THE IN VITRO EFFECTS OF GLUCOSAMINE ON EQUINE AND CANINE
PLATELET AGGREGATION AND THROMBOXANE SYNTHESIS

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Objective: To determine the effects of glucosamine on equine and canine platelet aggregation and thromboxane synthesis in vitro.

Procedures: Citrated blood samples were collected via jugular venipuncture and used to create platelet-rich plasma (PRP). PRP samples were incubated for 5 minutes with four concentrations of glucosamine: 0 µg/ml (control), 1 µg/ml, 10 µg/ml, and 100 µg/ml. Platelet aggregation was determined using turbidometric aggregometry using ADP or collagen as agonists. Thromboxane B2 concentrations were measured using a commercially available ELISA kit. The results were analyzed using by mixed model analysis. Significance was set at p<0.05.

Results: In equine samples, there was no change in the mean maximum aggegrometry amplitude at any glucosamine concentration when ADP or collagen was used as an agonist. In canine samples, there was a significant increase in maximum amplitude for the 100 µg/ml samples compared to the 0 µg/ml and 1 µg/mL samples when ADP was used as an agonist. There was no significant change in maximum amplitude when collagen was used as an agonist. There was no significant change in plasma thromboxane B2 concentration at any glucosamine concentration for either species.

Conclusions and Clinical Relevance: Glucosamine does not exert an in vitro anti-platelet effect on equine and canine platelet aggregation and thromboxane synthesis, even at superphysiologic plasma concentrations. However, it is unknown if long-term oral glucosamine administration will inhibit platelet aggregation or thromboxane synthesis.

Key words: glucosamine, equine, canine, platelet aggregation, thromboxane synthesis
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CHAPTER I

INTRODUCTION

As cliché as it sounds, I have surrounded myself with the company of animals for as long as I can remember, whether they are my own cats and dogs or the stray animals at the local animal shelter. Through various internships at clinics in Texas, Indiana, and Argentina, I have furthered my understanding of the veterinary field and gained greater insight into my specific interests. I have witnessed the day-to-day routine of large and small animal veterinarians, as well as the intense emergency cases that these clinics have encountered. Rather than deter me from this career path, these experiences have strengthened my desire to dedicate my life to improving the well being of animals. Therefore, when I arrived at Mississippi State University in the fall of 2013, I set my sights on achieving a successful undergraduate career centered on veterinary medicine, with the hopes of attending veterinary school after the completion of a bachelor’s degree in biological sciences. I began to accomplish this goal with the Shackoul Summer Study at the University of Oxford program, which led me to discover the world of tendinopathy and regenerative therapies.

After spending the summer studying equine and human tendinopathy with Dr. Stephanie Dakin at the Botnar Research Centre Institute of Musculoskeletal Sciences at the University of Oxford in Oxford, England, I was full of ambition and determination to further research in equine sports medicine. In my tutorial with Dr. Dakin, I studied the risk factors, pathogenesis, and treatment options for tendinopathy, including regenerative therapies. These therapies range from well-known and common treatments like platelet rich plasma (PRP) to innovative and novel remedies like embryonic stem cells and
mesenchymal stem cells. While studying these therapies and remedies, I developed a specific interest in platelets and platelet function, which led me to contact Dr. Fontenot and Dr. Thomason about research opportunities when I returned to Mississippi State University in the fall of 2014. From there, I began to explore the world of platelets and glucosamine in hopes of completing my honors thesis on the effect of glucosamine on equine and canine platelet function.

This thesis begins with a review analyzing osteoarthritis, glucosamine, and platelets. Then, the thesis explores the in vitro effects of glucosamine on equine and canine platelet aggregation and thromboxane synthesis. Finally, the thesis concludes with the accomplishments and clinical relevance of the research.
CHAPTER II
LITERATURE REVIEW

Osteoarthritis

Overview

Osteoarthritis (OA), otherwise known as degenerative joint disease, is a multifactorial syndrome where the cartilage of the joints deteriorates. Over time, the cartilage begins to erode due to normal repetitive forces or abnormal and high forces placed upon the joints of an animal. In addition, inflammation of the joint caused by an infection or autoimmune disease may lead to damaged cartilage. Chronic OA is progressive, potentially leading to severe lameness if left undiagnosed or untreated. However, acute OA can suddenly appear depending on the cause, for example, fracture or other severe injury to the joint. Both of these involve some sort of injury to the cartilage of a joint. Dogs and horses, especially those that have experienced traumatic damage to a joint, are frequently affected by OA.

