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Anticoagulant profile of subcutaneous enoxaparin in healthy dogs

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Anticoagulant profile of subcutaneous enoxaparin in healthy dogs

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Enoxaparin, a low-molecular-weight heparin, is commonly used as an anticoagulant in dogs, and is currently dosed at 0.8mg/kg every 6 hours. With an increase in individual enoxaparin doses, less frequent dosing may be possible, thereby reducing owner inconvenience and expense.

The three phases of this study investigated the appropriate dose (Phase one- 0.8mg/kg, SQ once; Phase two- 2mg/kg, SQ once; Phase three- 1.3 mg/kg, SQ q8h for 7 total doses) and dosing interval needed for maximum effectiveness of enoxaparin. A Sonoclot® analyzer and factor Xa activity were used to assess level of anticoagulation in six healthy dogs.

Anticoagulation was inconsistent at the 0.8mg/kg dose, while the 2mg/kg dose showed a high level of anticoagulation, and the 1.3mg/kg dose provided more reliable anticoagulation than the other dosages and dosing intervals. Small sample size and the use of same-breed healthy dogs potentially affected the strength of the results.

DEDICATION

I would like to dedicate this research to my loved ones who gave me unwavering support and encouragement when I needed it the most. During the most difficult times, they are the only reasons I kept going and pursued the life and career I knew would bring me the most fulfillment and joy. Everything I have accomplished is because of the incomparable support system I have in the people that surround me, and for them I am forever thankful.

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LITERATURE REVIEW

Background

Thromboembolism is a significant complication of many common diseases in veterinary

medicine, and can be a devastating complication of otherwise treatable conditions such as

immune-mediated hemolytic anemia (IMHA) and protein-losing nephropathy.[1] Despite our best

efforts, mortality rates for canine IMHA remain high, ranging from 50 to 70%.[2] The most

common cause of death in dogs with IMHA is thromboembolic disease, particularly pulmonary

thromboembolism (PTE). The mechanisms that cause thrombus formation in dogs are not fully

understood but are thought to include disruptions in platelet function (primary hemostasis) and

the clotting cascade (secondary hemostasis).

Unfortunately, predicting which patients will develop a life-threatening thromboembolism is

extremely difficult, and prophylactic medications are routinely administered to inhibit either

platelet function or the clotting cascade, or both, in an effort to prevent thrombus formation.

With the addition of anti-coagulant[3] or anti-platelet therapy[4] for the treatment of canine IMHA,

survival rates have improved, but patients continue to develop fatal thromboembolic

complications[3,4], signifying a need for improved prophylactic anticoagulant therapy.

Additionally, heparins, which are the preventative medications that are currently used most often

to inhibit the clotting cascade, can only be given by subcutaneous (SC) or intravenous injection,

typically at relatively short dosing intervals, and can be cost prohibitive for long term

administration. Therefore, these life-saving medications are commonly only administered in hospital or for short periods of time, potentially increasing the risk of developing a fatal thromboembolism once therapy is tapered or discontinued. Ideally, a dosing protocol requiring fewer injections would decrease drug costs and improve the likelihood of maintaining long-term therapy.

Hemostasis, Coagulation, and Thrombus Formation

The function of the hemostatic system is to create a response to a vascular injury, but to not impede continuous blood flow through intact vasculature or hinder tissue perfusion.[5] Hemostasis is divided into three components: primary hemostasis, secondary hemostasis, and fibrinolysis. Primary hemostasis is the response of the body to injuries in blood vessel walls by platelets. In this initial phase, once damage or injury to the vascular endothelium has occurred, vasoconstriction of the vessels in the region of the injury decreases the blood flow to the affected area. The exposed endothelial cells release endothelin and tissue factor, and the platelets release thromboxane. This step initiates the process of platelet adhesion that causes the platelets to bind to the exposed subendothelial matrix using the receptors that bind collagen and von Willebrand factor (vWF). vWF binds to the exposed endothelium and then binds to the glycoprotein-1b on the membrane. This glycoprotein-1b, vWF complex is the major platelet receptor that is capable of binding vWF, collagen, and thrombospondin-1. These different substances are what circulating platelets are attracted to, as well as coagulation proteins and fibrinolytic substances. The receptors on the surface of the platelet plug interact with circulating coagulation factors that will form the fibrin required to strengthen the plug. The most attractive coagulation factors to the platelet plug are factor XI-kininogen complex, factor XIa, factor Xa-factor Va complex, thrombin, and fibrinogen.[6] The platelets that are attracted to the site of injury decelerate along

the endothelium, allowing for additional receptor bindings of collagen to glycoprotein receptor IV and glycoprotein receptor 1a and IIa and the binding of vWF to the glycoprotein IIb-IIIa complex.[5] These attractive substances lead to the formation of the primary platelet plug via adhesion of the platelets to the subendothelial structures. After this adherence to the site of the damage has occurred, the platelet undergoes a change in its structure which leads to the attraction of additional platelets to the injured region. The vasoconstriction and the platelet plug are what provide the temporary seal in the blood vessels at the injury. Depending on the severity of the damage at the vessel or if there is high hydrostatic pressure in the vessels at that site, a more secure plug involving fibrin may be necessary, which leads to the need for secondary hemostasis.[5]

Secondary hemostasis is the process of forming complexes of fibrin by coagulation proteins on the surface of the activated and aggregated platelet plug. The receptors on the surface of the platelet plug interact with circulating coagulation factors that will form the fibrin required to strengthen the plug. The most attractive coagulation factors to the platelet plug are factor XI-kininogen complex, factor XIa, factor Xa-factor Va complex, thrombin, and fibrinogen. [6] The binding of the plug to these coagulation factors leads to the main event in the coagulation of blood, when fibrinogen is converted into fibrin under the influence of thrombin. [7] The coagulation proteins that affect this process are nearly all produced by the liver, other than factor VIII, which is produced by the endothelial lining of the vessels. These coagulation proteins are classified into three categories: the fibrinogen family, (factors V, VIII, XIII), the vitamin K-dependent family/prothrombin complex, (factors II, VII, IX, and X, and C and S proteins), and the contact group, (factors XI and XII, kininogen, and prekallikrein). [5] To begin transformation of fibrinogen to fibrin, the activation of the coagulation cascade has to occur.

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The coagulation cascade is the sequence of events that leads to the coagulation of blood and the formation of a stable fibrin clot.[5,8] The three pathways needed for coagulation to proceed are separated into intrinsic, extrinsic and common. They are divided based on the method of activation with the intrinsic pathway involving factors only found in the blood, and the extrinsic pathway requiring tissue factor.[8]

The extrinsic pathway is stimulated by the release of tissue factor from endothelial cells, leading to the interaction of factor VII and tissue factor. This complex directly activates factor X in the common pathway, and factor IX in the intrinsic pathway. Factor VII and the tissue factor complex also activate factor X. From this step forward, the common pathway takes place. [8]

The intrinsic pathway is activated by the alteration of endothelial surfaces leading to the exposure of collagen that initiates the binding of factor XII to prekallikrein, changing it to kallikrein, making plasma kallikrein that is involved in many steps of coagulation and fibrinolytic systems. [5] The binding of kallikrein to factor XII produces the complex kallikrein-XIIa, which in turn causes classic signs of inflammation. The plasma kallikrein binds factor XI, activating factor Xia, which along with calcium and factor VIII converts factor X to its activated molecule, leading to the start of the common pathway.[5]

In the common pathway, factor Xa, calcium, platelet phospholipid, and factor V convert factor II, also called prothrombin, to thrombin. Thrombin has many functions in the coagulation cascade, including amplifying effects on factor XIII to convert it to factor XIIIa and in effect stabilize the fibrin clot that has formed. This fibrin is stabilized by factor XIII due to cross-linking of the fibrin. Factor XIII is activated by thrombin, in the presence of calcium, in order to stabilize the fibrin, while at the same time further activation of factors V, VIII, and XIII and platelets are all affected by the presence of thrombin. [5] Although thrombin plays many important

roles in the coagulation cascade, the amount of thrombin generated by the activation of factor Xa is not in excess and is at risk of being easily terminated by the tissue factor (TF) pathway inhibitor. Because the amount of thrombin generated is not large, there are numerous positive feedback loops that exist that bind thrombin with platelets. The thrombin that is generated in the initial phase of coagulation further activates factor V and VIII, which serves as a cofactor in the prothrombinase complex and accelerates the activation of factor II (prothrombin) by factor Xa and by factor IXa respectively, serving as an amplifying step and increasing the amount of thrombin in circulation.[9]

The last step in the process of hemostasis is fibrinolysis. This step is essential in the removal of the clot formed by the activation of the coagulation pathway. The main modulator of fibrinolysis is plasmin. It breaks down the fibrin at specific locations to dissolute the clot. Plasmin is produced initially from the response to the presence of thrombin and venous occlusion. [8] The actions of plasmin directly affect fibrinogen as well as fibrin. And once the plasmin has addressed the fibrinogen or fibrin, it is released back into circulation. [5]

There are several pathologic processes that can overwhelm the regulatory mechanisms of hemostasis, such as liver damage or failure, vitamin K deficiencies, warfarin or other anticoagulant rodenticide toxicity, hemophilia and other hereditary coagulation disorders, and the loss of antithrombin 3 through the kidney in the presence of kidney disease. Hemostatic abnormalities can also include increased generation of prothrombotic elements due to inflammatory pathways, direct production of tissue factor, or other prothrombotic substances, activating the circulating tissue factor and platelets.[10] Decreased antithrombin, tissue factor inhibitor, or endothelial dysfunction can all lead to the inhibition of anticoagulation mechanisms. Alteration of thrombolysis through weakening of the process of fibrinolysis can also occur

leading to thrombosis.[10] The coagulation cascade is regulated at several levels by different anticoagulant pathways, however, the loss of a single anticoagulant pathway can lead to thrombosis formation. This is potentially due to different tissues each utilizing distinct anticoagulant pathways to regulate clotting. When a clot forms in a blood vessel, it is removed by proteolytic digestion of fibrin by plasmin, and plasmin is regulated by plasminogen activators and inhibitors, especially plasminogen activator inhibitor 1 (PAI-1). This is why elevated levels of PAI-1 are associated with thrombosis.[10]

