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Anticoagulant Activity of Inhaled Heparin in the Dog

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Respiratory disease represents an important component of small animal emergency medicine. The morbidity and mortality of respiratory disease and inflammation, although poorly defined, is considered to be significant. Much of the therapy used in the stabilization and management of respiratory disease in veterinary patients has been taken from human medicine, including inhalation therapy. Heparin has been shown to have substantial anticoagulant, anti-inflammatory, and anti-fibrotic effects within the lungs when administered via inhalation in human patients. To date, no studies have evaluated the use of nebulized heparin in dogs. This study is the first to attempt to generate pharmacokinetic data regarding nebulized unfractionated heparin in the dog.

DEDICATION

I would like to dedicate this research to my parents because they always supported me in the long path of becoming a veterinarian.

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CHAPTER I

INTRODUCTION

Respiratory distress is a common reason for presentation in small animal emergency and critical care medicine, and is associated with a significant mortality rate. The exact percentage of veterinary patients presenting in respiratory distress is poorly defined. Animals can present with true acute respiratory disease or an acute manifestation of chronic respiratory disease. Determining whether the animal has primary or secondary lung disease is important to aid in tailoring medical intervention and therapy to optimize a successful outcome for the patient.

Common causes of respiratory distress in small animal medicine include thoracic trauma, pneumonia (bacterial, viral, fungal, parasitic), interstitial lung diseases, hemodynamic diseases (coagulopathies, anemia, thromboembolic disease), upper airway obstructions, neoplasia, or respiratory manifestations of systemic disease. Regardless of the primary cause, as more of the lung becomes compromised, secondary inflammation can develop in the lungs, triggering acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). In both syndromes, noncardiogenic pulmonary edema develops secondary to pulmonary vascular endothelial or alveolar epithelial injury.[1] ARDS is considered a more severe manifestation of ALI on the basis of the degree of hypoxemia involved. Human and veterinary patients both tend to succumb to ALI/ARDS within two weeks of developing the condition.

Very little clinical or evidence-based medicine regarding the development and treatment of ALI/ARDS in veterinary medicine exists. Current therapy is contingent on early recognition of the disease process, is mostly based on supportive care, and is focused on survival rather than prevention of long-term sequelae. Severe inflammation, both in the respiratory tract as well as systemically, is a major contributing factor to morbidity and mortality. Therapy is directed at trying to control the underlying disease process as well as to limit the inflammatory response and inhibit fibrosis that may result in impairment of pulmonary function.

Various treatment options have been tried and are being evaluated in human medicine for the management and treatment of ALI/ARDS. Inhaled medications are gaining popularity for their ability to act locally in the lung without displaying marked systemic effects. One anti-inflammatory and anti-fibrotic treatment option that has shown promise in human ALI/ARDS is inhaled heparin. In veterinary patients, the use of heparin in an inhaled manner has not been fully evaluated. It has been shown that unfractionated heparin exhibits similar pharmacokinetic properties in both humans and dogs when administered subcutaneously.[2] The same parallel activity would be expected when heparin is administered via an inhaled route in the canine patient.

Acute Lung Injury/Acute Respiratory Distress Syndrome

ALI and ARDS have only recently been recognized as distinct disease entities in veterinary medicine. Specific risk factors have been documented in veterinary patients for the development of ALI/ARDS. In dogs, the most common associated diseases include bacterial pneumonia, aspiration pneumonia, sepsis, and shock.[3] Primary lung disorders that have resulted in the development of ARDS in dogs include lung lobe

torsions, smoke inhalation, pneumonia (bacterial, aspiration, parasitic), strangulation, pulmonary contusions, and hyperoxia.[4] Secondary disorders that have resulted in ARDS in dogs include babesiosis, pancreatitis, gastric and splenic torsion, sepsis, shock, disseminated intravascular coagulation (DIC), and parvovirus.[4] In cats, ARDS risk factors are harder to define. ARDS has been suspected based on pulmonary necropsy findings of septic feline patients.

The pathophysiology of ALI/ARDS has been documented in human medicine, and the same pathophysiology is thought to occur in the small animal patient. ARDS is thought to occur in three phases. The three phases are not distinct, but rather an overlapping process of continued damage. The first phase, the exudative phase, is characterized by increased pulmonary vascular permeability and infiltration by inflammatory cells, resulting in the development of noncardiogenic edema. Type I pneumocytes are irreversibly damaged. Type II pneumocytes replace the lost type I pneumocytes and attempt to repair damage locally. In doing so, the stressed type II pneumocytes decrease their production of surfactant. Surfactant deficiency results in hyaline membrane formation and decreased oxygen exchange at the level of the alveoli. Vascular endothelial damage results in local thrombosis and alveolar collapse.[4] In human patients with ARDS, this phase lasts approximately 7 days.[5]

The second phase of ARDS is the proliferative phase, in which the exudates from phase I organize and fibrosis develops. Type II pneumocytes and fibroblasts proliferate in an attempt to repair the on-going damage and inflammation. Initially, these changes take place in the pulmonary interstitium. As the disease process progresses, the alveolar lumen then becomes abnormal. These proliferative changes result in narrowing and,

ultimately, collapse of the airways and airspaces.[6] As more of the lung succumbs to the disease, pulmonary hypertension ensues.[5] An end-stage result of pulmonary hypertension in some ALI/ARDS patients is right-sided heart failure.

In the final phase, termed the fibrotic phase, collagen deposits in the alveolar, vascular, and interstitial beds. Microcysts, small cystic structures lined by pulmonary epithelium and filled with fluid and debris, develop in the lungs. In human patients, the amount of fibrosis that develops is thought to be a key predictor of survival of ARDS.[7] Interestingly, in people who survive ALI/ARDS, pulmonary function can return to normal in 6-12 months.[8] In veterinary medicine, little is known about this phase or the long-term effects of ALI/ARDS due to the high mortality rate in the earlier phases.[4]

Specific criteria for the diagnosis of ALI/ARDS have now been defined for veterinary patients. Five categories of criteria were established for the diagnosis of ARDS in veterinary patients. A patient must have at least 4, though ideally all 5, of the criteria to be diagnosed clinically with ARDS. The five criteria include: 1) acute onset of tachypnea/labored breathing at rest, greater than 72 hours in duration; 2) known risk factors for the development of ARDS; 3) evidence of pulmonary capillary leak without increased pulmonary capillary pressure, such as bilateral/diffuse infiltrates on thoracic radiographs or CT; 4) evidence of inefficient gas exchange as determined by the ratio of arterial oxygen tension to fractional inspired oxygen concentration (PaO_2:FiO_2), for ALI and ARDS, the PaO_2:FiO_2 is less than 300 mmHg and 200 mmHg, respectively; 5) evidence of diffuse pulmonary inflammation as based on an airway wash sample.[1]

Multiple therapies have been evaluated for the treatment of ALI/ARDS in human medicine. There is no single best treatment option; rather, a multifactorial approach is

often necessary for a successful outcome. Ventilatory support, in combination with drug therapy, provides the best survival results by allowing time for the damaged lung to recover. The treatment options that have been evaluated and that are most promising are based on the specific cytokines that have been implicated in the development and progression of ARDS. Such cytokines include tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), tumor growth factor-β (TGF-β), interleukin-6 (IL-6), platelet-activating factor, CXC chemokine ligand (IL-8), eicosanoids, and interleukin-10 (IL-10).[9] Some of these cytokines are primarily pro-inflammatory, and some are anti-inflammatory. Controlling the activity of these cytokines is difficult because of cross-over in their activity depending on the stage of ARDS. Some of the drugs that have been attempted in ARDS include albumin, corticosteroids, cyclooxygenase inhibitors, N-acetylcysteine, pentoxifylline, procysteine, prostaglandin E1, and surfactant. None of those drugs has been shown to significantly affect morbidity or mortality in human ARDS to date.[10] Many of the same or similar drugs have been used in experimental models of ARDS in dogs and cats with the same limited effects.[4] Because of the lack of positive response to therapy and substantial systemic side effects, inhalation therapy and immunomodulatory therapy have become major areas of investigation in the management of ALI/ARDS in both human and veterinary medicine.

Inhalation Therapy

Inhalation therapy has been the subject of intense research in the field of human respiratory medicine. Inhalation therapy is also becoming increasing more popular in veterinary medicine as more is learned from human medicine and research. The lung is an important target for administration of medications because of several key features.