The common clinical signs include pain, decreased activity, reluctance to move, and stiffness when moving. Effusion of the joint and decreased range of motion are other clinical signs often found on physical examination. OA may result in chronic pain, lameness, and/or gait abnormalities. In order to diagnose OA in horses and dogs, physical and lameness exams are performed. Radiographs are necessary to determine the extent of the damage and the level of joint deterioration. Less commonly, other modalities such as magnetic resonance imaging, nuclear scintigraphy, computed tomography, ultrasound, or arthroscopic surgery may be necessary to obtain a diagnosis.
While there is no cure for OA, there are therapies that aid in mitigating the pain and inflammation caused by the condition. Therefore, the condition can only be managed by palliative care. Non-steroidal anti-inflammatory drugs, such as aspirin, carprofen, meloxicam, firocoxib, flunixin, and phenylbutazone, offer relief from the pain. In addition, injections or intravenous administration of hyaluronic acid have proven to be effective.\textsuperscript{1} Adequan, a polysulfated glycosaminoglycan, has shown to be effective in tarsal and carpal joint arthritis.\textsuperscript{2} Non-drug related forms of treatment, such as dietary supplements or lifestyle changes leading to a reduction in weight, are also effective in reducing osteoarthritic pain and clinical signs.\textsuperscript{3} Nutritional supplements, commonly referred to as nutraceuticals, can potentially aid in the treatment of pain. A recent study found omega-3 fatty acids (in the form of dietary fish oil) affect synovial fluid by decreasing the expression and activity of matrix metalloproteinases\textsuperscript{4}, damaging enzymes expressed in osteoarthritic joint tissues.\textsuperscript{5} However, various formulations of glucosamine and chondroitin are the more frequently administered supplements.

**Glucosamine**

**Background**

Glucosamine is an abundant naturally derived molecule consisting of the monosaccharide glucose and an amino group. Glucosamine is practically a part of every cell in the body. The nutraceutical is naturally found within the synovial fluid\textsuperscript{6} and many other mammalian cells.\textsuperscript{7} Proteoglycans, large macromolecules found in cartilage, intertwine with collagen in the joints in order to aid with elasticity. Glucosamine is an integral part of the synthesis of these proteoglycans found in cartilage. The cartilage
matrix is comprised of various proteins and collagens that undergo glycosylation with glucosamine.\textsuperscript{7}

There are various forms of glucosamine such as glucosamine sulfate, glucosamine hydrochloride, N-acetyl-glucosamine and D-\(+\)-glucosamine. Each of these forms serve different purposes due to the slight alterations in their chemical structure. However, a majority of these forms are used in supplements for joints. Glucosamine supplements are rapidly modified in the liver into glucose and fructose derivatives then released into the blood stream to be used by the body in areas such as the joints.\textsuperscript{7}

\textbf{Glucosamine and Osteoarthritis}

Glucosamine supplements have been shown to be an effective form of prevention for osteoarthritis in horses. Many believe that the supplementation of glucosamine will slow the degradation of the cartilage and therefore alleviate some of the pain associated with osteoarthritis.\textsuperscript{8} In stressed equine cartilage models, glucosamine, in conjunction with chondroitin, was found to have partially mitigated the inflammatory reaction caused by osteoarthritis.\textsuperscript{9} Another study using normal cartilage models, found that glucosamine significantly reduced the translation of some mediators of osteoarthritis.\textsuperscript{10} Finally, in a cartilage model that was stressed using interleukin-1 (the primary inflammatory cytokine that is produced in arthritic joints), glucosamine inhibited cytokine-induced responses and therefore prevented degradation of the joint.\textsuperscript{11} In an equine clinical study, ten hunter/jumper horses that normally received distal tarsal injections were administered glucosamine.\textsuperscript{12} The results of this study showed that supplementation of a glucosamine/chondroitin mix lowers the need for injection to maintain soundness. In equine experimental studies, glucosamine was found to increase range of motion in the
elbow, stifle, and fetlock. Similar results were found in canine studies utilizing glucosamine. In an in vivo study using 35 dogs suffering from osteoarthritis in their hips or elbows, glucosamine (in a mixture with chondroitin) was found to have improved the weight bearing ability and overall condition of the dogs.

*Controversy Surrounding Glucosamine*

While there are proponents for the administration of glucosamine, there is strong evidence against the benefits of supplementation of the nutraceutical. Many have found the claims supporting glucosamine supplementation are weak and insubstantial. A majority of the evidence proving the efficacy of glucosamine with treating symptoms of osteoarthritis was studied *in vitro* rather than *in vivo*. When glucosamine was administered to horses in conjunction with chondroitin, there was a decrease in the amount of injections necessary to maintain soundness. However, lameness exams are based upon a subjective analysis. Therefore, the evaluator may observe an improvement, even if no clinical improvement actually exists. Ultimately, a majority of the in vivo studies utilized solely lameness exams to assess the effectiveness of glucosamine supplementation, which does not provide substantial evidence to support the claims about glucosamine. It is also important to note that the supplement administered to these horses was a combination of glucosamine and various other nutraceuticals. Therefore, the sole administration of glucosamine was not studied, but rather the combination of glucosamine and another molecule. When administered to dogs *in vivo*, glucosamine showed no improvement in objective gait analysis or subjective gait analysis when examined by an orthopedic surgeon or owner. In addition, the changes in the
ground reaction forces specific to the arthritic joint were not improved, whereas with the
treatment of carprofen or meloxicam they were significantly improved. Therefore, the
existing data on glucosamine-based nutraceuticals is considered to be inconclusive in
regards to evidence supporting the efficacy of these products in horses\textsuperscript{15} and dogs\textsuperscript{20}.

