The Effects of Prednisone and Prednisone Plus Ultralow-dose Aspirin on Coagulation Parameters in Healthy Dogs

Allison L. O'Kell

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David C. Grant

David L. Panciera

Gregory C. Troy

Nicole M. Weinstein

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ABSTRACT

Objectives: To determine the effects of prednisone and prednisone plus ultralow-dose aspirin on coagulation in healthy dogs, and to determine intra-individual variation in thromboelastography (TEG).

Animals: 14 healthy experimental dogs and 10 healthy client-owned dogs

Procedures: Prospective, randomized, blinded study. TEG was performed twice three days apart on each experimental dog prior to treatment and intra-individual variation was calculated. Dogs were given prednisone (2 mg/kg/day) plus aspirin (0.5 mg/kg/day) or prednisone (2 mg/kg/day) plus placebo for 14 days, after which TEG and other baseline tests were repeated. Changes from baseline between and within each group were compared using t-tests or Wilcoxon 2 sample tests. Client owned dogs had TEG performed twice three days apart to determine intra-individual variation.

Results: Intra-individual variation in TEG parameters were $\leq$10% for MA (maximum amplitude) and angle. For experimental dogs, MA and fibrinogen significantly increased from baseline whereas Ly30 (percent lysis 30 minutes after MA) and antithrombin activity significantly decreased within each group. For the prednisone plus placebo group, Ly60 (percent lysis 60 minutes after MA) significantly decreased from baseline. For all parameters, there was no difference between groups for change from baseline.

Conclusions and Clinical Relevance: Prednisone caused hypercoagulability in healthy dogs evidenced by increased MA and fibrinogen and decreased antithrombin activity. Concurrent use of ultra-low dose aspirin had no effect on measured TEG parameters. Intra-individual variation in some TEG parameters is high and may preclude routine clinical utility.

DEDICATION

I dedicate this work to my mentors, the two people who have had the most influence on my veterinary career thus far...

To Dr. David Panciera, for the unwavering encouragement, support, and patience you showed me from day one of my internship and that continued throughout all aspects of my residency and Master's degree. You have helped to shape me as a veterinarian more than any other, and although you believe in excellence, you never let me forget that sometimes "good enough is good enough."

To Dr. David Grant, for everything you have done for me. Your kindness, patience, encouragement, advice, realism, and friendship were instrumental in my success in both my residency and Master's degree. You helped me with so much more than veterinary medicine and I will be forever grateful.

...and I hope someday I will have the opportunity to influence others as you have

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ATTRIBUTIONS

David C. Grant, DVM, MS, DACVIM

Dr. David Grant is an Associate Professor of internal medicine in the Department of Small Animal Clinical Sciences at the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech. Dr. Grant is chair of this committee and was the principal investigator on a grant awarded by the Veterinary Memorial Fund. He was involved in the design, planning, and execution of the project, as well as the writing and editing of the thesis and manuscript for publication.

David L. Panciera, DVM, MS, DACVIM

Dr. David Panciera is a Professor of internal medicine in the Department of Small Animal Clinical Sciences at the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech. Dr. Panciera was involved in the design, planning , and financial support of the project, and contributed to and reviewed the manuscript for publication.

Gregory C. Troy, DVM, MS, DACVIM

Dr. Gregory Troy is a Professor of internal medicine in the Department of Small Animal Clinical Sciences at the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech. Dr. Troy was involved in the design, planning and financial support for a portion of the project, and contributed to and reviewed the manuscript for publication.

Nicole M. Weinstein, DVM, DACVP

Dr. Nicole Weinstein is an Assistant Professor of clinical pathology in the Department of Biomedical Sciences and Pathobiology. Dr. Weinstein was involved in the design and planning of the project and contributed to and reviewed the manuscript for publication.

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ABBREVIATIONS

aPTT	activated partial thromboplastin time
AT	antithrombin
CV	coefficient of variation
Hct	hematocrit
IMHA	immune mediated hemolytic anemia
K	K-time
Ly30	percent lysis 30 minutes after MA
Ly60	percent lysis 60 minutes after MA
MA	maximum amplitude
PA	prednisone plus aspirin
PP	prednisone plus placebo
PT	prothrombin time
R	R-time
ROTEM	rotational thromboelastometry
TAT	thrombin-antithrombin complexes
TEG	thromboelastography

CHAPTER I: LITERATURE REVIEW

A. Physiology of Hemostasis

i. Primary Hemostasis

Primary hemostasis consists of vascular events and formation of a platelet plug.[1] Damage to the blood vessel endothelium is the triggering event,[2] which causes vasoconstriction.[3,4] Cytokines, leukocyte adhesion molecules, or up-regulation of major histocompatibility complex antigens also activate endothelial cells.[4] Exposed collagen from the damaged endothelium leads to platelet adhesion.[5] Von Willebrand's factor (vWf), a plasma glycoprotein predominantly synthesized by endothelial cells, also causes platelet adhesion, along with fibrinogen, when vascular damage is less severe by binding to glycoprotein IIb/IIIa on platelets. [4] If shear stress due to blood flow is low, platelet binding via glycoprotein IIB/IIIa receptors by fibrinogen is adequate for platelet adhesion.[4] When shear stress is high, vWf also binds to glycoprotein Ib/IX/V receptors on platelets which allows for more efficient platelet adherence and activation.[4] Platelet glycoprotein Ib/IX/V also contains thrombin binding sites.[1] Platelet storage granules release agonists, such as adenosine diphosphate, serotonin, epinephrine, thromboxane A_2 and platelet activating factor (PAF), which are synthesized by the activated platelets.[4] These compounds induce activation and aggregation of additional platelets.[4] Conformational changes in glycoprotein IIb/IIIa after platelet adhesion assists with fibrinogen binding to the platelet.[1]

ii. Secondary Hemostasis

Secondary hemostasis, occurs on cell surfaces (endothelial cells and platelets) and involves many different coagulation, or clotting, factors.[1] The traditional cascade model (intrinsic and extrinsic pathways) of coagulation has fallen out of favor, as this model is not a

true reflection of *in-vivo* coagulation.[1,6] The cell-based model of coagulation more accurately reflects the interactions between clotting factors and cellular components,[6] and is divided into three stages: initiation, amplification, and propagation.[1,6] Tissue factor (Factor III), is the most important protein involved in initiation of coagulation.[6] This factor is located in the vascular adventitia, but not on the vascular surface of intact blood vessels.[4] Factor VIIa, the only clotting factor that circulates in an active form, then binds to exposed tissue factor on damaged blood vessels.[6] This VIIa-tissue factor complex subsequently activates more Factor VII, as well as activating Factors IX and X.[6] A small amount of Factor V is activated by Factor Xa, and these two activated factors form the prothrombinase complex, which cleaves prothrombin (Factor II) to form a small amount of thrombin (Factor IIa).[6]

In the amplification phase, the thrombin formed in the initiation phase binds to and activates platelets.[1,6] Thrombin also serves to cleave Factor VIII from vWf,[6] and activates factors V, VIII, and XI.[1,6]

Propagation occurs on the surface of activated platelets, and begins with the binding of activated Factor IX and VIII on the platelet, forming the intrinsic-tenase complex.[6] This complex then forms large amounts of Factor Xa on the platelet surface, which again joins with Factor Va to form the prothrombinase complex.[6] At this point, large amounts of thrombin are formed, which goes on to cleave fibrinogen and form fibrin.[6] This fibrin then polymerizes to form strands and an insoluble clot.[6] Activation of Factor XIII by thrombin leads to cross linking of fibrin, which enhances clot elasticity and strength.[6]

Calcium ions are important for binding of coagulation factors to phospholipids and are required for many of the enzymatic reactions,[4,7] as well as for the maintenance of fibrinogen stability and structure.[7]

iii. Fibrinolysis

The fibrinolytic system is responsible for dissolution of clots through degradation of fibrin by enzymes.[4] Plasminogen, an inactive proenzyme produced in the liver, is converted to an active serine protease, plasmin, by an activator.[4] Plasminogen activators are divided into two types – intrinsic and extrinsic.[4] Factor XIIa acts as an intrinsic activator, through either direct activation of plasminogen or indirectly by activating prekallikrein (PK) and high molecular weight kininogen (HMWK).[4,7] The indirect method leads to formation of kallikrein or urokinase (UK), which then converts plasminogen to plasmin.[4] The extrinsic activators are tissue plasminogen activator (tPA) and UK,[4,7] which both have very specific activity in forming plasmin from plasminogen.[4] tPA is primarily produced by endothelial cells[7] and has enhanced activity when fibrin is present, which helps to localize plasmin's activity to areas that fibrin has been deposited.[4,7] UK is predominantly produced in the kidney and excreted via the urine; however, some other cells (pneumocytes, fibroblasts, placental cells) also produce UK.[4] Fibrin is not necessary for activation of plasminogen by UK, and it is suggested that UK also has an important role in wound healing, tissue remodeling, tumor cell invasion, and embryogenesis.[4,7]

Plasmin then degrades fibrin, its principle substrate.[4] As plasmin's action is non-specific, it can also inactivate other components of the coagulation system, such as fibrinogen, (which forms fibrinogen degradation products (FDPs)), factors Va, VIIIa, IXa, XIa, PK, HMWK, and some parts of the complement system.[4]