The alveolar surface area of the lung is large, providing a huge area for absorption. In humans, the size of the alveolar surface varies between 80-140 m^2 depending on the level of distension of the lung.[11] The lung also has a thin alveolar epithelial surface of 0.1-0.2 μm in thickness.[12] Substances deposited in the lung have to travel a shorter distance between the epithelial surface and blood then in other areas of the body (for example, the intestinal epithelium) for absorption. The proteases and peptidases in the alveolar region of the lungs are in lower quantities and activity than in other areas of the body such as the gastrointestinal tract, meaning that the inhaled drugs will undergo slightly less proteolytic degradation.[13]

There are several factors that must be overcome when utilizing the lungs as a delivery vehicle for medications. The natural defense mechanisms of the upper airways and bronchi consist of the mucociliary apparatus, epithelium, and lymphoid structures. Particles of 2-3 μm or larger that are inhaled will primarily be deposited on the mucus of the mucociliary apparatus, transported to the oropharynx, and swallowed.[14] The presence of inflammation, fluid, increased mucus, or fibrosis will also alter the ability of the lung to absorb or clear particles. In the lower airways, the cell type encountered by the particle may determine if the particle is absorbed or not. The lower airways are lined by macrophages and lymphocytes with sparse neutrophils. Macrophages are normally the only phagocytic cells encountered in the lower airways, although neutrophils and lymphocytes can be induced to phagocytize particulate matter that enters the lower airways. A substantial portion of any substance administered in an inhaled fashion will be lost to the above defense mechanisms, leaving only a small amount to be absorbed for local or systemic effects.

The method of delivery is a major factor in the amount of absorption and bioavailability of inhaled drugs. Three major categories of delivery devices have been developed for aerosol therapy. The first category includes nebulizers. Nebulizers are available in three basic types: air-jet nebulizers, ultrasonic nebulizers, and vibrating plate nebulizers. Air-jet nebulizers utilize a compressor that forces compressed air or oxygen through a liquid substance, aerosolizing the liquid. With this type of nebulizer, the structure of the drug can be altered by denaturation, shear-stress, or desiccation. A large portion of the drug, as much as 95-99%, is recycled within the nebulization chamber before ever even leaving the chamber, requiring long inhalation times for full delivery of a specific dose.[11] Ultrasonic nebulizers work by an electronic oscillator that generates a high frequency ultrasonic wave on a vibrating element, disrupting the liquid surface of the medication which aerosolizes the liquid in high concentration. These nebulizers work best for larger substances that are not intended for deep lung delivery. Vibrating plate nebulizers function by the use of a fine, vibrating mesh overtop a liquid reservoir. The liquid is forced through the mesh, resulting in the production of a high fine-particle aerosol. The vibrating plate nebulizers are not as efficacious for liposomal formulations or suspensions of drug.[11]

The second category of aerosol delivery device is the metered dose inhaler (MDI). MDIs are composed of set amounts of dissolved or suspended drug in a pressurized propellant, most often chlorofluorocarbons. Spacing devices are often necessary with MDIs as the aerosol is expelled with high velocity. These devices are convenient because of their light weight and portability. However, for a drug to be used in an MDI, it must meet specific requirements regarding particle size, stability within the device and

propellant, and ability to withstand the nebulization process on expulsion. These requirements often limit the utility of the device for most drugs. MDIs are most commonly used in the management of asthmatic patients or patients with chronic obstructive pulmonary disease.

Powered aerosols are the third method of delivery for inhalation therapy. Powered aerosols are composed of preformed particles of a drug. The drug is stored as a dried powder in a vehicle device. The energy necessary for dispelling the drug from the device comes from the patient's own inhalation. A patient must be able to fully inhale for the drug to reach the deeper sections of the lung. The advantages of powdered aerosols include stability of the drug, ease of use, portability, and often decreased cost. Disadvantages include dependency on the patient's ability to fully inhale, discrepancies in consistent dosing, and susceptibility of the drug/device to environmental conditions such as humidity. Drugs currently available and established as powdered aerosols include steroids, β-mimetics, anticholinergics, antihistamines, and insulin. Powdered aerosols have more limited use in veterinary medicine because of the reliance on the patient fully inhaling the drug for dosing.

Once deposited in the alveolar space, a medication can be absorbed by four different mechanisms: phagocytosis by alveolar macrophages, diffusion through tight junctions, vesicular endocytosis or pinocytosis, and receptor-mediated transcytosis. The size of the particle often determines the mechanism utilized.[15] Previous studies have shown that for particles up to 30,000 Da, only 20-50% of the total amount of drug delivered to the alveoli will be available for systemic absorption and activity.[16,17] A substantial portion of the drug will be lost to the mucociliary epithelium on inhalation.

The remaining portion will be subject to phagocytosis by the alveolar macrophages before coming in contact with pneumocytes. Most of the drug absorption occurs at the level of the type I and II pneumocytes. Tight junctions located between the pneumocytes regulate the transport of small particles, fluid, and ions. The tight junctions in the lungs will accommodate diffusion of particles up to approximately 12 kDa.[11] Vesicular transport also occurs at the level of the pneumocytes. In type I pneumocytes, vesicular transport can occur independent of hydrostatic or oncotic pressure gradients, allowing the transport of both fluid and larger molecules. Type II pneumocyte vesicles are more dependent on receptors and binding proteins for absorption of molecules.

Inhaled drug therapy was originally evaluated for the beneficial local effects observed within the lungs. Early studies in inhalation therapy often focused on the maximum amount of drug that could be administered without the development of systemic side effects. More recently, investigations in inhalation therapy have focused on the use of inhaled drugs to alter the respiratory environment for increased efficacy of systemic medications as well as the use of medications in an aerosolized or nebulized manner for systemic absorption and activity.

Heparin Therapy for Respiratory Disease

Heparin is an acidic, sulfated mucopolysaccharide that is widely used for its anticoagulant properties. In the body, heparin is found in tissues with large amounts of glycosaminoglycans such as the lungs and the intestinal tract. Heparin is stored in the secretory granules of mast cells as well as in basophils. The most commonly used formulation of heparin is unfractionated heparin, or heparin sodium. Heparin enacts its anticoagulant activity by inhibiting the activated forms of factors II (thrombin) and X.

Heparin binds to plasma antithrombin, causing a conformational change in antithrombin. This conformational change is necessary for the inhibition of the target enzymes, activated factors X and IX (Xa and IXa), as well as for the formation of a complex of heparin, antithrombin, and thrombin. This complex formation results in inhibition of thrombin. Additional anticoagulant activities of heparin include its ability to reduce the activity of factors V and VIII as well as inhibit platelet function by binding to platelet factor 4. Other known activities of heparin include anti-inflammatory and immunomodulatory effects such as inhibition of leukocyte recruitment, neutrophil chemotaxis, neutrophil elastase, and numerous chemokines.[18,19]

Low-molecular-weight-heparin (LMWH) derivatives are also available for use as anticoagulants. Current LMWHs include enoxaparin, dalteparin, certoparin, and tinzaparin. They differ from unfractionated heparin in several important ways. LMWHs are smaller in size, averaging between 4,000 – 6,000 Da, where unfractionated heparin is approximately 15,000 Da.[20] LMWHs also have significantly reduced thrombin activity.[21] LMWHs also have a longer plasma half-life and higher bioavailability then unfractionated heparin. LMWHs target anti-Xa activity rather then anti-thrombin activity, resulting in a lower rate of side effects and a more predictable dose-response relationship. LMWHs have become the mainstay of therapy for venous thrombosis, while unfractionated heparin is the drug of choice for the acute management of arterial thrombosis. For both unfractionated heparin and the LMWHs, the overall negative charge and large size of the compounds prevent them from being absorbed when administered orally. Therefore, heparin and its derivatives are most often administered subcutaneously. Dosing intervals depend on the half-life of the drug. Unfractionated

heparin has a half-life in plasma of approximately 1-2 hours, resulting in the need for multiple injections to sustain the anticoagulant effects for a 24 hour period. The LMWHs have a half-life of approximately 4-5 hours and can be administered less frequently for sustained effect.[22]

The administration of unfractionated heparin and the LMWHs via an inhaled route has been evaluated for both local anticoagulant and anti-inflammatory properties as well as systemic anticoagulant effects. It has been determined that comparable blood levels of both heparin and the LMWHs can be achieved when administered via deep inhalation or given subcutaneously. Additionally, when administered via deep inhalation, the heparin products have a more rapid onset of action.[21] The dose of unfractionated heparin that will result in systemic anticoagulant effects as determined by prolongation of partial thromboplastin time (PTT) and anti-Xa activity when inhaled is 150,000 IU.[23] The dose of unfractionated heparin that will result in clinical bleeding when inhaled is greater than 400,000 IU.[24] For the LMWH certoparin, an inhaled dose of 9,000 IU will result in plasma anti-Xa levels greater than 0.2 U/mL, which is the level used for the prophylaxis of venous thrombosis.[20] For unfractionated heparin and the LMWHs, inhaled dosing has not been found to result in a marked plasma peak following administration. When the LMWH products are altered into liposomes or microspheres, their resultant activity after inhalation can be prolonged, necessitating fewer administrations for the same systemic anticoagulant effects.[25,26]