Since glucosamine is a nutraceutical, it does not require the same requirements in
regards to proving health claims as pharmaceuticals. In Britain, pharmaceuticals are
under the direct regulation of the Veterinary Medicines Directorate. Meanwhile,
nutraceuticals are not under the regulation of the Veterinary Medicine Directorate, unless
the company manufacturing the product makes specific health claims relating to a
specific disease.\textsuperscript{16} In the United States, supplements are regulated by the U.S. Food and
Drug Administration. However, the term nutraceutical has no regulatory definition for
humans\textsuperscript{21}, yet the veterinary usage of the term does have a descriptive definition. The
North American Veterinary Nutraceutical Council defines a nutraceutical as “a substance
produced in purified or extracted form which, when administered orally to patients, aims
to provide them the necessary elements for their structure and normal function to better
their health and well-being”\textsuperscript{22}, but this definition has no regulatory meaning.\textsuperscript{16} Therefore,
there are no regulations of glucosamine supplements.

**Platelets**

*Platelet Formation*

Platelets, otherwise known as thrombocytes, are an integral part of hemostasis in
the mammalian circulatory system. Platelets are non-nucleated cells composed of
fragments of cytoplasm, specifically the cytoplasm of megakaryocytes. As the name
suggests, megakaryocytes are large bone marrow cells. They are formed from hematopoetic stem cells (hemocytoblasts) and serve as the producers of platelets. Through the production of thrombopoietin, megakaryocytes form and differentiate.\textsuperscript{23} Once mature, megakaryocytes replicate and increase the number of chromosomes through endomitosis (mitosis without cellular division). When an adequate number of chromosomes are reached, platelets bud off from the megakaryocytes and released into systemic circulation.\textsuperscript{24} The platelets form a “platelet plug” during primary hemostasis after they are activated. Therefore, the platelets circulate in an inactive form in the bloodstream until an injury or damage to the blood vessel occurs. At that point, platelets will become activated and form a platelet plug to prevent excessive blood loss. The non-circulating platelets, or reserve platelets, are stored within the spleen and released into circulation when necessary (i.e. when circulating platelet levels are low). When the circulating platelets reach the end of their lifespan (about 6 to 9 days in horses\textsuperscript{25} and about 5 to 7 days in dogs\textsuperscript{26}), they are destroyed via phagocytosis within the liver and spleen.

\textit{Platelet Adhesion, Activation, and Aggregation}

During normal vasculature cellular turnover or damage to the vessel wall, platelets undergo a series of steps, adhesion, activation, and aggregation, to form a platelet plug to prevent further blood loss. Along with the formation of a platelet plug, the injured blood vessel will undergo vasoconstriction to prevent blood loss. This process is mediated by a nerve reflex, the presence of higher levels of endothelin secreted from the endothelium, and many platelet-derived molecules that cause vasoconstriction. Initially,
subendothelial bound von Willebrand factor binds to glycoprotein Ib, which serves as the start of platelet adhesion.\textsuperscript{27} Along with the reduced diameter of the vessel, circulating platelets are attracted by signaling molecules to the site of injury., where collagen, from the subendothelial matrix, is exposed and leads to additional platelet adhesion.\textsuperscript{28}

Platelet activation involves degranulation, shape change, aggregation with other platelets and cells to form a platelet plug. After activation, the production of thromboxane A\textsubscript{2} from the platelet increases causing further vasoconstriction and activation to amplify platelet aggregation. Thromboxane A\textsubscript{2} binds to the thromboxane receptors located on the platelet leading to the conversion of the GP IIb/IIIa from the inactive state to the active state. However, prior to this shape change the platelet releases two important granules. First, the platelet secretes dense granules full of various molecules such as serotonin, adenosine diphosphate, and calcium ions. Serotonin acts as a vasoconstrictor and further prevents blood loss, while adenosine diphosphate activates other platelets in order to form a platelet plug rapidly. After the contents of the dense granules are released, the alpha granules release their respective contents. Von Willebrand factor and fibrinogen are part of many molecules residing within the alpha granules. Von Willebrand factor will adhere more platelets to the exposed collagen, while fibrinogen will begin to link activated platelets.