Several inhibitors of fibrinolysis exist, which act through direct plasmin inhibition or through inhibition of plasminogen activation.[4,7] The most important direct plasmin inhibitor is α_2-antiplasmin, which is produced in the liver[7] and is present in plasma and platelets.[4] Another compound, α_2-macroglobulin, plays a smaller role in plasmin inhibition,[4] and is important when the inhibitory capacity of α_2-antiplasmin is maximized.[7] Plasminogen activator inhibitor 1 (PAI-1) is an important inhibitor of tPA and UK, and thus fibrinolysis.[4] It was initially thought to be produced in endothelial cells and hepatocytes,[4,7] but newer investigation has identified the adipocytes as the predominant source.[7] It is also present in platelet α granules,[4,7] which makes up the predominant portion of circulating PAI-1.[7] Various compounds, including endotoxin, thrombin and several cytokines, trigger the release of PAI-1.[4] PAI-1 is present in excess over tPA in circulation, which keeps the activity of tPA controlled.[7] PAI-1 activity is inhibited by activated protein C.[4]

iv. Anticoagulation

The two major pathways of anticoagulation are the antithrombin heparin pathway and the thrombomodulin-protein C-protein S pathway.[4] Antithrombin (AT) is formed in the liver and endothelial cells. It prevents the formation of fibrin from fibrinogen by binding thrombin,[4,7] in a reaction catalyzed by heparan sulfate proteoglycans present on the vascular endothelial surface.[7] AT also inhibits other activated coagulation factors, including IXa, Xa, XIa, and XIIa.[4,7] Thrombin-antithrombin complexes are formed as a result of AT's inhibition of thrombin, and act as a marker of coagulation activation.[4] Heparin cofactor II, the activity of which is enhanced by heparin and dermatan sulphate proteoglycans, also inhibits thrombin, but appears less important than AT.[4,7]

Protein C is activated by thrombin, particularly when thrombin is bound to thrombomodulin.[4,7] Activated protein C (APC)'s major function is inactivation of factors Va and VIIIa, in conjunction with ionized calcium, phospholipids, and protein S (PS).[4] Protein S acts as a cofactor to increase the rate of APC's inactivation of factors Va and VIIIa.[4] It exists in both free and bound forms in plasma, with the free form of protein S thought to be the more effective cofactor.[4]

B. Glucocorticoids

i. General physiology and side effects

Endogenous glucocorticoids, of which cortisol is most important, are synthesized in the zona fasciculata and zona reticularis of the adrenal cortex and mediate protein and carbohydrate metabolism.[8] Cortisol has predominantly glucocorticoid effects, but retains a small amount of mineralocorticoid activity.[3] Many synthetic glucocorticoids have greater glucocorticoid activity due to structural alterations, which ameliorates some of the unwanted side effects of mineralocorticoid activity. [8] Almost all cells in the body possess glucocorticoid receptors, which are steroid-hormone receptor proteins present in cell cytoplasm.[8,9] Once the glucocorticoid binds to the receptor, the complex migrates into the nucleus, interacts with DNA, and leads to the formation of proteins which mediate the specific response.[10]

Glucocorticoids have a multitude of effects on the body both at physiologic and supraphysiologic concentrations. At physiologic concentrations, metabolic effects include increased hepatic gluconeogenesis, decreased glucose utilization by cells, muscle catabolism and decreased protein synthesis, and stimulation of lipolysis.[3,8] Glucocorticoids also contribute to

vascular tone by enhancing the effects of other vasoconstrictive substances and increasing cardiac output.[8]

At supraphysiologic concentrations, glucocorticoids inhibit fibroblast synthesis of collagen, causing skin thinning and delayed healing, stimulate bone resorption and inhibit bone formation, inhibit the intestinal absorption of and increase urinary excretion of calcium, and inhibit growth in young animals.[8] Unique to dogs is polyuria and polydipsia due to either antagonism of the action of ADH on the renal tubules and/or decreased ADH secretion.[8] Chronic glucocorticoid excess can also decrease total thyroxine (T4) and free T4 without clinical manifestations of hypothyroidism.[8] Chronic use of exogenous glucocorticoids can also lead to iatrogenic hyperadrenocorticism and prolonged suppression of adrenocorticotropic hormone secretion and subsequent adrenocortical atrophy.[8] Increased liver enzyme activity, particularly alkaline phosphatase activity due to both liver and corticosteroid induced isoenzymes, is common with glucocorticoid treatment, along with structural hepatic changes in the form of vacuolar hepatopathy,[11] which is reversible.[12] Glucocorticoids may also lead to gastrointestinal ulceration by altering mucosal defenses, predispose to infections due to immune suppression, and cause insulin resistance.[8] An anecdotal increased risk of pancreatitis has been reported, but supportive evidence is lacking at present.[8] Glucocorticoids can lead to systemic hypertension via systemic vasoconstriction due to increased sensitivity to catecholamines and angiotensin II, increased endothelin secretion, and decreased activation of the nitric oxide system.[13]

ii. Anti-inflammatory and Immunosuppressive effects

Glucocorticoids have both anti-inflammatory and immunosuppressive effects on tissues.[8,14] These effects are somewhat species dependent, as humans and rodents are more

sensitive than dogs and cats.[8,14] Macrophages and monoctyes are very sensitive to glucocorticoids, and their phagocytic, bactericidal, and antigen presenting abilities are diminished.[8,14] Depressed macrophage aggregation at sites of inflammation and decreased tumor necrosis factor production contribute to their anti-inflammatory effects.[8] Bone marrow neutrophil release is increased, and along with an increased half-life and decreased migration into tissues, leads to neutrophilia.[8,14] The phagocytic capacity and bactericidal activity of neutrophils is also depressed.[8,14] Lymphocytes, particularly T-cells, redistribute from the vascular to the extravascular space, including the spleen, lymph nodes, and bone marrow.[8] Lymphocyte proliferation and cytotoxic abilities are also hindered.[8,14] The eosinopenia observed with glucocorticoid administration is likely due to redistribution of eosinophils and not cytotoxicity.[8] Basophil and mast cell number and activity are also depressed.[8] An important anti-inflammatory action of glucocorticoids is decreased arachidonic acid metabolism, leading to decreased levels of the pro-inflammatory substances prostacyclin, thromboxane, and lipoxygenase.[8,14] In general, glucocorticoid effects are somewhat dose dependent, with some immunosuppressive effects increasing as the dose increases.[8] For example, high doses of glucocorticoids also lead to decreased immunoglobulin concentrations (IgG, IgA, IgM) and therefore blunted immunologic response to antigenic stimulation.[8] The immunosuppressive effects are useful in treating various immune mediated conditions, including immune mediated hemolytic anemia (IMHA).[8]

iii. Glucocorticoids and hypercoagulability

a. Humans

Humans with endogenous glucocorticoid excess (Cushing's syndrome) are predisposed to thromboembolic complications.[15,16] Multiple human studies have documented various

abnormalities in hemostasis and fibrinolysis.[15-18] Increases in clotting factors, specifically factors II, V, VIII, IX, XI, XII, and protein C, protein S, C1-inhibitor[17] and PAI-1[15-17] have been documented. As well, antithrombin activity, antithrombin antigen, and plasminogen were increased, likely a secondary event due to coagulation activation.[17] Increased vWf is present in some humans with Cushing's syndrome.[15,16] Twenty percent of humans with Cushing's syndrome develop thromboembolic complications post-treatment, of which 10% die.[16] Patients treated prophylactically with heparin and/or warfarin have significantly fewer thromboembolic events and a decreased mortality rate .[16] Compared to patients with non-adrenocorticotropic hormone producing pituitary adenomas, patients with Cushing's syndrome have a higher incidence of venous thromboembolism post-operatively.[15]

High dose corticosteroid treatment has also been linked to cerebral venous thrombosis in patients with various disorders.[19] Chronic glucocorticoid administration, even at sub-immunosuppressive doses, in human transplant patients leads to hypercoagulability caused by a hypofibrinolytic state due to increased PAI-1 activity.[20-22] In healthy volunteers, shorter term prednisolone therapy (11 days) resulted in hypofibrinolysis and increases in Factor VIII in one study,[23] but no change in PAI-1 activity and increases in Factors VII, VIII, and XI in a study of short term (7 day) dexamethasone administration.[24] A systematic review of the published literature on glucocorticoid effects on hemostasis yielded variable results, however increased PAI-1 activity was commonly detected.[25] The lack of consistency evident among the included studies suggests that the definitive mechanism remains to be determined.[25]

b. Dogs

Although not as well documented as in humans, endogenous and exogenous glucocorticoids are associated with hypercoagulability and thromboembolism in dogs. Unlike in humans, hypofibrinolysis due to increased PAI-1 has not been documented in dogs with hyperadrenocorticism.[26] One study found increased activity of factors II, V, VII, IX, X, XII, and vWf, thrombin-anti-thrombin complexes, and decreased antithrombin activity as evidence of hypercoagulability,[26] while another documented increased factors V, X, and fibrinogen, compared to reference ranges, and increased antithrombin and plasminogen.[27] The latter study did not measure thrombin-antithrombin complexes or PAI-1. There is obvious discrepancy regarding the cause of hypercoagulability in hyperadrenal dogs, however hyperadrenocorticism is one of the most commonly identified concurrent diseases in dogs with pulmonary thromboembolism.[28-30] In a report of six dogs with aortic thromboembolism, three had hyperadrenocorticism (one each with pituitary dependent, adrenal dependent, and iatrogenic hyperadrenocorticism).[31] However, studies assessing hypercoagulability by thromboelastography (TEG) in dogs with naturally occurring hyperadrenocorticism are contradictory.[32-33] Healthy dogs treated with 1 and 4 mg/kg/day of prednisone for two weeks[34] and with 2 mg/kg/day of prednisone for 1 week[35] have several TEG parameters consistent with hypercoagulability. In addition, decreased clot lysis as measured by TEG, was present.[35]

C. Aspirin

i. Anti-platelet effects

Aspirin irreversibly acetylates the enzyme cyclooxygenase (COX) in platelets[36,37] in a dose dependent fashion.[37] COX transforms arachidonic acid to prostaglandin G_2 (PGG_2).[38,39]