Thrombosis is the formation of a clot within a blood vessel that can cause a reduction in blood flow or an infarct of the surrounding tissues supplied by that vessel. In certain disease processes, pathologic thrombosis can occur, whether due to excess clot formation, insufficient fibrinolysis, or both scenarios. There are many types of thrombus, but they can occur in arteries or veins.[11] The clot formed contains a mixture of platelets and fibrin and sometimes red blood cells. However, the makeup of arterial and venous thrombi are different. Arterial clots are formed under high shear stress. In people, this occurs typically after the rupture of an atherosclerotic plaque or other damage to the blood vessel wall.[10] Arterial clots tend to be platelet-rich and are generally treated with antiplatelet drugs. In contrast, venous thrombi form under lower shear stress on the surface of a largely intact endothelium.[10] Venous thrombi are fibrin-rich and tend to be more frequently treated with anticoagulant drugs.[11,12] Reduction in blood flow may cause the development of venous thrombosis, as this allows the accumulation of the procoagulant protease thrombin, that allows the clot to overcome the anticoagulant pathway to induce thrombus formation. [11] The previously mentioned intrinsic pathway potentially contributes to the formation of arterial thrombosis, with one study describing the activity of factor XII playing a role in thrombus formation. [13] Aortic thrombosis has been reported in both

dogs and cats, with the main pathology of the clot being due to parts of the thrombus breaking off, to form an embolus, leading to obstructions to blood flow to distal limbs.[14]

Anticoagulation Therapy and Benefits

Thrombotic disorders in dogs are well-recognized to be associated with significant morbidity and mortality, and it is vital that there are multiple treatment options available to prevent and treat thromboses. In human medicine, antithrombotic therapies for thrombosis are well-defined. In veterinary medicine, the approach to prevention and treatment of thrombotic disorder is focused on prophylactic antiplatelet agents that are designed to inhibit platelet function in primary hemostasis, and anticoagulant agents, that inhibit the activity of clotting factors in secondary hemostasis.[15,16] The most common antiplatelet drugs utilized are asprin and clopidogrel, while the range of available anticoagulants is more varied, and includes unfractionated heparins, low-molecular weight heparins (enoxaparin, dalteparin), and more recently direct Xa inhibitors (rivaroxaban).[15]

Antiplatelet Therapy

Aspirin therapy has been extensively studied for its effectiveness as an antiplatelet agent in dogs for thrombosis. Variations in dosing and timing of the dosing of aspirin play a role in its ability to prevent and treat thrombi, with its best results shown when it was started at least several days prior to thrombus formation.[17] The most evidence-based support for the use of oral aspirin in dogs at risk is for prevention of arterial thromboembolism. The reported dose range varies greatly, but low and medium oral doses of 0.5-1mg/kg once daily, appears to maintain a safe use of the drug with some prevention for clot formation. Aspirin is not recommended as a first line of defense for use against suspected predisposition or concurrent affliction in dogs for

venous thromboembolism.[17] Specifically, IMHA patients are typically at higher risk of forming

venous thrombi that are thought to be less dependent on platelet number or function.[16] Therefore,

for these patients, anticoagulant therapy is recommended, but if it is not available or owner funds

are limited, antiplatelet therapy is an appropriate option.

Clopidogrel therapy is another option for antiplatelet therapy in dogs at risk of

thromboembolism. Clopidogrel is recommended currently for the prevention of arterial

thromboembolism in dogs, with an oral loading dose utilized, following by regular oral dosing

after that of 1-4 mg/kg once daily. Like aspirin, clopidogrel is not currently recommended for a

first line therapy by itself for dogs at risk for venous thromboembolism. In dogs at risk for

arterial thromboembolism, clopidogrel has shown to be effective against provoked arterial

thrombosis, and to have significant platelet inhibitory effects. [17] Additionally, due to availability,

lack of need for monitoring, ease of administration and minimal cost to owners, clopidogrel has

become a standard therapy in combination with anticoagulation therapy in dogs with IMHA.[18]

Direct-Xa Inhibitor Therapy

Direct Xa inhibitors such as rivaroxaban have been gaining more widespread use as more

research is done on their overall effectiveness. It is thought, based on current research, that direct

Xa inhibitors could have equivalent efficacy to low-molecular weight heparins for the prevention

of arterial and venous thrombosis in dogs.[15]Rivaroxaban is a newly developed oral direct factor

Xa anticoagulant that is used to prevent thrombosis in human patients. Direct Xa inhibitors

anticoagulants have wide safety margins, and this can make their use a preferred choice for the

patient compared earlier anticoagulants, particularly as they do not require routine coagulation

monitoring.[19] Rivaroxaban functions by directly inhibiting activated factor Xa and therefore

prothrombin activity. It does not require a cofactor, and directly inhibits platelet aggregation

induced by thrombin. Assays that evaluate the activity of factor Xa can be used to evaluate plasma rivaroxaban concentration indirectly. Currently extrapolated dosages for rivaroxaban in dogs are still being optimized, but one study has evaluated a dose of 0.5-1mg/kg orally once daily, with results indicating minimal adverse effects, however more research is required to identify this type of drug's overall effectiveness in clinical patients.[20]

*** Heparin Therapy ***

Fractionated and unfractionated heparins (UFH) both inhibit coagulation by accelerating the formation of irreversible complexes between the anticoagulant protein antithrombin (AT) and coagulation factors IIa (thrombin) and Xa as well as several other factors involved in the coagulation cascade.[21] Animal studies have shown that, although equipotent dose regimens of enoxaparin and unfractionated heparin produce very comparable anti-Xa activity[22–24], there is a reduced risk of hemorrhage in patients receiving enoxaparin therapy compared with patients receiving UFH.[25,26] Unfortunately, unlike UFHs, low-molecular-weight heparins (LMWHs) can be cost prohibitive when administered at the current dosages, especially for long term therapy.

Unfractionated heparin in both the human and veterinary patients is complicated by the need for frequent administration (multiple subcutaneous injections daily or an intravenous constant rate infusion) with careful and regular monitoring of clotting times or Xa inhibition to avoid either under-treatment or bleeding complications. On the other hand, LMWHs have more consistent pharmacokinetic properties, more predictable bioavailability, and do not require routine monitoring to ensure appropriate anticoagulation.

Enoxaparin (Lovenox®) is one of several fractionated or LMWHs that were developed as an alternative to unfractionated heparin (UFH), the most commonly used form of heparin in both human and veterinary medicine. Unfractionated heparin has long been considered standard

therapy in the prevention of venous and arterial thromboembolism in humans, and is widely used prophylactically in veterinary medicine to guard against the development of PTE and disseminated intravascular coagulation secondary to diseases that may result in hypercoagulable states.[22]

Dalteparin (Fragmin®) is another treatment option for fractionated or LMWHs to use in dogs at risk of hypercoagulation. It was evaluated utilizing viscoelastometry in a study in healthy dogs, and it appeared to achieve adequate monitoring and anticoagulant activity in healthy dogs.[27] Minor bleeding has been noted with dalteparin use and, even with recent publications, there is little data to help determine an appropriate dose in dogs. Additionally, the overall cost and availability of dalteparin compared to other LMWHs, couple with the lack of evidence of an effective treatment plan, makes it less likely to be a first line choice of a LMWH for at risk dogs.[17]

Mechanism of Action of Enoxaparin

Enoxaparin is a low molecular weight heparin which has antithrombotic properties. By binding to and accelerating antithrombin, LMWHs enhance the inhibition of factor Xa and thrombin. The potential advantage to utilizing LMWHs over the UFHs is that they preferentially inhibit factor Xa and only have a minor impact on thrombin and activated partial thromboplastin clotting times (aPTT).[28]

Pharmacokinetics of Enoxaparin

Pharmacokinetic Modeling

Pharmacokinetics is a tool used to describe the movement of drugs through the body, and has been extensively utilized to calculate safe and effective drug dosages for many animal

species. Optimally, a complete pharmacokinetic profile is determined from an intravenous study in the target species.[29] Clearance, volume of distribution, and elimination half-life are the three most frequently extrapolated pharmacokinetic parameters. The use of pharmacokinetics in veterinary medicine is important for drug dosage selection, and despite the potential for error when extrapolating from human dosages, the use of scaling to the different animal species is commonly done. This is different than the term pharmacodynamics, which refers to the body's biological response to a drug. Pharmacokinetic studies are the most commonly conducted studies, and are performed through many types of modeling techniques, while pharmacodynamics is the actual data seen in the body in response to the drug. Pharmacodynamic studies would arguably be the preferred source of information about a certain drug and its effect on the body, however, such studies can be difficult to perform, which leads to the more frequent use of pharmacokinetics modeling in research settings.[29]