Aggregation, the final step of primary hemostasis, creates a platelet plug which contributes to the formation of a blood clot via secondary hemostasis. The activated and now dendritic platelets bind via GP IIb/IIIa receptors to form a temporary patch covering the injury.
**Platelet Aggregometry**

Various methods are utilized in order to assess platelet function. These methods range in complexity from the very simple (bleeding time) to the more complex (platelet aggregometry). However, even though platelet aggregometry is complex and requires special training and machinery, it is frequently used to evaluate platelet function. Turbidometric aggregometry is considered to be the “gold standard” when assessing platelet function. In this form of platelet aggregometry, light transmission is utilized to analyze aggregation. Two cuvettes are compared as a part of this test—one with platelet rich plasma and one with platelet poor plasma. In order to mimic in vivo aggregation, an agonist (collagen, adenosine diphosphate) is added to the platelet rich plasma cuvette to initiate platelet aggregation. After aggregation occurs, the turbid platelet rich plasma (due to the floating platelets) becomes a translucent liquid and the platelets are clumped together (platelet plugs). The changes in the transparency of the cuvette are recorded. If platelet aggregation is inhibited, the curve will be closer to a flat line than a curve.

**Thromboxane A₂ and B₂**

A part of the eicosanoid lipid family, thromboxane is a vasoconstrictor that facilitates platelet activation. When released by activated platelets, thromboxane A₂ (TXA₂) amplifies platelet aggregation as well as stimulates activation of other platelets. When platelets are activated, they release arachidonic acid, which is previously stored in the phospholipids of the platelet’s plasma membrane. Once released, arachidonic acid is converted into prostaglandin H₂ by cyclooxygenase-1. Finally, thromboxane synthase converts prostaglandin H₂ into TXA₂.
Thromboxane $A_2$ has a short half-life and will be quickly metabolized to a more stable form, thromboxane $B_2$ (TXB$_2$). TXB$_2$ is excreted in the urine but can also be found in plasma. Using a commercially available ELISA kit, the levels of TXB$_2$ can be determined, which is an accurate assessment of TXA$_2$ synthesis in vivo. Ultimately, the levels of TXA$_2$ can be used to examine platelet function since TXA$_2$ is involved during platelet activation and aggregation.

**Glucosamine and Platelets**

Recent studies have found glucosamine to have an inhibitory affect upon platelet function.$^{32,33}$ In guinea pigs, researchers found that after 22 days of in vivo administration of a glucosamine hydrochloride solution, platelet aggregation and thromboxane synthesis decreased.$^{33}$ Each animal received an average of 400mg of glucosamine each day, or rather 800mg/kg which is 40 times the commonly recommended dose of 20mg/kg. Platelet aggregation was analyzed via turbidometric aggregometry with ADP and collagen as the agonists. Glucosamine was determined to have reduced ADP-induced aggregation and thromboxane synthesis. ADP induced aggregation was reduced by 51%, whereas thromboxane synthesis was reduced by 96%. However, collagen-induced aggregation, body weights, bleeding time, and platelet counts were not affected by administration of glucosamine.

Similar results were found with humans.$^{32}$ When in vitro administration of glucosamine was studied to determine its effect on ADP-induced platelet aggregation, it was determined to be the most potent of aminosugars due to its ability to inhibit aggregation when ADP was used as an agonist. Various amounts of glucosamine were
found to inhibit platelet aggregation. For example, 10uM of glucosamine partially suppressed platelet aggregation by 30% when platelets were stimulated with 10uM of ADP. Then, when platelets were induced with 1μM of ADP, only 1mM of glucosamine inhibited aggregation by 20%. Other aminosugars, such as galactosamine and N-acetyl-galactosamine, did not have an inhibitory effect on platelet aggregation. Additionally, glucosamine was shown to have inhibited ADP induced platelet aggregometry (by 29%) when human volunteers were administered glucosamine for a week. In addition to these findings, glucosamine was noted to have inhibited the ADP-induced TXA₂ production at 0.1 and 1 mM glucosamine. Normal platelets induced by ADP produced approximately 30 ng/ml of thromboxane B₂, however the 0.1 and 1 mM glucosamine treated platelets produced approximately 23 ng/ml and 13 ng/ml. No inhibitory effect occurred when platelet aggregation was induced by collagen or thrombin. Therefore, glucosamine was determined to have only an inhibitory effect on ADP induced platelet aggregation and thromboxane production from ADP-stimulated platelets.
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CHAPTER III

THE IN VITRO EFFECT OF GLUCOSAMINE ON EQUINE AND CANINE PLATELET AGGREGATION AND THROMBOXANE SYNTHESIS*

Introduction

Osteoarthritis is a multi-factorial syndrome that ultimately leads to cartilage degeneration within diarthrodial joints that affects all species. The condition is prevalent in dogs and horses, especially in animals that are predisposed due to conformation abnormalities or that frequently participate in athletic events due to the higher rate of joint loading and frequency of impact.\(^1-3\) Unfortunately, there is no cure for osteoarthritis and treatments, such as non-steroidal anti-inflammatory drugs (NSAIDs), are intended to minimize or reduce the pain and discomfort associated with this condition.