Prostaglandin H_2 (PGH_2) is then formed from PGG_2 by the same enzyme, which is the direct precursor to various eicosanoids, including PGE_2, thromboxane A_2, prostacyclin (PGI_2), PGD_2, and PGF_2.[38-40] Various enzymes catalyze the formation of these eicosanoids.[38,39] There are two isoforms of COX, COX-1 and COX-2, each of which has many functions.[38-40] Classically, COX-1 and COX-2 are considered constitutively expressed and inducible, respectively.[39] COX-1 is expressed in many cells, and COX-2 was believed to be only induced by inflammation.[39] However, constitutively expressed COX-2 is present in the stomach, kidney, uterus, and brain.[39] Platelets produce thromboxane A_2 through activity of COX-1, causing vasoconstriction and platelet aggregation.[40,41] Endothelial cells produce PGI_2 through activity of both COX-1 and COX-2, leading to vasodilation and decreased platelet aggregation.[40,41] In humans, much lower doses of aspirin are required to inhibit COX-1 than COX-2, and these lower doses are utilized when anti-platelet but not analgesic or anti-inflammatory effects are desired.[41] Endothelial PGI_2 is predominantly formed by COX-2, and therefore low-dose aspirin does not significantly affect its formation and anti-thrombotic effects.[40,41] A low response to aspirin, as measured by inhibition of platelet aggregation, has a prevalence in human patients between 1-45% among different studies, depending on the assay and agonist used and the cut-off values used to define a low response.[42] Possible causes of this purported resistance include poor compliance, patient health factors (eg. concurrent disease), concurrent drug interactions, and genetic variability.[42]

Although much of the aforementioned work has been done in humans, anti-platelet effects of aspirin have also been studied in dogs. Aspirin inhibits platelet thromboxane B_2 (a product of thromboxane A_2 breakdown and a standard measure of COX-1 inhibition[43]) production *in-vitro*[44] and *in-vivo*[45] in canine blood. Administration of anti-inflammatory doses of aspirin (3.5-10 mg/kg q12h) to dogs has resulted in conflicting results. Decreased platelet

aggregation,[46,47] platelet function (using the PFA-100),[46] and platelet thromboxane B_2 concentration,[47] have been documented. Contradicting this, another study found no effect on platelet function and aggregation or plasma thromboxane B_2 concentration,[48] however circulating plasma thromboxane B_2 does not necessarily correlate with platelet thromboxane B_2[48] or with platelet function and aggregometry.[46] In another study using whole blood impedance aggregometry, platelet aggregation was impaired using arachidonic acid as an agonist, but not collagen.[49] Ultralow-dose aspirin (0.5 mg/kg/day) resulted in impaired platelet aggregation in the majority of healthy dogs using ADP and collagen as agonists in whole blood impedance aggregometry.[50] However, a study using platelet rich plasma for aggregometry found that an aspirin dose of 0.5 mg/kg every 12 hours effectively decreased platelet aggregation using ADP plus arachidonic acid as agonists, while a dose of 0.5 mg/kg every 24 hours did not.[51] Differences in results of these studies may also relate to differences in platelet function and aggregometry methodology (including type of agonist) between the studies, as well as inter-individual and breed variation.[46] Lack of platelet aggregation in response to arachidonic acid occurs in a variable number of dogs, and is inherited and varies with breed[52,53] However, canine platelets in some studies respond well to arachidonic acid,[49] and the impaired platelet aggregation present with aspirin therapy in that study, and others using varying methods of platelet function assessment, [46,47,50] suggest that aspirin does impair *in-vitro* platelet function in the majority of dogs.

ii. Clinical use in humans for treatment and prevention of thromboembolic disease

Aspirin is used extensively in human medicine for treatment and prevention of arterial thrombosis.[41,54] When taken within 24 hours of onset of an acute myocardial infarction and continued for 30 days, aspirin reduces premature death without an increased incidence of

bleeding.[54] A decreased mortality rate and recurrence of stroke was also found in patients with acute ischemic stroke who received aspirin within 48 hours of the event.[54] Low dose aspirin results in a decreased risk of nonfatal myocardial infarction and stroke, adverse cardiovascular events, and death in patients with stable cardiovascular disease.[55] Aspirin therapy (at varying dosages) is also beneficial in patients at high risk for vascular occlusive disease, including patients previously affected by myocardial infarction, stroke, transient ischemic attacks, or other vascular conditions.[41,54] However, an increased risk of major bleeding can also accompany these benefits,[41,55] particularly at higher dosages.[54] Aspirin has also been studied in combination with other anti-platelet agents, such as clopidogrel and dipyridamole, with a variable increase in preventative benefit and/or increased bleeding risk.[56] The use of aspirin for prevention of venous thromboembolism is less clear; some efficacy has been documented in high risk surgical patients, however therapy also leads to an increased risk of bleeding.[57]

iii. Clinical use in dogs for prevention of thromboembolic disease

Studies examining the use of aspirin to prevent thromboembolism in dogs are scarce, and the dose required is unknown. Aspirin (22 mg/kg/day) reduces pulmonary thromboembolism after thiacetarsamide treatment for experimentally induced *Dirofilaria immitis* infection.[58] Aspirin (10 mg/kg, IV bolus) reduces thrombus formation in experimental *in-vivo* and *ex-vivo* models of coronary artery occlusion in dogs.[59] Dogs undergoing carotid endarterectomy and receiving a single dose of 10 mg/kg of oral aspirin have significantly less thrombus formation than dogs receiving 0.5 mg/kg of oral aspirin and control dogs.[60] However, in a clinical retrospective study on canine immune mediated hemolytic anemia, ultralow-dose aspirin (0.5 mg/kg/day), administered with the intent to prevent thromboembolic complications, was shown to improve both short and long term survival in combination with glucocorticoids and azathioprine, although

a placebo treated control group was not included.[61] This has led to the use of ultralow-dose aspirin as anti-thrombotic therapy in IMHA. In a subsequent study comparing the effects of clopidogrel alone and in combination with ultralow-dose aspirin (0.5 mg/kg/day), along with immunosuppuppressive therapy, on survival in dogs with IMHA, no difference was found between the two groups.[62] However, a placebo treated control group was not used in this study either, and high survival rates (75-88%) and a small numbers of dogs may have precluded the detection of a difference. Ultralow-dose aspirin is also commonly used with the goal of preventing thromboembolism in idiopathic glomerulonephritis,[63] but studies to date have not demonstrated a beneficial effect.

D. Hypercoagulability and Immune Mediated Hemolytic Anemia in Dogs

i. Pathophysiology

IMHA is a common cause of anemia in dogs in which anti-erythrocyte antibodies lead to hemolysis.[64] Mortality rates in this disease are high, ranging from 24-70 %.[61,65-68] Thromboembolism is a common complication and contributor to mortality of IMHA in dogs, [61,65,66,69,70]and thromboemboli in various organs are detected in 40-100% of dogs with IMHA receiving post-mortem examinations.[61,65,71,72] In a study of 29 dogs with necropsy confirmed pulmonary thromboembolism, IMHA was a suspected predisposing factor in 10% of cases.[30] Thrombocytopenia, hyperbilirubinemia, elevated alkaline phosphatase, hypoalbuminemia, a negative Coomb's test, and intravenous catheterization are risk factors for thromboembolism.[65,69]

The pathogenesis of thromboembolism in IMHA is unknown, and there is great difficulty in confirming a diagnosis of thromboembolic disease ante-mortem in patients. Hypoalbuminemia may play a role in some cases, as platelet hypersensitivity has been documented in two hypoalbuminemic dogs with proteinuria; this hypersensitivity was reversed when canine albumin

was added to the hypoalbuminemic plasma.[73] Circulating activated platelets, as detected by greater P-selectin expression in platelet rich plasma compared to control dogs, have been detected in dogs with primary IMHA.[74] However, another study, using whole blood instead of platelet-rich plasma, detected increased platelet activation (increased P-selectin expression, fibrinogen binding, and proportion of platelet microparticles) only in a subset of dogs with IMHA and concurrent severe thrombocytopenia (<40,000 platelets/uL).[75]

Disseminated intravascular coagulation (DIC) has been suggested as a mechanism for thromboembolism in IMHA, and 12 – 45%[70,71] of dogs with IMHA have suspected or confirmed DIC. However, the presence of suspected or confirmed DIC has not been substantiated as a risk factor for thrombosis in IMHA.[65] Fibrinogen is increased in 85% of dogs with IMHA,[70] and in an experimental study that used intravenous thromboplastin to induce putative DIC, low levels of thromboplastin, suggested to cause "chronic DIC", led to increased fibrinogen levels in dogs.[76] In studies evaluating thromboelastography in critically ill dogs, those with hypercoagulable thromboelastograms had fibrinogen levels significantly higher than either dogs with normocoagulable thromboelastograms,[77,78] hypocoagulable thromboelastograms,[78] or control dogs.[77] This suggests that hyperfibrinogenemia may be involved in the pathogenesis of hypercoagulability. Fibrinogen is also an acute phase protein in dogs,[79-81] including in dogs with IMHA[82,83] but a causative relationship between elevations in fibrinogen due to the acute phase response and thromboembolic events has not been established. Approximately 50% of dogs with IMHA have low antithrombin, of which the majority were diagnosed with DIC.[70] There was, however, no difference in diagnosis of thromboembolic disease in dogs with low antithrombin compared to normal dogs, thus antithrombin deficiency alone is not the cause of thromboembolism.