The local activity of heparin after inhalation has been evaluated in the face of numerous respiratory diseases and conditions. Heparin has been shown to exert profibrinolytic effects in the lung via inactivation of plasminogen activator inhibitor-1

(PAI-1) when administered via nebulization; the same effects in the lungs were not noted when heparin was administered systemically.[27] Unfractionated heparin may enhance airway clearance by increasing sputum expectoration.[28] These findings have been elucidated in models of human cystic fibrosis, pulmonary fibrosis, and COPD. Heparin appears to increase the absorption and activity of itself and other nebulized drugs by stimulating opening of the tight junctions of the pneumocytes in a transient and reversible fashion.[21] In a model of ALI and smoke inhalation in sheep, concomitant administration of nebulized unfractionated heparin and antithrombin has been shown to prevent the formation of fibrin casts.[29] When administered with *N*-acetylcysteine via nebulization, unfractionated heparin has resulted in reduced pulmonary failure, atelectasis, and mortality in children with massive burn injury and smoke inhalation.[30] In asthma models, the LMWHs cause a reduction in allergen-induced bronchoconstriction and an inhibition of the influx of inflammatory cells, especially eosinophils, to the lower airways.[31] In a model of early ALI, nebulized heparin appeared to make modest improvements in pulmonary parenchymal inflammation, interstitial edema, microvascular thrombosis, alveolar fibrin deposition, and fluid accumulation. However, these findings were not statistically significant and the study population was small.[32] Heparin and the LMWH derivatives are able to exert their anticoagulant and anti-inflammatory properties without substantial local side effects in regards to lung function and compliance. It also appears that with chronic administration of inhaled heparin, the dose administered can be decreased while still maintaining steady-state activity in the alveolar space.[23]

Although the vast majority of the research that has been done in regards to inhaled heparin therapy has utilized animal models, the disease conditions investigated have been

simulated to mimic human respiratory disease states. The animal models used have most frequently been sheep, rabbits, mice, and rats. Canine models have been infrequently used considering the similarities in heparin activity between humans and dogs. Scant research has been done to establish the use, pharmacokinetics, and activity of heparin when administered via inhalation or nebulization in the dog as a potential therapeutic agent for canine patients. Inhaled heparin could serve as a novel therapy for respiratory diseases of both dogs and cats. The research presented in the following section of this manuscript reflects a preliminary step in developing an understanding of the use of inhaled heparin in the healthy canine lung. Once the pharmacokinetics of inhaled heparin have been established in the healthy dog, the use of inhaled heparin in different canine respiratory and systemic disease states can be evaluated.

References Cited

1. Wilkins P, Otto C, Baumgardner J, et al. Acute lung injury and acute respiratory distress syndromes in veterinary medicine: consensus definitions: The Dorothy Russell Havemeyer Working Group on ALI and ARDS in Veterinary Medicine. *J Vet Emerg Crit Care* 2007; 17(4):333–339.

2. Mischke R, Schuttert C, Grebe S. Anticoagulant effects of repeated subcutaneous injections of high doses of unfractionated heparin in healthy dogs. *Am J Vet Res* 2001; 62(12):1887-91.

3. Parent C, King L, Walker L, et al. Clinical and clinicopathologic findings in dogs with acute respiratory distress syndrome: 19 cases (1985-1993). *J Am Med Vet Assoc* 1996; 208:1419-1427.

4. DeClue A, Cohn L. Acute respiratory distress syndrome in dogs and cats: a review of clinical findings and pathophysiology. *J Vet Emerg Crit Care* 2007; 17(4):340-347.

5. Bellingan G. The pulmonary physician in critical care 6: the pathogenesis of ALI/ARDS. *Thorax* 2002; 57:540-546.

6. Anderson W, Thielen K. Correlative study of adult respiratory distress syndrome by light, scanning, and transmission electron microscopy. *Ultrastruct Pathol* 1992; 16:615-628.

7. Martin C, Papazian L, Payan M, et al. Pulmonary fibrosis correlates with outcome in adult respiratory distress syndrome. A study in mechanically ventilated patients. *Chest* 1995; 107:196-200.

8. Ware L, Matthay M. The acute respiratory distress syndrome. *N Engl J Med* 2000; 342(20):1334-1349.

9. Campbell, V. Respiratory complications in critical illness of small animals. *Vet Clin Small Anim* 2011; 41:709-716.

10. Adhikari N, Burns K, Meade M. Pharmacologic therapies for adults with acute lung injury and acute respiratory distress syndrome. *Cochrane Database Syst Rev* 2004; 4: CD004477.

11. Siekmeier R, Scheuch G. Systemic treatment by inhalation of macromolecules – principles, problems, and examples. *J Physiol Pharmacol* 2008; 59(6):53-79.

12. Patton J, Byron P. Inhaling medicines: Delivering drugs to the body through the lungs. *Nat Rev Drug Discov* 2007; 6:67-74.

13. Byron P, Patton J. Drug delivery via the respiratory tract. *J Aerosol Med* 1994; 7:49-75.

14. Nicod L. Pulmonary defense mechanisms. *Respiration* 1999; 66:2-11.

15. Niven R. Delivery of biotherapeutics by inhalation aerosol. *Crit Rev Ther Drug Carrier Syst* 1997; 14:395-453.

16. Wolff R. Safety of inhaled proteins for therapeutic use. *J Aerosol Med* 1998; 11:197-219.

17. Patton J, Fishburn C, Weers C. The lungs as a portal of entry for systemic drug delivery. *Proc Am Thorac Soc* 2004; 1:338-344.

18. Tyrell D, Horne A, Holme K et al. Heparin in inflammation: potential therapeutic applications beyond anticoagulation. *Adv Pharmacol* 1999; 46:151-208.

19. Matzner Y, Marx G, Drexler R et al. The inhibitory effect of heparin and related glycosaminoglycans on neutrophil chemotaxis. *Thromb Haemost* 1984; 52:134-137.

20. Scheuch G, Brand P, Meyer T, et al. Anticoagulative effects of the inhaled low molecular weight heparin certoparin in healthy subjects. *J Physiol Pharmacol* 2007; 58(5):603-614.

21. Yiwei Q, Zhao G, Liu D, et al. Delivery of therapeutic levels of heparin and low-molecular-weight heparin through a pulmonary route. *PNAS* 2004; 101(26):9867-9872.

22. Eikelboom J, Hankey G. Low molecular weight heparins and heparinoids. *Med J Aust* 2002; 177(6):379-383.

23. Markart P, Nass R, Ruppert C, et al. Safety and tolerability of inhaled heparin in idiopathic pulmonary fibrosis. *J Aerosol Med* 2010; 23(3):161-172.

24. Kohler D. Aerosolized heparin. *J Aerosol Med* 1994; 7:307-314.

25. Rawat A, Majumder Q, Ahsan F. Inhalable large porous microspheres of low molecular weight heparin: In vitro and in vivo evaluation. *J Control Release* 2008; 128:224-232.

26. Bai S, Ahsan F. Inhalable liposomes of low molecular weight heparin for the treatment of venous thromboembolism. *J Pharm Sci* 2010; 99(11):4554-4564.

27. Hofstra J, Cornet A, de Rooy B, et al. Nebulized antithrombin limits bacterial outgrowth and lung injury in *Streptococcus pneumoniae* pneumonia in rats. *Crit Care* 2009; 13(5):R145.

28. Ledson M, Gallagher M, Hart C, et al. Nebulised heparin in *Burkholderia cepacia* colonized adult cystic fibrosis patients. *Eur Respir J* 2001; 17:36-38.

29. Enkhbaatar P, Esechie A, Wang J, et al. Combined anticoagulants ameliorate acute lung injury in sheep after burn and smoke inhalation. *Clin Sci (Lond)* 2008; 114:321-329.

30. Toon M, Maybauer M, Greenwood J, et al. Management of acute smoke inhalation injury. *Crit Care Resusc* 2010; 12:53-61.

31. Duong M, Cockcroft D, Boulet L, et al. The effect of IVX-0142, a heparin-derived hypersulfated disaccharide, on the allergic airway responses in asthma. *Allergy* 2008; 63:1195-1201.

32. Dixon B, Santamaria J, Campbell D. A phase 1 trial of nebulised heparin in acute lung injury. *Crit Care* 2008; 12:R64.