Glucosamine is manufactured by chondrocytes from glucose and glutamine and is one of the key compounds necessary for healthy cartilage matrix production.\(^2,4\) The oral supplementation of glucosamine is believed to slow cartilage degradation and alleviate some of the pain associated with osteoarthritis.\(^5\) In veterinary medicine, glucosamine is commonly administered to both horses and dogs to maintain joint health and reduce the pain associated with osteoarthritis. Clinical and experimental studies in both species have demonstrated positive clinical effects of oral glucosamine administration, especially combined with chondroitin sulfate.\(^6-10\)

Toxicology studies in animals and clinical trial data from 3,063 human patients have failed to demonstrate significant adverse effects of glucosamine supplementation on

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hematologic, biochemical, or physical exam parameters, and patients receiving glucosamine supplementation reported fewer side effects than those receiving NSAIDs or a placebo.\textsuperscript{11,12} Despite the apparent safety of these supplements, they may have unexpected effects on platelet function. In humans, the \textit{in vitro} treatment of platelets with glucosamine caused a significant inhibition of platelet activation.\textsuperscript{13} Also, when glucosamine was administered orally to guinea pigs, there was a decrease in platelet aggregation when platelets were activated with adenosine diphosphate (ADP).\textsuperscript{14} In both humans and guinea pigs, thromboxane A\textsubscript{2} synthesis, a potent platelet activator and vasoconstrictor, and secretion of granule contents were suppressed, suggesting that this nutraceutical may have anti-platelet effects.\textsuperscript{13,14}

Since glucosamine supplementation is common in canine and equine patients, it is important to determine if the nutraceutical has anti-platelet effects. If glucosamine negatively impacts platelet aggregation, supplementation could theoretically lead to peri-operative bleeding or impair wound healing. The objectives of this study were to determine the \textit{in vitro} effects of glucosamine on platelet aggregation and thromboxane synthesis in horses and dogs at clinically relevant doses. Our hypotheses were that, similar to other species, the \textit{in vitro} treatment of equine and canine platelets with glucosamine would cause a decrease in platelet aggregation and thromboxane synthesis.

\textbf{Materials and Methods}

\textit{Study Population}

Eight healthy quarter horses (4 castrated males and 4 females) and 8 healthy adult walker hound dogs (4 neutered males and 4 intact females) were used for the study. The
mean age of the horses was 10 years (range, 5-15 years) and the mean age of the dogs was 3 years (range, 1.5-6.5 years). The horses and dogs did not receive any medications for at least 2 weeks prior to or during the study. The health status was established via physical examination, complete blood count, and serum chemistry analysis. Animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee.

**Study Design and Sample Collection**

Blood samples were collected via jugular venipuncture with a 20 gauge needle into a 4.5 mL vacutainer tube containing 3.2% sodium citrate anticoagulant\(^a\). Platelet-rich plasma (PRP) was created from whole blood via centrifugation and collected for *in vitro* treatment with three concentrations of glucosamine plus a control. The three treatment concentrations were selected based on approximate plasma concentrations of glucosamine.\(^{12,15-17}\)

To create equine PRP, whole blood samples were placed into the centrifuge and spun at 450 x g at room temperature for 5 minutes. The PRP supernatant was removed and the remaining blood samples centrifuged at 1,800 x g at room temperature for 8 minutes to create platelet poor plasma (PPP).\(^\text{18}\) A previously published technique\(^\text{19}\) was used to create canine PRP. Briefly, whole blood was centrifuged at 1,200 x g at room temperature for 3 minutes and the PRP supernatant was removed, and the remaining blood sample was centrifuged 1,800 x g at room temperature for 8 minutes to create PPP.
Glucosamine Incubation

Previously published protocols\textsuperscript{20,21} were modified to evaluate canine and equine platelet function following the \textit{in vitro} treatment of PRP using 3 concentrations of D-(+)-glucosamine\textsuperscript{b} plus a control. Briefly, 3 concentrations of glucosamine working solutions, 1 \(\mu\)g/mL, 10 \(\mu\)g/mL, and 100 \(\mu\)g/mL, were created from a stock solution containing 10 mg/mL of glucosamine in sterile water. To determine the dose effect on platelet aggregation, 5 \(\mu\)L from each working solution was added to 245 \(\mu\)L of PRP, inverted 3 times and incubated, at room temperature, for 5 minutes. For the control sample, 5 \(\mu\)L of sterile water was added to the same volume of PRP. Following the incubation, the treated PRP was transferred to a glass cuvette for analysis.

