EFFECTS OF DIETARY CALCIUM FRUCTOBORATE SUPPLEMENTATION ON JOINT COMFORT AND FLEXIBILITY AND SERUM INFLAMMATORY MARKERS IN DOGS WITH OSTEOARTHRITIS

BY

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THESIS

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ABSTRACT

Symptoms of osteoarthritis (OA) afflict approximately 20% of adult dogs in North America. Clinical signs consistent with OA include decreased range of motion of a joint, reduced physical activity, difficulty climbing stairs or onto furniture, and a reduced ability to rise from a lying position. A safe and effective nutraceutical supplement may benefit dogs suffering from OA. Calcium fructoborate (CFB), a mimetic of a naturally occurring molecule, has previously been reported to be safe and effective in humans with joint problems. The objective of this randomized, double-blinded, placebo-controlled study was to evaluate the short-term effects of CFB alone, or in combination with a blend of glucosamine hydrochloride (GH) and chondroitin sulfate (CS), on gait analysis, goniometry, serum inflammatory markers, and owner perception of pain in client-owned dogs. Sixty-four dogs with joint discomfort were recruited and 59 dogs (mean age = 8.42 ± 0.37 yr.; mean BW = 31.11 ± 1.28 kg) completed the study. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee, and pet owners signed an informed consent prior to study initiation. Dogs were randomly assigned to one of four treatments: placebo (60 mg fructose; n = 15), low dose (69 mg CFB; n = 14), high dose (127 mg CFB; n = 14), or combination (69 mg CFB, 500 mg GH and 200 mg CS; n = 16). Treatments were provided once daily as dietary supplements. Small dogs weighing up to 22.9 kg received 1 capsule/day, while large dogs weighing 23 to 50 kg received 2 capsules/day for 28 days. A physical examination, radiographs, goniometry measurements, gait analysis, blood sample collection, and the canine brief pain inventory (CBPI) questionnaire were performed and administered on days 0 and 28. As expected, a majority (69%) of the dogs were overweight or obese, with a body condition score (BCS) > 6 on a 9-point scale. Dogs fed the low dose (-2.93) and high dose (-2.21) of CFB were shown to improve ($P < 0.05$) in their ability to rise from a

lying position from day 0 to day 28 compared to dogs fed the placebo (0.00), but no difference was observed for dogs fed the combination treatment. Dogs assigned the low dose of CFB also tended to have an improved pain severity score (PSS; -1.46; P = 0.08) and pain at its worst score (-2.14; P = 0.06) from day 0 to day 28 compared to dogs fed the placebo (0.05 and 0.00, respectively). Dogs fed the high dose of CFB had a greater increase (P = 0.05) in serum concentration of soluble receptor for advanced glycation end products (sRAGE) from day 0 to day 28 (7.88 ng/mL) compared to dogs fed the placebo (0.83 ng/mL). All blood metabolites were within reference range except total alkaline phosphatase and corticosteroid-induced alkaline phosphatase, which started and ended at concentrations greater than the upper reference range. Dogs assigned the high dose of CFB tended to have a greater reduction (P = 0.07) in serum chloride from day 0 to day 28 (-1.64 mmol/L) compared to dogs fed the low dose of CFB (0.08 mmol/L). Given the low number of small dogs recruited and the increased variability noted as a result of their inclusion, a sub-analysis of large dogs only was performed. Large dogs fed the low dose were shown to have decreased (P < 0.05) scores for PSS (-1.77) and pain at its worst (-2.45) from day 0 to day 28 compared to the placebo group (0.19 and 0.42, respectively). Large dogs assigned the low dose of CFB tended to have improved scores for pain at its least (-1.27; P = 0.08) and pain on average (-1.82; P = 0.07) from day 0 to day 28 compared to dogs fed the placebo (0.25 and -0.08, respectively), but no difference was observed for dogs fed the high dose or combination groups. Large dogs fed the low dose also were shown to improve (P < 0.05) in their ability to rise from a lying position (-3.09) compared to the placebo treatment (0.25) from day 0 to day 28. Overall, supplementation of CFB alone was well-tolerated and appeared to have potential for joint discomfort mitigation in canines.

For my family.

Thank you for always believing in me.

This achievement could not have been accomplished

without your constant love, prayers, and encouragement.

