephrine closure time. The remaining 24 dogs received aspirin for 10 days with no reported adverse effects.

Platelet Function Analysis

The mean PFA-100® collagen/epinephrine closure time on Day 0 was 140.2 seconds (range, 72-248 seconds), while the mean closure times on Days 3 and 10 were 227.3 seconds (range, 91-300 seconds) and 227.7 seconds (range, 64-300 seconds), respectively, an increase of 62.1% from baseline values. When compared to Day 0, there was a significant increase in closure times at both Day 3 ($P < 0.001$) and Day 10 ($P < 0.001$). There was no difference in closure times between Days 3 and 10 ($P = 0.991$).

There was an equal distribution among the three groups of aspirin responsiveness, with a total 8 dogs per group.

In the aspirin non-responder group, mean closure time on Day 0 was 122.6 seconds (range, 91-203), while the mean closure times on Days 3 and 10 were 162 seconds (range, 91–235) and 166.5 seconds (range, 92 – 219), respectively. In this group, no dog attained the machine cut-off closure time of greater than 300 seconds at any time point. In the incomplete aspirin responder group, mean closure time on Day 0 were 125.4 seconds (range, 72-155). 4 dogs had closure times of greater than 300 seconds on Day 3, and the other 4 dogs had closure times of greater than 300 seconds on Day 10. In the aspirin responder group, the mean closure time on Day 0 was 172.5 seconds (range, 90 – 248). In this group, all dog attained the machine cut-off closure time of greater than 300 seconds on both Day 3 and Day 10. Mean closure times during aspirin administration could not be calculated in the incomplete aspirin responder and the aspirin responder groups because, at each time point, a number of dogs exceeded the machine cut-off closure time.

Platelet COX-1 and COX-2 Expression

Platelet expression of COX-1 and COX-2 was measured prior to and at all tested time points during aspirin administration. There was a significant increase in platelet COX-1 expression from Day 0 to Day 3 by 19% (range, -29.7%-136.1%) ($P = 0.047$) and from Day 0 to Day 10 with an mean increase of 71.9% (range, -0.37- 210.36%) ($P < 0.001$). (Figure 3.1) There was also a significant ($P < 0.001$) increase in mean COX-1 expression from Day 3 to Day 10 by 56.5% (range, -24.7-241.4%). There were no

significant (P = 0.958) differences in COX-1 expression between the three groups of aspirin responsiveness.

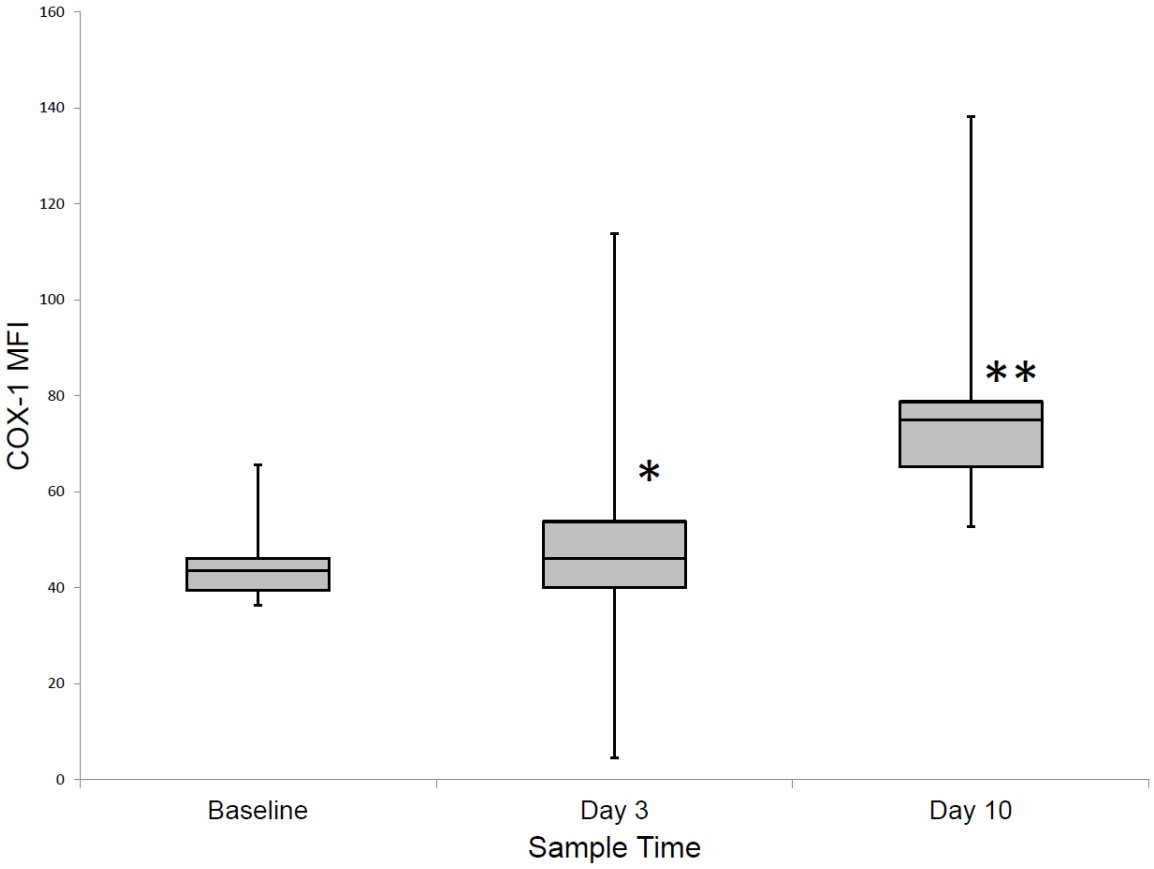

Figure 3.1 Mean platelet COX-1 expression during treatment of 24 healthy dogs with low dose aspirin (1 mg/kg PO every 24 hours for 10 days). There was a significant increase in COX-1 expression from Day 0 to Day 3 (P = 0.047) (*) and from Day 0 to Day 10 (P < 0.001) (**). There was also a significant (P < 0.001) increase between Day 3 and Day 10.

Platelet COX-2 expression was significantly (P = 0.003, P <0.001 respectively) increased from Day 0 to Day 3 by 43.82% (range, -29.2- 270.4%) and from Day 0 to Day 10 by 75.1% (range, -19.7- 226.2%). (Figure 3.2) COX-2 expression was also significantly (P = 0.014) increased between Days 3 and 10 by 26.5% (range, -27.3- 117.2%). Similar to the COX-1 expression, there were no significant (P = 0.479) differences in COX-2 expression between the three groups of aspirin responsiveness.

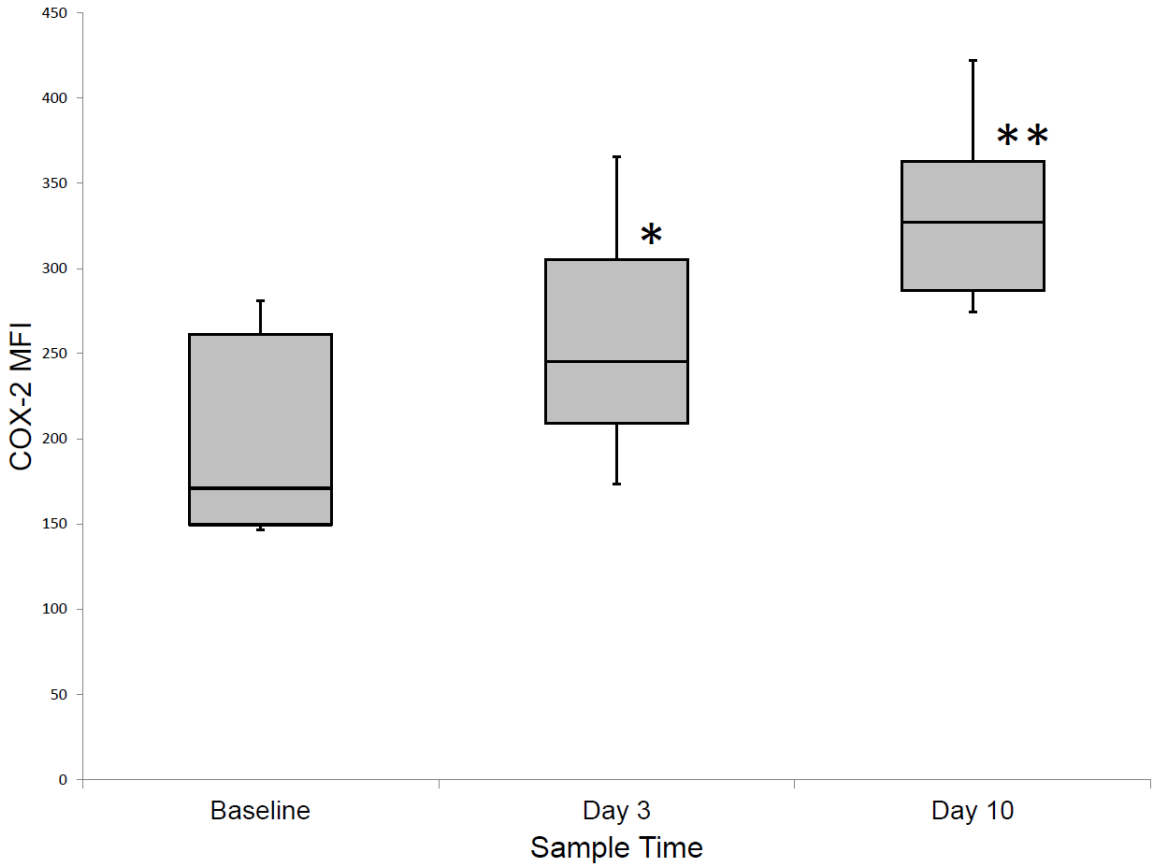

Figure 3.2 Mean platelet COX-2 expression during treatment of 24 healthy dogs with low dose aspirin. There was a significant increase in COX-2 expression from Day 0 to Day 3 (P = 0.003) (*) and from Day 0 to Day 10 (P < 0.001) (**). There was also a significant (P = 0.014) increase between Day 3 and Day 10.

Urinary Thromboxane Concentration

The mean urine 11-dTXB$_2$ to creatinine ratio on Day 0 was 10.8 (range, 4.0-38.5), while the mean ratios for Day 3 and Day 10 were 7.5 (range, 3.2-18.2) and 5.2 (range, 1.2-11.8), respectively. There was a 23% decrease in the urine 11-dTXB$_2$ to creatinine ratio between Day 0 and Day 3, while there was a 43.9% decrease between Day 0 and Day 10 and 23.9% decrease between Day 3 and Day 10. (Figure 3.3) Significant decreases in the urinary 11-dTXB$_2$ to creatinine ratio occurred at all time points, from Day 0 to Day 3 ($P = 0.005$), from Day 0 to Day 10 ($P < 0.001$), and from Day 3 to Day 10 ($P = 0.014$). Only 4 dogs on Day 3 and 2 dogs on Day 10 had an increase in the 11-dTXB$_2$ to creatinine ratio. There were no significant ($P = 0.885$) differences in the urine 11-dTXB$_2$ to creatinine ratio between the three groups of aspirin responsiveness.