Platelet Aggregometry

\textit{Turbidometric Aggregometry}

A 2 channel turbidimetric platelet aggregometer\textsuperscript{c} was used to analyze platelet aggregation. For each sample, time point, and glucosamine concentration, 3 total samples were analyzed, and the results were averaged to yield a single value. Aggregation, for both horses and dogs, was assessed using ADP (40 \(\mu\)M) or collagen (10 \(\mu\)g/mL) as agonists, with a temperature of 37°C, and a stirring speed of 1,000 rpm for equine samples and 1,200 rpm for canine samples. Samples were analyzed based on the manufacturer's standard guidelines\textsuperscript{d}. Briefly, 247 \(\mu\)L of glucosamine treated PRP was placed into a glass cuvette with a stir bar and 250 \(\mu\)L of PPP was placed into a cuvette without a stir bar. Samples were incubated for one minute at 37°C, placed into the aggregometer, and stable baseline values corresponding to 0% and 100% aggregation
were obtained using PRP and PPP, respectively. ADP or collagen was added to the PRP, and platelet aggregation was monitored for 6 minutes and 8 minutes respectively. The maximal percentage aggregation was calculated and recorded using computer software. The samples were analyzed in triplicate and all samples were analyzed within 4 hours of collection. Based on recommendations published by the International Society of Thrombosis and Haemostasis Platelet Physiology and Scientific and Standardization Committee, the platelet count in the PRP was not adjusted to a standardized count by dilution with PPP prior to analysis.²²⁻²⁴

*Thromboxane B₂ Concentration*

Using the same technique as mentioned previously, for each horse and dog, PRP was incubated for 5 minutes at all glucosamine concentrations. Following incubation, the samples were centrifuged and the PPP supernatant was collected and stored at -80°F until analysis. The thromboxane B₂ concentration was determined using a thromboxane B₂ ELISA kit that has previously been used for analysis in horses and dogs and reported as pg/mL.²⁵⁻²⁷ Samples were analyzed according to the manufacturer’s instructions. Briefly, prior to analysis, the samples were thawed to room temperature and the assay buffer was used to dilute each sample. A correction factor was applied to account for these dilutions. A 96-well plate was prepared by adding 50 μL of each dilute sample, 50 μL of TXB₂ monoclonal acetylcholinesterase tracer, and 50 μL of TXB₂ monoclonal antibody to each well. The plate was incubated overnight at 4°C, washed developed using Ellman’s Reagent. Using a plate reader, each plate was analyzed at a wavelength of 412 nm.
Statistical Analysis

The effect of in vitro glucosamine supplementation on platelet aggregation and thromboxane B₂ synthesis in dogs and horses were analyzed by mixed model analysis using a computer program\(^9\). Separate models were made for each marker and species. The model included concentration as a fixed effect. Dog or horse was included as a random effect. Tukey’s adjustment for multiple comparisons was made if concentration was found to have a significant effect. Conditional residual plots were assessed to ensure the assumptions of the statistical methods had been met. In the interpretation of results, P-values ≤ 0.05 were considered significant.

Results

Turbidimetric Aggregometry

Equine

Based on turbidimetric aggregometry, the mean and standard deviation of the maximum amplitude for ADP- and collagen-induced platelet aggregation are summarized (Table 1). Compared to the control sample, there were no significant changes in the maximum amplitude at any glucosamine concentration when ADP (p=0.096) or collagen (p=0.86) was used as an agonist.

Canine

The mean and standard deviation of the maximum amplitude for ADP- and collagen-induced platelet aggregation are summarized (Table 1). Compared to the control sample, with ADP-induced aggregation, there was a significant (p=0.0013) increase in maximum amplitude for 100 μg/mL glucosamine concentration.
Additionally, compared to the 1 μg/mL concentration, there was a significant increase (p=0.0244) in maximum amplitude with 100 μg/mL glucosamine concentration. Compared to the control sample, there was no significant changes in the maximum amplitude at any glucosamine concentration when collagen (p=0.2925) was used as an agonist.

**Plasma Thromboxane B₂ Concentration**

The mean and standard deviation of thromboxane B₂ concentration in PRP after the *in vitro* treatment with glucosamine are summarized (Table 1). Compared to the control sample, no significant differences in plasma TXB₂ concentrations were detected in the both equine (p=0.7818) and canine (p=0.8458) samples with three concentrations of glucosamine.