CHAPTER II

PHARMACOKINETICS OF INHALED HEPARIN IN THE DOG

Introduction

Inflammation, both systemically and locally, is a major contributing factor to morbidity and mortality for many diseases in both human and veterinary medicine. Therapy is typically directed at controlling the underlying disease process, while concurrently limiting the inflammatory response and inhibiting the fibrosis that may subsequently develop. Although more commonly considered an anticoagulant therapy, heparin (including unfractionated heparin and low-molecular weight heparin derivatives) has anti-inflammatory properties, and is a mainstay in the treatment of certain inflammatory conditions in people, including rheumatoid arthritis, asthma, and inflammatory bowel disease.[1] Besides high affinity binding to antithrombin, which greatly enhances antithrombin's anticoagulant properties, heparin has potential to bind many other proteins, including pro-inflammatory molecules.[2,3] The mechanisms responsible for the anti-inflammatory effects of heparin are not completely understood, but are thought to include neutralization of the release of pro-inflammatory mediators from inflammatory cells, binding to chemokines, cytokines, and complement factors, prevention of interactions with pro-inflammatory receptors, and inhibition of several endothelial adhesion molecules.[4,2,1,5] Numerous studies in veterinary medicine have

evaluated the use of heparin in inflammatory conditions; however, this medication is still primarily used for its anticoagulant properties.

In both human and veterinary medicine, heparin is most commonly administered via an intravenous or subcutaneous route. With the administration of heparin via injection, the systemic effects are significant. Unfortunately, especially for unfractionated heparin, the duration of action is short lived, requiring either frequent injections or continuous rate infusions. The anti-inflammatory and anti-fibrotic benefits of heparin administered via an inhaled route are currently being evaluated in human patients, specifically for use in the management of acute respiratory distress syndrome (ARDS), acute lung injury (ALI), and venous thromboembolism.[6,7] The use of inhaled heparin in human patients with chronic lung diseases such as pulmonary fibrosis, asthma, and cystic fibrosis has also been evaluated.[8,9,10] Inhaled heparin appears to have a longer duration of action when compared to the subcutaneous route.[3] The inhaled route of administration may also have a more rapid onset of systemic anticoagulant activity.[11]

Unfractionated heparin exhibits similar pharmacokinetic properties in both humans and dogs.[12] The dosage for subcutaneous administration of unfractionated heparin is similar for both dogs and humans, at 200 U/kg.[13] Additionally, in both species, there is a wide range of inter-individual variation in heparin's pharmacokinetic and pharmacodynamic profile. This variation has resulted in the recommendation of treating patients with an individually adjusted heparin dose.[14,15] In all previous studies, the pharmacokinetics of heparin in dogs have been evaluated following administration via an intravenous or subcutaneous route. Only one study has evaluated the use of heparin in an inhaled manner in dogs. That study evaluated the effects of inhaled heparin on

bronchoconstriction in the canine model.[16] The local and systemic anticoagulant effects of inhaled heparin in the dog have not been fully evaluated.

Recently, several studies have evaluated the use of inhaled heparin in rabbits and humans. In a study using rabbits, administration of heparin via the respiratory tract produced blood levels comparable to those achieved via the subcutaneous route.[11] In humans, a recent study determined the threshold dose and tolerability of inhaled unfractionated heparin in healthy adults and patients with idiopathic pulmonary fibrosis. Both groups were administered a heparin dose of 150,000 IU via inhalation. Despite evidence of systemic effects (prolonged partial thromboplastin time and increased anti-factor Xa activity), no serious adverse events were noted. Additionally, based on bronchoalveolar lavage fluid (BALf) analysis, the local anticoagulant effect persisted as long as 72 hours after heparin administration. This local effect is considerably longer than the systemic effect as indicated by the increase in the plasma anti-Xa activity.[8]

The goals of this study were to establish the threshold dose of inhaled heparin for systemic effects in healthy dogs, and to generate pharmacokinetic data for inhaled heparin. The main hypothesis was that the effects of inhaled heparin would be prolonged when compared to the systemic anticoagulant effects, that the threshold dose for spillover of anticoagulant effects from the alveolar space to the plasma would be similar to that seen in people, and that inhaled heparin would exert effects on canine BALf similar to those observed in humans.

Materials and Methods

Animals

Six healthy female intact Walker hounds were used in the study. The mean age of the dogs was 3 years (range 2 to 5 years), and their mean body weight was 25.8 kg (range 20.2 to 68.6 kg). Body weight was obtained at the beginning of the study and used to calculate all subsequent doses. The dogs were not exposed to any medications or vaccines for at least 1 month before initiation of the study. Prior to beginning the study, the dogs underwent a health screen that included complete blood counts with differential, serum chemistry panel, prothrombin time (PT), activated partial thromboplastin time (aPTT), occult heartworm testing and thoracic radiographs. The dogs were acclimated to the face masks that were used in the study to administer the heparin over a period of 3 weeks prior to beginning data collection. Animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee, and was in compliance with the requirements at a facility accredited by the American Association for Accreditation of Laboratory Animal Care.

Assay Development

Two dogs were anesthetized for the collection of BALf. This fluid was utilized in the generation of a calibration curve which would relate the clotting time endpoint in the aPTT assay to corresponding heparin concentration (IU/mL) in BALf. Anesthesia was induced and maintained using acepromazine[a] (0.02 mg/kg IM), butorphanol[b] (0.2 mg/kg IM), and propofol[c] (5 mg/kg IV). An 8 mm sheath diameter flexible endoscope[d] was used to evaluate the airways for any gross abnormalities prior to sample collection. The endoscope was then used to guide collection of BALf samples from the right middle lung

lobe. The lung lobe was lavaged with a total volume of 60 milliliters of sterile saline[e] in three 20 milliliter aliquots. The fluid collected was pooled, filtered through sterile gauze, centrifuged at 200*x*g for 10 minutes at 4ºC to remove cells, and frozen at -40ºC prior to analysis. Additionally, plasma was collected into sodium citrate[f] from healthy dogs to act as a control and to be used in the assessment of heparin activity. Samples were stored in plastic sample vials and shipped on dry ice to Cornell University's Comparative Coagulation Laboratory.

Unfractionated heparin[g] was diluted in BALf from two individual dogs in increasing increments of 0.125 IU of heparin/mL of BALf, through a range of 0 IU/mL to 1.0 IU/mL heparin. The heparinized BALf dilution was combined with an equal volume of pooled canine plasma as a source of clotting factors and fibrinogen, and an aPTT was performed on the reaction mixture. As a control, unfractionated heparin was added to imidazole buffered saline (pH 7.4) through the same dilution series and then combined with plasma. The aPTT assessment on the collected BALf samples was measured similar to routine plasma aPTT measurement. Briefly, 75 µL of BALf or heparin standard solution was added to 75 µL of pooled citrated canine plasma from healthy donors and 100 µL of Actin® FS reagent[h]. Samples then were incubated for 3 minutes at 37ºC, and clotting was initialized by adding 100 µL of $CaCl_2$[i] (0.025 M). Heparin activity was then calculated based on a standard curve generated for this project and reported in International Units (IU)/mL.

Threshold Dose

Two different dogs were used to determine the threshold dose of inhaled heparin that would prolong systemic aPTT. A face mask was placed around the muzzle of each

dog, and 50,000 IU of sodium heparin was administered via inhalation using an ultrasonic nebulizer[j]. The appropriate heparin dose was combined with sterile saline to a total of 15 mLs, loaded into the nebulizer, and administered for 15 minutes. Following heparin administration, blood was collected via jugular venipuncture and placed in a 3 mL glass vacutainer tube containing 3.2% sodium citrate for immediate aPTT evaluation. The aPTT was assessed with a standard bead coagulometer[k] according to the manufacturer's specifications. If the aPTT was not prolonged outside the normal range, each dog was administered additional heparin via inhalation, in increments of 10,000 IU. Following each dose increase, blood was collected for immediate systemic aPTT evaluation. This dose escalation continued until either the systemic aPTT was prolonged, or a total heparin dose of 200,000 IU was administered. The total heparin dose was determined based on the maximum total volume the nebulizer trough would hold.

In order to absolutely ensure direct delivery of the heparin to the pulmonary vasculature, the same two dogs were anesthetized, intubated, and administered inhaled heparin via the endotracheal tube. Each dog was placed under general anesthesia using the previously mentioned anesthetic protocol, and administered inhaled sodium heparin with the same ultrasonic nebulizer in a reverse dose escalation fashion. A reverse dose escalation was chosen to minimize the anesthetic time while allowing for any systemic prolongation of the heparin to be observed. Positive pressure ventilation was used to aid in delivery of the nebulized heparin to the lower airways. The initial heparin dose was 200,000 IU, and the dosage decreased in 25,000 IU increments, with the lowest administered dose being 175,000 IU. Following 15 minutes of heparin administration,

blood was collected from the jugular vein and placed in 3.2% sodium citrate for aPTT evaluation.