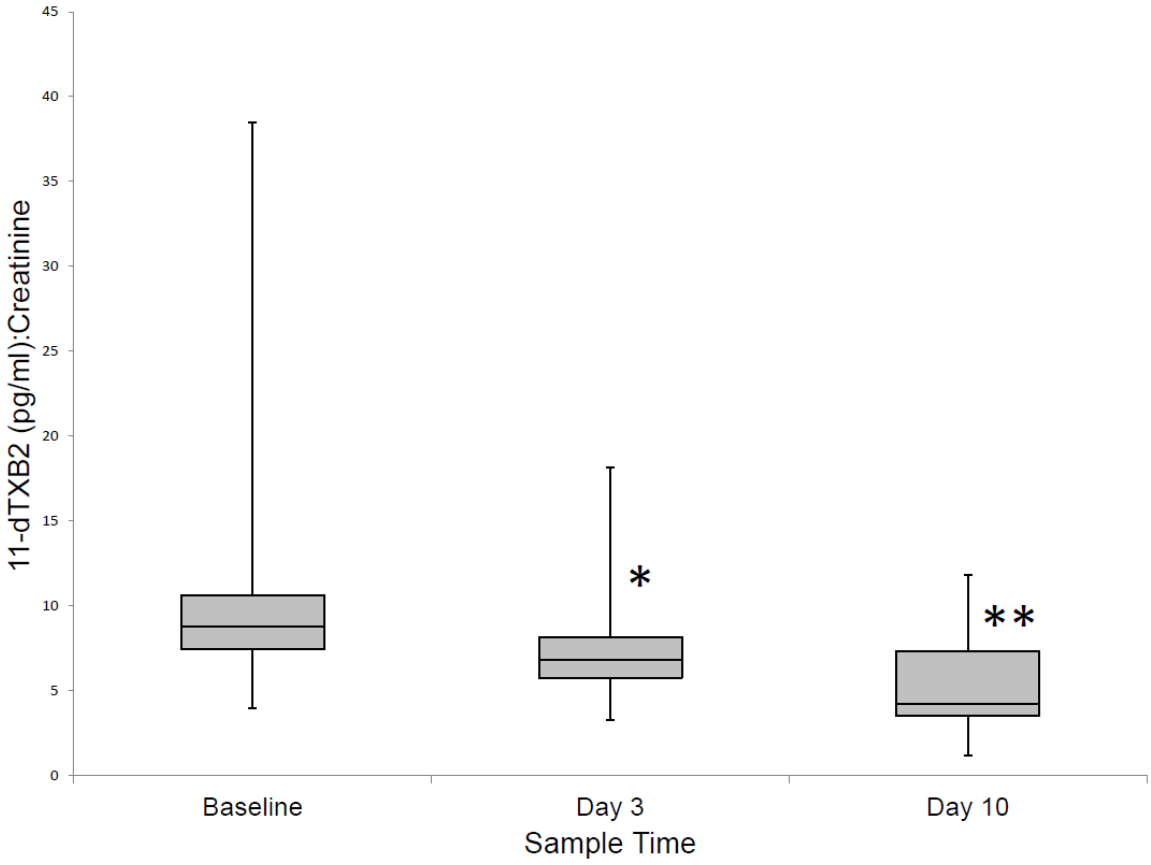

Figure 3.3 Mean urinary 11-dTXB$_2$ to creatinine ratios during treatment of 24 healthy dogs with low dose aspirin. There was a significant decrease in the urinary 11-dTXB$_2$ to creatinine ratio from Day 0 to Day 3 ($P = 0.005$) (*) and from Day 0 to Day 10 ($P < 0.001$) (**). There was also a significant ($P = 0.014$) decrease between Day 3 and Day 10.

Discussion

Anti-inflammatory doses of aspirin reliably inhibit platelet function in dogs.[6,7,19] Our study revealed that low dose aspirin, in contrast, does not inhibit platelet function in all dogs as measured using the PFA-100® collagen/epinephrine cartridge, suggesting that aspirin resistance may occur in dogs as well as in humans. Chronic low dose aspirin administration did inhibit thromboxane synthesis in most dogs: although 4 dogs on Day 3 and 2 dogs on Day 10 demonstrated increased urine 11-dTXB$_2$ to creatinine ratios, all of the dogs in the study at least once had a decreased 11-dTXB$_2$ to creatinine ratio. The inhibition of thromboxane by low dose aspirin did not appear to correlate with aspirin responsiveness as measured by the PFA-100®. With no differences in urinary thromboxane concentrations between aspirin responders, non-responders, and inconsistent responders, our study suggests that COX-1 independent thromboxane synthesis, either through the COX-2 enzyme pathway or alternative pathways, is unlikely to be the mechanism for aspirin resistance in dogs.

One possible explanation for aspirin resistance in dogs may be variable responsiveness of platelets to thromboxane. Previous studies have suggested that about 70% of canine platelets are insensitive to thromboxane stimulation.[29-31] The thromboxane insensitivity observed in some canine platelets may be due to impaired platelet thromboxane A$_2$ receptor linked G proteins.[31] If only 30% of dogs have platelets that are sensitive to the effects of thromboxane, this may explain why only one third of the animals in our study responded to low dose aspirin with a consistent increase in PFA-100 closure time. In those dogs that have platelets that are insensitive to thromboxane-induced activation, other agonists such as ADP or serotonin may play a more important

role in platelet activation, and such dogs may therefore be relatively resistant to the inhibitory effects of low dose aspirin. A recent human study suggests that resistance to aspirin may be due to a COX independent pathway and that, in affected people, inhibition of platelet function is more readily achieved using an ADP antagonist.[12]

We measured urine levels of the thromboxane metabolite 11-dehydro-thromboxane B_2 to evaluate the effects of aspirin on platelet thromboxane synthesis. Measurement of this metabolite has been utilized in previous studies as an indicator of aspirin-induced COX inhibition.[32-34] Following synthesis by platelets, thromboxane A_2 has an immediate physiologic effect, and is then metabolized to thromboxane B_2 and further metabolized into 11-dehydro-thromboxane B_2 and 2,3-dinor-thromboxane B_2 (2,3-dinorTXB_2) before being eliminated in the urine.[8,34,35] Levels of thromboxane metabolites in plasma or serum can be artifactually increased due to platelet activation during collection of blood and sample processing. The use of urine to measure thromboxane metabolites avoids the problem of *in-vitro* platelet activation associated with blood collection, and is therefore considered to be a more reliable indicator of platelet thromboxane synthesis.[34,35]

Although most dogs in this study had a persistent decrease in urine 11-dTXB_2 to creatinine ratios after administration of low dose aspirin, and every dog had a decreased ratio for at least one time point, a few dogs demonstrated an increased ratio at a single time point. Similar inconsistent inhibition of urine 11-dTXB_2 levels has previously been reported in dogs in a study using the same low dose of aspirin.[8] This previous study also evaluated urinary 2,3-dinor-thromboxane B_2 and demonstrated that, compared to 11-dTXB_2, urine 2,3-dinorTXB_2 levels were a more sensitive indicator of aspirin-induced

thromboxane inhibition in dogs.[8] However, although the urine 11-dTXB$_2$ assay used in our study is less sensitive for detecting aspirin-induced thromboxane inhibition compared to the urine 2,3-dinorTXB$_2$ assay, our assay was still sufficiently sensitive to detect a significant decrease in urine thromboxane metabolites at both measured time points after aspirin administration.

While cyclooxygenase function was demonstrably inhibited by low dose aspirin as indicated by prolongation of PFA-100® collagen/epinephrine closure times and decreased urine 11-dTXB$_2$ levels, platelet expression of both COX-1 and COX-2 progressively increased throughout the course of aspirin administration. The COX-1 enzyme isoform was originally thought to be strictly constitutively expressed, but recent studies have shown that COX-1 expression can be induced in certain tissues, including human megakaryoblasts during thrombocytopoiesis.[15,36,37] Similar to the results of this study, a previous study has demonstrated that anti-inflammatory doses of aspirin also cause increased platelet COX-1 expression, decreased thromboxane synthesis, and inhibition in platelet function.[19] One possible explanation for increased platelet COX-1 expression could be a compensatory up-regulation of production of the COX-1 isoform by megakaryocytes in response to a reduction in platelet thromboxane A$_2$ production. Platelet COX-2 expression also increased during administration of low dose aspirin, a finding that contrasts with a previous study in dogs treated with an anti-inflammatory dose of aspirin, in which platelet COX-2 expression decreased in most treated dogs.[19] Since COX-2 expression is typically induced by inflammatory mediators,[15,38,39] it is feasible that the anti-inflammatory effects of high doses of aspirin are sufficient to decrease COX-2 expression, whereas the low dose of aspirin used in our current study

was insufficient to exert the same effect. At low doses of aspirin, the mechanism of increased platelet COX-2 expression could be similar to the mechanism of increased COX-1 expression.

Pre-treatment patient-to-patient variations in platelet COX-1 and COX-2 expression have been proposed as one potential mechanism for variable aspirin responsiveness. Our current study, however, did not demonstrate a difference in pre-treatment platelet COX-1 or COX-2 expression between the three categories of aspirin responsiveness. Our results suggest that differences in pre-treatment platelet COX expression do not play a major role in aspirin resistance in dogs, and indicate that pre-existing COX expression cannot be used to predict aspirin responsiveness

Platelet aggregometry has long been considered the gold standard methodology for the evaluation of platelet responsiveness to aspirin.[7,40] Platelet aggregometry is, however, expensive and requires specialized equipment and training, thus making it difficult to utilize this technology in a clinical setting. The PFA-100®, in contrast, is an instrument that is practical for routine clinical use, and prolongation of the PFA-100® closure time as measured with the collagen/epinephrine cartridge has become well established as a sensitive indicator of aspirin-induced platelet dysfunction in both humans and dogs.[7] The PFA-100® closure times in our present study revealed that only one third of healthy dogs responded adequately and consistently to low dose aspirin therapy. If our study is an accurate representation of the canine population, the administration of a 1 mg/kg per day dose of aspirin may not be adequate to create a consistent and reliable preventive therapy for thromboembolic complications in a majority of dogs. Anti-inflammatory doses of aspirin (10 mg/kg bid), in contrast, have been shown to

consistently inhibit platelet function in dogs.[19] Commencement with low dose aspirin at 1 mg/kg per day and subsequent utilization of periodic measurement of platelet function via the PFA-100® to individually dose escalate 'to effect' could increase the population of aspirin responders and thereby more effectively prevent thromboembolic complications. The aspirin responders in our study had inhibition of platelet function by Day 3 of therapy, suggesting that platelet function analysis after three days of aspirin should be adequate to detect whether a patient will respond to the current dose of aspirin, or require a higher dose. However, as the aspirin dose increases, so does the chance of developing adverse side effects. Furthermore, as aspirin dosage approaches anti-inflammatory doses, the likelihood of concurrent inhibition of prostacyclin production by the vascular endothelium increases, potentially negating the anti-platelet effects of aspirin.

There were several limitations to our study. While our study population consisted of a variety of breeds and ages, we evaluated aspirin effects only in healthy dogs. Responsiveness to aspirin could vary in the presence of endogenous platelet activation, and a similar study in dogs with pro-inflammatory or prothrombotic diseases would be necessary to determine the incidence of aspirin resistance in sick patients. Additionally, our study used no methods to ensure that all patients received the appropriate daily dose. Although it is unlikely, a lack of owner compliance might explain why some dogs either did not respond or inconsistently responded to the aspirin dose. Due to unexpected drop-out of several owners at the start of the study, and the exclusion of one dog because of a prolonged baseline PFA-100® collagen/epinephrine closure time, the study was completed with 24 dogs rather than the minimum of 27 dogs suggested by preceding

power analysis. However, despite this slight reduction in the number of enrolled dogs, animal numbers were still sufficient to detect significant post-aspirin changes in platelet function, platelet COX expression, and thromboxane production.

Our study demonstrates that low dose aspirin inhibits platelet thromboxane synthesis and increases platelet expression of both COX-1 and COX-2, while inhibition of platelet function is less consistently observed in individual dogs. Platelet COX expression before and during therapy was not related to aspirin responsiveness. Additional research is needed to better define the mechanism of aspirin resistance in dogs.

Materials, Instruments, and Supplies

a. Shearer L, Kruth SA, Wood D. Effects of aspirin and clopidogrel on platelet function in healthy dogs (abstr). *J Vet Intern Med* 2009; 23: 745.

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

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

d. Aspirin, Major Pharmaceuticals, Livonia, MI

e. PFA Collagen/Epinephrine Cartridge, Siemens Healthcare Diagnostics, Duluth, GA

f. 3.8% sodium citrate, Vacutainer tube, Becton Dickinson, Franklin Lakes, NJ

g. FITC-conjugated monoclonal COX-1, Cayman Chemical Co, Ann Arbor, MI

h. Monoclonal anti-human CD9:RPE, Clone MM2/57, AbD Serotec, Raleigh, NC

i. Paraformaldehyde, Biolegend Inc., San Diego, CA

j. Triton X-100, Sigma-Aldrich, St. Louis, MO

k. FITC-conjugated monoclonal COX-2, Cayman Chemical Co, Ann Arbor, MI

l. Monoclonal CD61-purified, Clone JM2E5, Accurate Chemical, Westbury, NY

m. Goat anti-mouse IgG:RPE, AbD Serotec, Raleigh, NC

n. FACSCalibur, Becton Dickinson, San Jose, CA

o. CellQuest software, Becton Dickinson, San Jose, CA

p. Luminex® 200 System xMAP Technology, Luminex Corporation, Austin, TX

q. Luminex® 11-dehydro Thromboxane B_2 Kit, Cayman Chemical Co, Ann Arbor, MI

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

s. SAS for Windows version 9.2, SAS Institute, INC., Cary, NC, 2008

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CHAPTER IV

THE EFFECTS OF CYCLOSPORINE ON PLATELET FUNCTION AND CYCLOOXYGENASE EXPRESSION IN NORMAL DOGS

Introduction

Cyclosporine has become a popular treatment for allergic dermatitis,[1,2] anal furunculosis,[3-6] and several other immune-mediated and inflammatory diseases in dogs, including immune-mediated hemolytic anemia (IMHA). This is due partly to the perception that cyclosporine has a more favorable side effect profile when compared to other immunosuppressive agents.[7] Recent human studies, however, have shown that cyclosporine can alter the platelet plasma membrane, increase the thrombogenic properties of platelets, and accelerate thrombin generation.[8-11] Approximately 50% of dogs with IMHA have been shown to be in a hypercoagulable state, predisposing these patients to complications such as pulmonary thromboembolism (PTE).[12] The incidence of PTE in dogs with IMHA has been determined to be over 30%,[5,13] however, due to the difficulty in making an antemortem diagnosis, it is possible that the incidence of PTE in such patients may be as high as 80%.[13] Minimizing risk factors for thromboembolic complications plays an important role in the treatment of IMHA patients.