Absorption

Pharmacokinetic trials were conducted using the 100mg/ml formulation of enoxaparin in humans, however, it is unclear if these studies can be directly related to its antithrombotic actions in dogs. [28] After subcutaneous administration, enoxaparin has a maximum anti-factor Xa and antithrombin activity at 3 to 5 hours. The mean peak anti-factor Xa activity was 0.16IU/ml and 0.38 IU/ml after 20mg and 40 mg of enoxaparin were injected subcutaneously, respectively. The mean peak anti-factor Xa activity was 1.1 IU/ml at a steady state in human patients receiving 1mg/kg SC q12hrs. The mean absolute bioavailability of enoxaparin after 1.5 mg/kg SC, based on the anti-factor Xa assay, is approximately 100% in healthy humans.[30] In dogs, after SC administration, enoxaparin has a shorter duration of inhibitory activity on factor Xa than in humans, and likely needs to be dosed more frequently than in humans.[28] In healthy greyhounds it

was shown that enoxaparin at 0.8mg/kg SQ q6h was required to reach an effective and consistently inhibited factor Xa activity.[21,28] However, a subsequent study of beagles showed a transient inhibition of factor Xa activity in only 3 or 8 dogs, and suggested that the dose of enoxaparin was not uniform across different breeds of dog.[28,31]

Distribution

The volume of distribution of anti-factor Xa activity in people is about 4.3L. [30]

Metabolism

Enoxaparin is primarily metabolized in the liver by desulfation and depolymerization to lower molecular weight species with a reduced biologic potency. Renal clearance of the active fragments of enoxaparin represents about 10% of the administered dose, with the total renal excretion of active and non-active fragments being 40% of those.[30]

Excretion

Following intravenous dosing, the total body clearance of enoxaparin is 26 mL/min in humans. After IV dosing of enoxaparin, there was 8-20% of anti-factor Xa activity recovered in urine in 24 hours. Elimination half-life based on anti-factor Xa activity was 4.5 hours after a single SC dose to about 7 hours after repeated dosing. There was detected anti-factor Xa activity persisting in the plasma of humans for approximately 12 hours following a 40mg SC once a day dose. Additionally, following SC dosing in humans, the apparent clearance of enoxaparin is approximately 15ml/min.[30]

Use of Enoxaparin in Dogs

Enoxaparin has been extensively studied in dogs, both in dogs at risk of hypercoagulation and in healthy dogs, to attempt to fully understand its overall safety and effectiveness.

Enoxaparin was initially studied in humans to determine its use in comparison with UFH for preventing venous thromboembolism in acutely ill medical inpatients. In one such study, either enoxaparin or an UFH was administered, and afterwards the incidence of deep-vein thrombosis and pulmonary embolism were compared, and it was assessed that the human patients receiving enoxaparin had a 74% lower risk of venous thromboembolism than those treated with UFH.[32] As veterinary medicine often does, it frequently extrapolates what is already known in human medicine to determine its translational effectiveness in animals. An enoxaparin dose of 1mg/kg subcutaneously every 12 hours used in humans was utilized in a pharmacodynamic study of healthy cats, for example, in which the cats were given this dose for several doses, and then their caudal vena cava was surgically ligated. The vena cava was then injected with fibrinogen to stimulate the formation of a thrombus. The thrombus was then removed and studied, and it was shown that the enoxaparin administered to the cats at this dose was effective in decreasing clot formation. However, with this study, one major pitfall was that the patients were part of terminal research where they did not survive the procedure performed.[33]

In a research study in dogs, enoxaparin was administered to healthy greyhounds that were part of a research population. The goal of this study was to determine the effective dose of enoxaparin and to dtermin if the anti-factor Xa assay that served as a gold standard for monitoring coagulation in humans could also be used in dogs on enoxaparin. Through pharmacokinetic modeling and the use of varied doses, this study established an optimal subcutaneous dose of enoxaparin of 0.8mg/kg given every 6 hours. While this was considered safe for these healthy dogs in the study, with minimal side effects of unintended bleeding, there remained a concern that this dosing regimen could be a treatment limiting factor with owners due

to cost and frequency of administration.[21] Because of this, additional research has continued regarding optimal dosing of enoxaparin.

Following the greyhound study, a comparable study was repeated in a group of healthy research beagles, given at the same dose as published. However, in this study, it was determined that the beagle dogs were not adequately anticoagulated, and that the 0.8mg/kg dose was not shown to be effective.[31] This finding raised questions regarding the true effective dose of enoxaparin, and of the difficulty of knowing the drug's true potential for success in at-risk clinical dogs.

Assessment of Anticoagulation

Prothrombin Time and Activated Partial Thromboplastin Time (aPTT)

Measurement of prothrombin time (PT) and activated partial thromboplastin time (aPTT) is most frequently performed on whole blood in citrated plasma, and are some of the most commonly utilized laboratory tests in dogs suspected to have a coagulopathy.[34] PT is a screening test for the activity of coagulation factors II, V, VII, and X of the extrinsic and common coagulation pathway, while aPTT tests for factors II, V, VIII, IX, X, XI, and XII of the intrinsic and common pathway.[34] While these are common tests are often available at diagnostic laboratories and referral centers, some veterinary hospitals do not have the ability to perform these tests in house. Previous studies have looked into the effects of storage and handling of sent out samples for PT/ aPTT and it as determined that 24-48 hours post-sampling the values of PT were similar to fresh samples, however the aPTT results were significantly shorter than the fresh samples.[34] This leads to the conclusion that this send out test is not a reliable or ideal monitoring solution for clinically affected patients that need their coagulation assessed in a timely fashion so

that treatments can be adjusted in a reasonable timeframe.[17] Additionally, there is not significant

evidence that PT/ aPTT are altered notably following treatment with enoxaparin.[17]

Activated Clotting Time (ACT)

The activated clotting time (ACT) evaluates the intrinsic and common pathways of the

coagulation cascade. The ACT is the actual time that it has taken for whole blood to form a

detectable clot when in contact with a contact activant.[35] The contact activators can be anything

with a large surface area, and they are used to shorten the activation time and to allow the

stimulus for activation to be repeatable. This is the first and least clinically important stage of the

intrinsic pathway. There are often inconsistencies in the activation time due to variations in

sample handling and due to the fact that the ACT endpoint is determined visually by detection of

the clot forming or using automated devices. This makes ACT dependent on the method used to

read it, and the consistency of the procedures and personnel performing it.[35] There is evidence

that prolongation of ACT can occur with the use of enoxaparin in healthy dogs, but ACT is still

not considered the most sensitive test for monitoring of anticoagulation for the use of LMWHs.[17]

Thromboelastography (TEG)

Thromboelastography (TEG) is a viscoelastic test that can be utilized to assess

hemostasis in animals. It incorporates all blood components and allows evaluation of all phases

of coagulation. A graph is produced that represents the rate of fibrin polymerization, and the

overall clot strength and fibrinolysis.[36] The information derived from the TEG tracing includes

the time to initial fibrin formation, (the reaction time; R), the time needed to reach a

predetermined clot strength, (clotting time; K), the rate of the clot formation (angle; alpha), and

maximum clot strength (MA). MA is the maximum amplitude of the tracing measured in

millimeters, and can be converted to G, which expresses clot strength in units of force. The prolonged data collection phase of TEG can also provide information regarding fibrinolysis.[37] The main focus of the use of TEG has been to identify hypercoagulable states in patients, and then to monitor effectiveness of treatment protocols for the hypercoagulability. TEG can be performed as a patient-side test using fresh whole blood, but in veterinary medicine this test typically uses blood anticoagulated with sodium citrate. TEG has been evaluated in order to monitor the use of enoxaparin in healthy dogs, with the most reliable changes seen being a prolonged R , implying that TEG could be used as a reliable avenue for evaluation of anticoagulation. However further studies would have to be performed to provide more supportive evidence of TEG's effectiveness for monitoring enoxaparin.[17]

Viscoelastometry (Sonoclot®)

In humans, a viscoelastic coagulation monitoring instrument, the Sonoclot® analyzer, measures the entire coagulation process by detecting changes in viscosity during clot formation. The Sonoclot® is primarily used to monitor the adequacy of heparin anticoagulation in extracorporeal circulation (cardiopulmonary bypass)[38], but has also been used for perioperative hemostatic management and prediction of postoperative hemorrhage.[39] Additionally, the Sonoclot® is a bedside instrument that can provide a rapid assessment of the level of heparinization or anticoagulation. During bypass, if there is inadequate anticoagulation, there is an elevated thrombotic risk. However, excessive anticoagulation potentially impairs hemostasis restoration. That is why the availability of a tool to immediately determine anticoagulation in a critically at risk patient is such a necessary tool.[38]

The principle of operation within the Sonoclot® Analyzer is a microviscometer. This instrument is sensitive to any resistance to motion that an oscillating probe encounters as it

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moves within the test sample of blood as it forms a clot. The greater the viscosity of the medium the probe is in, the greater the signal generated by the Sonoclot®. The Sonoclot® provides information such as coagulation, fibrin formation, and clot retraction. The viscoelastic measurement of the Sonoclot® changes over time as the clot forms, creating the Sonoclot Signature. This is generated for each test. Several types of reagents can be used for the Sonoclot, but for assessment of heparin therapy, a standard activator such as celite or kaolin is used. In most studies using Sonoclot in dogs, glass beads (gbACT+) are used to generate the traditional Sonoclot signature displaying activated clotting time (ACT) and clot rate (CR) over time.[38]

The Sonoclot® was utilized in a study of randomly sourced healthy dogs in a hospital setting in an attempt to establish a standardized protocol for analysis of canine whole blood and to generate reference intervals. This study was able to establish a protocol for dogs with the Sonoclot® that included letting blood rest for a period of 30 minutes and then adding 340µL of citrated blood added to 20µL of 0.2 M CaCl2 in two Sonoclot® cuvettes that were warmed to 37degrees C. The magnetic stir-bar with glass beads (gbACT+) was used, and reference intervals were able to be established that correlated to several TEG parameters.[27]

Although the Sonoclot® has not been used to determine the anticoagulant effects of enoxaparin in dogs, this instrument has been used to determine the pharmacodynamic effects of dalteparin, another LMWH, in dogs.[27] In this previous study, the Sonoclot® provided adequate monitoring during dalteparin therapy, suggesting that this instrument might be the ideal instrument to pharmacodynamically assess the anticoagulant effects of enoxaparin in dogs. Although enoxaparin and dalteparin have similar mechanisms of actions, these two medications do not have the exact same pharmacological properties, and therapeutic interchange between

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these two LMWHs may not provide the same therapeutic results.[40] Work with dalteparin therefore cannot be directly extrapolated to enoxaparin.