Pharmacokinetics

Two different dogs were placed under general anesthesia using the previously mentioned anesthetic protocol. As performed in Phase I, an 8 mm endoscope was used to evaluate the airways for gross abnormalities and conduct a bronchoaveolalar lavage of the right middle lung lobe using 60 mLs of saline (three 20 mL aliquots). The collected BALf was processed and stored in the same fashion as described in Phase I. After the collection of this initial BALf, the ultrasonic nebulizer was used to administer the heparin. Each dog was administered 200,000 IU of heparin through the endotracheal tube. BALf was then collected 30 minutes post-heparin administration using the previously described collection protocol, and the dogs were recovered from anesthesia. BALf was subsequently collected 24, 48, 72, and 96 hours post-heparin administration, with the dogs re-anesthetized each time using the previously described anesthesia protocol.

The local anticoagulant pulmonary effects of the inhaled heparin were evaluated by analysis of BALf. In the collected BALf samples, the anti-Xa activity and aPTT, as previously described, were assessed. At Cornell, a commercial kit[l] was used to measure anti-Xa activity, which has previously been validated in dogs.[17] The samples were analyzed according to the manufacturer's instructions, including standards and quality control materials. This one-step competitive inhibition assay is configured with a bovine factor reagent and a chromogenic substrate of factor Xa, and does not require exogenous antithrombin. Briefly, 50 μL of test plasma (diluted 1:2 in sample buffer) was added to

125 µL of substrate. Following 4 minute incubation, 125 µL of factor Xa reagent was added to the sample. After a 30 second equilibration period, optical density (OD) readings were taken at 2 second intervals for 60 seconds, and the delta OD of test plasma was converted to Units anti-Xa activity per milliliter (U/mL) by regression on a three point log-linear standard curve derived using calibrators containing known quantities of low-molecular-weight heparin in pooled normal canine plasma. In addition, a canine plasma control sample spiked with unfractionated heparin to contain 0.7 U/mL anti-Xa activity was assayed simultaneously with each run of test samples.

Results

Assay Development

From the two dogs used in this portion of the study, ten mls of BALf was collected from one dog, and eight mls was collected from the second dog. For both dogs, increasing concentrations of heparin in BALf caused increasing prolongation of the aPTT. (Figure 2.1) The heparin-spiked BALf from one dog generated a similar curve to that obtained from heparin diluted in buffer, with close to a 10-fold prolongation at 1.0 IU/mL heparin concentration. The same 1.0 IU/mL concentration of heparin in BALf from the second dog generated an approximately 3-fold prolongation of aPTT. Although the source of BALf influenced the aPTT, spiked BALf from both dogs generated a heparin dose-response curve. The generated curve of heparin activity in the BALf demonstrates that the anticoagulant activity of heparin can be measured in canine BALf.

Threshold Dose

After the initial dose of inhaled heparin, administered via face mask, the systemic aPTT did not prolong in either dog. In fact, even at the maximal dose of 200,000 IU, and at all heparin doses in between, the systemic aPTT did not exceed the normal reference range of the analyzer. With the same dogs anesthetized, and a more direct delivery of heparin to the lower airways, there was no prolongation of the plasma aPTT at any dose, including the maximal dose of 200,000 IU. One dog received the initial 200,000 IU dose, and the second dog received an initial 200,000 IU dose and a second dose of 175,000 IU.

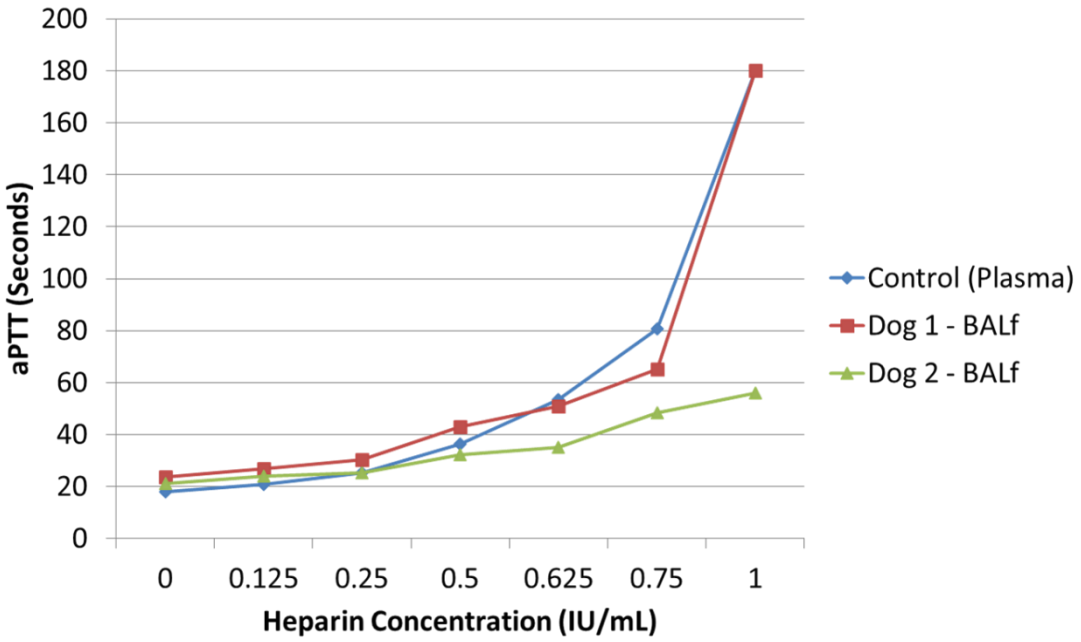

Figure 2.1 Heparin activity (as reflected by activated partial thromboplastin time, aPTT, in seconds) in experimental dog bronchoalveolar lavage fluid (BALf) compared with healthy canine plasma (control). The generated curve demonstrates the activity of heparin can be detected in normal canine BALf in a dose-response relationship.

Pharmacokinetics

The pre-heparin aPTT for BALf from the two dogs were 16.2 and 17.3 seconds, respectively. The aPTT for the control plasma diluted in buffer rather than BALf was 12.1 seconds. After the addition of unfractionated heparin (1 IU/mL) in buffer, the control plasma sample exceeded 180 seconds, the upper limits of the analyzer. The BALf aPTT results did not exceed the establish reference range, at any time point, after the inhalation of unfractionated heparin.

Prior to heparin administration, there was no detectable anti-Xa activity (0 U/mL) in plasma or BALf from either study dog or in the control plasma sample. The assayed anti-Xa activity in the control plasma spiked to contain 1 IU/mL of heparin was 1.2 U/mL. Thirty minutes after heparin inhalation, the anti-Xa activity in BALf from Dog 1 was 0.1 U/mL, the lower limit of assay detection. At all other time points, there was no detectable anti-Xa activity in BALf from either dog. (Table 1) (Table 2.1)

Table 2.1 Anticoagulant activity (aPTT and anti-Xa) in canine bronchoalveolar lavage fluid (BALf)

Sample #	ID	Timepoint	aPTT (sec)	anti-Xa (U/mL)
1	Dog 1	Baseline	16.2	0
2	Dog 1	30 mins	17.3	0.1
3	Dog 1	24 hrs	16.7	0
4	Dog 1	48 hrs	16.2	0
5	Dog 1	72 hrs	16.1	0
6	Dog 1	96 hrs	15.6	0
7	Dog 2	Baseline	17.3	0
8	Dog 2	30 mins	16.7	0
9	Dog 2	24 hrs	16.1	0
10	Dog 2	48 hrs	18.8	0
11	Dog 2	72 hrs	15.9	0
12	Dog 2	96 hrs	16	0
	Canine control plasma:		12.1	0
	Heparin in plasma:		> 180	1.2

Discussion

Most commonly utilized for its anticoagulant properties, unfractionated heparin has been extensively studied for use in numerous conditions in both human and canine patients. Dogs and humans demonstrate similar pharmacokinetics of unfractionated heparin whether administered intravenously or subcutaneously. The administration of heparin via inhalation has rarely been reported in dogs, and the pharmacokinetic profile of inhaled heparin in this species has not been established. In humans, when compared to subcutaneous administration, inhaled heparin has a more rapid onset of systemic anticoagulant activity and a longer duration of action.[18] This study attempted to establish the pharmacokinetic profile and anticoagulant effects of inhaled heparin in normal healthy dogs.