In humans, hemolytic anemia has been associated with antiphospholipid antibodies and the antiphosphoplipid syndrome (APS), a cause of hypercoagulability and venous and/or arterial thrombosis.[84] Antiphospholipid antibodies are autoantibodies that recognize phospholipids and/or phospholipid binding proteins.[84] These antibodies lead to thrombosis in the APS, and although the pathogenesis has not been definitively determined, hypotheses include activation of endothelial cells, oxidant mediated damage to the vascular endothelium, or interference with phospholipid proteins involved in the regulation of coagulation.[84] There has been limited study on the antiphospholipid syndrome in dogs. A case report describes a single dog with hemolytic anemia, thrombocytopenia, glomerulonephritis, polyarthropathy, and pulmonary thromboembolism with testing consistent with the presence of lupus anticoagulant, an antiphospholipid antibody.[85] One study measured dilute Russell viper venom time and kaolin clotting time, to detect lupus anticoagulant, which were prolonged in 35% and 15% of dogs, respectively.[70] In humans, diagnosis of APS can be difficult and guidelines have evolved over the past decade.[84,86] Kaolin clotting time is no longer recommended to detect lupus anticoagulant due to poor reproducibility.[86] Attempts to detect other antiphospholipid antibodies, such as anticardiolipin and anti-β_2-glycoprotein I,[86] were not made in the dogs in either of these studies, limiting their meaningfulness. Anticardiolipin antibodies were measured in both healthy dogs and dogs with a variety of diseases in a separate study; 52% of diseased dogs, including one dog with IMHA, had elevated levels (based on a reference interval produced from the healthy dogs).[87] However, the dogs had a wide variety of diseases and no effort was made to determine the significance of these elevations. Anti-endothelial antibodies were not detected in dogs with diseases predisposing to thromboembolism, including IMHA,[88] and anti-endothelial antibodies are commonly detected in human patients with APS.[89] Although to date there is no evidence to

support APS as a cause for hypercoagulability in IMHA in dogs, investigation into this syndrome has been extremely limited and further studies are necessary before discounting APS as a cause.

Another proposed mechanism of hypercoagulability relates to free hemoglobin released during intravascular hemolysis, which scavenges nitric oxide.[90] Nitric oxide deficiency leads to decreased inhibition of platelet aggregation and adhesion.[90] However, since intravascular hemolysis, and therefore free hemoglobin release, occurs less often than extravascular hemolysis in canine IMHA,[4,91] this mechanism seems unlikely to play a major role.

As mentioned previously, corticosteroids, either endogenous or exogenous, are associated with thromboembolic disease in dogs,[28-31] and healthy dogs treated with prednisone have hypercoagulable thromboelastograms.[34,92] The majority of dogs in two studies with IMHA had hypercoagulable thromboelastograms.[93,94] However, some dogs were being treated with corticosteroids and/or other immunosuppressive therapies for unspecified periods of time at the time of sampling,[93,94] and there was no report of the degree of anemia, both of which can cause hypercoagulable thromboelastograms.[95,96] The contribution of corticosteroids to hypercoagulability and thromboembolic disease in IMHA patients remains to be determined.

ii. Anti-thrombotic therapy in dogs with IMHA

Despite the high prevalence of thromboembolic disease in IMHA, studies evaluating the use of anti-coagulant or anti-platelet medications as prevention are limited. A study in 18 dogs with IMHA treated with 300 IU/kg of unfractionated heparin (UFH) subcutaneously (SC) every 8 hours had 83% survival to discharge and 61% 1 year survival rates.[72] The anti-Xa activity used to monitor heparin therapy was below the human therapeutic range in the majority of dogs, and of 6 dogs that died that had necropsies performed, 50% had thromboemboli.[72] A control group

was not included, so no conclusions could be made regarding heparin's effect on mortality or thromboembolic disease. Thirteen dogs treated with a fresh frozen plasma transfusion combined with UFH (100 IU/kg SC every 6 hours, with dosing adjustments made based on every 24-48 hour PTT measurement) had a short term mortality rate of 38%, and 46% of dogs that had necropsies performed had thromboemboli present.[97] Antithrombin levels did not increase in the dogs at 30 minutes or 48 hours post transfusion.[97] Again, there was no contemporary control group with which to compare survival and/or prevalence of thromboembolism. In a prospective study evaluating constant dose (CD; 150 U/kg every 6 or 8 hours) compared with individually adjusted dose (IAD; based on anti-Xa activity human therapeutic range) unfractionated heparin showed a higher case fatality rate for dogs in the CD group.[98] Although the authors conclude that the result was due to either a positive effect of individual adjustment of heparin dosing or a negative effect of subtherapeutic heparin dosing, the small sample size (n=7 for CD group, n=8 for IAD group) and lack of a control group (without heparin treatment) are significant limitations. However, this treatment protocol warrants further investigation. A large retrospective study of dogs with IMHA, all treated with glucocorticoids and azathioprine, found significantly increased survival in dogs treated concurrently with ultralow-dose aspirin (0.5 mg/kg/day) compared with concurrent mixed molecular weight heparin (mHEP), mHEP plus ultralow-dose aspirin, or no concurrent medication.[61] Although the mHEP group had characteristics associated with negative survival, which may have imparted a bias as to choice of treatment by the clinician, the mHEP plus ultralow-dose aspirin group had significantly better survival than the former group, suggesting that aspirin conferred a benefit.[61] There was no difference in survival in dogs with IMHA treated with immunosuppressives and given either clopidgrel alone, ultralow-dose aspirin alone, or both drugs in combination, on survival in dogs

with IMHA.[62] However, a control group that solely received immunosuppressive therapy was not evaluated in this study. Prospective, randomized, controlled studies are lacking with respect to anti-thrombotic therapy, in particular ultralow-dose aspirin.

E. Thromboelastography

i. Mechanics and physiology

Thromboelastography provides information, in a graphical form, regarding the overall state of clotting and fibrinolysis at a single point in time.[99] TEG measures the *in-vitro* viscoelastic changes that occur during coagulation,[100,101] and includes assessment of both plasma clotting factors and the cellular components of clotting, namely platelets and leukocytes.[78] Whole blood is added to a heated cup (37° C), a pin attached to a torsion wire is inserted into the blood, and then the cup is oscillated back and forth.[99-101] When the blood clots and fibrin strands attach the pin to the cup, altered cup motion affects the pin oscillation, and these changes are transformed into a computer generated curve. [99-102] Thromboelastometry (ROTEM) uses similar principles to TEG; however with ROTEM the pin oscillates relative to the cup instead of the cup oscillating relative to the pin (as with TEG).[103] The nomenclature used for ROTEM is slightly different, and the tracings and information gleaned from ROTEM are similar to, but not interchangeable with, TEG.[103] The remainder of the discussion will focus on TEG.

The tracing has three general phases: the initial linear zone of precoagulation, the coagulation zone that extends from the end of precoagulation to point of greatest separation of the two lines of the tracing, and the fibrinolytic zone that extends from the end of coagulation until the end of the tracing.[104] Several numerical parameters are derived from the TEG tracing. The R-time (R; reaction/clotting time) begins at test initiation and ends when the clot formation begins (denoted when the amplitude of 2 mm is reached on the TEG tracing).[99,100] The reaction

time evaluates factors VIII, IX, XI, and XII and provides information on thrombin production.[104,105] The K-time (K) is measured from the end of R until the amplitude of 20 mm is reached on the TEG tracing.[99,100] The K-time measures the speed of clot formation and describes thrombin activity and fibrin production. It is influenced by factor II, fibrinogen concentration, platelet number/function, and hematocrit.[104,105] Alpha (angle or α) is the angle between the baseline of the tracing and the slope of the line between R and K.[99,100] Alpha represents the speed of clot formation and clot strength, and is influenced by fibrinogen function.[104] Maximum amplitude (MA) is the greatest amplitude of the TEG tracing and represents overall clot strength.[99,100,104] The MA is influenced by thrombin and fibrinogen concentration, fibrin, factor XIII, and hematocrit, as well as platelet number and function.[104] Ly30 represents percentage of fibrinolysis at 30 minutes after MA is reached and is based on the decrease in amplitude of the TEG tracing over time measured by the reduction in the area under the curve.[100] Ly60 is similar, but reflects the percentage of fibrinolysis 60 minutes after MA is reached.[106] The clot index (CI) is a calculated value derived from R, K, α and MA,[100] and is used to categorize patients as hyper-, hypo-, or normocoagulable.[102] Global clot strength (G) is a calculated value using MA, and reflects overall clot strength.[107] It is also used as a measure of overall coagulation status to identify patients as hyper-, hypo-, or normocoagulable.[78,108] Aside from calculated values to assess overall coagulation status, individual TEG parameters also reflect pathophysiologic changes. For example, a prolonged R indicates coagulation factor deficiency, and a decreased angle is a signal of reduced fibrin.[100,101] K-time and angle can also be abnormal with clotting factor deficiencies, and platelet abnormalities (functional or numerical).[101] A decreased MA can reflect decreased platelet function and number[100,101,107] or hypofibrinogenemia.[101] Increased Ly30 and Ly60 reflect an increase in fibrinolysis.[100]

ii. Sample types and activators

Native whole blood is traditionally used for TEG analysis, but comes with the limitation that it must be used within 6 minutes after collection[109,110] to achieve the most stable values.[111] The use of citrated whole blood to allow for storage has been evaluated in several human studies using both healthy and clinical patients,[109,110,111 112-114]with varying results on whether citrate storage significantly changes thromboelastograms and the stability of the TEG values at varying time points of storage. An overall trend towards hypercoagulability has been demonstrated, however.[115] Repeated sampling of blood from the same tube also leads to hypercoagulable thromboelastograms.[110,112] A concurrent increase in thrombin-antithrombin complexes and prothrombin 1 and 2 fragments detected in one study suggests gradual activation of thrombin and incomplete inhibition of clotting by the citrate.[111] Increased β-thromboglobulin levels (released by activated platelets) were also detected, which may indicate early platelet activation.[111] The conflicting results underscore the importance of developing reference ranges for each individual laboratory and sample type, and for not extrapolating results between different sample types or laboratories.