Anti-factor Xa Assay

The Comparative Coagulation Section of the Diagnostic Laboratory at the Cornell University College of Veterinary Medicine has developed and validated a chromogenic assay that measures heparin activity based on its inhibition of factor Xa (anti-Xa activity), which can be used to monitor the therapeutic effects of LMWHs such as enoxaparin in the dog. LMWH concentrations can be indirectly measured by quantifying the presence of anti-factor Xa for dose adjustment. LMWHs predominantly acts on factor Xa, unlike UFH, which exerts its effect on both factor II and factor Xa, which is why LMWH activity is monitored using serum anti-factor Xa levels instead of activated partial thromboplastin time.[41] A target therapeutic range of 0.5 to 1 U/mL anti-Xa activity is considered appropriate for human patients at risk for development of venous thromboembolism.[42]

Unfortunately, the anti-Xa activity assay is not always available for immediate therapeutic adjustments. Additionally, in a study of critically ill dogs receiving the LMWH dalteparin, where anti-Xa monitoring was used to gauge the level of anticoagulation, it was shown that the dose adjustment response to anti-Xa activity interpretation inconsistently resulted in the attainment of the targeted anti-Xa range.[43] Additionally, although the anti-Xa activity assay is considered to be an acceptable surrogate marker of heparin levels, it may not be a specific indicator of the desired end result of adequate heparinization, which is inhibition of coagulation. While the anti-Xa activity assay is currently the most supported monitoring tool for LMWHs, there is also the concern that the human target range for anticoagulation that is being applied to dogs is not accurate for level of anti-factor Xa activity in treated dogs.[17]

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CHAPTER II

ANTICOAGULANT PROFILE OF SUBCUTANEOUS ENOXAPARIN IN HEALTHY DOGS

Introduction

Thromboembolism is a significant complication of many common disorders in veterinary

medicine, including immune-mediated hemolytic anemia (IMHA), hyperadrenocorticism,

neoplasia, cardiac disease, and protein-losing nephropathy.[1] Prophylactic medications are

routinely administered to inhibit either platelet function or the clotting cascade, or both, in order

to prevent thrombus formation. With the addition of anti-coagulant[2] or anti-platelet therapy[3] for

the treatment of canine IMHA, survival rates have improved, but affected patients still continue

to develop fatal thromboembolic complications[2,3], signifying a need for improved prophylactic

anticoagulant therapy.

Compared to a fixed dose, the use of an individually adjusted dose of unfractionated heparin

(UFH), with adjustments based on anti-Xa activity, has been shown to decrease case fatality in

dogs with IMHA. [2] Unfortunately, dogs have a variable response to UFH, and the use of UFH

therefore requires monitoring through either measurement of anti-Xa activity or prolongation of

the activated partial thromboplastin time (aPTT).[2] The use of UFH without monitoring may

predispose a dog to inadequate or excessive anticoagulation, leading to thrombosis or

hemorrhage, respectively. Enoxaparin (Lovenox®) is one of several fractionated or low-

molecular-weight heparins (LMWHs) that were developed as an alternative to UFW. LMWHs

have more consistent pharmacokinetic and pharmacodynamic profiles and a reduced risk of

predisposing to hemorrhage compared to patients receiving UFH.[4,5]. Additionally, in humans, the relatively predictable anticoagulation achieved with LMWH therapy does not require frequent monitoring.

In dogs, the current dosage recommendation for enoxaparin (0.8 mg/kg, SQ, q6h) necessitates frequent administration, which may not be feasible for at-home care. This dosage is based on pharmacokinetic modeling utilizing anti-Xa activity,[6] but the length of effective anticoagulation post-dosing for enoxaparin has not been comprehensively determined in dogs. Additionally, although a pharmacokinetic modelling study estimated that a dosage of 0.8 mg/kg, SC, q6h should maintain anti-Xa activity within a target range (0.5-1 U/ml) in most dogs, one in six dogs studied did not demonstrate effective anticoagulation when enoxaparin was administered at this dose.[6] Therefore, to ensure consistent and adequate anticoagulation with the currently recommended dose, frequent monitoring with individually adjusted doses of enoxaparin, similar to UFH, should be considered, or a different, potentially higher dose of enoxaparin should be administered.

The most common assay used to monitor the therapeutic effects of LMWHs in dogs is the inhibition of factor Xa (anti-Xa activity) assay. In dogs, the most commonly recommended therapeutic target range for anti-Xa activity with LMWH therapy is 0.5-1 U/ml, which was extrapolated from humans receiving LMWH to prevent the development of venous thromboembolism. Unfortunately, the anti-Xa activity assay is not usually immediately available for therapeutic dose adjustments, and a more readily available assay to allow for individual enoxaparin dose adjustments would be beneficial for hypercoagulable patients. In humans, a viscoelastic coagulation monitoring instrument, the Sonoclot® analyzer, measures the entire coagulation process by detecting changes in viscosity during clot formation. The

Sonoclot® is primarily used to monitor the adequacy of heparin anticoagulation in extracorporeal circulation in human patients, and can be used as a bedside instrument that can provide a rapid assessment of the level of heparinization or anticoagulation. [7] In a previous canine study, the Sonoclot® provided adequate monitoring during administration of dalteparin, another LMWH.

The objective of our study was to determine the anticoagulant effects of various doses of enoxaparin in healthy dogs using measurement of anti-Xa activity, a pharmacodynamic assay (Sonoclot®) and a comprehensive panel of measures of secondary hemostasis. Our hypotheses were that a single subcutaneous (SC) 0.8 mg/kg dose of enoxaparin will provide inconsistent anticoagulation in healthy dogs, that higher enoxaparin doses would provide more consistent anticoagulation over a greater duration of time, and that the Sonoclot® would be an effective instrument for detecting the anticoagulant effects of enoxaparin.

Materials and Methods

Study Design, Animals

The study used healthy adult walker-hound dogs. Their health status was confirmed to be normal by physical examination and baseline testing including a complete blood count (CBC), serum biochemistry profile, urinalysis, heartworm testing, prothrombin time (PT) and aPTT. While receiving enoxaparin, all dogs had a daily physical examination to identify evidence of hemorrhage or other side effects. Body weights were obtained at the beginning of each phase of the study to calculate all drug doses. This study was approved by the Mississippi State University Institutional Animal Care and Use Committee and was in compliance with the requirements of a facility accredited by the American Association for Accreditation of Laboratory Animal Care.

Hematologic Testing

Blood was collected from the jugular vein (catheter or venipuncture) into two 4.5 mL vacutainer tube containing 3.2% sodium citrate anticoagulant[a] (anti-Xa activity, antithrombin [AT] activity, aPTT, PT, fibrinogen concentration, and viscoelastometry) and EDTA[b] (CBC). Citrated blood was centrifuged, and the plasma supernatant was removed and frozen at -80°C. Frozen plasma samples were shipped overnight on dry ice to the Comparative Coagulation Section of the Diagnostic Laboratory at the Cornell University College of Veterinary Medicine for analysis. The following coagulation assays were performed: anti-Xa activity, AT activity, aPTT, PT, and fibrinogen concentration. All samples were analyzed within two months of collection.

A commercial kit[c] was used to measure the bioactivity of enoxaparin's anticoagulant action based on inhibition of a bovine factor Xa reagent added in excess to the assay mixture. Residual uninhibited factor Xa cleaves a chromogenic substrate so that the inverse of color change is proportional to drug concentration (expressed as U/ml anti-Xa) in sample plasma. The assay is calibrated with a standard containing defined concentrations of LMWH[d]. The therapeutic target range for anti-Xa activity is 0.5-1 U/ml.[8]

Plasma AT activity was measured in a chromogenic substrate kit[e] according to the manufacturer's recommendations for assay method and instrumentation[f]. The assay is modified by use of pooled normal canine plasma, rather than human plasma, for the calibration standard. The AT levels in test samples are reported as the percentage of the canine standard (assigned value of 100% activity). The aPTT and PT were measured with an automated, mechanical endpoint clot detection instrument using commercially available coagulation reagents. Clottable

fibrinogen was measured in an automated coagulation instrument via the Clauss method using 100 NIH U/mL human thrombin reagent.[9,10]

Viscoelastometry (Sonoclot®)

A 2-channel Sienco SonoClot® Analyzer[g] was used to provide information on coagulation, fibrin gel formation, and clot retraction by detecting mechanical changes within whole blood as a clot formed. Following collection, a second citrated whole blood was held at room temperature for a 30-minute rest period prior to analysis.[11] Coagulation analysis with the SonoClot® was performed based on the manufacturer's recommendations. Briefly, 330μl of citrated whole blood and 15μl 0.2% $CaCl_2$ were added to a cuvette that had been warmed to 37°C. An oscillating tubular probe, which detects the resistance to motion during clot formation, was placed in the whole blood sample, generating a clot signal. Clotting was triggered using the gbACT+ Kit[h], and the results were calculated by the Signature Viewer Program[i]. The clot signal includes the activated clotting time (ACT; the time until the first detectable clot) in seconds, the clot rate (CR; the slope of the SonoClot® signature during the fibrin gel formation) in clot signal units per minute, and platelet function (an index which reflects the role of platelets in clot formation). Samples were analyzed in duplicate and averaged. All samples were analyzed within 1 hour of collection. Quality control analysis was performed at the beginning of each day that samples were analyzed. Based on similar criteria used in humans during hemodialysis, a change in the ACT compared to baseline value (ΔACT) of ≥40 seconds and a CR of <20 U/min (CRpost) was considered excessive anticoagulation.[12]