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The exact mechanisms of PTE formation are not completely understood, but it is hypothesized that predisposing pro-thrombotic conditions involve both blood platelets and the clotting system.[12,14] Dogs with IMHA have been shown to have hyperactive platelets associated with platelet plasma membrane alteration and increased expression of P-selectin, an adhesion protein with procoagulant properties.[14] Results of several studies have suggested that cyclosporine may enhance platelet reactivity,[9-11] a finding that raises doubt about the safety of administering the drug to IMHA patients already at increased risk for PTE formation. A recent pilot study performed in our laboratory using a small number of dogs found increased platelet P-selectin expression after administration of cyclosporine.[a] The administration of cyclosporine has also been associated with increased platelet expression of phosphatidylserine, which acts as a catalyst for thrombin generation and expedites thrombus formation.[8] Cyclosporine has also been shown to increase human platelet production of thromboxane A_2.[15-18] Thromboxane A_2 is synthesized and released by activated platelets, and triggers vasoconstriction, enhanced platelet aggregation, and further platelet activation. Platelet thromboxane A_2 is generated via the enzyme cyclooxygenase (COX), which exists in two major forms, COX-1 and COX-2. Although it was long believed that platelets did not contain COX-2, recent studies have identified platelet COX-1 and COX-2 expression in humans and dogs.[19-21]

Our current study was designed to thoroughly evaluate the effects of cyclosporine on hemostasis in normal dogs, concentrating on platelet function, thromboxane production and expression of P-selectin, phosphatidylserine, COX-1 and COX-2, and thereby ascertain whether treatment with cyclosporine could feasibly put canine IMHA patients at an increased risk of thromboembolic complications.

Material and Methods

Study Design

Eight healthy female intact Walker hound dogs were used in this study. The dogs were not exposed to any medications or vaccines for at least one month prior to initiation of the study. Two dose rates of oral cyclosporine[b] were administered for 7 days each, a high (immunosuppressive) dose followed by a low (atopy) dose, with a 2 week washout period between dosing. For the high dose, cyclosporine was commenced at a dose rate of approximately 10 mg/kg twice daily, and blood samples were collected from each dog on Day 4 of drug administration 12 hours after the previous dose in order to determine trough cyclosporine blood levels using high-performance liquid chromatography (HPLC) analysis. A target immunosuppressive trough blood level was set at 600 ng/ml,[22] and drug doses were adjusted upwards if needed to ensure that the trough blood levels exceed this target level by the time of sample collection on Day 7. The mean administered dosage of the high dose of cyclosporine was 10.1 mg/kg $\pm$ 0.48 (mean $\pm$ SD) mg/kg, PO, q 12 hours, at the time of drug commencement, but mean dosage had increased to 19 $\pm$ 9 mg/kg, PO, q 12 hours, by Day 7. The mean administered dosage for the low dose of cyclosporine was 5.1 $\pm$ 0.21 mg/kg, PO, q 24 hour. Drug doses were not adjusted during administration of the low dose of cyclosporine.

Blood and urine samples were collected at regular intervals for platelet function testing, flow cytometric evaluation of platelet P-selectin, phosphatidylserine, COX-1 and COX-2 expression, and urinary thromboxane analysis. For both drug doses, blood and urine samples were collected at baseline prior to cyclosporine administration, and on Days 1 and 7 of drug administration. Samples were collected at 2 time points on each

day of collection, at the estimated times of peak and trough cyclosporine levels. Samples were collected at estimated peak blood drug levels 2 hours after drug administration for both doses, and at estimated trough blood drug levels 12 hours after drug administration for the high dose, and 24 hours after drug administration for the low dose.

Animals

The mean age of the dogs was 5.5 years (range, 1.5-6.5 years), and their mean body weight was 22.7 kg (range, 18.2 to 28.6 kg). Body weight was obtained at the beginning of the study and used to calculate all subsequent dosing. Normal health status was established by detection of no abnormalities on physical examination, complete blood count (including manual platelet count), serum biochemistry, urinalysis, prothrombin time, partial thromboplastin time, fibrinogen, buccal mucosal bleeding time, vonWillebrand factor (ELISA method), *Babesia* and rickettsial serology, and heartworm testing. One dog developed pyometra during the washout period between doses, and did not continue in the study. Animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee and was in compliance with the requirements at a facility accredited by the American Association for Accreditation of Laboratory Animal Care.

Flow Cytometry

Blood was collected via jugular venipuncture with a 20 gauge needle directly into a glass vacutainer tube containing 3.8% sodium citrate.[c] Sample preparation for flow cytometry was initiated within 2 hours of collection.

P-selectin labeling*:* Previously described protocols[14,23,24] were modified for the detection of platelet P-selectin (CD62). Briefly, 5 µL of citrated whole blood was added to 45 µL of FACS-PBS and incubated in the dark at room temperature with an affinity-purified, monoclonal, mouse anti-canine CD62 antibody.[d] After incubation, samples were washed with PBS, pelleted via centrifugation, and re-suspended. Goat, anti-mouse-PE[e] conjugate was added to all samples and incubated in the dark at room temperature for 30 minutes. For platelet identification, samples were incubated with monoclonal FITC-conjugated mouse anti-porcine CD61[f], and fixed with 1% paraformaldehyde[g] for ten minutes at 4°C in the dark.

Phosphatidylserine labeling*:* A previously described protocol was used to detect platelet phosphatidylserine.[23] Briefly, 5 µL of citrated whole blood was added to 250 µL of annexin-binding buffer.[h] Samples were incubated with annexin-V-FITC[i] and mouse anti-porcine CD61-purified antibody[j] for 30 minutes in the dark at room temperature. After incubation, goat anti-mouse IgG:RPE[k] was added to the samples, followed by a 30 minute incubation. Samples were fixed with 1% paraformaldehyde for 10 minutes.

COX-1 labeling*:* A previously described protocol[21] was used to quantify platelet COX-1 expression. Briefly, 5 µL of citrated whole blood was added to 45 µL of FACS-PBS and incubated with ovine COX-1 specific monoclonal antibody.[l] To identify platelets, samples were incubated with a monoclonal antibody to human CD9.[m] Samples were fixed for 10 minutes at 4°C in the dark with 1% paraformaldehyde.

COX-2 labeling*:* Analysis of canine platelet COX-2 expression was performed using a modification of a human protocol[19] that has been previously reported in dogs.[21] Briefly, 5µL of citrated whole blood was added to 45 uL of FACS-PBS and fixed at 4°C

in the dark with 1% paraformaldehyde. Samples were washed, pelleted, re-suspended, and incubated in 0.3% Triton X-100[n] followed by another wash. Samples were then incubated with a monoclonal FITC-conjugated mouse anti-human-COX-2 antibody[o] in the dark at room temperature. Platelets were identified with a monoclonal antibody to porcine CD61 and goat anti-mouse IgG:RPE antibody.

All samples were stored in the dark at 4°C prior to flow cytometric analysis. Isotype matched monoclonal antibodies were used for all marker specific antibodies. Flow cytometric analysis was performed with a flow cytometer[p] with CellQuest Pro software.[q] Platelet populations were displayed on log forward-scatter versus log side-angle light scatter plots. Gates were adjusted to baseline platelet populations, and a total of 5,000 gated events were recorded for each labeling. Expression was quantified by the intensity of antibody fluorescence and expressed as mean fluorescence intensity (MFI). A histogram was created with MFI on the x-axis and platelet number on the y-axis.

Urinary Thromboxane

Urinary 11-dehydro-thromboxane B_2 (11-dTXB_2) concentration was analyzed using a commercially available competitive enzyme immunoassay kit[r] for a multiplex analyzer[s] that has been previously validated in the dog.[25] Urine was collected via cystocentesis, batched, and stored at -80°C until analysis. The assay buffer was used to standardize the urine specific gravity of each sample to fit within the working range (1.003 to 1.012) of the analyzer, and a correction factor was applied to account for these dilutions. Samples were analyzed in duplicate according to the manufacturer's instructions, and reported in picograms per milliliter of urine. Briefly, a 96-well plate was

prepared by adding 100 µl of each sample and 50 µl of both 11-dTXB$_2$ phycoerythrin tracer and 11-dTXB$_2$ XMAPR® Beads to each well. Prior to analysis, each plate was incubated at room temperature, in the dark and on an orbital shaker, for 4 hours. Urine creatinine concentration was measured using a biochemistry analyzer[t] and the 11-dTXB$_2$ concentration was normalized to create a urinary 11-TXB$_2$to creatinine ratio.[25,26]

Platelet Function Analysis

A commercial point-of-care platelet function analyzer (PFA-100®)[u] that has been previously evaluated for use in dogs was used to analyze platelet function.[27-30] The PFA-100® is an *in vitro* platelet function analyzer that mimics an environment similar to blood vessels and stimulates platelet function with several platelet agonists to measure the time, in seconds, needed to form a platelet plug and inhibit blood flow. The cut-off time for the instrument is greater than 300 seconds.

The instrument was used according to manufacturer's instructions. Briefly, samples were collected directly into 5 ml blood collection tubes containing 3.8% sodium citrate, and 800 µl of whole blood sample was transferred into PFA-100® cartridges (both collagen/ADP and collagen/epinephrine)[v,w] and analyzed. All samples were analyzed within three hours of collection. An automated hematologic analyzer[x] was used to determine an accurate platelet count and packed cell volume (PCV) on each sample. Cartridges were stored at 4^{o}C and warmed to room temperature before analysis.

Cyclosporine Assay

Measurement of peak and trough blood cyclosporine levels was performed via HPLC analysis based on a modification of the therapeutic drug monitoring assay used at the University of California at Davis.[y] Briefly, blood was collected into tubes containing EDTA[z] anticoagulant, batched, and stored at -80°C until analysis. Samples were thawed and underwent an extraction procedure that mixed 2 mLs of the whole blood sample with a protein precipitating solution that consisted of 5% zinc sulfate, 20% acetonitrile, 30% methanol, water, and an internal standard of 400 ng/ml of cyclosporine D.[aa] Samples were mixed, centrifuged, and the supernatant was added to a prepared C18 solid phase extraction (SPE) column.[bb] The SPE column, under vacuum, was washed with 5 mls of 50% acetonitrile and then the cyclosporine was eluted by 1 ml of 100% methanol. To the eluent, 200 µL of water followed by 300 µL of hexane was added. This sample was centrifuged at 14.5 revolutions per minute for 90 seconds and 200 µL of the aqueous layer was extracted and prepared for HPLC analysis. Only 100 µL of the sample was injected for HPLC analysis. Blank EDTA whole blood was used for a standard curve with cyclosporine[cc] at 0, 200, 400, 800, and 1600 ng/ml.

Cyclosporine analysis was performed using an Agilent 1100 HPLC system[dd] with degasser (G1322A), quaternary pump (G1311A), autoinjector (G1313A), and diode array detector (G1315). The reverse phase column was a Phenomenex Luna 5u C18 (2) (150 x 2.00 mm 5 micron) which was maintained at 75°C. A 1 mL per minute gradient mobile phase consisted of acetonitrile (A) and water adjusted to pH 3.1 (B). The gradient transitioned linearly from 65% A and 35% B to 70% A and 30% B over 5 minutes, and held for 15 minutes. After each injection, there was a 5 minute re-equilibration time

period. Detection was at 200 nm. The retention time for cyclosporine and cyclosporine D was 4.2 min and 5.6 minutes, respectively. The assay was linear over the standard curve range with an r^2 of 0.98. The assay had an average coefficient of variation of 6.7% (range 3.7 to 9.9%) and an average accuracy of 94.4% (range 92 to 98%).