Various activators can be added to blood with the intention of increasing the speed of thromboelastogram generation.[116-118] Kaolin and celite are used as contact surfaces to activate the "intrinsic" pathway, and tissue factor (TF) is used to activate the "extrinsic" pathway.[117] Kaolin,[115-117] tissue factor,[115,117] and celite[117,118] have been studied in humans, with conflicting results regarding the correlation between TEG parameters using different activators. The studies are also difficult to compare due to a lack of comparison to a standardized method, such as native or non-activated citrated whole blood TEG, in some. The choice of activator may depend on the clinical situation. Aprotinin, a serine protease inhibitor of both plasmin and kallikrein, alters

thromboelastograms using kaolin and celite, but not tissue factor, in humans.[117] Therefore, TF-activated TEG may be chosen in human patients receiving aprotinin so that underlying coagulopathies can still be detected.[117] Tissue factor activation in dogs only partially inhibits the effects of LMWH using heparinase cups, while kaolin activation almost completely inhibits these effects.[119] Thus, kaolin activation is a better choice to detect underlying coagulopathies in dogs receiving heparin.

In dogs, both citrated whole blood and various activators have been used in different studies. TEG was initially validated in dogs using human recombinant TF as an activator on citrated blood measured after 30 and 120 minutes of storage at room temperature.[107] A trend towards hypercoagulability was demonstrated with longer storage time, similar to humans, and low analytical variation was present at each time point.[107] A recent abstract found no significant differences between TEG performed 15 minutes after venipuncture versus 1 hour (using both citrated whole blood and kaolin activation).[120] The same study also evaluated TEG parameters over three consecutive days and found no significant difference between the days for each dog. However, actual values were not presented in the abstract and therefore these findings must be interpreted with caution.

Citrated non-activated,[94,121-124] citrated TF activated,[77,78,108,119,122,125] and citrated kaolin activated[126,127] TEG has been evaluated in various states of health and disease. However, although reference intervals have been developed, to date no study has compared these different techniques in the same population of dogs. Significant differences in TEG parameters between some sampling methods (blood drawn into syringes containing sodium citrate versus into plain syringe and blood then transferred to a vacuum tube) also occur,[128] which potentially could affect interpretation of results.

iii. Other factors affecting TEG

Factors such as anemia, thrombocytopenia, fluid dilution, and hemolysis affect TEG parameters, most of which have been studied only in humans. Anemic chemotherapy patients have increased R and MA with decreasing hemoglobin levels; these findings were reversed after transfusion with whole blood.[129] A correlation between K and hemoglobin concentration has been found, with a shorter K in patients with lower hemoglobin.[114] Using dog blood diluted *in-vitro* with platelet-rich plasma, strong direct correlations were detected between hematocrit and R-time and K-time, while strong inverse correlations were detected between hematocrit and angle, MA, and G.[96] Another study in dogs also detected increased MA values when blood was diluted *in-vitro*, using a similar method to the previous study, in samples with decreased hematocrit.[95] *In-vitro* dilution with normal saline, Ringer Acetate, 4% albumin, Dextran 70, and 6 and 10% hydroxyethylstarch (HES) resulted in hypercoagulable thromboelastograms at low and moderate dilutions.[130] At high dilutions (40% for dextran and HES and 50% for crystalloids and albumin) hypocoagulability was observed.[130] These findings have been confirmed *in-vivo* in pre-operative human patients[131] and healthy volunteers[132] that had 20-30% dilution of blood volume with saline, in which thromboelastograms had characteristics of hypercoagulability compared to baseline. These effects quickly resolved (within 45 minutes after saline infusion) in the healthy patients.[132] Although it has been hypothesized that lower hematocrits may lead to less mechanical interference of red blood cells with platelets and fibrin during clot formation,[133] it is unknown whether the effects of anemia and hemodilution on TEG are artifactual or truly affect *in-vivo* coagulation.[134]

Platelet count also affects TEG, and *in-vitro* studies in humans have shown that a decreasing platelet count correlates with decreasing MA and K, but not with R.[135,136] In dogs,

experimental hemolysis of citrated blood, created by mechanical methods or by freezing, led to overall hypocoagulable kaolin activated thromboelastograms.[127] Although the hemolysis in this study was greater than what generally would occur clinically,[127] these findings warrant further study into the effects of clinically relevant hemolysis on TEG. The effects of hemolysis have not been studied in humans.

In humans, gender can also influence TEG, as healthy human females have hypercoagulable thromboelastograms compared to males. [114,115] A single study in dogs evaluated the effects of gender on kaolin activated TEG and found no significant differences between males and females.[126] A small number of castrated males and females were included, but the effects of castration were not evaluated due to small numbers. Also in this study, German Shepherds and Beagles constituted a large portion of the dogs,[126] which could confound the results if breed differences exist. The effects of breed have been evaluated in only two studies. In healthy greyhounds, using citrated whole blood, the K-time was significantly prolonged and the angle, MA, and G value were significantly lower than healthy dogs of a variety of other breeds.[121] No significant differences in the R-time or Ly60 were detected.[121] A breed specific reference interval for Bernese Mountain dogs was also established using citrated whole blood with tissue factor activation. The reference intervals for MA and G were different than the standard laboratory reference interval (MA reference interval included higher values and G had a wider range), while the reference intervals for R, K, and angle (Ly30 and Ly60 not investigated) were the same.[137] These differences in greyhounds and Bernese Mountain dogs indicate that breed related differences in TEG exist and deserve further investigation.

iv. TEG Modifications

There are several methods to modify the TEG analyzer that can be useful clinically. Heparinase, an enzyme that inactivates heparin, has been used in humans to detect coagulopathies in liver transplant patients,[138] as well as to detect effects of various anti-coagulant medications.[139] In dogs, using heparinase cups, kaolin activated TEG resulted in nearly complete reversal of heparin effects and TF- activated TEG resulted in partial and dose-dependent reversal when low-molecular weight heparin was added *in-vitro* to whole blood. [119] Although there are limitations due to the *in-vitro* nature of the study, it suggests that TF-activated TEG may be useful to monitor the effects of heparin therapy in dogs. Modified TEG has also been used to evaluate platelet activation (platelet mapping) by using blood treated with reptilase and Factor XIII to form a clot without thrombin generation, and assessing the response to platelet agonists (arachidonic acid and ADP).[140,141] The ability to predict clopidogrel induced platelet dysfunction and predict clinical bleeding in human patients undergoing coronary artery surgery, [141] and decreased platelet aggregation in healthy dogs receiving clopidogrel,[142] has been demonstrated using this method. The technology requires special software and reagents, however, and is not available as widely as traditional TEG.

v. Use in Humans

TEG is used commonly in human medicine, and can provide rapid information about coagulation status.[99] Its most important applications are to monitor coagulation status in surgical patients, particularly liver transplant and cardiac patients, and guide transfusion choices.[99,100,103,143] During cardiac surgery, TEG is used to guide heparin therapy.[143] TEG is used to detect coagulopathies, as well as hyperfibrinolysis, in trauma patients.[100,143] An increased 30

day mortality rate was detected in patients with hypocoagulable thromboelastograms on admission to an intensive care unit.[144] It has also been used to detect hypercoagulability in pregnant women,[103] cancer patients[100] and those undergoing prostate[99,143] and other surgery.[103,145] Elevated G values (indicating hypercoagulability) predict thromboembolic events in surgical patients in the intensive care unit.[145] TEG is also used in pregnant women to detect preeclampsia, thrombocytopenia, and predict risk of repeated abortion.[99,100] TEG does have major limitations, including, the inability to predict postoperative bleeding,[103,145] a lack of standardization of sample types, activators, and storage time of blood[103,145], and an inability to monitor treatment with vitamin K antagonists[103] or detect bleeding risks with oral anti-platelet drugs.[100,103]

vi. Use in Dogs

Expanding interest in the use of TEG in dogs has developed only recently, despite a long history of use in human medicine. Dogs with varying diseases admitted to an ICU had hypercoagulable (11/27), normocoagulable (13/27) or hypocoagulable (3/27) thromboelastograms.[77] Dogs diagnosed with DIC by expert panel using traditional coagulation tests had hypercoagulable (44%), normocoagulable (34%) and hypocoagulable (22%) thromboelastograms based on the G value. Dogs with hypocoagulable TEG values had a higher case fatality rate than those with hypercoagulable values.[78] In dogs with various types of neoplasia, 50% of dogs with malignant neoplasia are hypercoagulable (based the G value) and 17% are hypocoagulable, and 31% of dogs with benign neoplasia are hypercoagulable.[125] Dogs with carcinoma are hypercoagulable based on TEG_{TG}, which represents thrombin generation, or total area under the TEG curve.[146] Puppies with parvovirus are also hypercoagulable, based on a higher MA than age-matched controls.[123] Hypercoagulability, based on CI, was documented in the majority of dogs with IMHA in two studies,[93,94] although in each many dogs had received

immunosuppressive corticosteroids at the time of sampling and the effect of hematocrit was not accounted for. One hundred percent mortality was present in dogs with a normal CI in both studies, and it was postulated that this may represent a "relative" hypocoagulability given the results in the other dogs.[93,94] TEG has higher positive and negative predictive values for identifying dogs with clinical bleeding compared to a conventional coagulation profile, based on categorizing the dogs as hypo-, hyper-, or normocoagulable using the G value.[108] Non-activated, but not tissue factor activated, TEG (using citrated whole blood) detected prolonged K time and TMRTG (time to maximum rate of thrombus generation) in dogs with Scott syndrome, a hereditary defect in which platelets do not have appropriate procoagulant activity and cannot support assembly of coagulation factors.[122]