Phase One (0.8 mg/kg, SC, single dose)

Three healthy, intact female dogs with a mean age of 2.4 years (range, 1.8-3.4 years) and a mean weight of 21.7 kg (range, 19.1-29.9 kg) were used during this phase of the study. The dogs were lightly sedated with butorphanol[j] (0.4 mg/kg, IV) and midazolam[k] (0.5 mg/kg, IV). A 14-gauge, 13 cm intravenous sample catheter[l] (priming volume, 0.3 ml) was inserted into the either the right or left jugular vein and flushed with 0.9% sterile saline. Following recovery, the dogs were placed in the Mississippi State University College of Veterinary Medicine Small Animal Intensive Care Unit for monitoring and assistance with catheter protection until all samples had been collected. The catheter was flushed every 4 hours with 3 ml of sterile saline. The dogs did not receive any food overnight, and the following pre-treatment samples were collected the following morning: CBC, aPTT, PT, anti-Xa activity, and AT activity. Following collection of baseline samples, enoxaparin (0.8 mg/kg, SC) was administered along the left thorax of each dog. Using the jugular catheter, blood samples were collected every other hour for 6 hours for the measurement of anti-Xa activity and hourly for 12 hours for viscoelastometry.

Phase Two (2 mg/kg, SC, single dose)

Six healthy dogs, five intact females and one intact male, with a mean age of 3.6 years (range, 2.2-4.8 years) and a mean weight of 27 kg (range, 19.1-29.9 kg) were used during this phase of the study. Three of the six dogs used in this phase of the study were also used during Phase 1, and there was at least a 4-week washout period between the completion of Phase 1 and commencement of Phase 2. Following the same protocols as Phase 1, the dogs were sedated, a jugular sampling catheter was placed, and baseline samples were collected. Enoxaparin (2 mg/kg, SC), was administered along the left thorax of each dog, and blood was collected every other hour for 12 hours for the measurement of anti-Xa activity and hourly for 12 hours for

viscoelastometry. The results of the anti-Xa activity from Phases 1 and 2 were used to generate a biological effect curve, which was used to calculate the optimum enoxaparin dose and dose interval to maintain anti-Xa activity within a therapeutic target range of 0.5-1 U/mL. The dose that was determined to most likely achieve consistent anticoagulation was 1.3 mg/kg, SC, q8h.

Phase Three (1.3 mg/kg, SC, q8h)

The same six dogs used in Phase 2 were then used in Phase 3, with at least a 4-week washout period between the completion of Phase 2 and commencement of Phase 3. All blood samples were collected via jugular venipuncture using a 20-gauge needle and syringe, with blood immediately transferred into 3.2% sodium citrate anticoagulant. The following pre-treatment samples were collected: aPTT, PT, anti-Xa activity, AT activity, and fibrinogen concentration. Following the collection of baseline samples, enoxaparin (1.3 mg/kg, SC, q8h for 48 hours) was administered over the left thorax, for a total of 7 doses. After the first enoxaparin injection (Day 1), blood was collected every 2 hours for the first 10 hours for the measurement of anti-Xa activity and viscoelastometry. Immediately before the fourth injection (Day 2), and 2 hours after the injection, blood samples were collected for anti-Xa activity and viscoelastometry. Finally, before and every 2 hours after the seventh injection (Day 3), blood samples were collected for anti-Xa activity and viscoelastometry.

Data Analysis

The pharmacokinetics of anti-Xa activity were determined using standard pharmacokinetic approaches as provided by a commercially available software programs.[m,n] In all phases, enoxaparin was dosed as mg/kg; to allow for calculation of the volume of distribution divided by the fraction absorbed (Vd/F), anti-Xa activity, represented as U/mL, was converted to

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μg/mL using a conversion of 100 U/1000 μg of enoxaparin. Parameters generated from all three phases of the study were fit by noncompartmental analysis for the first dose and by compartmental population-pharmacokinetics using mixed-effect methodology for all doses. Parameters from the final Monolix fit were transferred to its companion product[o], to create population percentile simulations for 1.3 mg/kg SC at q8hr and q12hr dosings. Pearson correlations between anti-Xa activity with ACT and CR were performed with the ACT or CR acting as a surrogate for anti-Xa activity in the model. The strength of correlation was based on the following scale: 0.0-0.0.19, very weak; 0.2-0.39, weak; 0.4-0.59, moderate, 0.6-0.79, strong; 0.8-1, very strong. A P-value of <0.05 was considered significant.

Results

Daily Physical Examinations

During Phases 1 and 2, no excessive or overt hemorrhage was noted. During Phase 3, two dogs developed hematomas over the jugular vein where blood samples had been previously collected. Following the development of hematomas, additional blood samples were collected on the opposite jugular vein or cranial to the hematoma on the same jugular vein. Hematomas resolved within 48 hours of first detection.

Phase One

The pre-treatment values for aPTT, PT, AT, and anti-Xa are represented in Table 2.1. The ACT and CR results determined via viscoelastometry are represented in Tables 2.2 and 2.3, respectively. The ACT values remained outside of previous established reference intervals[11] for 6 hours in one dog and 2 hours in another dog. In the third dog, ACT values remained within established reference intervals for 12 hours. After enoxaparin administration, and based on the

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ΔACT, one dog was classified as having excessive anticoagulation during the first 2 hours and another dog had excessive anticoagulation for 4 hours. The third dog was not considered to have excessive anticoagulation. Most of the CR values remained within the established reference intervals.[11] Based on the CRpost, only one dog was classified as having excessive anticoagulation for the initial 2 hours after enoxaparin administration.

The anti-Xa activity results are represented in Figure 2.1. The anti-Xa activity values remained within the targeted range (0.5-1 U/ml) for 6 hours in one dog and 4 hours in another dog. In the third dog, there was no change in anti-Xa activity values for 6 hours after enoxaparin administration. One dog was excluded from the pharmacokinetic analysis because there was no change in anti-Xa activity after the enoxaparin injection.

Phase Two

The pre-treatment values are represented in Table 2.1. The ACT and CR results determined via viscoelastometry are represented in Tables 2.2 and 2.3, respectively. The ACT and CR values remained outside of previous established reference intervals for 8 hours in all dogs, and 3 dogs, had values outside of reference range for at least 12 hours. After enoxaparin administration, and based on the ΔACT and CRpost, all dogs were classified as having excessive anticoagulation, but the length varied between 6 and 12 hours.

The anti-Xa activity results are represented in Figure 2.1. The anti-Xa activity values exceed the targeted range (0.5-1 U/ml) for the initial 8 hours after enoxaparin injection in all dogs expect for one dog (anti-Xa activity at hour 8, 0.96 U/mL). All dogs demonstrated anti-Xa activity within the target range for 10 and 12 hours after drug administration.

Based on the anti-Xa activity data from Phases 1 and 2, a pharmacological projection was generated that predicated that an enoxaparin dose of 1.3 mg/kg SC q8hr would maintain anti-Xa activity between 0.5 and 2 U/mL at steady state (Table 2.4).

Phase Three

The pre-treatment values are represented in Table 2.1. The mean and standard deviation of fibrinogen concentration was 382 ± 226 mg/dL (reference interval, 150-190). The ACT and CR results determined via viscoelastometry are represented in Tables 2.2 and 2.3, respectively.

On Day 1, the ACT values remained outside of previous established reference intervals for all dogs between the first and second enoxaparin injection. Based on the ΔACT, all dogs were classified as having excessive anticoagulation during the first hour after enoxaparin administration, but the length of excessive anticoagulation was variable (1-6 hours). No dogs were considered to have excessive anticoagulation at the time of the second enoxaparin injection. On Day 3 of enoxaparin administration, all dogs experienced at least 1 hour of excessive anticoagulation (1-6 hours). On Days 1 and 3, CR values remained below the established reference intervals for all dogs for 4-8 hours. On Day 1, three dogs had CR values below reference range when the second enoxaparin injection was administered. Based on the CRpost, all dogs experienced variable lengths of excessive anticoagulation between the first and second injections. On Day 3, all dogs were considered to have excessive anticoagulation, for variable lengths of time.

The anti-Xa activity results for Days 1 and 3 are represented in Figure 2.2. On Day 1, the anti-Xa activity reached or exceeded the target range of 0.5-1 U/ml in 5 of the 6 dogs. For 1 dog, there was change in the anti-Xa activity following the initial enoxaparin administration (mean $\pm$ standard deviation for the first 8 hours was 0.18 ± 0.008). On Day 3, all dogs at all sampled time

points were within or exceeded the target range. One dog on Day 1 was excluded from the pharmacokinetic analysis because there was no change in anti-Xa activity after the enoxaparin injection.

At a dose of 1.3 mg/kg SC, the mean (n=6) apparent specific Vd/F was 90 mL/kg, and the proximal and terminal half-lives were 0.73 hours and 5.73 hours, respectively (Table 2.5). There was weak positive correlation between anti-Xa activity and ACT values (r = 0.452; p<0.001) and a weak negative correlation between anti-Xa activity and CR values (r=-0.554; p<0.001).