Statistical Analysis

A single population, repeated measures design was utilized in this study. Visual assessment of the data using Q-Q plots and histograms with UNIVARIATE procedure of SAS for Windows 9.2 (SAS Institute, Inc., Cary, NC, USA) indicated the data was not consistently normally distributed for all of the outcomes. Consequently, nonparametric methods for analysis of repeated measures were utilized. For all four flow cytometry assays, PCV, platelet count, PFA-100®, and urine 11-dTBX$_2$, the data were ranked and then analysis of variance type statistics were obtained through the MIXED procedure of SAS for Windows 9.2 by using the ANOVAF option and the MIVQUE0 estimation method for the covariance parameters and a REPEATED statement specifying an unstructured covariance structure. Each dose of cyclosporine was analyzed separately from the other dose, and the results were not compared. Sample time was included in the model as a fixed effect. For outcome in which time had a significant effect ($p \leq 0.05$), comparisons were made between baseline and the other time points using differences in least square means. Paired T-Tests using the UNIVARIATE procedure of SAS for Windows 9.2 were conducted to compare baseline levels of outcomes prior to high dose and low dose administration and between baseline levels prior to high dose

administration and levels following the washout period A p-value of less than or equal to 0.05 was considered to be significant for all analyses.

Results

Flow Cytometry

Platelet expression of P-selectin, phosphatidylserine, COX-1 and COX-2 was measured both prior to and at all tested time points during drug administration, and percent change in MFI compared to baseline for all four markers is represented in Table 4.1.

There was a significant decrease in platelet P-selectin expression at all time points during administration of the high dose of cyclosporine, compared to baseline levels. During the administration of the low dose, P-selectin expression only significantly decreased at both trough time points (Figure 4.1).

Table 4.1

The percent change in MFI from baseline for all flow cytometry markers (P-selectin, phosphatidylserine, COX-1 and COX-2) during administration of both cyclosporine doses

	High Dose Cyclosporine		Low Dose Cyclosporine	
	Percent (%) MFI Change compared to baseline	**P-value**	**Percent (%) MFI Change compared to baseline**	**P-value**
P-selectin				
Day 1 – Peak	-48.0	0.003*	-12.0	0.587
Day 1 – Trough	-56.3	0.004*	-43.5	0.007*
Day 7 – Peak	-36.7	0.019*	3.0	0.600
Day 7 – Trough	-35.0	0.025*	-28.8	0.013*
Phosphatidylserine				
Day 1 – Peak	-69.7	<0.001*	-14.1	0.022*
Day 1 – Trough	-70.1	<0.001*	31.8	0.022*
Day 7 – Peak	-66.6	<0.001*	5.9	0.496
Day 7 – Trough	-68.2	<0.001*	6.4	0.780
COX-1				
Day 1 – Peak	-14.7	0.250	-21.2	0.029*
Day 1 – Trough	-27.6	0.035*	-25.6	0.022*
Day 7 – Peak	-38.5	0.004*	-51.2	<0.001*
Day 7 – Trough	-40.8	0.002*	-2.0	0.555
COX-2				
Day 1 – Peak	148.9	<0.001*	-32.4	<0.001*
Day 1 – Trough	9.4	0.349	5.7	0.458
Day 7 – Peak	-42.3	<0.001*	-17.9	0.114
Day 7 – Trough	-33.5	0.001*	-30.5	0.003*

MFI = Mean Fluorescence Intensity Statistical Significance *

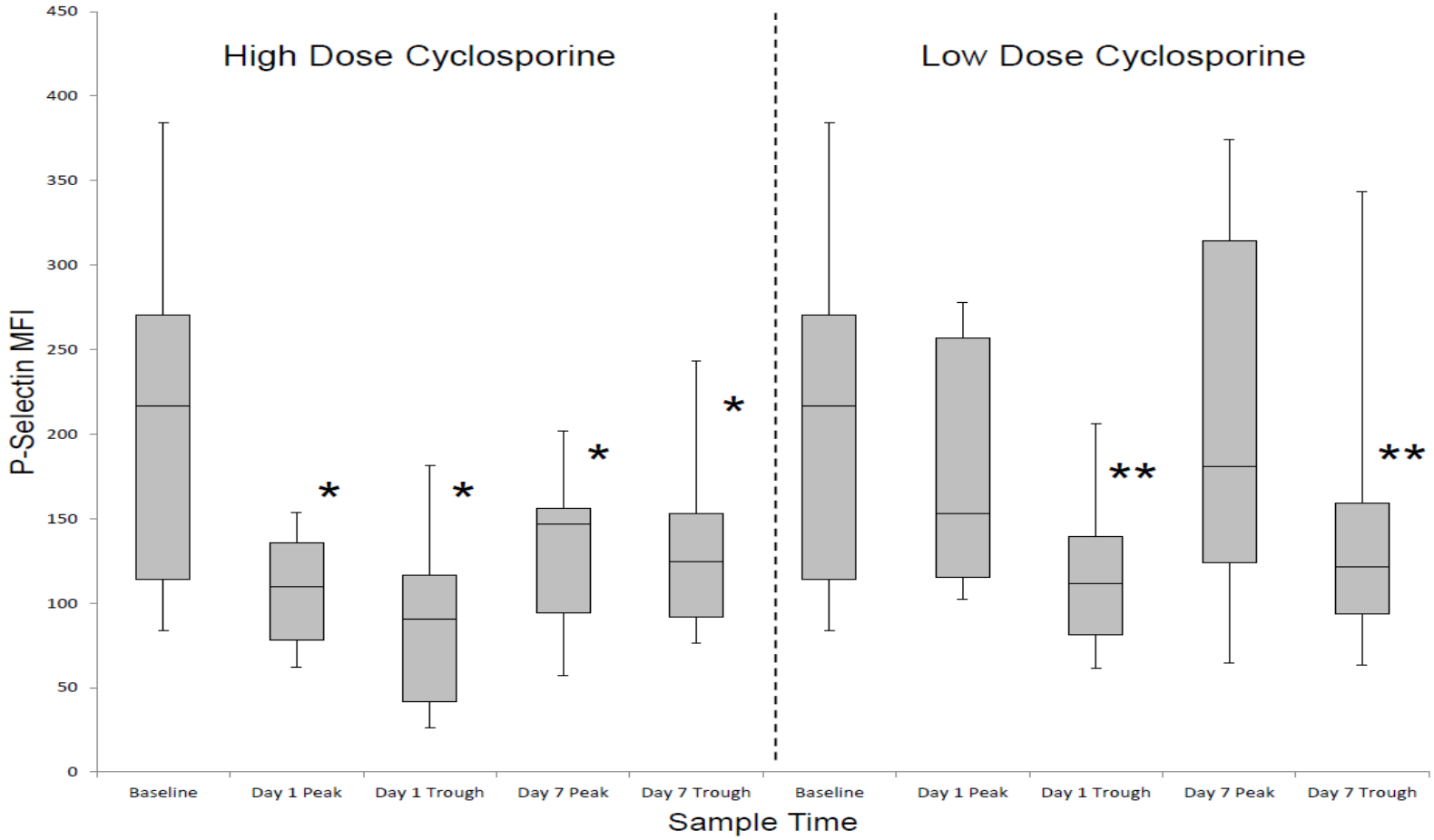

Figure 4.1 Mean P-selectin expression for all dogs treated with two doses of cyclosporine (High Dose = mean 19 mg/kg PO every 12 hours, and Low Dose = 5 mg/kg PO every 24 hours). There was a significant decrease from baseline values in P-selectin expression at all time points after the high dose of cyclosporine (*). However, only the two trough time points showed a significant decrease during the low dose (**).

There was also a significant decrease in platelet phosphatidylserine expression at all time points during administration of the high dose of cyclosporine, compared to baseline levels. The initial baseline result prior to the high dose trial was considerably greater than all subsequent time points, including the low dose trial baseline. In order to confirm that this elevated initial baseline result was not erroneous, blood samples were obtained for repeat analysis of platelet phosphatidylserine expression on two different days after a prolonged washout time period. The mean of these two repeat samples were greater than all other time points, but the MFI was still significantly ($p=0.008$) less than the initial baseline result. Significant changes in phosphatidylserine expression observed during the administration of the low dose of cyclosporine were a decrease at the peak time point and an increase at the trough time point on Day 1 (Figure 4.2).

Compared to baseline levels, there was a significant decrease in platelet COX-1 expression at both trough time points and at the peak time point on Day 7 during administration of the high dose of cyclosporine. During the administration of the low dose, there was a significant decrease in COX-1 expression at both time points on Day 1, and Day 7 peak (Figure 4.3).

Compared to baseline levels, there was an immediate significant increase in platelet COX-2 expression two hours after initiation of the high dose of cyclosporine (Day 1 peak time point). However, there was then a decline in platelet COX-2 expression until there was a significant decrease in COX-2 expression at both the peak and trough time points on Day 7. During administration of the low dose of cyclosporine, there was a significant decrease in platelet COX-2 expression two hours after drug

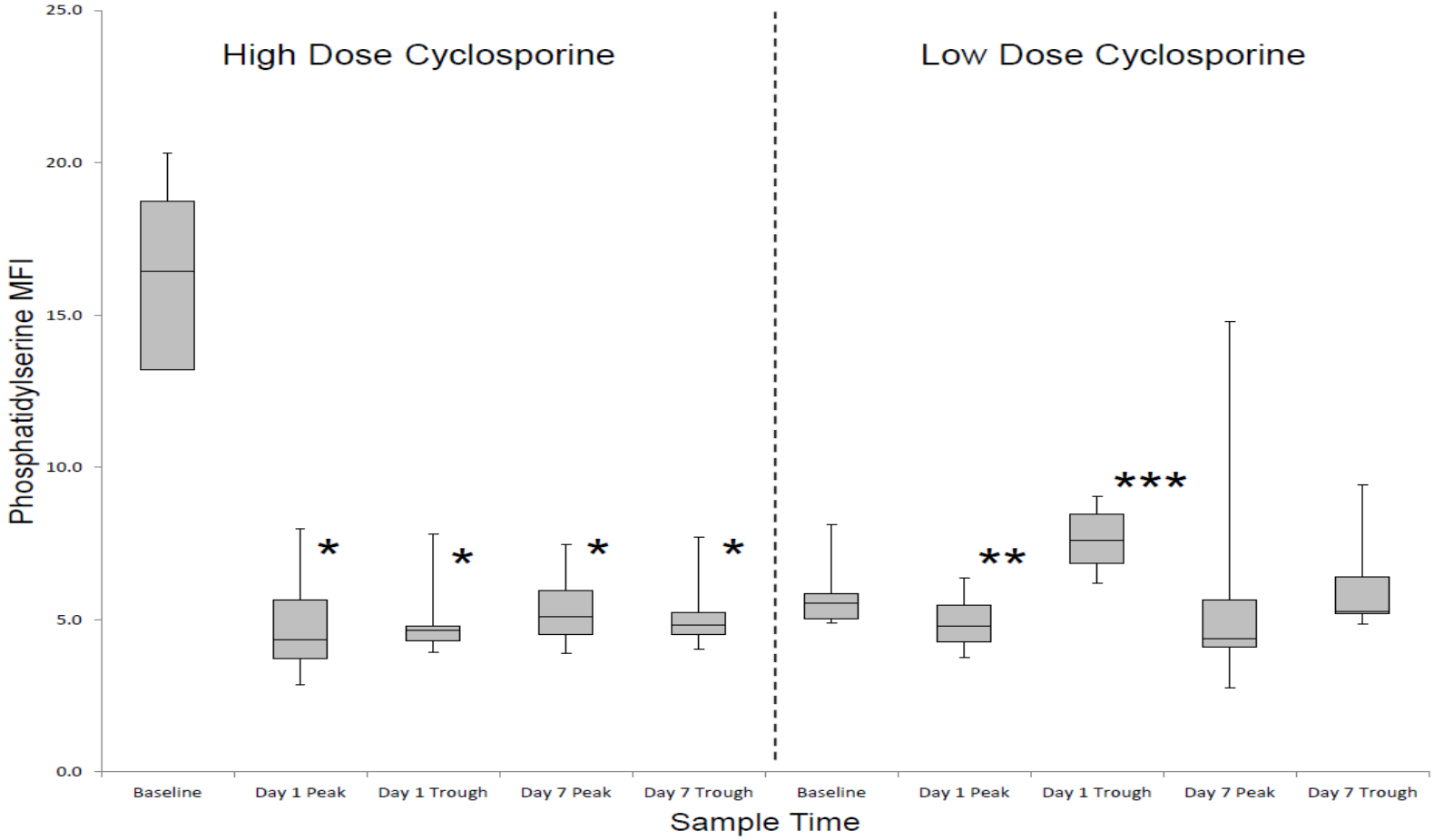

Figure 4.2 Mean phosphatidylserine expression for dogs treated with two doses of cyclosporine. There was a significant decrease from baseline values in phosphatidylserine expression at all time points after the high dose of cyclosporine (*), while during the low dose study the Day 1 peak showed a significant decrease (**), and Day 1 trough showed a significant increase (***).