**Discussion**

Our results are in sharp contrast to similar studies performed in humans and guinea pigs.⁵,⁶ Lu-Suguro et al demonstrated that compared to pretreatment aggregation, there was a 51% decrease in ADP-induced platelet aggregation in guinea pigs treated with glucosamine; however, glucosamine supplementation did not adversely inhibit collagen-induced platelet aggregation.⁶ Additionally, in humans, following a week of glucosamine supplementation, there was a 29% decrease in ADP-induced platelet aggregation, without adversely inhibiting collagen-induced platelet aggregation.⁵ In these studies, it was suggested that glucosamine antagonized the binding of low and high affinity ADP platelet receptors in a noncompetitive and dose-dependent manner.⁵,⁶
In both humans and guinea pigs, the inhibition of ADP-induced aggregation was observed when platelets were activated with low concentrations of ADP (2.5 μM and 2 μM respectively). However, similar results could not be reproduced when higher concentrations of ADP (10 μM) were used for platelet activation,\textsuperscript{13,14} suggesting that the ADP-associated anti-platelet effects of glucosamine was dose dependent and could be overcome with the addition of a higher concentration of ADP. In our study, 40 μM of ADP was used for platelet activation, which was the lowest concentration of ADP that consistently yielded 50% platelet aggregation in untreated horses and dogs. Therefore, it is possible that with the use of a lower ADP concentration, glucosamine-associated platelet dysfunction could be detected in both horses and dogs. However, with the use of lower ADP concentrations, it is possible that glucosamine associated platelet dysfunction could be mistaken for inadequate platelet activation. One possible explanation for the differences between our study and those performed in humans and guinea pigs is a species difference in the response and sensitivity to platelet agonists. Other authors have also demonstrated species-related differences in platelet responsiveness to ADP.\textsuperscript{28,29}

With the assessment of canine platelet function, the maximum amplitude for the control samples and the three concentrations of glucosamine were below the standard reference range for both ADP- and collagen-induced platelet aggregation. One possible explanation for this difference was that the addition of sterile water, with and without glucosamine. Prior to this study, blood was collected from the same dogs that participated in this study, and using the same techniques, platelet aggregation was within reference range. Additionally, using the same aggregometry techniques, several of these dogs have participated in other studies that did not involve \textit{in vitro} incubation with sterile
water, and platelet aggregation was considered to be within reference range.\textsuperscript{1} Additionally, compared to the control sample and the 1 μg/mL concentration, there was a significant increase in the maximal amplitude when samples were incubated with 100 μg/mL of glucosamine. However, the amplitude results for this superphysiologic concentration are still below the standard reference range of ADP-induced aggregation, and does not necessarily indicate an increase in platelet aggregation. However, additional studies are required to determine the effects of glucosamine on increase platelet aggregation.

Additional differences between our study and the previous studies was the length and method of glucosamine treatment. In both the human and guinea pig studies, glucosamine was administered \textit{in vivo} for an extended length of time.\textsuperscript{13,14} This long-term administration and exposure of glucosamine to platelets could have contributed to the inhibition in platelet aggregation. In our study, we utilized previously published protocols that were capable of detecting drug associated platelet inhibition following a brief \textit{in vitro} incubation period.\textsuperscript{19-21} Therefore, in horses and dogs, it is possible that oral administration of glucosamine over an extended period of time could have an accumulative anti-platelet effect. Additional studies are required to determine the anti-platelet effects of glucosamine following long-term, oral glucosamine therapy.

Based on previous pharmacokinetic analysis of oral glucosamine administration in horses\textsuperscript{12,15} and dogs\textsuperscript{16,17}, the concentrations of glucosamine used in this study were based on approximate plasma concentrations in horses and dogs. Unfortunately, there is a wide variation in plasma concentrations for both species in these studies. There are several factors that contribute to the wide variation in glucosamine plasma
concentrations, such as differences glucosamine bioavailability, methodology used to measure the plasma concentration, and glucosamine formulation. The 1 µg/mL and 10 µg/mL glucosamine concentrations represent the range of achievable plasma concentrations reported following oral glucosamine administration in dogs and horses.\textsuperscript{12,15,16} The 100 µg/mL glucosamine concentration used in this study is 9.4 and 14.7 times greater than the documented mean peak plasma concentrations following oral glucosamine administration in horses\textsuperscript{12} and dogs\textsuperscript{16}, respectively. In horses, plasma concentrations can be as high as 349 µg/mL after intravenous glucosamine administration\textsuperscript{12}, however given the variation in glucosamine oral bioavailability, approximately 5% in horses\textsuperscript{12} and 12-26% in dogs\textsuperscript{16,30}, oral administration of standard glucosamine doses are unlikely to achieve plasma concentrations of 100 µg/mL.