Discussion

Our original hypothesis that the Sonoclot analyzer, and its values for ACT and CR, could be used to replace the need for the send-out test of anti-Xa activity to determine anticoagulant effects in dogs after dosing them with enoxaparin appears to not be supported, since there was not a strong correlation between anti-Xa and ACT/CR. This has been previously noted that when comparing anti-Xa and ACT/ CR produced by Sonoclot in humans, that they did not positively correlate when investigating the effects of a LMWH.[13]

It remains unclear if the canine target range, extrapolated from human anti-Xa activity of 0.5-1 U/ml, is the appropriate range for dogs. It has been determined in human medicine that the target range for anti-Xa activity can vary greatly based on dosing interval and comorbidities of the patient[8], therefore even though some dogs in our study were outside of the accepted target range, clinically, they may still be experiencing an appropriate level of anticoagulation necessary to prevent clot formation.

Another point of interest is that 5 of the 6 dogs used in this study had a non-zero baseline (time zero) concentration of anti-Xa activity present at the time of the anti-Xa assay. Our results

suggest that there are some animals, including dogs, where a pre-treatment low level of anti-Xa activity is detected. The assay's reported limit of detection is 0.1U/mL[14], however, these low baseline samples may be displaying a low level non-heparin Factor Xa inhibitory activity in the plasma.

During this research project, there was the benefit of studying the effects of enoxaparin and the use of monitoring with the Sonoclot analyzer on healthy dogs. Due to this wide safety margin that would not be present in a clinically affected patient, our patients' LMWH dose was not adjusted based on their clinical signs observed, such as hematomas at the site of blood draws. However, in humans being evaluated by the Sonoclot during UFH therapy during hemodialysis, the values of CR and ACT produced by Sonoclot were monitored closely to determine if a dose adjustment was necessary to prevent either unintended hemorrhage or thrombotic complications. In these human patients, a CR of less than 20U/min, or an ACT greater than 40 seconds were considered at risk for hemorrhage, and their UFH dose was decreased.[12] In this same study, a CR value of greater than 30 U/min was considered at risk for thrombotic complications, and the heparin dose was increased. The goal in humans was to maintain a CR within a range of 20-30 U/min to be effectively managing unintended adverse effects of heparin therapy in clinical human patients.[12] At this time, it is unclear if this target range for CR is appropriate for dogs as well, especially due to the fact that this research was based on the responses of clinically affected humans and not healthy subjects. In our study, some pre-treatment values of CR for our dogs were less than 20 U/min, and after treatment, the CR for some dogs maintained between 35-40 U/min. This raises the question of the benefit of monitoring CR for potential dose adjustments in the future. If the target CR range is extrapolated from human medicine, we could potentially be undertreating our canine patients. In the future, further investigation would be necessary into

what the appropriate CR range for dogs would be to utilize Sonoclot to make in the moment dose adjustments.

Based on anti-Xa activity results, there does not appear to be dose linearity with enoxaparin. With higher doses of enoxaparin, although there is an increase in anticoagulation, the increase did move not in a linear fashion. One possible explanation for this would be that as the dose increases, the ability of the body to eliminate the drug is saturated, leading to a longer half-life of the drug at the higher doses. Future research into intravenous dosing of enoxaparin would be necessary to understand the lack of dose linearity more fully.

Several dogs in our study did not reach an appropriate level of anticoagulation after a single dose of the 1.3mg/kg of enoxaparin based anti-Xa activity, however, after the second dose, they reached an acceptable level. There was also evidence based on Sonoclot® analysis that there was adequate anticoagulation following the first dose of enoxaparin. While anti-Xa activity results could indicate that a loading dose of enoxaparin would be more appropriate for patients at risk of hypercoagulability, Sonoclot® analysis results in contrast suggest that the anti-Xa activity assay may not be an appropriate predictor of enoxaparin's anticoagulant effects, and that a loading dose could predispose to hemorrhage. In a study of the use of enoxaparin in cats, where thrombi were experimentally created, measured and compared, an anti-Xa assay did not necessarily correlate with the physical data being recorded.[15] While there were dosage differences in the feline study compared to our study, it is possible that, in dogs as in cats, the anti-Xa assay is not a strong predicter of the effects of enoxaparin. Additional studies would need to be conducted to determine an effective loading dose that would allow the dog to be anticoagulated, without being overtreated to the point of concern of uncontrolled bleeding.

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Finally, the most consistently effective dose and dosing interval for enoxaparin as determined by our study was different from previously published studies. Based on our multi-phase study, our recommended for subcutaneous enoxaparin in a dog is 1.3 mg/kg SC every 8 hours. While this dosing interval is more convenient than every 6-hour dosing interval suggested with the 0.8mg/kg dose, and appears to provide more consistent anticoagulation, it is still a relatively minor extension in dosing intervals compared to a more optimal dosing frequency of once or twice daily.

Through Sonoclot analysis and anti-Xa activity, it was evident that in some dogs, while eventually reaching an adequate level of anticoagulation, this effect was not achieved until the second dose. At the 1.3mg/kg dose, appropriate anticoagulation was reached for a dosing interval of every 8 hours, but not longer.

In Phase 1, based on anti-Xa activity and ACT and CR, there was a lack of consistent anticoagulation, and this enoxaparin dose was unable to consistently persist in the anti-Xa activity therapeutic target range. Based on anti-Xa activity, the duration of anticoagulation for Phase 1 was 12 hours for Phase 2.

Our study had a number of limitations. First, a relatively small number of dogs were used, and all were of the same breed. Potentially, the use of different breeds would have revealed different responses to the drug. Another limitation is that we only studied enoxaparin's effects on anticoagulation in healthy dogs. It is possible that repeating a similar study on hypercoagulable dogs would return data that would give a better insight to the clinical effectiveness of enoxaparin in affected dogs.

The first two phases of this study evaluated the effectiveness of enoxaparin after only single injections, while our third phase results were based on reaching a steady state of

anticoagulation after multiple doses of enoxaparin. While this meant that the third phase of the study could not be directly compared with the first two phases, this approach was pursued in order to determine the kind of response the dogs would have at the initial injection versus after several injections. This was to see if the level of anticoagulation reached was adequate immediately after one dose or if it would appear to require several doses to ready the steady state.

At the 0.8mg/kg dosing of enoxaparin, anticoagulation was not consistently adequate. However, an increased dose of 2 mg/kg revealed ACT, CR, and the anti-Xa activity data that increased concern regarding the risks of unintended hemorrhage. A middle dose of 1.3 mg/kg administered subcutaneously every 8 hours appeared to have sufficiently and consistently anticoagulated 90% of the dogs used in this study based on their anti-Xa activity assay results. Due to poor correlation between the Sonoclot analysis of ACT and CR and anti-Xa activity, ACT and CR do not appear to be suitable replacements for anti-Xa activity when monitoring enoxaparin anticoagulation in dogs.

Additional studies examining the effectiveness of enoxaparin in clinically hypercoagulable patients and determining whether or not a loading dose of enoxaparin would help reach the appropriate level of anticoagulation sooner, are needed to further understand the true effectiveness of enoxaparin in a clinical setting.

Table 2.1   Mean and standard deviation of hemostasis indices before enoxaparin therapy in Phase 1 (0.8 mg/kg, SC, once), Phase 2 (2 mg/kg, SC, once), and Phase 3 (1.3 mg/kg, SC, q8h).

	aPTT (seconds)	PT (seconds)	AT Activity (%)	Anti-Xa Activity (U/ml)
Phase 1	13.2±1.4	12.5±0.7	106.7±9.9	0.13±0.04
Phase 2	13.1±1.3	12.6±0.5	110.2±9.7	0.22±0.04
Phase 3	12.7±1.4	13.0±1.3	122.3±16.8	0.12±0.08

Reference intervals: aPTT, 8.5-15.5 seconds; PT, 11-15.5 seconds; AT activity, 65-145%; Anti-Xa Activity, 0.5-1.0 U/ml

Table 2.2   Mean and standard deviation of ACT values determined by viscoelastometry before and during enoxaparin therapy in Phase 1 (0.8 mg/kg, SC, once, 3 dogs), Phase 2 (2 mg/kg, SC, once, 6 dogs), and Phase 3 (1.3 mg/kg, SC, q8h, 6 dogs).

Hour	0.8 mg/kg	2 mg/kg	1.3 mg/kg - Day 1	1.3 mg/kg - Day 3
0	107.8±42	160.6±15	135.7±21	164±26
1	131.3±64	233.7±48	---	---
2	155.5±54	284.9±62	218.7±43	212.5±50
3	131.3±63	274.4±60	---	---
4	148.8±56	316.3±59	205.8±28	206.3±61
5	146±33.5	284.5±62	---	---
6	132±28	284.5±62	186±19	189.2±44
7	120.5±13	246±56	---	---
8	123±48	203.5±57	141.8±35	165.8±38
9	122.5±59	195.2±34	---	---
10	134.3±39	192.9±40	222.3±35	180.1±42
11	101.3±51	184.6±36	---	---
12	98.7±39	173.9±20	---	156.3±27

Table 2.3   Mean and standard deviation of CR values determined by viscoelastometry before and during enoxaparin therapy in Phase 1 (0.8 mg/kg, SC, once, 3 dogs), Phase 2 (2 mg/kg, SC, once, 6 dogs), and Phase 3 (1.3 mg/kg, SC, q8h, 6 dogs).

Hour	0.8 mg/kg	2 mg/kg	1.3 mg/kg - Day 1	1.3 mg/kg - Day 3
0	31.2±4	24.4±6	27.3±7	22±5
1	31±13	13.4±5	---	---
2	27±13	8.1±2	13.1±5	11.8±4
3	33.2±10	10.4±5	---	---
4	27.3±9	6.9±3	12.7±6	14.4±5
5	29.8±8	9±4	---	---
6	35.3±9	11.6±5	16.1±3	18.5±11
7	41±9	11.5±4	---	---
8	34.7±8	18.4±10	28±11	22.7±10
9	33.2±8	16.1±6	---	---
10	34.8±7	18.1±8	11.9±2	20.8±9
11	38.3±7	21.6±7	---	---
12	41±10	21.5±5	---	24.8±8

Table 2.4   Noncompartmental PK Analysis by dose. Note that Tmax is median and range, and t1/2 is harmonic mean with pseudo-standard deviation.