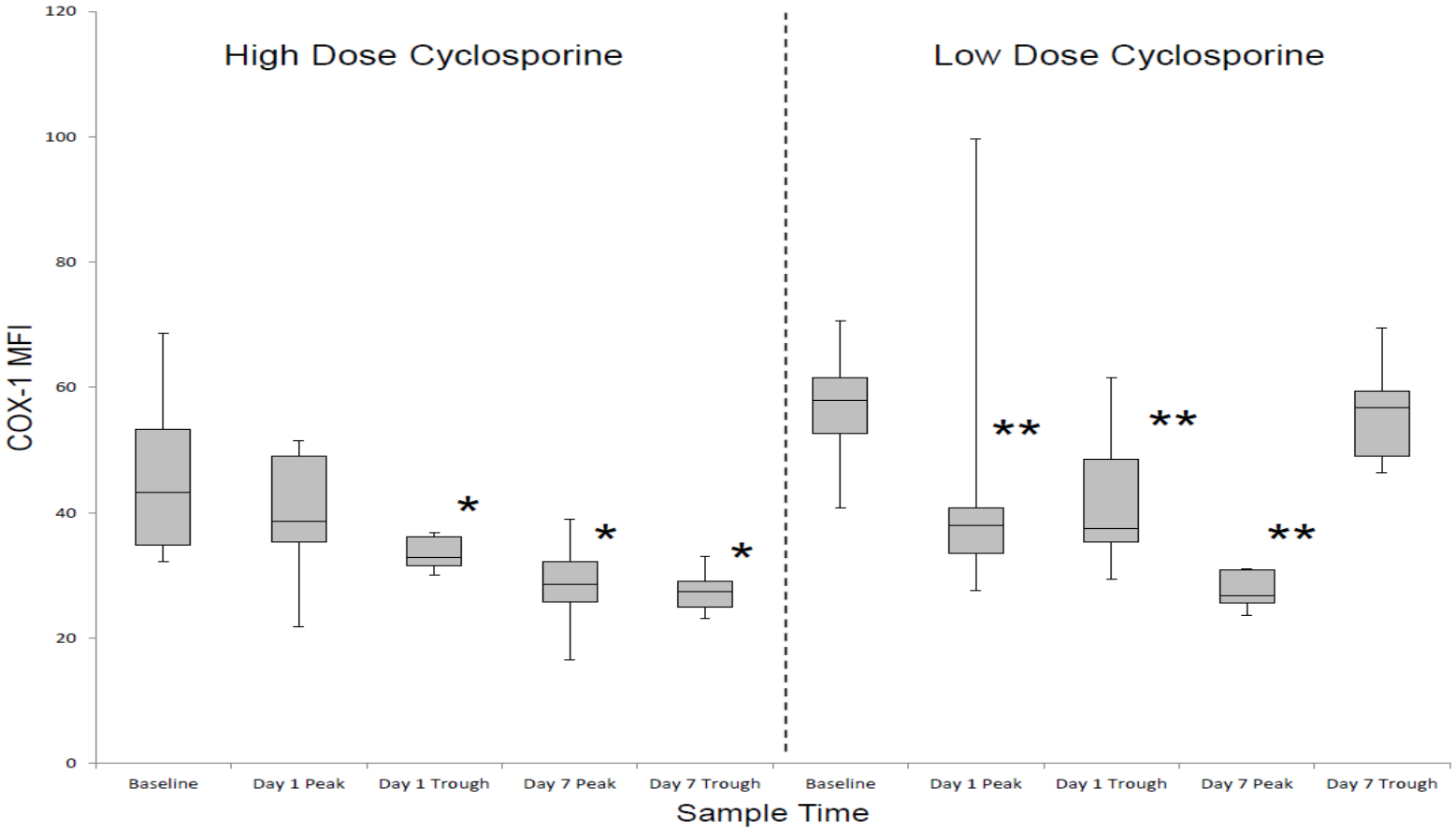

Figure 4.3 Mean COX-1 expression for dogs treated with two doses of cyclosporine. During the high dose of cyclosporine, COX-1 expression was significantly decreased from baseline values by the Day 1 trough time point, and remained significantly decreased for the remainder of drug administration (*). Three time points (Day 1 peak and trough and Day 7 peak) were significantly decreased during the low dose (**).

initiation (Day 1 peak time point). Platelet COX-2 expression then returned to levels comparable with baseline values 24 hours after administration of the first dose. Platelet COX-2 expression was again significantly decreased at the final time point (Day 7 trough) (Figure 4.4).

Urinary Thromboxane

The mean baseline urinary 11-dTXB$_2$ to creatinine ratio was 18 (range 10.7-24.7) prior to the high dose of cyclosporine. Following administration of the high dose, there was a significant increase in the urinary 11-dTXB$_2$ to creatinine ratio compared to baseline values at all time points. The peak time points on Day 1 and 7 had the highest percent increase in the mean 11-dTXB$_2$ to creatinine ratio, with increases of 1,027% (mean ratio 203; range 15.6-623) ($p=0.004$) and 1,461% (mean ratio 281.2, range 47.8-799.3) ($p<0.001$), respectively. There was also a significant increase in the mean baseline urinary 11-dTXB$_2$ to creatinine ratio at trough time points on Day 1 and 7, with increases of 142.7% (mean ratio 43.71; range 19.2-69.39) ($p=0.001$) and 107.6% (mean ratio 37.38; range 13.83-67.77) ($p<0.047$), respectively (Figure 4.5). Urine was not available from one dog at the peak time period on Day 1.

The mean baseline urinary 11-dTXB$_2$ to creatinine ratio was 16.7 (range 8.3-28.2) prior to the low dose of cyclosporine. There was no significant change in the urinary 11-dTXB$_2$ to creatinine ratio compared to baseline values at any time point (Figure 4.5). Urine was not available from one dog at the peak time point on Day 1, from three dogs at the peak time point on Day 7, and from two dogs at each trough time point.

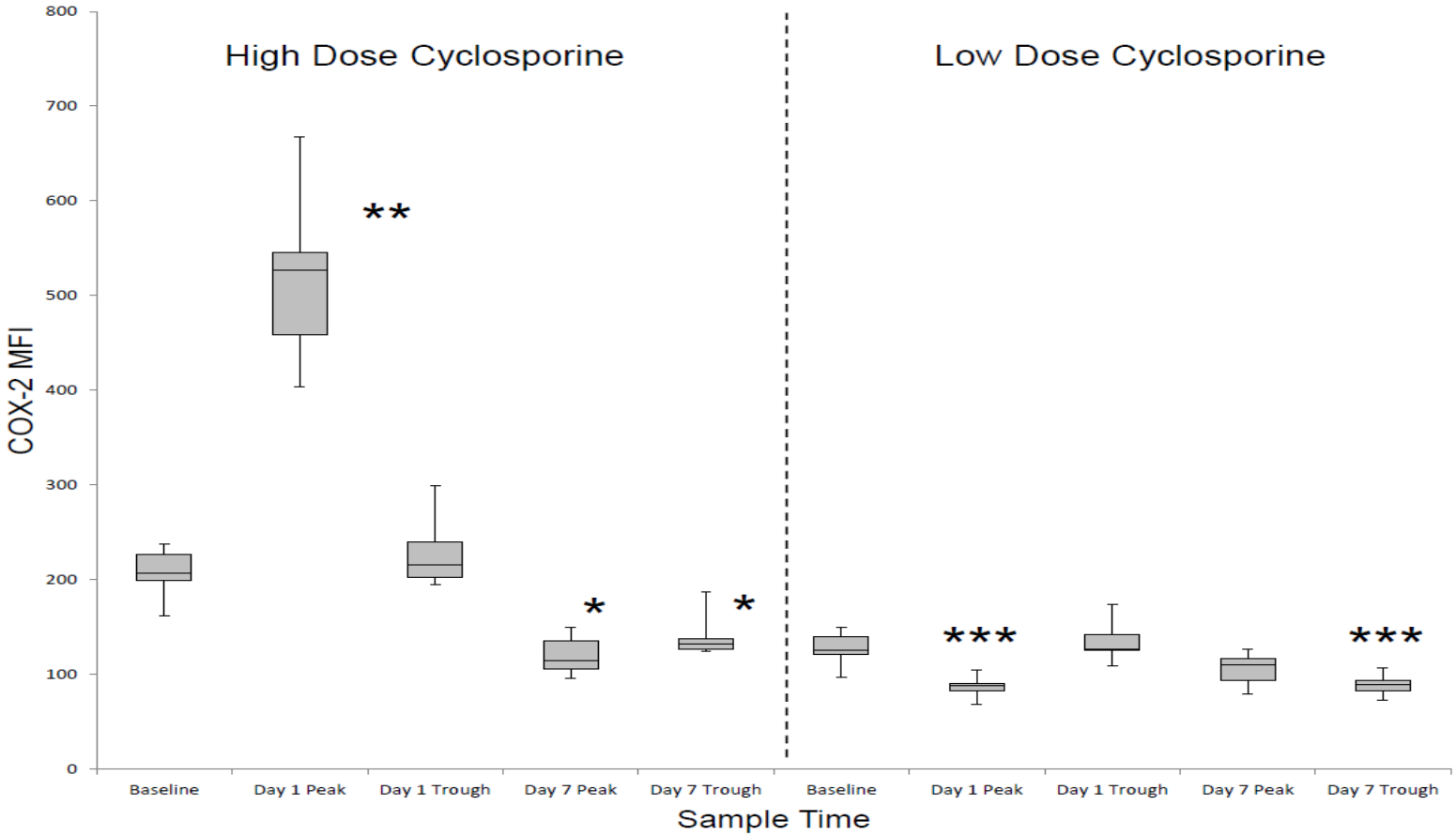

Figure 4.4 Mean COX-2 expression for dogs treated with two doses of cyclosporine. COX-2 expression was significantly decreased from baseline values by the end of administration (Day 7 peak and trough time points) of the high dose of cyclosporine (*). However, there was also a significant increase in COX-2 expression within 2 hours of initiating the high dose (**). With the low dose, there was an overall decrease in COX-2 expression, but only two time points (Day 1 peak and Day 7 trough) reached significance (***).

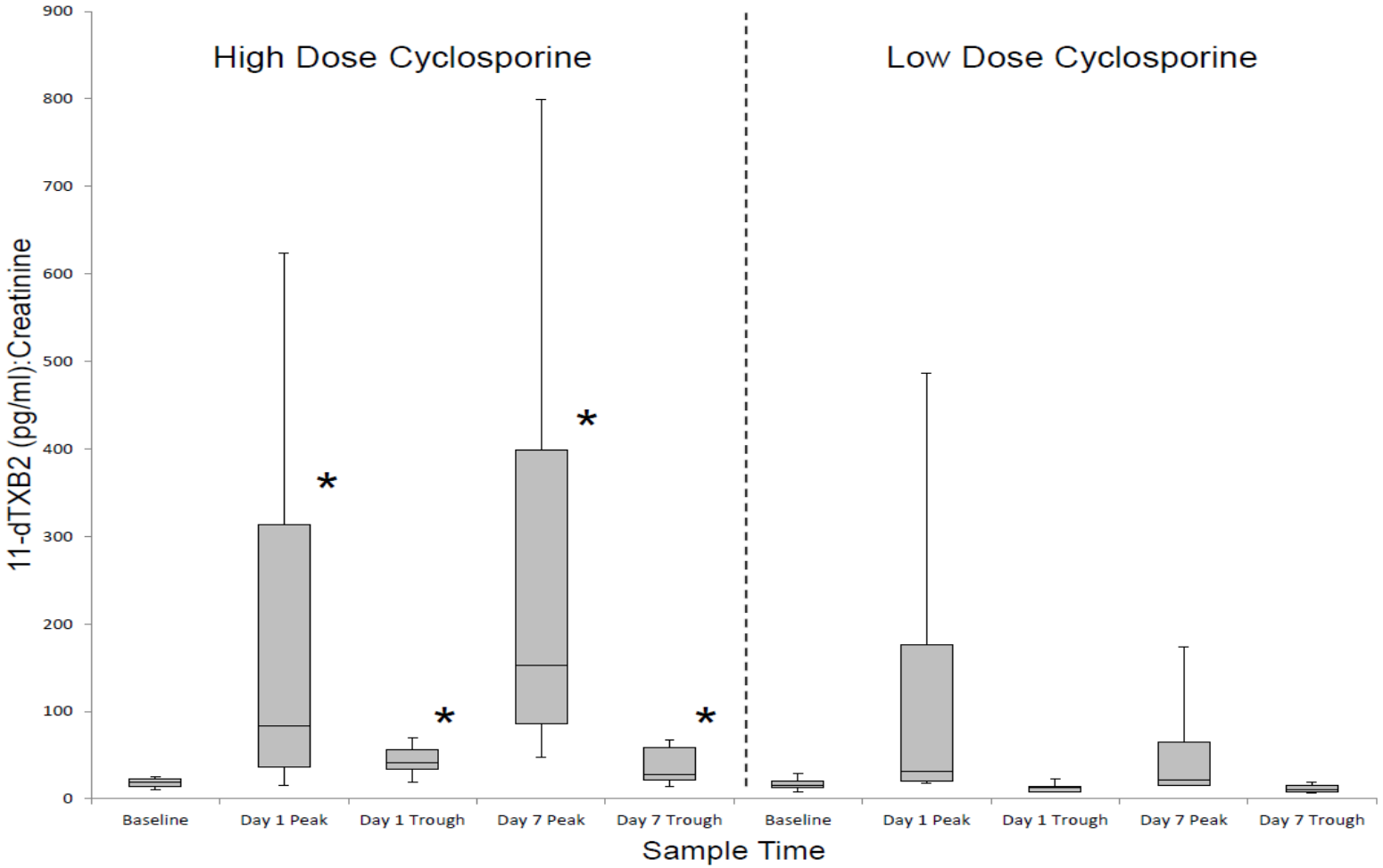

Figure 4.5 Mean urinary 11-dTXB$_2$ to creatinine ratios for dogs treated with two doses of cyclosporine. Urinary 11-dTXB$_2$ to creatinine ratios significantly increased from baseline values at all time points during the high dose of cyclosporine (*). No significant changes were detected during the low dose of cyclosporine.