Once synthesized and released by activated platelets, thromboxane A\textsubscript{2} has an immediate physiologic effect, triggers vasoconstriction, enhances platelet aggregation, and induces further platelet activation, and is then metabolized to the stable thromboxane B\textsubscript{2} metabolite.\textsuperscript{31-33} Measurement of this metabolite has been used in previous studies as an indicator of drug-associated thromboxane inhibition.\textsuperscript{34-36} In both humans and guinea pigs, glucosamine has been shown to inhibit ADP-induced thromboxane B\textsubscript{2} synthesis,\textsuperscript{13,14} suggesting an additional mechanism of glucosamine-associated platelet dysfunction. In our study, there was no change in equine or canine platelet thromboxane B\textsubscript{2} synthesis at any glucosamine concentration, suggesting that treatment with glucosamine does not alter resting platelet thromboxane synthesis. However, the addition of platelet agonists, ADP or collagen, to the incubated PRP samples would have determined the concentration of ADP- or collagen-induced platelet thromboxane synthesis.
The results of our study indicate that, unlike humans and guinea pigs, glucosamine does not exert an *in vitro* anti-platelet effect on equine and canine platelet aggregation and thromboxane synthesis, even at superphysiologic plasma concentrations. However, it is unknown if long-term oral glucosamine administration will inhibit platelet aggregation or thromboxane synthesis.
Footnotes:
a. 3.2% sodium citrate, Vacutainer tube, Becton Dickinson, Franklin Lakes, NJ
b. D-(+)-Glucosamine hydrochloride, BioReagent, Sigma Aldrich, St. Louis, MO
c. Chronolog 700 Whole Blood/Optical Lumi-Aggregometer, Chronolog Corporation Haverton, PA
d. Chronolog 700 Manual, Chronolog Corporation, Haverton, PA
e. AGGRO/LINK 8, Chronolog Corporation, Haverton, PA
f. Thromboxane B₂ EIA Kit. Cayman Chemical Company, Ann Arbor, MI
g. SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, California
h. SAS for Windows 9.4 SAS Institute, Inc., Cary, NC, USA
References:


CHAPTER IV

CONCLUSION

The information provided in this thesis is relevant in terms of veterinary clinical application. Ultimately, this research will be beneficial in understanding more about how glucosamine affects equine and canine platelets in vitro. The lack of inhibition of platelet function could have real world applications to veterinarians by providing a greater knowledge of canine and equine platelet function, as well as lowering the possible risk of complications during surgery by educating veterinarians about the effect glucosamine has on platelet function when given at a low dose, high dose, and an extremely high dose. The results of this thesis suggest that glucosamine is safe, in terms of affecting platelet aggregation and thromboxane synthesis, when given at any of the three concentrations. Therefore, according to these results, a veterinarian should not worry about an elective surgical procedure on a patient receiving glucosamine.

Although there was not a statistically significant decrease in platelet aggregation or thromboxane synthesis at any of the concentrations studied, glucosamine could still have an effect on these parts of platelet function when studied in vivo. Hopefully, further research will be completed in vivo to fully explain any and all possible effects that glucosamine has on canine and equine platelets.

Through the process of writing this thesis, I gained skills that will help in my future career goals. In the beginning of this project, I wrote a lengthy proposal for the Shackouls Honors College Summer Fellowship, in order to fund the research. This was almost a mock grant and will aid in my future endeavors if I decide to pursue more research. Prior to this experience, I had never completed an in-depth scientific literature
search or used EndNote software. However, now I am able to complete a literature search using Google Scholar and the Mississippi State Library system. As I sifted through countless articles and books, I decided that I needed a more efficient way to record notes and information from my sources. Through some assistance, I was able to utilize EndNote in order to keep an accurate record of my sources and citations. When I first started writing this project, I only had a few of citations so citing them by hand was not a big feat. However, when there were over 15 citations all in various parts of the thesis, EndNote became my trusted friend. The software generated citations based upon which journal I selected and kept track of these citations for easy referencing. Finally, I had the opportunity to write my own scientific paper. While it was a difficult task, I learned how to write with a scientific tone rather than a creative writing tone. All of these academic skills were useful during this project but will be helpful when I go on to become a veterinarian and pursue more research opportunities.

In addition to these skills, I learned valuable laboratory techniques, such as pipetting with one tip or with multiple tips. I learned how to operate the turbidometric aggregometer and use a commercially available ELISA kit to determine platelet function. These skills may be focused on understanding platelets however, it demonstrated my ability to step out of my comfort zone and learn unfamiliar techniques.

Ultimately, I believe that the Honors Thesis experience has adequately prepared me for my future by allowing me to work with scholarly mentors in pursuit of relevant and important research. The opportunities provided by the Honors College has led me to develop new skills that will be helpful in my future academics and career as a veterinarian.