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Chapter 1

Introduction

Classifying pets as members of the family is a growing trend in the United States. In 2011, 63.2% of owners viewed their pets as members of the family (American Veterinary Medical Association, 2012). With that trend, a pet's health, lifespan, and quality of life (QOL) have become increasingly important to owners. The American Pet Products Association reported that 65% of the households in the United States own a pet, including a population of over 77 million dogs (American Pet Products Association, 2015). As man's best friend, owners closely monitor their dogs and will go to great lengths to maintain health and avoid pain.

Osteoarthritis (OA), also known as degenerative joint disease or osteoarthrosis, is a debilitating, irreversible, lifelong disease that has no cure. In North America, over 20% of dogs over the age of 1 suffer from OA (Johnston, 1997). Based on the results of a lifelong study conducted on Labrador Retrievers that noted a linear development of OA with age, the incidence in aged dogs is much greater (Smith et al., 2006). Therefore, the prevalence of OA is even higher in geriatric dogs. Osteoarthritis affects mainly the hips, elbows, and stifles of dogs, but also can cause problems in vertebral, carpal, and tarsal joints. Common clinical signs include pain, tenderness, decreased range of motion (ROM), swelling, stiffness, muscle atrophy, crepitus, and effusion. Due to the pain and other symptoms, an affected dog's behavior may change and lead to aggression, decreased activity, limping, problems rising from a lying position, and difficulty climbing stairs or onto furniture. Age is the main risk factor of OA, but other predisposing factors include genetics, size of the breed, obesity, joint deformity, trauma and fractures, surgery, and elbow or hip dysplasia (MacPhail, 2000; Rychel, 2010; Sandell, 2012).

Although there is no cure for OA, modalities exist such that the disease may be managed, resulting in slowed progression and decreased pain and signs associated with the disease. Treatment of OA aims to improve QOL of the animal by relieving pain, decreasing inflammation, increasing activity level, and increasing ROM of the joint (MacPhail, 2000). Nutraceuticals, food or dietary supplements that offer a health or medical benefit have become popular treatments for OA. Some treatments already exist, but novel nutraceuticals are being sought by the industry. Calcium fructoborate (CFB) is a naturally-occurring plant-mineral complex found in certain fruits, nuts, and legumes. It is commercially manufactured from a proprietary reaction of fructose, calcium, and boric acid that produces a nature-identical molecule composed of calcium bound to mono- and di-fructoborate complexes. Calcium fructoborate has been shown to positively affect humans suffering from symptoms of OA in several clinical studies, but has yet to be tested in dogs. Therefore, the objective of this study was to evaluate the short-term effects of CFB alone, or in combination with a blend of glucosamine hydrochloride (GH) and chondroitin sulfate (CS), on serum inflammatory markers, goniometry, gait analysis, and owner perception of pain in client-owned dogs. It was hypothesized that the CFB treatment would improve joint mobility, decrease pain, and decrease inflammatory markers.

Chapter 2

Literature Review

DOG POPULATION AND INCIDENCE OF OSTEOARTHRITIS

The American Pet Products Association reported that 65% of the households in the United States own a pet, including a population of over 77 million dogs (American Pet Products Association, 2015). In 2011, 63.2% of owners classified their animals as members of the family (American Veterinary Medical Association, 2012). Because of that classification, a pet's health, in addition to their length and quality of life (QOL), have become more important to owners. As man's best friend, owners closely monitor their dogs and will go to great lengths to maintain health and avoid pain.

Osteoarthritis (OA), also known as degenerative joint disease or osteoarthrosis, is a debilitating, irreversible, lifelong disease that has no cure. In North America, more than 20% of dogs greater than the age of 1 suffer from OA (Johnston, 1997). Based on the results of a lifelong study conducted on Labrador Retrievers that noted a linear development of OA with age, the incidence in aged dogs is much greater (Smith et al., 2006). Therefore, the prevalence of OA is even higher in geriatric dogs. More recent data agree, as the Banfield Pet Hospital State of Pet Health 2012 Report stated that nearly 1 in 4 geriatric (> 10 years) large (22.7 to 40.9 kg) and giant breed dogs (≥ 41 kg) are diagnosed with arthritis.