Platelet Function Analysis

The mean baseline PFA-100® closure time using the collagen/ADP cartridge was 95.6 seconds (range 69-172 seconds) prior to the high dose of cyclosporine. There was an immediate significant (p=0.006) increase in closure time two hours after the administration of the initial high dose of cyclosporine (Day 1 peak time point), on average by 100%. However, after this first time point, the mean closure time dropped to 30%, 35%, and 62% above baseline at the remaining three time points, respectively. The Day 7 peak and trough time point closure times were still significantly increased (P= 0.005 and 0.008, respectively) (Figure 4.6).

The mean baseline PFA-100® closure time using the collagen/ADP cartridge was 140.6 seconds (range 67-234 seconds) prior to the low dose of cyclosporine. There was a significant (p=0.037) decrease in closure time twenty four hours after the administration of the initial low dose of cyclosporine (Day 1 trough time point), on average by 42.9% (Figure 4.6). Closure times remained significantly decreased for all remaining time points.

The mean baseline PFA-100® closure time using the collagen/epinephrine cartridge was 164.8 seconds (range 117-229 seconds) prior to the high dose of cyclosporine, and 174.9 seconds (range 130-254) prior to the low dose. There was no significant association between closure times and sample time for either high dose ($p \geq 0.054$) or low dose ($p \geq 0.472$) cyclosporine (Figure 4.7).

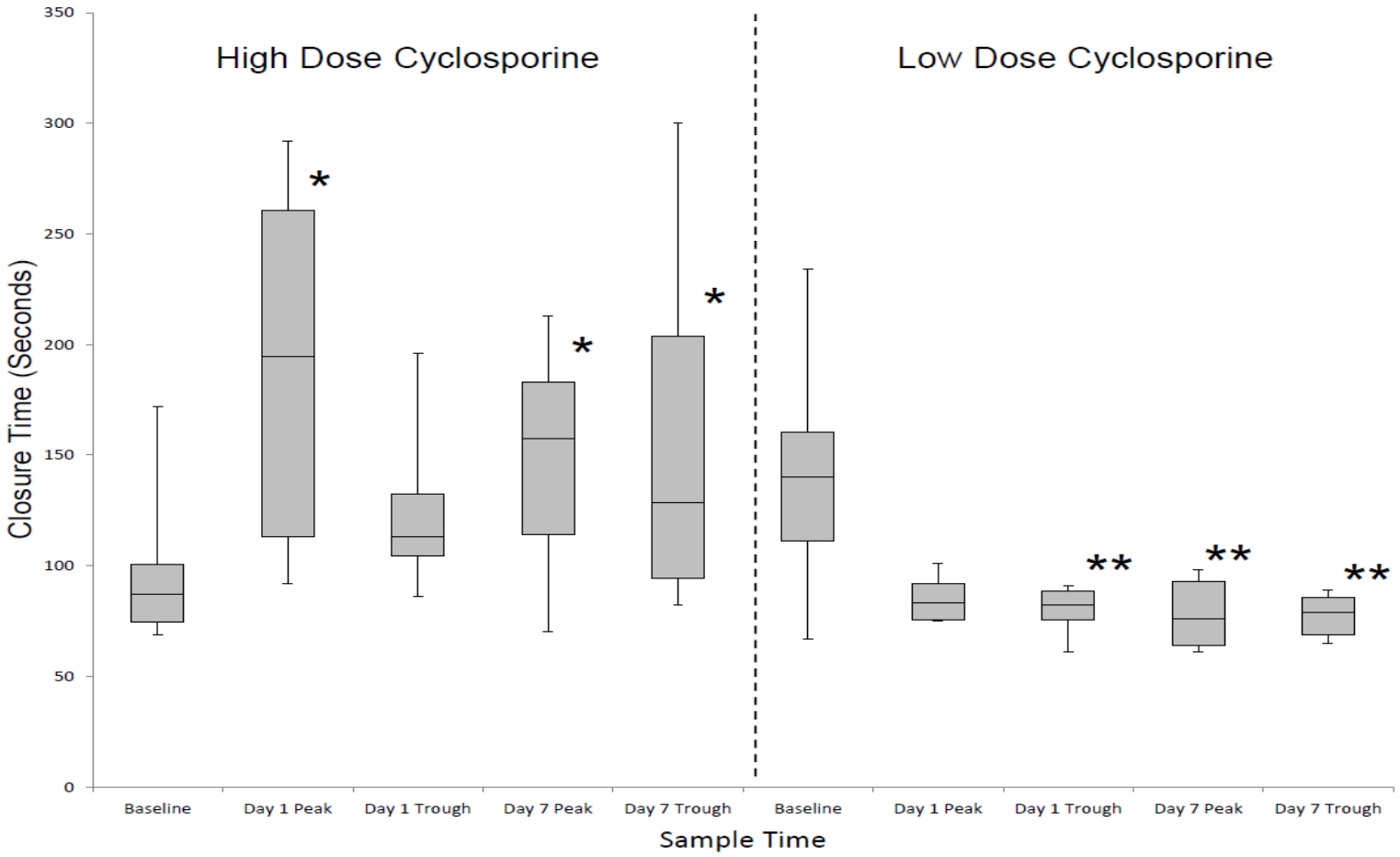

Figure 4.6 Mean PFA-100® closure times (seconds) for the collagen/ADP cartridge for dogs treated with two doses of cyclosporine. Closure times demonstrated a significant increase from baseline values at most time points during the high dose of cyclosporine (*). In contrast, during the low dose of cyclosporine, there was a significant decrease in closure times at three time points (**).

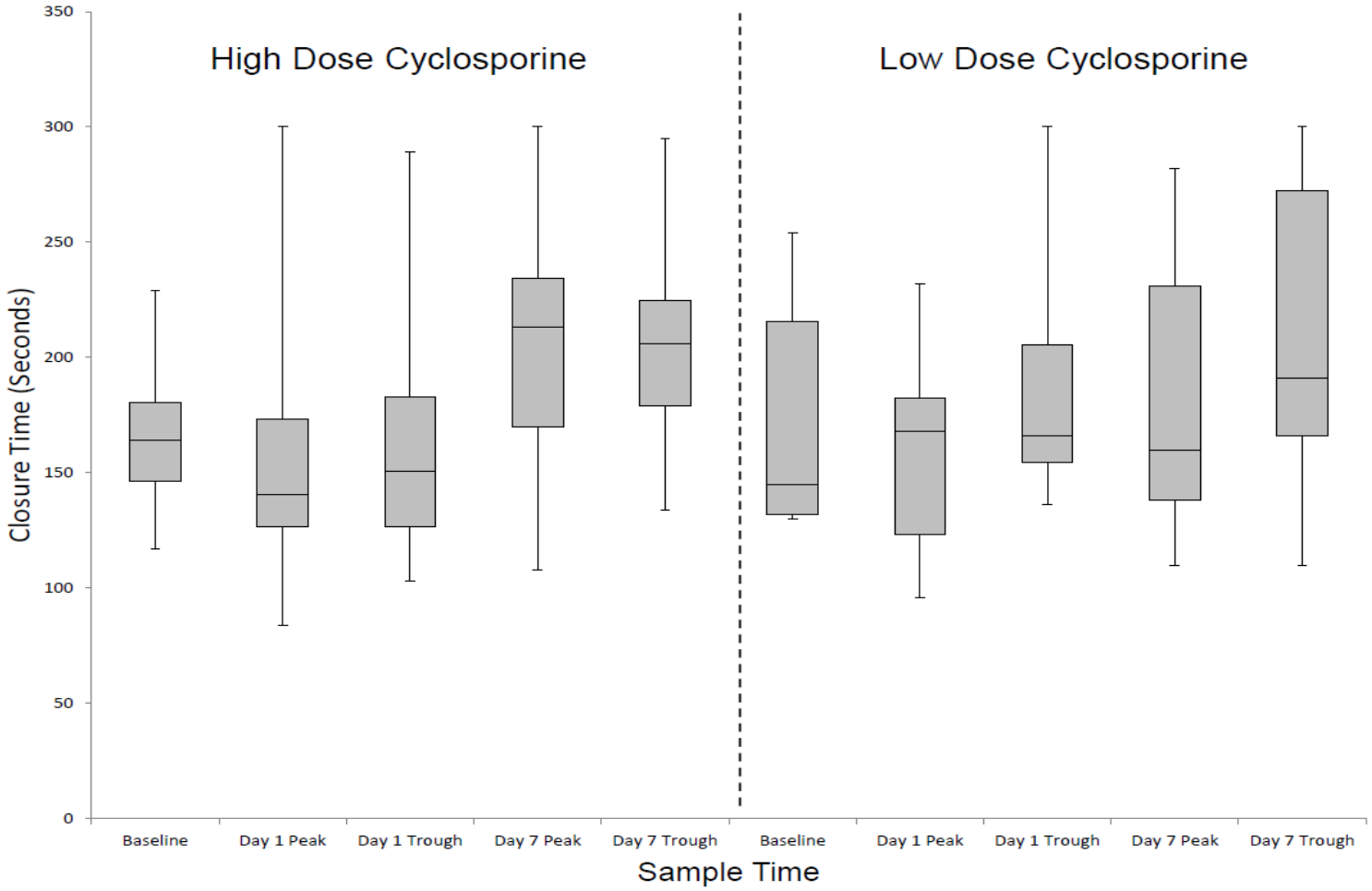

Figure 4.7 Mean PFA-100® closure times (seconds) for the collagen/epinephrine cartridge for dogs treated with two doses of cyclosporine. There were no significant changes in closure times at any time point with either dose of cyclosporine.

Other Testing

The mean baseline PCV prior to the high dose of cyclosporine was 45.5% (range 41.1-52.6). Significant differences in mean PCV during the high dose occurred at the peak time point on Day 1, with a 4.8% decrease (P=0.020), and peak time point on Day 7, with a 9.2% decrease (P=0.028); while during the low dose, only the peak time point on Day 1 was significantly different, with an 11% increase (P=0.019). There was no significant association ($p \geq 0.068$) between platelet count and sampling time for either cyclosporine dose

Discussion

Cyclosporine is one of the more commonly used immunosuppressive agents in dogs, partly due to the perception of relatively minimal side effects. Several human studies, however, have suggested that cyclosporine can increase the risk of thromboembolic complications by altering the platelet surface membrane and increasing platelet thromboxane production.[8-10,31-33] In our study, we showed that cyclosporine does alter the platelet membrane, and is associated with a significant increase in platelet thromboxane production.

Our study demonstrated a significant increase in the urinary 11-dTXB$_2$ to creatinine ratio during the administration of high doses of cyclosporine, especially at peak cyclosporine blood levels. This effect appears to be dose-dependent, since a similar but lesser (not sufficient to attain statistical significance) pattern was seen with low doses of cyclosporine. Our results are similar to previous studies in humans that demonstrate an increase in thromboxane production during cyclosporine administration in renal

transplant and coronary artery disease patients.[9,10,31-33] When compared to renal transplant recipients administered azathioprine, patients receiving cyclosporine had increased thromboxane production and increased rates of thrombus formation within the renal parenchyma.[10] The mechanism for the increase in platelet generated thromboxane is undetermined, and additional research would be needed to define the effects of cyclosporine on thromboxane production. Urinary 11-dTXB$_2$ is considered to be a reliable marker for platelet thromboxane A$_2$ production.[25,34,35] Our results therefore strongly suggest that cyclosporine at standard immunosuppressive doses triggers enhanced platelet activation in dogs.