Dog	Dose	CMax	Cmax/D	Tmax	AUC0-Inf	AUC/D	AUMC0-Inf	MRT0-Inf	Cl/F	Vss/F	Lambda-z	t1/2
1	0.8	7.40	9.25	2.00	43.82	54.78	238.75	5.45	0.018	0.099	0.218	3.18
2	0.8	9.00	11.25	2.00	71.54	89.42	532.51	7.44	0.011	0.083	0.152	4.56
	mean	8.20	10.25	2	57.68	72.10	385.63	6.45	0.015	0.091	0.185	3.75
	SD	1.13	1.41	2	19.60	24.50	207.72	1.41	0.005	0.011	0.05	0.99
	CV	13.80%	13.80%		33.98%	33.98%	53.86%	21.83%	33.98%	12.56%	25.23%	26.45
2	1.3	9.60	7.38	2.00	70.75	54.42	525.02	7.42	0.018	0.136	0.158	4.39
3	1.3	13.80	10.62	2.00	108.63	83.56	750.59	6.91	0.012	0.083	0.177	3.92
4	1.3	13.20	10.15	4.00	172.53	132.71	1929.69	11.18	0.008	0.084	0.101	6.86

Table 2.4 (continued)

Dog	Dose	CMax	Cmax/D	Tmax	AUC0-Inf	AUC/D	AUMC0-Inf	MRT0-Inf	Cl/F	Vss/F	Lambda-z	t1/2
5	1.3	8.50	6.54	4.00	79.40	61.08	624.41	7.86	0.016	0.129	0.159	4.36
6	1.3	10.70	8.23	2.00	105.20	80.93	888.54	8.45	0.012	0.104	0.138	5.02
	mean	11.16	8.58	2	107.30	82.54	943.65	8.36	0.013	0.107	0.15	4.73
	SD	2.28	1.76	(2-4)	39.92	30.71	567.83	1.67	0.004	0.025	0.03	0.908
	CV	20.46%	20.46%		37.20%	37.20%	60.17%	20.00%	31.65%	23.06%	20%	19
1	2	17.60	8.80	4.00	226.90	113.45	2588.80	11.41	0.009	0.101	0.1	6.93
2	2	15.20	7.60	4.00	213.63	106.82	2586.79	12.11	0.009	0.113	0.092	7.53
3	2	19.50	9.75	2.00	212.87	106.44	2245.27	10.55	0.009	0.099	0.103	6.73
4	2	18.50	9.25	2.00	179.07	89.53	1461.50	8.16	0.011	0.091	0.144	4.81

Table 2.4 (continued)

Dog	Dose	CMax	Cmax/D	Tmax	AUC0-Inf	AUC/D	AUMC0-Inf	MRT0-Inf	Cl/F	Vss/F	Lambda-z	t1/2
5	2	16.60	8.30	2.00	206.87	103.44	2231.27	10.79	0.010	0.104	0.103	6.73
6	2	14.50	7.25	4.00	316.07	158.03	6178.48	19.55	0.006	0.124	0.054	12.83
	mean	16.98	8.49	3	225.86	112.93	2894.30	12.13	0.009	0.106	0.099	6.98
	SD	1.92	0.96	(2-4)	47.63	23.81	1692.18	3.92	0.00	0.01	0.03	
	CV	11%	11%		21%	21%	58%	32%	18%	11%	29%	

Table 2.5   Population parameter estimates

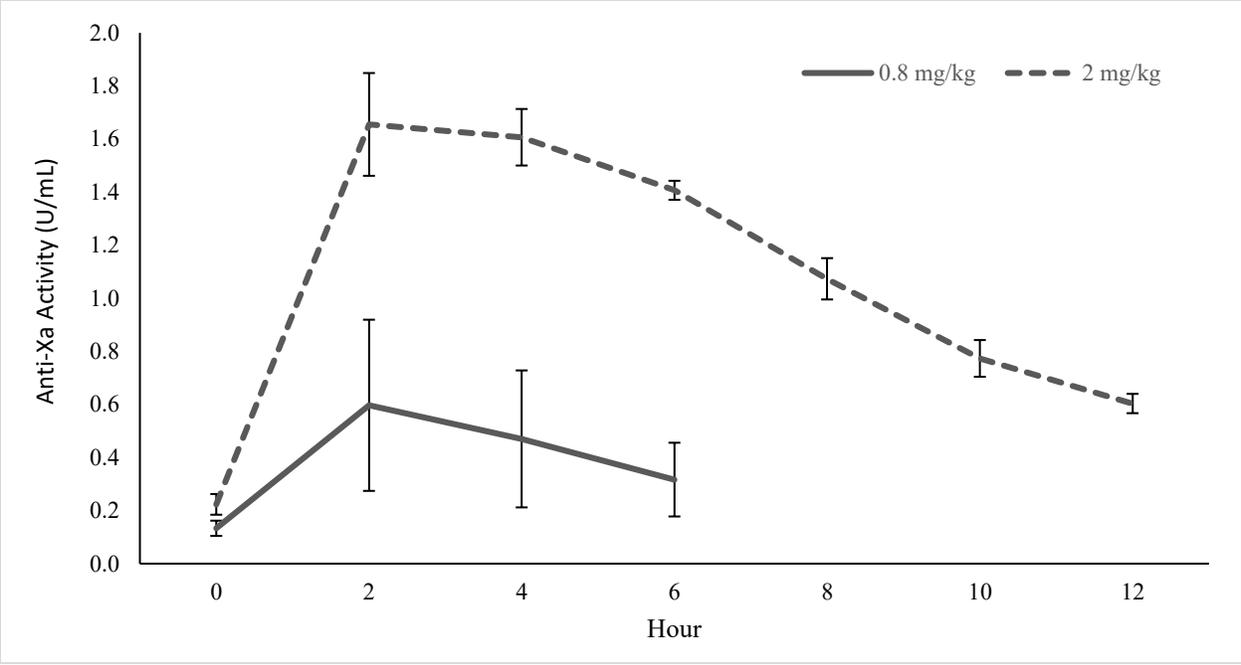| | VALUE | STOCH. APPROX. | | |
		S.E.	R.S.E.(%)	
Fixed Effects				
ka_pop	0.952	0.209	21.9	half-life=0.73 h
V_pop	0.0972	0.0129	13.2	
k_pop	0.121	0.0137	11.3	half-life=5.73 h
Standard Deviation of the Random Effects				
omega_ka	0.0396	0.136	343	
omega_V	0.186	0.061	32.7	
omega_k	0.0274	0.126	461	
Error Model Parameters				
b	0.158	0.0144	9.12	

Figure 2.1   A. Anti-Xa activity results for Phases 1 and 2, at doses 0.8 mg/kg and 2mg/kg, respectively.
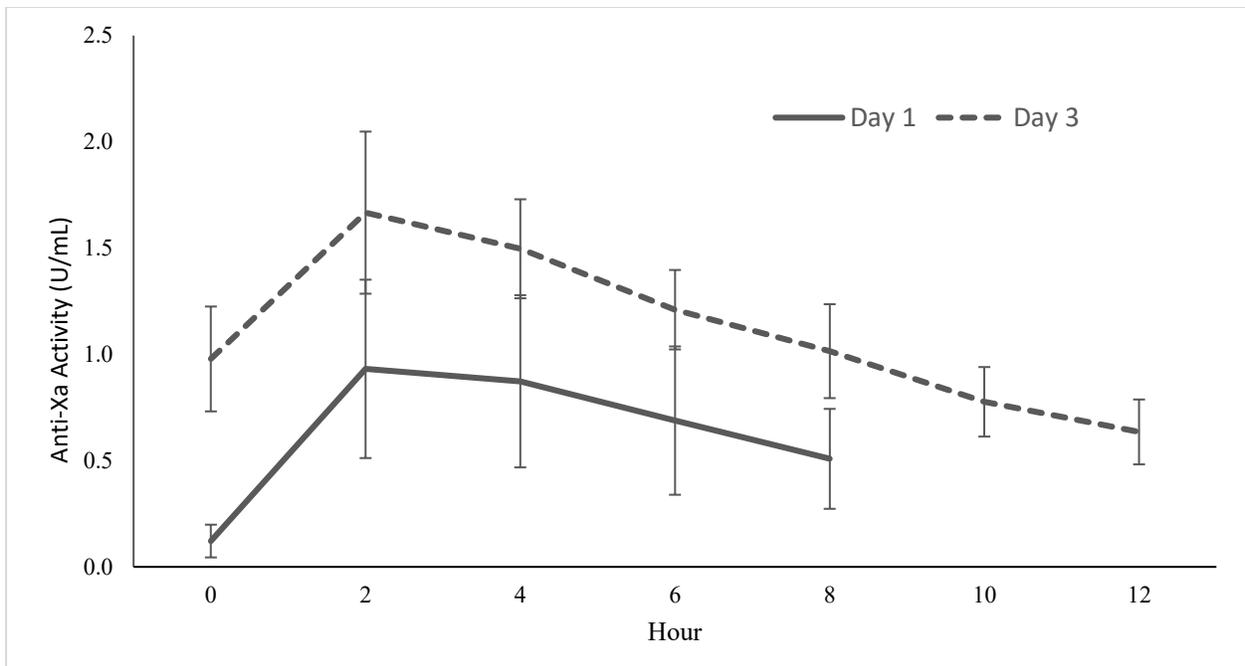
Figure 2.2   Anti-Xa activity results for days 1 and 3 at dose of 1.3mg/kg every 8 hours for seven total doses

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CHAPTER III

CONCLUSIONS AND FUTURE WORK

In this thesis, the initial research was performed to optimize the dosage and pharmacodynamic monitoring for enoxaparin therapy in healthy dogs. In the beginning, our goals were to evaluate the effectiveness of the previously published dose of 0.8mg/kg SQ, q6h, in healthy dogs[1] and compare anticoagulant monitoring using viscoelastometry (Sonoclot®), to the previously published most effective monitoring tool, the anti-factor Xa assay. As our first phase progressed, it became clear that while the Sonoclot® appeared effective preliminarily, the 0.8 mg/kg dosage was not consistently and effectively attaining an anticoagulated state in all of the dogs in the study. Due to these findings, a dose escalation study was pursued to further determine the appropriate and effective dose of enoxaparin and the usefulness of the Sonoclot® in measuring the level of anticoagulation achieved. Initial results of Phase Three (1.3mg/kg SQ enoxaparin q8hr for 7 doses) showed evidence of anticoagulation within the targeted range of the Sonoclot® ACT values, and also appeared to exceed or fall within the targeted range of 0.5- 1.0 U/ml in the anti-factor Xa assay for all but 10% of dogs studied. However, once pharmacokinetic analysis was performed, it was clear that there were some concerns with the study and its design.