TEG has also been used to evaluate the effects of anticoagulant and anti-platelet drugs in dogs. Standard anti-inflammatory doses of aspirin and meloxicam have no effect on TEG, while significantly decreased K-time, angle, and MA are seen with carprofen and significantly increased MA with deraxocib in dogs with osteoarthritis.[47] Potential causes of these findings include differences in COX selectivity between drugs, inter-individual differences in thromboelastographic response to non-steroidal anti-inflammatory drugs, and a lack of sensitivity of TEG in the detection of subtle platelet dysfunction.[47] Low and high dose clopidogrel in healthy dogs do not affect citrated non-activated whole blood thromboelastograms, although platelet inhibition was documented using TEG platelet mapping.[142] An *in-vitro* study of the low molecular weight heparin delteparin found significant and dose-dependent changes in R, K, angle, and MA with increasing concentrations of delteparin using TF activated TEG with heparinase cups, suggesting that this method may be useful in the monitoring of heparin therapy.[119] In the same study, kaolin activated TEG with heparinase cups

showed only significant increases in R-time with delteparin, which limits the use as a monitoring tool for therapy. However, the almost complete reversal of heparin's effects makes it possible to detect coagulation disturbances not related to the presence of heparin. The *in-vitro* nature and the supraphysiologic doses of delteparin used unfortunately limit this study's clinical utility.[119] In an *in-vivo* study of a commonly used dose of unfractionated heparin given subcutaneously to healthy dogs, TEG (citrated non-activated samples with standard cups) tracings showed marked changes peaking 3-5 hours after administration.[124] The R-time was so prolonged in some dogs that MA was not determined even though concurrent anti-Xa activity had not reached the human target range for most dogs. As well, aPTT was within the reference range for most dogs throughout the study. It was concluded that either TEG (without use of heparinase) is too sensitive to be a useful monitoring tool for unfractionated heparin therapy, or that therapeutic levels are lower in dogs than in humans.[124]

At this time, much of the difficulty of mainstream use of TEG in canine medicine lies in the variability of blood sample and activator types used in various studies. As well, the effects of fluids, platelet count, hematocrit, breed, and gender have not been thoroughly studied. Further research in these areas, as well as standardization of technique, is needed in veterinary medicine for TEG to be a widespread and clinically useful test.

vii. Effects of glucocorticoids and aspirin on TEG in dogs

Analysis of the use of TEG in dogs receiving glucocorticoids and aspirin is limited. Aspirin at a dose of 5 mg/kg orally every 12 hours results in no change in thromboelastograms in dogs with osteoarthritis.[47] Ultralow-dose aspirin (0.5 mg/kg/day orally) also has no effect on

TEG parameters in healthy dogs.[35] Healthy dogs treated with 1 and 4 mg/kg/day[34] and 2 mg/kg/day[35] of oral prednisone for two and one week(s), respectively, have increased K,[34] angle,[34] MA,[34,35] and G[35] values. After 6 days of 2 mg/kg/day prednisone, decreased clot lysis is also present.[35] The latter study also combined low dose aspirin with prednisone for one week, and found similar results to the prednisone only group. As mentioned previously, dogs with IMHA treated with glucocorticoids, with or without concurrent low dose aspirin, have hypercoagulable thromboelastograms.[93,94] However, hypercoagulabilty caused by the disease is essentially impossible to separate from that caused by glucocorticoids in these patients.

CHAPTER II: The effects of prednisone and prednisone plus ultralow-dose aspirin on coagulation parameters in healthy dogs.

Introduction

Hypercoagulability has been documented using thromboelastography (TEG) in healthy dogs given prednisone. [34,35] Additionally, administration of excess endogenous and exogenous glucocorticoids increases the risk of thromboembolic disease in humans.[15,16,19] This hypercoagulability may be important in diseases in dogs commonly treated with glucocorticoids, such as immune mediated hemolytic anemia (IMHA), which induces a hypercoagulable state[147] and predisposition to thromboembolic complications.[65,69] Ultralow-dose aspirin is commonly used in dogs with IMHA for its anti-platelet activity and putative thromboprophylaxis.[61] Recently, a retrospective study showed increased survival when aspirin was administered in conjunction with prednisone and azathioprine.[61]

Hypercoagulability has been documented with TEG in dogs with IMHA, although these results were confounded by treatment with glucocorticoids prior to sampling in many

cases.[94,147,148] In addition, the effect of hematocrit, a factor known to affect TEG, was not accounted for in these studies. The effects of glucocorticoids and aspirin on coagulation are difficult to separate from those of IMHA. One study of healthy dogs found that ultralow-dose aspirin, with or without prednisone, had no effect on TEG.[35]

Thromboelastography is being used frequently to investigate coagulation in veterinary medicine, and appears to be a more sensitive indicator of hypercoagulability than more traditional hemostatic tests.[77,78,149] Although tissue-factor activated TEG was recently validated in dogs,[107] and TEG (using various activators) has been used in multiple clinical studies,[77,78,94,108,125,147] intra-individual variation in TEG has not been thoroughly investigated in dogs. A study found no significant difference over three consecutive days for the TEG parameters R, K, angle, and MA in healthy dog using citrated re-calcified whole blood.[120] However, the actual methodology and results were not presented in the abstract.

The primary objective of this study was to determine the effects of prednisone and prednisone plus ultralow-dose aspirin on coagulation in healthy dogs. A secondary goal was to determine the intra-individual variation in TEG parameters in healthy dogs using citrated, re-calcified whole blood. We hypothesized that prednisone would cause hypercoagulablity in dogs, the addition of aspirin would have no effect, and that intra-individual variation in TEG parameters would be $\leq$ 10%.

Materials and Methods

The study was approved by the Institutional Animal Care and Use Committee of Virginia Tech. Fourteen healthy dogs (4 male Beagles and 10 mixed breed females) with a mean age of 1.9 years (range 1.25-2.5 years) and a mean body weight of 11.0 kg (range 7.9 - 13.1 kg) were

included in the study. Dogs were determined to be healthy based on results of physical examination, complete blood count, serum biochemistry profile, urinalysis, and zinc sulfate fecal flotation that were performed at the beginning of a two week period in which they were acclimated to the research housing. During the first week of this period, all dogs received fenbendazole (50 mg/kg PO q 24 hours x 3 consecutive days). Dogs were fed a maintenance commercial dog food twice daily, except on sample collection days when they were fasted for a minimum of 12 hours prior to sample collection.

After the 2 week acclimation period, dogs were randomly divided into two groups of seven. Using jugular venipuncture, two milliliters of blood was collected into a glass serum vacuum tube (subsequently discarded), followed by 2.7 mL into a 3.2 % sodium citrate vacuum tube for measurement of prothrombin time (PT), activated partial thromboplastin time (aPTT), d-dimer, fibrinogen, antithrombin activity (AT), and thrombin-antithrombin complexes (TAT). The tubes were carefully inverted five times to allow mixing of the blood and anticoagulant. Citrated samples were centrifuged at 1418 g for 10 minutes and the plasma was separated and collected immediately and frozen at -70 C until testing was performed at the Animal Health Diagnostic Center, Cornell University College of Veterinary Medicine. The PT, aPTT, and clottable (Clauss) fibrinogen were performed using an automated clot detection instrument,[a] commercial reagents,[b-d] and reaction conditions as previously described.[150] A pooled canine plasma (prepared from 20 healthy, adult dogs) was used as the fibrinogen assay standard. The fibrinogen content of the standard (330 mg/dL) was measured by a gravimetric method.[151] Antithrombin activity was measured in a functional assay configured to measure thrombin inhibition (anti-IIa assay) using a commercial chromogenic kit[e] and the manufacturer's automated analyzer.[a] Antithrombin activities of the test samples were reported as a percentage of the pooled canine plasma, which

had an assigned value of 100%. D-dimer concentration in ng/mL was measured using a quantitative, immunoturbidometric method as previously described,[152] using a commercial kit and the manufacturer's human D-dimer standards.[f] The thrombin-antithrombin complex concentration was measured using a commercial sandwich enzyme immunoassay.[g] The assay is configured with cross-reactive rabbit anti-human antibodies and a human TAT standard.[153,154] The TAT concentration of test plasmas was reported (after log-log transformation) as ug/L.[154]

Baseline TEG was performed twice on blood from each dog three days apart to determine intra-individual variation. Each sample was run in duplicate in a separate channel of the TEG analyzer[i] as previously described.[47] All phlebotomies and TEGs were performed by a single individual who was blinded to the treatment groups. Level I and II quality controls and an e-test were run daily on the TEG analyzer, per the manufacturer's recommendations.[h] Following sample collection as described above, citrated blood was allowed to sit at room temperature for 30 minutes and each tube was then inverted five times immediately prior to performing TEG. Briefly, 20 µL of $CaCl_2$ was placed into plain TEG cups pre-warmed to 37 C, and then 340 µL of citrated blood was added to the cup. TEG analyses were run for 120 minutes and the parameters R (R-time), K (K-time), angle, MA (maximum amplitude), Ly30 (percent lysis 30 minutes after MA), and Ly60 (percent lysis 60 minutes after MA) were measured and recorded as the mean of the duplicate samples for each dog.

After the baseline samples were obtained, both groups began receiving medications. The PA (prednisone plus aspirin) group received prednisone at a median dose of 2.2 mg/kg (range 2.0 – 2.5 mg/kg) PO q 24 and aspirin (compounded using pulverized aspirin tablets with methylcellulose 4000 cps to appropriate capsule sizes) at a median dose of 0.53 mg/kg (range 0.50-0.63 mg/kd) PO q24h. The PP (prednisone plus placebo) group received prednisone at a

median dose of 2.3 mg/kg (range 2.0-2.5 mg/kg) PO q 24 and an aspirin placebo capsule (containing methylcellulose 4000 cps) identical in appearance to the aspirin capsules. After 14 days of treatment, the following tests were repeated: CBC, biochemistry profile, TEG, PT, aPTT, d-dimer, fibrinogen, TAT, and AT as described above. The latter six tests were performed along with baseline samples.