In the initial phase of the study, our results indicate that canine BALf does not eliminate the anticoagulant activity of unfractionated heparin detected by prolongation of in vitro clotting time in the aPTT. The aPTT dilution series demonstrated that the anticoagulant activity of heparin in BALf was dose-dependent. The aPTT values for heparin-spiked BALf and canine control plasma were similar at heparin concentrations in the range of 0.25 to 0.75 IU/mL. At a high concentration of 1 IU/mL, we observed some inter-individual variability in aPTT prolongation suggesting differences among dogs in heparin-protein binding in the BALf fluid, or non-heparin compounds in BALf that might influence contact pathway activation in the aPTT. Additional experiments are needed to further define the cause of this variability.

In the second phase of the study, we attempted to establish a threshold dose of inhaled heparin that would produce systemic anticoagulant activity. As in humans, the

threshold dose used in the study was defined as the dose of inhaled unfractionated heparin that would cause a measurable increase in plasma aPTT or detectable (> 0.1 IU/mL) anti-factor Xa activity. In healthy humans, using the same starting dose (50,000 IU) as in this study, and the same incremental dose increase (10,000 IU), the inhaled heparin threshold dose was found to be 150,000 IU.[8] Because dogs and humans appear to share similar heparin pharmacokinetic profiles after intravenous and subcutaneous administration, it was anticipated that the canine threshold dose for inhaled heparin would be at most 150,000 IU. However, the exact threshold dose of inhaled heparin in the dog could not be determined because there was no measurable increase in plasma aPTT values at any time point. Based on the results of this study, it does not appear that inhaled heparin in healthy dogs, even at high drug dosages, has a pharmacokinetic profile similar to humans. It is possible that a threshold dose of inhaled heparin in dogs could be identified, however it was not clinically feasible to use doses above the maximal dose of 200,000 IU in our study (higher doses would have required more prolonged nebulization times). We therefore did not attempt to administer inhaled heparin doses of greater than 200,000 IU.

This initial attempt to establish the canine threshold dose of inhaled heparin was based on the standard human method of delivering inhaled heparin via ultrasonic nebulization. After administering very high inhaled heparin doses via a face mask and nebulizer without any change in systemic aPTT, there was concern that the anatomical differences between dogs and humans, particularly in the size of the oral and nasal cavities, may have prevented the nebulized heparin from reaching the lower airways for adequate drug absorption. In humans, it is estimated that 80-90% of inhaled

glucocorticoids are deposited in the oropharynx and swallowed, with only the remaining 10-20% of the medication entering the respiratory tract.[19,20] Approximately eight percent of an aerosolized unfractionated heparin dose reaches the lower respiratory tract in humans.[21] Given failure to achieve systemic effects using nebulized heparin inhaled via a face mask, heparin was then administered with the assistance of positive pressure ventilation through an endotracheal tube directly to the lower airways, ensuring direct delivery of nebulized heparin at the greatest concentration to the respiratory tract for absorption. However, despite the revised delivery method, the results of the systemic aPTT were similar to results obtained using the face mask method, and comparable to a previous study conducted in dogs. The previous study used a considerably lower heparin dose (10,000 IU) than this study, which dose escalated up to a maximum dose of 200,000 IU.[16]

In the final phase of the study, a high dose of inhaled heparin was administered to evaluate the local pharmacokinetic effects of the inhaled heparin on normal canine BALf. In a recent human study, the inhalation of aerosolized heparin created a significant prolongation in the BALf aPTT 24 hours post-nebulization, an effect that persisted for 72 hours post-inhalation.[8] The aerosolization of heparin appears to provide a significant improvement in the length of local anticoagulant effects when compared to parenteral injection. Even with this prolongation in anticoagulant effects, the incidence of hemorrhage in humans during inhaled heparin therapy, both locally within the respiratory tract and systemically, is minimal. If heparin's anticoagulant effects in dogs persisted for 72 hours, as in humans, the use of an aerosolized form of this medication could provide substantial improvement in local anticoagulant efficacy in canine patients prone to

conditions such as pulmonary thromboembolism. However, despite using a higher heparin dose than used in humans, we did not detect persistent heparin activity in the BALf in either dog studied. Only one dog, at one time point, demonstrated heparin anticoagulant activity as evidenced by a slight increase in anti-Xa activity. However, since only a single time point demonstrated trace anti-Xa activity, it is unlikely that this transient effect would be of clinical benefit.

Given that pharmacokinetic responses with injectable heparin in humans and dogs are comparable, it was surprising that there was a lack of detectable systemic anticoagulant effects for the nebulized heparin protocol used in this study. This difference in results for inhaled heparin could be related to the type of nebulizer used or the particle size of the drug that reached the alveolar space. In this study, an ultrasonic nebulizer was used. This type of nebulizer uses ultrasound to disrupt the surface of the liquid and produce a high concentration of aerosol. With this type of nebulizer, the temperature of the liquid will increase and potentially create some degree of denaturation of inhaled medications. Fortunately, most medications used clinically for nebulization, including heparin, are stable and do not typically undergo denaturation when administered with an ultrasonic nebulizer.[22] Another form of nebulizer, the air-jet nebulizer, uses compressed air at high velocity to convert liquids into aerosols. The shear-stress generated with this form of nebulizer can compromise the stability of certain molecules. Using air-jet nebulizers, only one percent of the droplets produced will leave the nebulizer, while the remaining liquid will undergo nebulization multiple times.[22] The size of the droplets generated with various types of nebulizers differs; the air-jet nebulizer generates smaller particle droplets, and the ultrasonic nebulizer generates larger droplets.

The mass median diameter (MMD) of particles generated with an air-jet nebulizer is approximately 2 µm, while the MMD of particles generated with an ultrasonic nebulizer is greater than 5 µm.[9] Mimicking a study that assessed the safety and tolerability of inhaled heparin in humans[8], an ultrasonic nebulizer was used in this study. An air-jet nebulizer, at a driving rate of 10 L/min, has been shown to provide the greatest heparin dose to the lower respiratory tract due to the smaller particle size generated by this type of nebulizer.[21] However, aerosolized heparin particles can still be efficiently absorbed with an aerodynamic diameter of greater than 5 µm, the size typically generated by an ultrasonic nebulizer.[18] Ultrasonic nebulizers are more commonly used in clinical veterinary practice; as such, this type of nebulizer was chosen for the study. Particle size was not measured in this study, and additional research would be necessary to determine if the particle diameter and type of nebulizer would influence canine responses to inhaled heparin. Although an air-jet nebulizer was not used to generate smaller aerosolized heparin particles, this project reproduced an inhalant protocol using an ultrasonic nebulizer that has been shown to effectively heparinize human patients.[8]

Despite the lack of any detectable inhibitory effects on coagulation, neither local nor systemic, inhaled unfractionated heparin may still potentially provide beneficial anti-inflammatory activity. In humans, heparin has been used for its anti-inflammatory properties in rheumatoid arthritis[23] and inflammatory bowel disease.[24] Additionally, inhaled heparin has been used as an effective therapy in humans to control chronic inflammatory pulmonary diseases such as pulmonary fibrosis[8], asthma[9], and cystic fibrosis.[10] The inhalation of heparin has improved respiratory function in humans suffering from asthma that has become refractory to other more conventional therapies.

The dose used in human patients with inflammatory pulmonary diseases is 100,000 IU. This dose is half of the maximal dose used in this study. In dogs, a much lower inhaled heparin dose of 10,000 IU per sublobar segment resulted in a decrease in several pro-inflammatory eicosanoids (thromboxane B2 and the leukotrienes C4, D4, and E4) without any evidence of anticoagulant effects.[16] Given these previous findings and the fact that the high doses of heparin used in this study had no detectable in vitro anticoagulant effects, further exploration of the potential anti-inflammatory properties of inhaled heparin in dogs is warranted. As in people, inhaled heparin could have the potential to be a therapeutic option in dogs with inflammatory pulmonary diseases without the risk of excessive anticoagulation and hemorrhage.