Due to the lack of response of some of dogs in separate phases of the study, this data had to be eliminated from the analysis. Because of this, a mixed-effect, compartmental population pharmacokinetic analysis was utilized. The first area of concern was that for 5 of the 6 dogs used in our study, animals showed some low level amounts of anti-factor Xa present at Time Zero

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prior to the first dosage of enoxaparin, even though in previous studies performed, the Time Zero anti-factor Xa was reported to be zero.[1] This was thought to be due to the limit of detection, (LOD), of the anti-Xa assay, which is reported to be 0.1U/mL in humans and 0.126 U/mL in dogs.[2] It has been reported that occasionally a low anti-Xa activity is seen in untreated dogs due to other non-heparin factor Xa inhibiting activity in their plasma. There is also some suspected lot to lot variation in detection of activity in the low-level detection of activity, therefore leading to minor assay variability contributing to this issue. The LOD of an assay is typically 3 times the standard deviation of the background noise of the assay. While this should make up for the detection of anti-factor Xa in circulation at Time Zero, there is some concern the assay's LOD is not accurate for dogs specifically and that it may need to be adjusted in future analysis.

Using this same analysis model, some calculations were performed in order to create potential simulations for the patient population. While these are guarded simulations as they were only based on the 6 dogs, they did demonstrate that, using the current target range of 0.5-1.0U/mL for the anti-Xa assay, that 50% of the dogs, or the median, showed a steady state trough of enoxaparin at 0.937U and a peak at 1.71U/mL. These findings may be associated with an increased likelihood of unintended bleeding. The upper 10% of dogs showed a steady state trough of 1.21-1.65 U/mL and a peak at 2.19 – 2.98 U/mL, showing that this group was potentially particularly at risk of bleeding due to overshooting our intended target range of 0.5-1.0U/mL. In contrast, the lower 10% of the dogs showed a steady state trough of enoxaparin of 0.52 -0.73 U/mL, and a peak of 0.95 -1.34 U/mL. While this initially seems to indicate that even the lower 10% of the dogs would be adequately anticoagulated to within the target range of the anti-Xa assay, there were a small portion of the dogs that were below target at 8 hours post-dosing. After 2 doses, those lower ranges increased to 0.62 and 0.69 U/mL, so it can potentially

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be assumed that after more than one dose, all dogs would be appropriately anticoagulated. This further demonstrates the need for future studies in a higher number of dogs, and also that there is potentially a need for a loading dose of enoxaparin in dogs to reach peak drug concentration and maximum effectiveness. It would be maximally beneficial to continue to study the use of the Sonoclot® compared to the anti-factor Xa assay in this type of future research incorporating more dogs and using a loading dose to finally determine if the Sonoclot® is a potential replacement for anti-factor Xa as, according to our study, at this time it does not appear to be a sufficient replacement.

It was evident through this research that while 6 dogs can be an acceptable number to start with for evaluating the pharmacokinetic aspects of a drug, the level of pharmacodynamic variability between each dog can lead to some inconsistencies when such a small number of animals is used. As previously discussed, when there were some dogs that were non-responders to the medication in one of the phases of our study, it necessitated having to remove that data in order to perform the data analysis. This had an overall effect on the final data. Therefore, in the future, a higher number of dogs should ideally be used when performing dose optimization studies and pharmacokinetic analysis of enoxaparin in order to ensure findings can be applied to the general dog population.

We also only used healthy dogs from the same breed (Walker Hounds) in this study. While this is justifiable due to cost and availability of research dogs at hand, there have been other studies of enoxaparin in healthy dogs of one type of breed that yielded varying results between different breeds[1,3], and therefore one valid question is how much does the breed of the dog have an effect on the results? One study looked only at enoxaparin pharmacokinetics in greyhounds.[1] Greyhounds have known clinical pathologic differences when compared to non-

greyhound dogs, including a higher hematocrit percentage, a higher hemoglobin concentration, a higher glomerular filtration rate, and a lower number of circulating platelets. It is unclear at this time the effects those breed-related clinical pathological differences may have on the pharmacokinetic profile enoxaparin.[4] In future studies of enoxaparin effectiveness, it may be beneficial to incorporate a range of breeds of dogs to assess any effect that may have on enoxaparin pharmacokinetics.

In analyzing the results of this study, we also became aware of an apparent lack of dose linearity with enoxaparin. With the increase of the dosage of enoxaparin from 0.8mg/kg to 2mg/kg, and then with a decrease back down to 1.3mg/kg, we saw an increase in the drug half-life at the 2mg/kg dose, but not an overall increase in the length of anticoagulation. This is potentially because the dose increase does not equate to a linear anticoagulant response. This could be due to the fact that an increase in dose may be saturating the means of the body to eliminate the drug. So, while dose increases seemingly increase the level of anticoagulation to concerning levels where the dog is at risk of inadvertent bleeding, the drug is still is effectively out of their system within the 8-hour timeframe. Future studies looking intravenous enoxaparin dosing would be necessary to further evaluate this finding.

While this research showcased the need for further studies looking at optimizing the dose of enoxaparin in dogs and the potential use of the Sonoclot® Analyzer to truly establish if it can replace the currently accepted anti-factor Xa assay as a measurement of anticoagulation, it also identified the need for further research into anticoagulant therapy and monitoring in dogs clinically at-risk of hypercoagulation.[5,6]

As mentioned previously, another subcutaneous dosing enoxaparin study could be pursued with a different study design that incorporated a greater number of dogs, of varying

breeds, that were actually clinically at risk for hypercoagulation. This study would be more arduous on the investigators as it would likely comprise of all client-owned dogs, and the number of patients presenting at a time would determine how quickly the data would be collected, but it would allow a better understanding of the proposed subcutaneous enoxaparin dosage of 1.3mg/kg q8hrs, and if the change in signalment of the dogs studied had an overall effect on the efficacy of enoxaparin. This would be an ideal future study to continue to compare the use of the Sonoclot® with the anti-factor Xa assay with these greater numbers of clinical dogs to fully assess if the Sonoclot® is an effective bed-side monitoring tool with these patients.

Still focusing on enoxaparin and its ideal dosing regimen, based on our results indicating that a small portion of dogs were not adequately anticoagulated after a single dose of enoxaparin, but after the second dose nearly all dogs were within the anti-factor Xa target range of 0.5-1.0U/mL, it would be ideal to perform an intravenous enoxaparin study. This could be performed first with a healthy research dog population to determine initial results. The goal of this study would be to investigate the possibility of needing a loading dose of enoxaparin to immediately achieve the appropriate anticoagulation, and then to also see if by dosing enoxaparin intravenously, if it would ultimately lead to a lower amount of drug needed over the course of hospitalization. As seen in our data, there currently does not appear to be dose linearity with enoxaparin given subcutaneously, but if there is a change in bioavailability when dosed intravenously, it may be that not only the dogs are anticoagulated sooner, but then the dose can be lowered overall to maintain anticoagulation, which could potentially keep total costs down for owners in the long term.

Finally, another direction that should be investigated in the future for anticoagulant therapy in dogs that were clinically at risk of thrombus formation would be to shift focus to a

different anticoagulant altogether. There have been few studies into the use of direct factor Xa inhibitory drugs in dogs, but the studies that do exist on the surface appear promising. A recently published study performed in dogs with primary IMHA in hospital utilized a once daily oral dosage of rivaroxaban (a direct Xa inhibitory drug), in 12 clinical dogs.[7] This study focused solely on the comparison of rivaroxaban results with other dogs given clopidogrel and low-dose aspirin. Overall the dogs showed no significant changes compared to the other groups, with no unintended bleeding or side effects attributed to rivaroxaban. However, the researchers only monitored PT/aPTT for changes in prolongation in coagulation, and only focused on a small number of dogs. Due to this, their results are not helpful in showing efficacy of rivaroxaban[7], however further research into this drug protocol could be extremely beneficial. Future research regarding this drug could focus on the currently accepted oral dosage but incorporate pharmacodynamic monitoring using anti-factor Xa assays and/or viscoelastometry to fully determine its effectiveness. This medication showing successful utilization in dogs at risk for thrombus formation could lead to a major change in protocol as oral medication is typically much easier on owners to continue post-hospitalization and, additionally, rivaroxaban is much more affordable than enoxaparin, potentially leading to better owner compliance upon taking their dogs home.

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