Due to unexpectedly high intra-individual variation in some TEG parameters, intra-individual variation was determined in 10 healthy client owned dogs with a mean age of three years (range 1.5-4 years) and a mean body weight of 23 kg (range 9.5 – 65 kg). The dogs were determined to be healthy based on physical exam, CBC and biochemistry profile, no history of major health problems in the past three months, no history of any bleeding or hematologic disorder at any time, no general anesthesia in the two weeks prior to the study, and no health problems in the month following the study. The only medications allowed were flea/tick and heartworm preventatives. Phlebotomy and TEG analyses were performed (as described above) twice on each dog two days apart. Owner consent was obtained prior to sampling.

Statistical Methods

Using the 2 baseline measurements, the intra-individual variation for R, K, angle, MA, Ly30, and Ly60 for each dog (both experimental and client owned) was calculated using the formula standard deviation ÷ mean. Intra-individual variation was summarized as mean with a 95% confidence interval for each variable. Values of $\leq$ 10% were considered acceptable.

In the experimental dogs, for changes from baseline measurements that followed a normal distribution, post-treatment measurements were compared to mean baseline measurements within each group using a paired t-test, while a 2 sample t-test was applied to

compare the change from baseline between groups. For changes from baseline that were skewed, post-treatment measurements were compared to mean baseline measurements within each group using a Wilcoxon one sample test. A Wilcoxon 2 sample test was applied to compare the change from baseline between groups. Statistical significance was at $\alpha=0.05$. All analyses were performed using commercial software.[j]

Results

Within both the PP and PA groups, MA and fibrinogen significantly increased and Ly30 and AT significantly decreased from baseline (Table 1). For the PP group, Ly60 significantly decreased from baseline (Table 1). All other within group changes from baseline were not statistically significant (Table 1). For all parameters, the groups did not differ for change from baseline (Table 1).

The mean intra-individual variation in experimental and client-owned dog groups was $\leq$ 10% for angle and MA, and >10% for K, R, Ly30 and Ly60 (Tables 2 and 3). Day to day variation in the quality control samples over both study periods was also calculated. The results for R, K, angle, and MA for Level I were 11.8%, 0%, 0.4%, and 2.8% respectively, and for Level II were 5.4%, 16.4%, 1.9%, and 5.3% respectively, which is similar to those listed in the TEG manual precision calculations (Level I 7%, 0%, 0%, 3% respectively; Level II 10%, 14%, 7%, 5% respectively).[l] All TEG parameters for all quality control samples were within the manufacturer's reference range throughout the study.

Discussion

Our results are similar to two preliminary reports evaluating the effects of prednisone on thromboelastography in healthy dogs which identified increased MA using tissue factor activated TEG[34] and unspecified activation,[35] respectively. Increases in K and angle[34] as well as decreases in clot lysis[35] were also found after treatment with prednisone. The mechanism for hypercoagulability due to exogenous glucorticoids in dogs has not been fully evaluated. In dogs with naturally occurring hyperadrenocorticism, increases in some individual coagulation factors,[26,27] TAT,[26] and fibrinogen.[27,155] have been documented that could lead to hypercoagulability. In our study, fibrinogen concentration increased and AT activity decreased with prednisone administration. Hyperfibrinogenemia has been associated with a hypercoagulable state based on TEG in dogs in ICU with various disorders,[77] dogs in DIC,[78] dogs with a clinical suspicion of a bleeding disorder,[108] and dogs with carcinoma,[146] which supports hyperfibrinogenemia as a possible factor involved in the hypercoagulability in the present study. Plasma AT activity has varied among different studies of dogs with spontaneous hyperadrenocorticism, with some dogs having elevated[27] and some dogs having decreased[26] AT activity. In contrast, Cushing's syndrome in humans results in increased AT[15,156] that decreases after disease remission.[15] In the present study, it is unknown whether decreased AT contributed to, or was the result of, hypercoagulability and subsequent subclinical thrombosis.

In human transplant patients, chronic glucocorticoid administration leads to hypercoagulability, due in part to a hypofibrinolytic state caused by increased plasminogen activator inhibitor 1 (PAI-1) activity.[20,22 21] This effect is also observed after prednisolone administration for as short as 11 days in healthy humans. [23] Unlike in humans, increased PAI-1 has not been documented in dogs with spontaneous hyperadrenocorticism.[26] However, percent

fibrinolysis decreased after prednisone administration in the present study, which was expected based on these studies in humans as well as a study in dogs.[35] Although the high intra-individual variation in fibrinolytic parameters in our study makes it difficult to interpret the significance of this finding, given the findings of decreased clot lysis in another study of prednisone administration in healthy dogs,[35] further investigation into hypofibrinolysis as a cause for hypercoagulability with glucocortioid administration is warranted.

There was no difference between groups and between baseline and post-treatment values for TAT. Thrombin-antithrombin complexes indicate thrombin generation and are suggested to be a marker of thrombosis in humans.[157] There was a wide range of TAT concentrations in the dogs of the present study both before and during treatment that may have masked significant differences between and within the groups. Marked inter-individual variability was also found in healthy dogs in another study that found no difference in TAT between dogs with carcinoma and healthy controls.[146] It was hypothesized that the use of a human TAT assay rather than a canine specific assay may be a cause of their findings, as well as those in the present study. Variability in the sensitivity of individual dogs to glucocorticoids is another possibility for the wide range of TAT concentrations. Alternatively, dosage or duration of treatment with glucocorticoids in this study may have prevented significant differences given that dogs with spontaneous hyperadrenocorticism have increased TAT.[26]

The lack of effect of ultra-low dose aspirin combined with prednisone on TEG is consistent with a study in healthy dogs.[35] Given that higher dosages of aspirin (5 mg/kg BID) alone also had no effect on TEG, it may be that TEG is not a sufficiently sensitive indicator of platelet function to detect these changes.[47] Although it is also possible the dose of aspirin given was too low to have an effect on platelets,[51] a study using whole blood impedance aggregometry

documented impaired platelet aggregation in healthy dogs receiving 0.5 mg/kg/day of aspirin.[50] Another possible explanation is that the effects of prednisone may have negated the anti-platelet effects of aspirin. Platelet function was not independently measured and is one limitation of this study.

While the primary purpose of this study was to investigate the effects of prednisone and prednisone plus ultralow-dose aspirin on coagulation parameters in healthy dogs, some potentially clinically relevant shortcomings of TEG were revealed. The large intra-individual variation in R, K, Ly30 and Ly60 noted in the experimental study was further investigated via a separate study using healthy client owned dogs. Intra-individual variation was similar in these dogs and remained >10% for R, K, Ly30 and Ly60. Biological (intra-individual or within subject) variation has been reported for some coagulation tests in humans,[158] and values for PT and PTT are consistently less than 10%. One study determined day to day variation (intra-individual variation) in both kaolin and tissue factor activated TEG in healthy humans and found variability between 4 and 14 %, with the highest variability in R.[115] Intra-individual variation for several TEG parameters in dogs was determined in one study, with values for R, MA and angle between 10 and 20%. [159] However, the results cannot be directly compared to those of our study as TEG was performed on frozen plasma with the use of tissue factor activation and different methodology[160] was used to calculate intra-individual variation. The acceptable level of intra-individual variation (CV $\leq$10%) was chosen based on these studies and the authors' opinion that greater variation could lead to misinterpretation of results. Given that TEG must be performed on whole blood within a standard period of time after collection to avoid changes to parameters over time,[107,115] and that the use of plasma abrogates the advantages of TEG in evaluating the whole coagulation system, including cellular components, TEG analyses were performed over multiple

days. In the present study, the same operator (ALO) performed all TEGs in an identical manner using the same reagents throughout the entire study in an attempt to minimize variability in conditions between days. Day to day variability in the quality control samples over both study periods was also calculated, and these results were similar to those listed in the TEG manual precision calculations.[l] The variability in quality control samples throughout the study illustrates the inherent variability in the analyzer, reagents, and operator, which explains some of the variability in the results of the present study. The similarity in quality control variation between the TEG manual and the present study suggests that both analyzer and operator error are unlikely to be causes of the marked intra-individual variation in some TEG parameters. Studies on intra-individual variation in TEG using whole blood in dogs is limited to a single abstract[120] that found no significant differences existed over 3 days among any parameter using both citrated re-calcified whole blood with or without kaolin activation. However, details of the methods and results were not stated. Coefficient of variation of duplicated samples (two channels of the same TEG analyzer) have been evaluated in dogs using different activators,[107,126] although neither study evaluated fibrinolysis parameters and this is not equivalent to intra-individual variation that was the focus of this study.

Other factors that can affect TEG results include timing after sample collection,[107] sampling method,[128] operator variability,[161] decreased hematocrit,[95,96] platelet count [101,135,136] and function,[100] sample hemolysis,[127] and breed.[121,137] We attempted to minimize these variables with careful jugular venipuncture, having a single operator perform all TEG analysis at a uniform time after sample collection, by ensuring that all hematocrits were normal both pre- and post-treatment, and by using predominantly mixed breed dogs. Due to the high incidence of platelet clumping in the present study, accurate platelet counts could not be determined for many dogs

and therefore were not included in the statistical analysis. However, all platelet counts were estimated to be in the normal range both at baseline and post treatment.