Our study also demonstrated that the sustained administration of cyclosporine at both low and high doses to dogs leads to decreased platelet COX-2 expression. COX-1 is the primary cyclooxygenase isoform responsible for platelet production of thromboxane, while COX-2 is the main isoform that controls tissue prostacyclin (prostaglandin I$_2$) production.[36,37] Compared to thromboxane, prostacyclin has the opposite physiologic effect on primary hemostasis, and these two prostaglandins work in concert to maintain a balanced, normal hemostatic system. COX-2 derived prostacyclin has been associated with a protective response to experimentally-induced thrombus formation.[38] Decreased COX-2 expression, in contrast, could lead to an imbalance between thromboxane and prostacyclin, resulting in relatively higher levels of thromboxane. Previous studies in rats have demonstrated that cyclosporine suppresses COX-2 expression in a number of different tissues.[39,40] Our study is the first to demonstrate that cyclosporine similarly decreases COX-2 expression in canine platelets. Additional research is needed to better define the role of COX-2 in platelets, and the effects of cyclosporine on this enzyme.

The COX-2 selective NSAIDs, celecoxib and rofecoxib, have been associated with an increased rate of thromboembolic complications and cardiovascular events in humans.[36,38,41] Several potential explanations have been proposed for this problem, including inhibition of prostacyclin production,[37,42] decreased nitric oxide synthesis, and enhanced free-radical production.[42] Similar to the mechanism proposed with the COX-2 selective NSAIDs, cyclosporine's inhibitory effect on COX-2 expression could contribute to an imbalance between prostacyclin and thromboxane production. In fact, previous studies in rats have demonstrated that prostacyclin production decreased with cyclosporine administration.[33] Also analogous to the mechanism proposed with the COX-2 selective NSAIDs, cyclosporine has been shown to inhibit the release of endothelium-derived relaxing factor and epicardial nitric oxide in dogs, leading to a potential vasoconstrictor effect.[43-46] Cyclosporine has also been associated with increased free-radical production.[47-49] Cyclosporine's effects on prostacyclin, nitric oxide and free-radical levels could contribute to a hypercoaguable state in a manner comparable to the COX-2 selective NSAIDs, and could thereby increase the likelihood of thromboembolic complications.

Our study demonstrated that there was a steady decrease in platelet COX-1 expression with sustained cyclosporine administration, and a similar but less consistent effect with lower drug doses. As platelet thromboxane production increased as measured by the urinary 11-dTXB$_2$ to creatinine ratio, platelet COX-1 expression tended to decrease. Interestingly, an opposite pattern was recently observed in an aspirin study in our laboratory: when an anti-inflammatory dose of aspirin was administered to dogs, a decrease in platelet thromboxane production was associated with an increase in platelet

COX-1 expression.[21] Platelet function was concurrently inhibited in the dogs receiving aspirin, as demonstrated by prolonged PFA-100®collagen/epinephrine cartridge closure times, and platelet dysfunction was presumed to be due to COX-1 enzyme inhibition. The results of our aspirin and cyclosporine studies, when considered in combination, suggest that there may be a negative feedback system between platelet thromboxane production and platelet COX-1 expression. Additional research would be required to determine the exact relationship between platelet COX expression and thromboxane production.

Platelet function was evaluated by the PFA-100® using two different cartridges containing two different platelet agonist combinations. In humans, the collagen/epinephrine cartridge has been shown to be more likely to be influenced by medications, while the collagen/ADP cartridge is considered to be a more general screen platelet function. In our study, cyclosporine had no significant impact on collagen/epinephrine cartridge closure times. However, although the collagen/epinephrine cartridge has been shown to be an excellent assay for drug-induced decreases in platelet function, it is not known how useful the same cartridge would be at detecting drug-associated increases in platelet reactivity. Interestingly, with the collagen/ADP cartridge, high doses of cyclosporine were associated with an increase in closure times at most time points, while low doses were associated with a decrease in closure times at all time points (closure times, however, did not depart from reference ranges in any dog). The reason for these apparently contradictory results with the collagen/ADP cartridges is unknown.

In our study, there was an immediate and sustained decrease in platelet phosphatidylserine expression after administration of high doses cyclosporine. Our findings are in contrast to the findings reported in a recent human study, which demonstrated that cyclosporine increased platelet phosphatidylserine expression.[8] One possible explanation for the different results is that in our study we evaluated the effects of cyclosporine administered *in vivo*, while the previous human study incubated platelets with cyclosporine *in vitro*. While we cannot explain the difference in results, we believe that an *in vivo* study such as ours is more likely to reflect what is truly occurring in patients receiving cyclosporine. However, it also possible that the decrease phosphatidylserine expression observed in our study is erroneous, since the initial baseline value prior to our high dose cyclosporine trial was considerably greater than all other time points, a result that could be explained by a non-repeatable complication with sample handling or preparation. We also observed slight but statistically significant changes in platelet phosphatidylserine expression (decrease at the peak time point and an increase at the trough time point) during the first day of low dose cyclosporine, findings that are unlikely to be of any clinical relevance.

Upon platelet activation, phosphatidylserine is exposed on platelet surface membranes and binds to prothrombin, converting prothrombin to thrombin and enhancing secondary hemostasis.[8] Phosphatidylserine is also expressed during apoptosis of nucleated cells.[50-52] Similar apoptotic pathways have been associated with platelet activation, primarily through opening of the mitochondrial permeability transition pore (MPTP).[53-55] During apoptosis, the MPTP transiently opens and releases several pro-apoptotic mitochondrial contents into the cell cytoplasm, mediating caspase-3 activation

and ultimately leading to increased phosphatidylserine expression on the cell surface.[52,53] Cyclosporine has been shown to inhibit platelet MPTP function,[52] thereby decreasing the release of mitochondrial pro-apoptoic contents and potentially limiting the amount of phosphatidylserine expressed on the platelet surface.[52,53] Our findings in the high dose trial, if real, would be consistent with cyclosporine-induced inhibition of MPTP function and a resultant reduction in platelet phosphatidylserine expression.

Our study demonstrated a significant, immediate and sustained decrease in platelet P-selectin expression during administration of high doses of cyclosporine. This effect appears to be dose-dependent, since a similar but less consistent pattern was seen with the low dose of cyclosporine. Several human studies, in contrast, have demonstrated an increase in soluble P-selectin in renal transplant recipients treated with cyclosporine.[11,56] In one study comparing renal transplant patients to hypertensive control patients, P-selectin expression increased with all immunosuppressive agents, including cyclosporine, although platelet aggregation was not measurably affected.[56] Another study also demonstrated an increase in P-selectin expression in renal transplant recipients treated with cyclosporine, but similarly found no measurable increase in platelet aggregation.[11] Since our study evaluated the effects of cyclosporine on platelet P-selectin expression in normal dogs, and the human studies evaluated P-selectin expression in renal transplant recipients, it is possible that cyclosporine has a different effect in diseased compared to healthy individuals.

Platelet P-selectin is a cell adhesion molecule that mediates platelet and leukocyte aggregation and generates procoagulant microparticles that contain active tissue factor and enhance fibrin deposition.[57,58] Therefore, like platelet phosphatidylserine, platelet P-

selectin is believed to have procoagulant effects that enhance secondary hemostasis. Platelet P-selectin expression has been shown to be expressed in higher concentrations in canine patients with primary IMHA,[14] and could contribute to the high rate of thromboembolic complications seen in IMHA patients. Our previous pilot study, albeit performed in a small number of animals, demonstrated an increase in platelet P-selectin expression in dogs receiving cyclosporine, a result that raised concern because cyclosporine is increasingly used by veterinarians to treat canine IMHA. It is reassuring that, in this current study using a larger number of dogs and more sustained cyclosporine dosing, the effect reported in our pilot study was not reproducible and, in fact, a decrease in platelet P-selectin expression was observed.

One possible explanation for the decrease in platelet P-selectin expression seen in our study could be a drug-associated inhibition of the pro-inflammatory mediators TNF-α and IL-1β, which may act as stimulators of P-selectin expression.[59-61] Cyclosporine-induced inhibition of TNF-α and IL-1β has been associated with down-regulation of E-selectin, a similar adhesion protein found on vascular endothelium.[62,63] Additional research would be required to evaluate the effect of cyclosporine on platelet P-selectin expression, soluble plasma P-selectin concentration, and platelet-leukocyte aggregation in dogs with various diseases, particularly conditions such as IMHA that predispose to a hypercoagulable state.

Recent human studies have suggested that the use of cyclosporine could lead to an increase in thromboembolic complications, which would be of considerable concern if the same phenomenon occurred in canine patients with prothrombotic diseases such as IMHA. Our study revealed that cyclosporine has multiple effects on canine platelets,

causing altered expression of proteins on the platelet surface membrane, altered platelet COX expression, and increased platelet thromboxane production. Since platelets play an important role in contributing to thrombus formation, it is particularly concerning that the administration of cyclosporine was associated with marked increases in platelet thromboxane production, suggesting that exposure to the drug induces platelet activation. On the other hand, our study also revealed that cyclosporine reduces platelet expression of the procoagulant proteins P-selectin and phosphatidylserine. Our study was performed in normal dogs, and our results may not be directly applicable to diseased canine patients. However, these results strongly suggest that there is a pressing need to evaluate the effects of cyclosporine on platelet thromboxane production and expression of COX enzyme isoforms, P-selectin and phosphatidylserine in canine patients that have diseases that are associated with a hypercoaguable state and predispose to thromboembolic complications.

Materials, Instruments, and Supplies

a. Thomason J, Lunsford K, Mackin A, et al. Effects of Cyclosporine on Canine Platelet Procoagulant Activity. *J Vet Intern Med*. 2009; 23(3): 692.

b. Atopica, Novartis Animal Health, Greensboro, NC

c. 3.8% sodium citrate,Vacutainer tube, Becton Dickinson, Franklin Lakes, NJ

d. Clone MD6, IgG1, generously provided by Dr. C. Wayne Smith, Baylor College of Medicine, Houston, TX

e. Goat, anti-mouse-PE, Lot Number LXP07, R&D Systems, Minneapolis, MN

f. FITC-conjugated monoclonal CD61, Clone JM2E5, AbDSerotec, Raleigh, NC

g. Paraformaldehyde, Biolegend Inc., San Diego, CA

h. Annexin-V Binding Buffer, Invitrogen, Camarillo, CA

i. Annexin-V FITC Conjugate (Recombinant), Invitrogen, Camarillo, CA

j. Monoclonal CD61-purified, Clone JM2E5, Accurate Chemical, Westbury, NY

k. Goat anti-mouse IgG:RPE, AbDSerotec, Raleigh, NC

l. FITC-conjugated monoclonal COX-1, Clone CX111, Cayman Chemical Co, Ann Arbor, MI

m. Monoclonal anti-human CD9:RPE, Clone MM2/57, AbDSerotec, Raleigh, NC

n. Triton X-100, Sigma-Aldrich, St. Louis, MO

o. FITC-conjugated monoclonal COX-2, Clone CX299, Cayman Chemical Co, Ann Arbor, MI

p. FACS Calibur, BD Biosciences, San Jose, CA

q. CellQuest software, BD Biosciences, San Jose, CA

r. Luminex® 11-dehydro Thromboxane B_2 Kit, Cayman Chemical Co, Ann Arbor, MI

s. Luminex® 200 System xMAP Technology, Luminex Corporation, Austin, TX

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

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

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

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

x. Abbott Cell-Dyn® 3700, Abbott Laboratories, Abbott Park, IL

y. Personal communication, John D Patz, 2008

z. 7.5% EDTA Blood Collection Tubes, Tyco Healthcare, Mansfield MA

aa. Generous gift from Novartis Pharmaceuticals, East Hanover, NJ

bb. Varian Bond Elut 100 mg; Agilent Technologies, Santa Clara, CA

cc. Sigma-Aldrich, St. Louis, MO

dd. Agilent Technologies, Santa Clara, CA

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

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CHAPTER V

CONCLUSION

Immune-mediated hemolytic anemia (IMHA) is a common cause of anemia in dogs. Despite aggressive therapy, there is a 50% mortality rate in IMHA patients, and the most common cause of death is thromboembolic disease. Treatment consists of both preventative anti-platelet therapy and immunosuppressive medications. Aspirin is commonly administered to dogs, both for its anti-inflammatory properties and for its anti-platelet effects in order to prevent thromboembolic complications. Cyclosporine has become a popular immunosuppressive therapy, but in humans has been associated with an increase in platelet reactivity and thrombus formation. Understanding the relationship between COX expression and thromboxane synthesis could provide a better understanding of the interaction between platelets and these two medications.