Materials, Instruments, and Supplies

a. Vedco Acepromazine maleate injection, Boehringer Ingelheim, St. Joseph, MO
b. Butorphanol tartrate injection, USP, Hospira, Lake Forest, IL
c. Propoflo (propofol), Abbott Animal Health, Abbott Park, IL
d. Evis Exera Flexible Endoscope, Olympus America, Center Valley, PA
e. 0.9% sodium chloride injection, USP, Hospira, Lake Forest, IL
f. 3.8% sodium citrate, Vacutainer tube, Becton Dickinson, Franklin Lakes, NJ
g. Heparin sodium injection, USP, APP Pharmaceuticals, Schaumburg, IL
h. Actin® FS reagent, Dade Behring, Newark, DE
i. Calcium chloride anhydrous, Sigma-Aldrich, St. Louis, MO
j. Invacare® Stratos™ Compact Aerosol Compressor, Invacare Corp., Elyria, OH
k. Stago STA Compact CT Coagulation Analyzer, Diagnostica Stago, Parsippany, NJ
l. Rotachrom Heparin, Diagnostica Stago, Parsippany, NJ

References Cited

1. Elsayed E, Becker R. The impact of heparin compounds on cellular inflammatory responses: A construct for future investigation and pharmaceutical development. *J Thromb Thrombolys* 2003; 15:11-18.

2. Young E. The anti-inflammatory effects of heparin and related compounds. *Thrombosis* Research 2008; 122:743-752.

3. Scheuch G, Brand P, Meyer T, et al. Anticoagulative effects of the inhaled low molecular weight heparin certoparin in healthy subjects. *J Physiol Pharmacol* 2007; 58(5):603-614.

4. Nissen N, Shankar R, Gamelli R, et al. Heparin and heparin sulphate protect basic fibroblast growth factor from non-enzymatic glycosylation. *Biochem J* 1999; 338:637-642.

5. Ludwig R. Therapeutic use of heparin beyond anticoagulation. *Curr Drug Discovery Technol* 2009; 6:281-289.

6. Dixon B, Santamaria J, Campbell D. A phase 1 trial of nebulised heparin in acute lung injury. *Crit Care* 2008; 12:R64.

7. Bai S, Ahsan F. Inhalable liposomes of low molecular weight heparin for the treatment of venous thromboembolism. *J Pharm Sci* 2010; 99(11):4554-4564.

8. Markart P, Nass R, Ruppert C, et al. Safety and tolerability of inhaled heparin in idiopathic pulmonary fibrosis. *J Aerosol Med* 2010; 23(3):161-172.

9. Bendstrup K, Jensen J. Inhaled heparin is effective in exacerbations of asthma. *Respir Med* 2000; 94:174-175.

10. Serisier D, Shute J, Hockey P, et al. Inhaled heparin in cystic fibrosis. *Eur Respir J* 2006; 27:354-358.

11. Yiwei Q, Zhao G, Liu D, et al. Delivery of therapeutic levels of heparin and low-molecular-weight heparin through a pulmonary route. *PNAS* 2004; 101(26):9867-9872.

12. Mischke R, Schuttert C, Grebe S. Anticoagulant effects of repeated subcutaneous injections of high doses of unfractionated heparin in healthy dogs. *Am J Vet Res* 2001; 62(12):1887-91.

13. Diquelou A, Barbaste C, Gabaig A, et al. Pharmacokinetics and pharmacodynamics of a therapeutic dose of unfractionated heparin (200 U/kg) administered subcutaneously or intravenously to healthy dogs. *Vet Clin Pathol* 2005; 34:237-242.

14. Breuhl E, Moore G, Brooks M, et al. A prospective study of unfractionated heparin therapy in dogs with primary immune-mediated hemolytic anemia. *J Am Anim Hosp Assoc* 2009; 45:15-133.

15. Helmond S, Polzin D, Armstrong P, et al. Treatment of immune-mediated hemolytic anemia with individually adjusted heparin dosing in dogs. *J Vet Intern Med* 2010; 24:597-605.

16. Suzuki R, Freed A. Heparin inhibits eicosanoid metabolism and hyperventilation-induced bronchoconstriction in dogs. Am J Respir Crit Care Med 2000; 161:1850-1854.

17. Brooks M. Evaluation of a chromogenic assay to measure the factor Xa inhibitory activity of unfractionated heparin in canine plasma. Vet Clin Pathol 2004; 33:208-214.

18. Yiwei Q, Zhao G, Liu D, et al. Delivery of therapeutic levels of heparin and low-molecular-weight heparin through a pulmonary route. PNAS 2004; 101(26):9867-9872

19. Barnes P. Inhaled glucocorticoids for asthma. N Engl J Med 1995; 332:868-875.

20. Johnson M. Pharmacodynamics and pharmacokinetics of inhaled glucocorticoids. J Allergy Clin Immunol 1996; 97:169-176.

21. Bendstrup K, Chambers C, Jensen J, et al. Lung deposition and clearance of inhaled 99mTc-Heparin in healthy volunteers. Am J Respir Crit Care Med 1999; 160:1653-1658.

22. Siekmeier R, Scheuch G. Systemic treatment by inhalation of macromolecules – principles, problems, and examples. J Physiol Pharmacol 2008; 59(6):53-79.

23. Imiela J, Nosarzewski J, Gorski A. Oral heparin in the treatment of rheumatoid arthritis. Arch Immunol Ther Exp 1995; 43:313-315.

24. Papa A, Danese S, Gasbarrini A, et al. Potential therapeutic applications and mechanisms of action of heparin in inflammatory bowel disease. Aliment Pharmacol Ther 2000; 14:1403-1409.

CHAPTER III

CONCLUSION

Respiratory disease represents a substantial proportion of small animal emergency medicine cases. The exact morbidity and mortality of patients presenting with respiratory disease is ill-defined. Many of the causes of respiratory distress, both in regard to primary lung disease and systemic processes, have been shown to be risk factors for the development of acute lung injury(ALI)/acute respiratory distress syndrome(ARDS) either through direct lung injury or as part of a generalized inflammatory process.[1] ARDS is associated with a 25-40% mortality rate in human medicine.[2] In veterinary medicine, mortality associated with ALI/ARDS is close to 100%.[3] ARDS is considered a substantial risk factor for the development of systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS).[4] More recently, it has been proposed that ARDS is not a distinct disease process but rather the first organ system to fail in MODS, which is then followed by the liver.[5] Given the high mortality rates and severity of these syndromes, novel therapies are being evaluated for use in both human and veterinary medicine.

One group of medications that have become widely used in numerous respiratory conditions in human medicine are heparin and the low-molecular-weight heparin derivatives (LMWH). Heparin has been demonstrated to have significant anti-inflammatory and anti-fibrotic effects beyond its traditionally accepted anticoagulant

effects. In several recent studies evaluating the use of unfractionated heparin in ALI/ARDS, heparin was shown to decrease ventilator-associated lung injury as well as decrease markers of inflammation within the lungs.[6,7] Heparin administered systemically and via nebulization has also been documented to reduce pulmonary fibrosis.[8,9,10] Only one previous study evaluating inhaled heparin has used the dog as a model. That study used the dog as a model for exercise-induced asthma in people, and assessed the role of heparin in reducing bronchoconstriction.[11] No pharmacokinetic information regarding the use of inhaled heparin in the dog was generated in this previous study.

The study presented in this thesis is the first to evaluate inhaled heparin for use as a potential therapy in the dog. In this study, unfractionated heparin was administered via nebulization using an ultrasonic nebulizer in order to generate a pharmacokinetic profile for inhaled heparin in the dog. The first requirement for evaluating inhaled heparin in the dog was to determine if the activity of unfractionated heparin could be assessed in normal canine bronchoalveolar lavage fluid (BALf). Heparin activity was assessed through determination of activated partial thromboplastin time (aPTT). We found that heparin activity could be detected in normal canine BALf, and that the activity of unfractionated heparin in BALf appears to be similar to its dose-dependent activity in canine plasma. Normal canine BALf did not appear to complex with or neutralize the activity of heparin.

In the second portion of this study, determination of a threshold dose of nebulized heparin that would result in systemic anticoagulant activity (that is, prolonged aPTT and anti-factor Xa activity in canine blood samples) was attempted. Our study modeled a study performed in people where a threshold dose of inhaled heparin was determined and then used in patients with idiopathic pulmonary fibrosis.[8] The human study found that an

inhaled dose of 150,000 IU of heparin resulted in increased aPTT and anti-factor Xa activity. Given that unfractionated heparin has a similar pharmacokinetic profile in people and dogs when administered via a subcutaneous route[12], we anticipated a similar threshold dose would be found in normal dogs. However, we were not able to document a threshold dose of inhaled heparin, as the plasma aPTT did not prolong in any dog at any dose. The highest dose of heparin we were able to administer was 200,000 IU, as this was the maximum volume the trough of the nebulizer would hold. Additionally, administration of the highest doses of heparin required nebulization times as long as 45 minutes. Although it is possible that the aPTT would have prolonged at even higher heparin doses, the clinical applicability of administering heparin via nebulization in this manner would be lost if extremely high doses were truly necessary for systemic anticoagulant activity.