That the variability in R was high was not entirely unexpected, as this parameter is most influenced by operator factors via variability in starting data collection after blood is placed in the TEG cup.[l] In a study on rotational thromboelastometry (ROTEM) comparing the use of re-calcification alone and with different activators, coagulation time (CT; equivalent to R) was significantly different between all activators, and was most prolonged using re-calcification as the only activator.[162] In addition, the use of re-calcified whole blood alone was most affected by sample handling compared to use of re-calcified whole blood with other activators. Significant differences in results between operators using citrated re-calcified whole blood and the same TEG analyzer for the parameters R, K and angle have been documented in a study on horses, which was minimized when tissue-factor activation was used.[161] The use of re-calcification only without other activators may have contributed to the high variability of our results. A study using TEG in dogs similar to the aforementioned ROTEM study[162]is therefore imperative in order to properly interpret the results of clinical studies using TEG as well as standardize TEG methodology for future studies. The human medical field is facing similar challenges with both TEG and ROTEM, and a TEG-ROTEM Working Group has been established in an attempt to standardize methodology and evaluate reproducibility and consistency of results.[163]

The extreme variation in the fibrinolysis parameters was unexpected. Some dogs had markedly increased Ly30 and Ly60, which in some cases was not present on repeat sampling. There is a notable lack of reported Ly30 and Ly60 values in veterinary studies, even though TEG analysis was reported to have run for 90-120 minutes (the time needed to generate lysis parameter(s)) in several.[77,78,107,108,125] While outlying percent lysis values could have been

excluded from our analysis, we felt it was most appropriate to include all values given the lack of information on fibrinolysis in the veterinary literature. A single veterinary study comparing TEG between greyhounds and non-greyhounds published values for Ly60, and although variability was not studied, the range of values was large (0-19.2% for greyhounds and 0-8.6% for non-greyhounds).[121] In healthy cats using citrated re-calcified TEG reported Ly60 values are 7.86±10.21%, and some cats have a markedly increased fibrinolytic pattern while others have minimal fibrinolysis.[164] It was theorized that platelet retraction led to this result. Further studies are needed to determine if this fibrinolytic pattern is common and if fibrinolytic parameters are reliable in dogs and cats.

In conclusion, the hypercoagulability caused by prednisone found in this study is consistent with other studies, and further investigations to confirm the mechanism of this hypercoagulability and the significance to clinical patients are warranted. Ultralow-dose aspirin had no effect on TEG using citrated, re-calcified whole blood and further studies using more sensitive determinants of platelet function should be performed. Additionally, this study emphasizes the need for a thorough investigation of pre-analytical variables for TEG, including the use of different activators to determine which demonstrates the least intra-individual variability, as well as further investigation into the validity of fibrinolysis parameters in veterinary medicine.

CHAPTER III: CONCLUSIONS

This study demonstrates that prednisone, given for two weeks at a standard immunosuppressive dosage, causes hypercoagulability in healthy dogs and that, based on thromboelastographic analysis, ultra-low dose aspirin does not diminish this effect. These results

are similar to those of other studies in healthy dogs. The clinical significance of hypercoagulability in clinical patients treated with prednisone is unknown and deserves further investigation. Additionally, the true effects of ultralow-dose aspirin to counteract this hypercoagulability remain unknown. Further studies are warranted, including a similar evaluation in healthy dogs using more sensitive indicators of platelet function (PFA, platelet mapping TEG, whole blood aggregometry) and higher dosages of aspirin (1 mg/kg/day). Measurement of PAI-I after prednisone administration is also indicated to further investigate hypofibrinolysis as a cause of hyperocoagulability. Prospective, placebo controlled studies in clinical patients with IMHA are warranted to determine if dogs with hypercoagulable TEG values are at increased risk of thromboembolism, and also to determine if ultralow-dose aspirin is effective in prevention.

A secondary objective of the study was to determine intra-individual variation in TEG, and the results of this investigation demonstrated high intra-individual variability in some TEG parameters. This raises concern regarding clinical utility of TEG with the methods used in this study. Further investigation into TEG variability is warranted before TEG can be used with confidence in a clinical setting.

FOOTNOTES

a. STA Compact, Diagnostica Stago, Parsippany, NJ

b. Dade Actin FS, Dade Behring, Newark, DE

c. Thromboplastin LI, Helena Diagnostics, Beaumont, TX

d. Fibrinogen, Diagnostica Stago, Parsippany, NJ

e. Stachrom ATIII, Diagnostica Stago, Parsippany, NJ

f. HemosIL, D-dimer Calibrator, Instrumentation Laboratory, Bedford, MA

g. Enzygnost TAT Micro, Dade Behring, Marburg, Germany

h. Haemoscope Corporation. TEG 5000 User Manual. Niles, IL: Haemoscope Corporation, 1999-2007.

i. TEG 5000 Hemostasis Analyzer, Haemoscope Corporation, Niles, IL, USA

j. SAS version 9.2 (Cary, NC, USA).

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TABLES

Table 1. Change from baseline within and between groups for coagulation parameters, TEG, and hematocrit

Measurement	Group	Baseline (mean ±SD)	Change from baseline (mean, 95% CI)	P-value for change from baseline	Difference between groups	P-value for Difference between groups
R (minutes)	PP	7.1 ± 0.9	-1.1 (-3.3 – 1.1)	0.27	-3.0 (-6.2 – 0.1)	0.058
	PA	6.0 ± 1.0	1.9 (-0.8 – 4.7)	0.13		
K (minutes)	PP	2.8 ± 0.5	-0.5 (-1.3 – 0.3)	0.20	-0.3 (-1.7 – 1.1)	0.63
	PA	2.8 ± 1.0	-0.2 (-1.5 – 1.2)	0.77		
A (degrees)	PP	54.2 ± 4.5	5.5 (-2.7 – 13.7)	0.152	4.6 (-7.8 – 16.9)	0.438
	PA	55.6 ± 7.9	0.9 (-10.3 – 12.1)	0.845		
MA (mm)	PP	51.3 ± 4.4	8.47 (4.5 – 12.4)	**0.00190**	-2.5 (-8.2 – 3.2)	0.357
	PA	51.1 ± 8.3	10.98 (5.9 – 16.0)	**0.00180**		
Ly30 (%)	PP	3.2 (0.0 – 11.0)*	-2.8 (-11.0 – 0.0) *	**0.031**	-1.9 (N/A)	0.90

	PA	1.7 (0.1 – 9.3) *	-0.9 (-9.3 – -0.1) *	**0.0156**		
Ly60 (%)	PP	10.6 ± 9.4	-9.4 (-17.5 – -1.3)	**0.030**	-3.9 (-12.6 – 4.9)	0.355
	PA	7.2 ± 5.6	-5.5 (-11.0 – 0.0)	0.050		
Hct (%)	PP	49.4 ± 4.6	-1.7 (-6.3 – 3.0)	0.417	2.4 (-3.8 – 8.7)	0.416
	PA	49.2 ± 5.9	-4.1 (-9.4 – 1.2)	0.108		
aPTT (seconds)	PP	12.4 ± 1.1	-0.2 (-0.9 – 0.5)	0.496	-1.5 (-3.2 – 0.2)	0.0711
	PA	12.4 ± 1.0	1.3 (-0.4 – 3.1)	0.115		
PT (seconds)	PP	13.3 (12.1 – 14.6) *	0.0 (-0.7 – 0.5) *	0.563	0 (N/A)	0.617
	PA	13.8 (3.7 – 14.4) *	0.0 (-0.4 – 9.4) *	0.9063		
Fibrinogen (mg/dL)	PP	349.0 ± 57.1	107.7 (20.2 – 195.2)	**0.0236**	-32.9 (-116.2 – 50.5)	0.407
	PA	342.2 ± 78.7	140.6 (107.2 – 173.9)	**<0.000100**		
AT (%)	PP	113.7 ± 11.7	-17.7 (-31.8 – -3.6)	**0.022**	-0.7 (-16.3 – 14.8)	0.922
	PA	115.9 ± 14.4	-17.0 (-27.3 – -6.7)	**0.0067**		
D-Dimer	PP	143.6 ±	-71 (-209.2 – 65.5)	0.25	16.4 (-	0.84

(ng/mL)		142.5			159.0 – 191)	
	PA	162.4 ± 154.4	-88.3 (-229.4 – 52.9)	0.18		
TAT (ug/L)	PP	7 (3 – 193) *	-1 (-190 – 42) *	1.0	6 (N/A)	0.1
	PA	10 (4 – 91) *	-7 (-78 – -1) *	-0.02		

* Values are medians (range)

PP=prednisone plus placebo group; PA= prednisone plus aspirin group; R=R-time; K=K-time; A=angle/α; MA=maximum amplitude; Ly30=percent lysis 30 minutes after MA; Ly60= percent lysis 60 minutes after MA; Hct=hematocrit; aPTT=activated partial thromboplastin time; PT=prothrombin time; AT=antithrombin; TAT=thrombin-antithrombin complexes

Table 2. Intra-individual coefficients of variation for TEG parameters

(Experimental dogs, n=14)

TEG parameter	Mean CV (%)	Standard deviation	95 % Confidence Interval
R (minutes)	28.3	21.3	(16.0-40.6)
K (minutes)	20.8	16.9	(11.0-30.5)
Angle (degrees)	10.0	7.8	(5.5-14.5)
MA (mm)	5.1	4.4	(2.5-7.6)
LY30 (%)	75.3	58.8	(41.3-109.2)
LY60 (%)	58.5	41.3	(34.6-82.3)

R=R-time; K=K-time; A=angle/α; MA=maximum amplitude; Ly30=percent lysis 30 minutes after MA; Ly60= percent lysis 60 minutes after MA; CV=coefficient of variation

Table 3. Intra-individual coefficients of variation for TEG parameters

(Client-owned dogs, n=10)

TEG parameter	Mean CV (%)	Standard deviation	95 % Confidence Interval
R (minutes)	16.2	13.6	(6.46-25.94)
K (minutes)	10.8	6.8	(5.95-15.65)
Angle (degrees)	4.5	2.9	(2.5-6.5)
MA (mm)	3.7	2.1	(2.23-5.17)
LY30 (%)	66.0	53.3	(27.9-104.1)
LY60 (%)	38.9	29.5	(17.8-60)

R=R-time; K=K-time; A=angle/α; MA=maximum amplitude; Ly30=percent lysis 30 minutes after MA; Ly60= percent lysis 60 minutes after MA; CV=coefficient of variation