This research identified the expression of both COX-1 and COX-2 in circulating canine platelets, and evaluated the changes in both isoforms during the administration of aspirin and cyclosporine. The COX-1 enzyme isoform was originally thought to be strictly constitutively expressed, but recent studies have shown that COX-1 expression can be induced in certain tissues.1 Both of the aspirin studies suggest that exposure to aspirin induces COX-1 expression in platelets and inhibits thromboxane synthesis. However, only an anti-inflammatory dose of aspirin consistently inhibits platelet function, as assessed by the PFA-100® closure times and urinary 11-dTXB$_2$ levels, while

lower doses of aspirin only inhibit platelet function in about a third of dogs, confirming the presence of aspirin resistance in normal dogs. One possible explanation for increased platelet COX-1 expression during aspirin therapy could be a compensatory up-regulation of the COX-1 isoform by megakaryocytes in response to a reduction in platelet thromboxane A_2 production.

The low dose aspirin study did not demonstrate a difference in pre-treatment platelet COX-1 expression between the three categories of aspirin responsiveness. Our results suggest that differences in pre-treatment platelet COX-1 expression do not play a major role in aspirin resistance in dogs, and indicate that pre-existing COX expression cannot be used to predict aspirin responsiveness. With no differences in urinary thromboxane concentrations between aspirin responders, non-responders, and inconsistent responders, our study suggests that COX-1 independent thromboxane synthesis is unlikely to be the mechanism for aspirin resistance in dogs.

Sustained cyclosporine administration caused a steady decrease in platelet COX-1 expression, with a similar but less consistent effect with lower drug doses. Unlike the aspirin studies, platelet thromboxane production increased during cyclosporine administration. The results of our aspirin and cyclosporine studies, when considered in combination, suggest that there may be a negative feedback system between platelet thromboxane production and platelet COX-1 expression.

The high dose aspirin study revealed that, in contrast to COX-1, platelet COX-2 expression decreased from baseline levels during aspirin administration, although the change was not statistically significant. After discontinuing the medication, however, there was a significant increase in platelet COX-2 expression, suggesting that high dose

aspirin does truly decrease platelet expression of this COX isoform. Pro-inflammatory mediators such as cytokines, endotoxins, and growth factors have been shown to increase cellular COX-2 expression.[2,3] Since inflammation is known to induce cellular COX-2 expression, and we administered a relatively high dose of aspirin, it is possible that the anti-inflammatory drug dose used served to decrease levels of some of the inflammatory mediators that induce COX-2.

Interestingly, platelet COX-2 expression increased during administration of low dose aspirin, a finding that contrasts with the anti-inflammatory dose of aspirin, but that is comparable to the effects of low dose aspirin on platelet COX-1 expression. It is possible that the anti-inflammatory effects of high doses of aspirin are sufficient to decrease or inhibit COX-2 expression, whereas low doses of aspirin were insufficient to exert the same effect. The COX-2 induction seen at low aspirin doses could possibly be associated with a negative feedback system with platelet thromboxane production, similar to COX-1.

Corticosteroids, dexamethasone and other glucocorticoids, have been shown to decrease cellular COX-2 expression as a result of decreased levels of the inflammatory cytokines IL-6 and TNF-α.[4] Similarly, aspirin has been shown to decrease levels of IL-6 and TNF-α, and would therefore be expected to, like glucocorticoids, decrease cellular COX-2 expression.[5-7] Interestingly, steroid administration has been associated with an increased risk of thromboembolic complications and in dogs with IMHA, the exact opposite effect that low dose aspirin has in IMHA patients.[8-10] It would interesting to determine the effect of steroids on platelet COX expression in both healthy dogs and

IMHA patients, and better understand the role these isoforms play in promoting a prothrombotic state and predisposing patients to thromboembolic complications.

At this time, the functions of the COX-2 isoform in canine platelets are currently unknown and additional research is required to better define the role platelet COX-2 plays in hemostasis. The expression of COX-2 in platelets could, like COX-1, contribute to thromboxane production. Evaluating platelet COX-2 expression in hypercoagulable patients, with corresponding thromboxane concentrations, would provide a better understanding of how platelet COX-2 contributes to hemostasis. Similar to the vascular endothelium, platelet COX-2 could contribute to prostacyclin synthesis and the prevention of platelet aggregation.

Pre-treatment patient-to-patient variations in platelet COX-2 expression have been proposed as one potential mechanism for variable aspirin responsiveness. The low dose aspirin study, however, did not identify a difference in pre-treatment platelet COX-2 expression between the three categories of aspirin responsiveness. Our results suggest that differences in pre-treatment platelet COX-2 expression do not play a major role in aspirin resistance in dogs, and indicate that pre-existing COX-2 expression cannot be used to predict aspirin responsiveness.

During the low dose aspirin study, there was a significant and consistent decrease in urinary thromboxane concentration, indicating that low dose aspirin is effective at inhibiting thromboxane generation, despite the fact that the drug did not provide consistent inhibition of platelet function in all dogs. The high dose of aspirin had a similar effect on thromboxane concentration, but was able to consistently inhibit platelet function. One possible explanation for variable inhibition of platelet function despite

consistent decreases in thromboxane production could be due togenetic polymorphisms in thromboxane receptors. Platelets express two subtypes of thromboxane receptors, TPα and TPβ, that originate from the alternative splicing of a gene.[11] Genetic differences in these subtypes of thromboxane receptors could explain why only some patients respond to the anti-platelet effects of aspirin.

Once stimulated, thromboxane receptors will lead to the activation of phospholipase C by coupling to several G-proteins, which in turn, increases cytosolic calcium, activates protein kinase C, and promotes platelet aggregation.[11] Previous studies have suggested that about 70% of canine platelets are insensitive to thromboxane stimulation.[12-14] The thromboxane insensitivity observed in some canine platelets may be due to impaired platelet thromboxane A_2 receptor linked G proteins.[14] If only 30% of dogs have platelets that are sensitive to the effects of thromboxane, this may explain why only one third of the animals in the low dose of aspirin study responded with a persistent increase in PFA-100® closure time.

In humans, it has been demonstrated that both thromboxane receptors and ADP receptors are dependent on receptor linked G proteins and activation of protein kinase C. It has also been demonstrated that there is a "cross-talk" between these two types of receptors, so that stimulation of one receptor could result in the desensitization of the other type of receptor.[11] It has been proposed that this cross-talk between receptors could lead to increased activity of the receptor that has not been inhibited.[11] In the dogs that have platelets that are insensitive to thromboxane-induced activation, other agonists such as ADP or serotonin may play a more important role in platelet activation, and such dogs may therefore be relatively resistant to the inhibitory effects of low dose aspirin.

Cyclosporine administration increases thromboxane synthesis and decreases platelet expression of both COX-1 and COX-2. Interestingly, this is the opposite pattern observed in the aspirin studies, where aspirin caused a decrease in platelet thromboxane production with an increase in platelet COX-1 expression for both doses and COX-2 expression for the low dose. The results of our aspirin and cyclosporine studies, when considered in combination, suggest that there may be a negative feedback system between platelet thromboxane production and platelet COX-1 expression. In contrast to aspirin, platelets exposed to cyclosporine exhibit a decrease in COX-2 expression, a phenomenon that could possibly be explained by the anti-inflammatory properties of the medication. Platelet P-selectin and phosphatidylserine expression decreased with cyclosporine administration, which is not consistent with a cyclosporine-induced increase in platelet reactivity. The concurrent dramatic increases in urine thromboxane concentrations however is concerning, suggesting that cyclosporine could induce platelet activation and potentially predispose to thrombus formation.

The results of these studies have generated many additional questions. It has been well established that COX-2 is present in megakaryocytes, and its expression can be altered by a medical condition or drug administration. The change in platelet COX-2 expression, induced or inhibited, could influence platelet function and the coagulation cascade. A better understanding of the rate and degree of change in COX expression could better explain aspirin resistance.

We demonstrated that some dogs do not consistently respond to the anti-platelet effects of low dose aspirin. With the potentially devastating complications of poor responses to aspirin, it is important to better understand the role aspirin plays in platelet

function. While platelet-derived thromboxane plays a critical role in vasoconstriction and promoting platelet aggregation, prostacyclin originating from the vascular endothelium and mainly through the COX-2 enzyme pathway, could also greatly influence platelet function. Low dose aspirin, unlike higher anti-inflammatory doses, will alter platelet function but continue to allow prostacyclin production to continue and contribute to the prevention of thrombus formation. Analyzing prostacyclin's role on hemostasis and platelet function, especially if synthesized by the platelet, may explain why some patients do not respond to aspirin. Other proposed mechanisms of aspirin resistance include poor drug bioavailability, alternative sources for platelet activation, and genetic polymorphisms of platelet glycoproteins and receptors needed to bind collagen.

Interestingly, isoprostanes, which are prostaglandin-like compounds, have been proposed as a mechanism of aspirin resistance. Isoprostanes are *in vivo* indicators of oxidant stress and will be elevated with cardiovascular dysfunction. These molecules are mainly produced through oxygen free radical induced peroxidation of arachidonic acid, but unlike prostaglandins, isoprostanes arise through a cyclooxygenase-independent pathway. 8-iso-prostaglandin $F_{2\alpha}$ will mobilize calcium in platelets and along with 8-iso-prostaglandin E_2 can initiate platelet activation through the TPα receptor. However, with inhibition of thromboxane receptors, isoprostanes may stimulate platelet activation through an alternative pathway.[15]

For those patients that do not respond to the effects of aspirin, there are several other medications that could inhibit platelet function. Clopidogrel which irreversibly blocks the platelet ADP receptor P2Y12, and abciximab which antagonizes the final common pathway by inhibiting the glycoprotein integrin αIIbβ3, are other anti-platelet

therapies are slowly becoming popular in veterinary medicine. Several of the techniques to evaluate platelet function used in these studies could also be used to evaluate these novel anti-platelet therapies. Monitoring the response to these medications will become necessary to allow appropriately inhibit platelets, but minimize bleeding complications.

With cyclosporine, our research identified a potentially serious platelet effect that could influence the rate of thromboembolism in hypercoagulable patients, especially IMHA patients. Using similar techniques, evaluating the effects of other immunosuppressive agents, such as azathioprine, mycophenolate, and leufonmide, could help determine which medications are safe to use in hypercoagulable patients. Dantrolene, a medication historically used to treat malignant hyperthermia, has recently been suggested to have immunosuppressive effects on T-lymphocytes, and could provide a less expensive immunosuppressive option in dogs. Interestingly, dantrolene has also been shown to inhibit platelet aggregation in rabbits, by inhibition of thromboxane formation and calcium mobilization.[16] If dantrolene is an effective inhibitor of T-lymphocytes and platelets, this medication would be a safe and less expensive therapy for dogs with IMHA, patients that typically require immunosuppression and anticoagulant therapies.

Since glucocorticoids have been associated with an increase incidence of thromboembolism in IMHA patients, determining influences of steroids on platelet function may help us understand and prevent life-threatening complications such as a pulmonary thromboembolism (PTE). Naturally occurring hyperadrenocorticism, or Cushing's Disease, is a commonly seen endocrinopathy in dogs that is characterized by excessive secretion of cortisol. It is generally accepted that both humans and dogs that

are affected with Cushing's are in a hypercoagulable state and could development a devastating PTE.[17] Since the exogenous administration and the excessive endogenous production of glucocorticoids could contribute to a hypercoagulable state, the techniques described in this manuscript could be very useful in evaluating effects of glucocorticoids on platelet function, and may help us understand and prevent any thromboembolic complications.

Finally, all of our studies have been performed in healthy dogs. However, the true evaluation of aspirin and cyclosporine will require evaluation in dogs in pro-inflammatory states. The next step for these research projects will be to use similar methodologies in patients with naturally occurring diseases, especially IMHA. Assessing thromboxane concentrations in IMHA patients receiving cyclosporine could better determine the safety of the medication, and adding low dose aspirin to the treatment plan could help us determine if aspirin is capable of counter-acting the pro-inflammatory effects of cyclosporine. Another potential clinical trial in dogs with naturally occurring disease would involve the use of an escalating dose of aspirin in procoagulant patients to determine a dose that will more consistently inhibit platelet function and thromboxane synthesis, without inhibiting prostacyclin through the COX-2 pathway.

The establishment of different populations of aspirin responders and non-responders could allow us to better understand the influences of thromboxane receptors on platelet function. With these different groups, we could evaluate the genetic make-up of these receptors, allowing us to determine if certain dogs and dog breeds are less susceptible to the anti-platelet effects of aspirin. The results of these studies would allow us to determine which anti-platelet therapy and what dose would provide the most

consistent inhibition of platelet function. If inhibition of thromboxane receptors could result in an increase in expression of ADP receptors, the use of multiple anti-platelet therapies may be beneficial to these patients. If multiple medications, or higher than normal doses, are required to provide consistent preventative therapy, it becomes important to have an effective and clinically useful ability to assess platelet function. By combining the techniques used in these studies, we will ideally be able to determine which drug and dose will provide the most ideal preventative therapy for hypercoagulable patients and the best method to monitor the effects anti-platelet therapies.

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