Initially, we administered the nebulized heparin with the use of a face mask attached directly to the nebulizer. When systemic anticoagulant activity was not detected, the route of administration was then changed as we suspected that a large portion of the nebulized heparin was not reaching the lower airways because it may have been trapped in the nasal cavity or upper airways. The possibility that the dogs were breath-holding or taking insufficient breaths was also considered. However, the dogs had been acclimated to the use of the face masks for a substantial time period prior to starting the study, and breath-holding or insufficient breaths were therefore not suspected. In order to investigate the possibility of upper airway heparin trapping, we then placed the dogs under injectable anesthesia and administered the nebulized heparin via the endotracheal tube with the assistance of positive pressure ventilation. Administration via

the endotracheal tube eliminated the oral and nasal cavities as a potential source of the problem. The use of positive pressure ventilation should theoretically have delivered the heparin at higher doses into the lower airways. Again, no detectable systemic anticoagulant activity was shown despite these changes in the method of administration.

In the final phase of this study, we attempted to evaluate any local anticoagulant effects of inhaled heparin in the lungs themselves. In humans, the local anticoagulant effects of inhaled heparin have been found to persist for up to 72 hours and have a half-life of approximately 28 hours.[8] A high dose of 200,000 IU was chosen as it was the maximum amount we could practically deliver with the nebulizer, and was higher then our proposed threshold dose of 150,000 IU. Unfortunately, no biologically relevant prolongation in either aPTT or anti-factor Xa activity was noted in the BALf at any time point.

The lack of detectable local and systemic heparin activity in our study was unexpected. It seems unlikely that heparin delivered directly to the alveoli via the inhaled route is not effective in dogs. The problem more likely lies in the anatomical differences between the upper airway of dogs and humans as well as the differences in ease of inhalation therapy in general between the two species. Two possible areas that warrant further investigation in the use of inhaled heparin would be the type of nebulizer used as well as the particle size of the nebulized heparin. We elected to use an ultrasonic nebulizer as it is the most common clinically used type of nebulizer in veterinary medicine.[13] Previous studies have also reported increased lung deposition of nebulized substances with the use of an ultrasonic nebulizer as compared to an air-jet nebulizer.[14,15] However, it has also been suggested that ultrasonic nebulizers are not appropriate for

deep lung delivery because they produce particles of a larger size.[16,17] An air-jet nebulizer may have been able to deliver the heparin to the lower airways more reliably. A previous study in cats was able to demonstrate that a radiopharmaceutical agent was found in all lung fields and the lower airways after administration via an air-jet nebulizer.[18] The use of a different nebulizer type may also overcome the limitation of the nebulizer trough size in this study, allowing for administration of higher doses of heparin (above 200,000 IU) over a shorter period of time.

An additional area warranting further investigation in the use of inhaled heparin relates to the particle size of the heparin once it is nebulized. The delivery and location of aerosolized particles in the respiratory tract depends on the size of the particle as well as the tidal volume, inspiratory flow rate, and ability to breath hold of the patient to whom the medication is being administered. For delivery to the trachea, optimal particle size is approximately 2-10 μm; for delivery to the lower airways, the ideal particle size is 0.5-5 μm.[19] In the study with the radiopharmaceutical agent administered to cats, the particle size of the agent was 1.3 μm.[18] In regards to aerosolized heparin specifically, an air-jet nebulizer has been shown to deliver a heparin particle size of 2.01-3.64 μm while an ultrasonic nebulizer has been shown to deliver heparin particles in the range of 5.61-7.03 μm.[20] Particle size of the nebulized heparin was not determined in this study. Differences in particle size of various medications are overcome in human inhalation therapy by having the patient take deep breaths and breath hold. Dogs are not amenable to performing those procedures on demand, so particle size could be a limiting factor in the use of certain medications in veterinary inhalation therapy.

Inhalation therapy is becoming increasingly more popular and feasible in small animal practice because of its many benefits. Drugs administered via inhalation can often be administered in smaller amounts than would be required for systemic administration. The risk of potential negative side effects is often lower when drugs are given via inhalation, because systemic absorption can be minimized while directing therapy at the problem area. Conversely, the lungs can also serve as a new route of delivery for systemic administration of medications. Drugs that are currently being used in an aerosolized manner in veterinary medicine include various antibiotics (especially the aminoglycosides), glucocorticoids, and bronchodilators. Inhaled heparin has shown promise for its anticoagulant, anti-inflammatory, and anti-fibrotic effects in human medicine in numerous respiratory conditions. Despite an absence of local anticoagulant effects, inhaled heparin could still be a beneficial therapeutic option in canine respiratory diseases because of potential anti-inflammatory and anti-fibrotic effects. For this reason, further research to establish the pharmacokinetic profile of inhaled heparin in the dog is warranted.

References Cited

1. DeClue A, Cohn L. Acute respiratory distress syndrome in dogs and cats: a review of clinical findings and pathophysiology. *J Vet Emerg Crit Care* 2007; 17(4):340-347.

2. Zambon M. Vincent JL. Mortality rates for patients with acute lung injury/ARDS have decreased over time. *Chest* 2008; 133(5):1120-1127.

3. Orsher A, Kolata R. Acute respiratory distress syndrome: case report and literature review. *J Am* Anim *Hosp* 1982; 18:41-46.

4. Demling R. The modern version of adult respiratory distress syndrome. *Annu Rev Med* 1995; 46:193–202.

5. Khadaroo R, Marshall J. ARS and multiple organ dysfunction syndrome. Common mechanisms of a common systemic process. *Crit Care Clin* 2008; 18(1):127-141.

6. Hofstra J, Levi M, Schultz M. HETRASE study-Did heparin treatment benefit patients with ALI/ARDS? *Crit Care Med* 2009; 37(8):2490-2491.

7. Li L, Huang C, Lin H, et al. Unfractionated heparin and enoxaparin reduce high-stretch ventilation augmented lung injury; a prospective, controlled animal experiment. *Crit Care* 2009; 13(4):R108.

8. Markart P, Nass R, Ruppert C, et al. Safety and tolerability of inhaled heparin in idiopathic pulmonary fibrosis. *J Aerosol Med* 2010; 23(3):161-172.

9. Piguet P, Van G, Guo J. Heparin attenuates bleomycin but not silica-induced pulmonary fibrosis in mice: possible relationship with involvement of myofibroblasts in bleomycin and fibroblasts in silica-induced fibrosis. *Int J Exp Pathol* 1996; 77:155-161.

10. Guenther A, Luebcke N, Ermert M, et al. Prevention of bleomycin-induced lung fibrosis by aerosolization of heparin or urokinase in rabbits. *Am J Respir Crit Care Med* 2003; 168:1358-1365.

11. Suzuki R, Freed A. Heparin inhibits eicosanoid metabolism and hyperventilation-induced bronchoconstriction in dogs. *Am J Respir Crit Care Med* 2000; 161:1850-1854.

12. Mischke R, Schuttert C, Grebe S. Anticoagulant effects of repeated subcutaneous injections of high doses of unfractionated heparin in healthy dogs. *Am J Vet Res* 2001; 62(12):1887-91.

13. Cohn L. Inhalant therapy: Findings its place in small animal practice. *Vet Med* 2009.

14. Harvey C, O'Doherty M, Page C, et al. Comparison of jet and ultrasonic nebulizer pulmonary aerosol deposition during mechanical ventilation. *Eur Respir J* 1997; 10:905-909.

15. O'Doherty M, Thomas S, Page C, et al. Delivery of a nebulised aerosol to a lung model during mechanical ventilation: effect of ventilator settings and nebuliser type, position, and volume of fill. *Am Rev Respir Dis* 1992; 146:383-388.

16. Siekmeier R, Scheuch G. Systemic treatment by inhalation of macromolecules – principles, problems, and examples. *J Physiol Pharmacol* 2008; 59(6):53-79.

17. Bendstrup K, Gram J, Jensen J. Effect of inhaled heparin on lung function and coagulation in healthy volunteers. *Eur Respir J* 2002; 19(4):606-610.

18. Schulman R, Crochik S, Kneller S, et al. Investigation of pulmonary deposition of a nebulized radiopharmaceutical agent in awake cats. *Am J Vet Res* 2004; 65:806-809.

19. Rozanski E, Bach J, Shaw S. Advances in respiratory therapy. *Vet Clin Small Anim* 2007; 37:963-974.

20. Bendstrup K, Newhouse M, Pederson O, et al. Characterization of heparin aerosols generated in jet and ultrasonic nebulizers. *J Aerosol Med* 1999; 12:17-25.