DEFINING OSTEOARTHRITIS

Osteoarthritis is a slowly progressive disease of the joint that is affiliated with an imbalance of the synthesis and breakdown of articular cartilage and characterized by degradation of articular cartilage, osteophyte formation, bone remodeling and subchondral bone thickening,

and inflammation of varying degree accompanied by pain and disability (Howell, 1986; Johnston, 1997; Sanderson et al., 2009). The specific etiology of OA is unknown, as it is considered a complex condition with a multitude of interacting biochemical and biomechanical factors. It can be considered an idiopathic disease, or it can be secondary to trauma and cause joint deformities. Osteoarthritis may occur from exogenous trauma that puts stress on a joint or may occur from normal forces that exacerbate problems on an abnormal joint, such as hip dysplasia or osteochondrosis (Johnston, 1997).

JOINT STRUCTURE AND DYSFUNCTION

Articular Cartilage

Hyaline, or articular, cartilage is a smooth, white tissue covering the end of long bones in a joint that allows almost frictionless motion and transmits load and shearing force to the subchondral bone. Chondrocytes and extracellular matrix are the primary components in cartilage. Chondrocytes, the metabolically active cells of cartilage, are responsible for the maintenance of the extracellular matrix. Collagen, proteoglycans, and water make up the extracellular matrix and work together to distribute force over subchondral bone and allow smooth movement of the joint. Structural support of the extracellular matrix in cartilage is provided by collagen fibrils and the distribution of proteoglycans among the fibrils. Proteoglycans have an affinity for water, which creates a swelling pressure necessary for the proper function and compression resistance of the joint. When compression is exerted on a joint, water slowly moves through the densely packed, extracellular matrix. Water then will leak onto the surface for hydrostatic lubrication. When cartilage fibers are damaged or the connections between proteoglycans and collagen fibrils are broken and a compressive force is applied, it can

cause damage to the extracellular matrix. This leads to the reduction of the cushioning capacity of cartilage and subsequent development of OA (Johnston, 1997).

Subchondral Bone

Subchondral bone is a deformable, thin layer of bone in contact with cartilage on one side and cancellous bone on the other. It helps support the cartilage by reducing peak load through a large contact area distribution (Radin and Paul, 1970). Thickening of the subchondral bone layer occurs when the extracellular matrix is damaged and loses its cushioning ability, causing the subchondral bone to be exposed to extra force (Johnston, 1997). Recurring trauma to a joint may also cause micro-fractures within the subchondral bone, leading to stiffness and decreased deformability (Henrotin et al., 2005).

Osteophytes

The presence or absence of osteophytes is thought to be a defining point for diagnosing OA (Spector et al., 1993). An osteophyte, also called a bone spur, is a bony outgrowth from the subchondral bone layer. Osteophytes have been speculated to form because of joint instability. In such cases, it is thought that the body attempts to increase the surface area by forming the spur to increase joint stability. The exact reason and the method by which this process occurs, however, is unknown. Despite the reason for their development, osteophytes tend to cause pain during motion because they extend toward the periosteum of bones and alter the normal movement of a joint (Johnston, 1997).

Joint Cavity

The joint cavity, or space between two articulating bones, is comprised of three layers, including the synovial membrane (innermost), subsynovial layer (middle), and the fibrous joint capsule (outermost). Synoviocytes remove debris from joints and produce cytokines. Synovial fluid is composed of electrolytes and other small molecules in similar proportions as plasma. Inflammation causes increased permeability of the synovium vasculature, which results in an increased protein concentration in synovial fluid and increased exchange of molecules across the synovial membrane. Production of cytokines by synoviocytes attracts extra inflammatory cells and releases prostaglandins, which cause further damage and continues the degradation of cartilage (Pelletier et al., 1985). Also, as articular cartilage is damaged, fragments are broken off and initiate an inflammatory response (Ghosh and Smith, 1993). Inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), or interleukin-1beta (IL-1β), stimulate fibroblasts to increase collagen production, causing joint capsule thickening due to increased collagen production (Krasnokutsky et al., 2008). Joint capsule thickening directly relates to stiffness, decreased range of motion (ROM) of a joint, and pain all of which are clinical signs of OA.

OSTEOARTHRITIS DIAGNOSIS

Physical Examination

Diagnosing an animal with OA is most accurate with the use of multiple measurements. Along with a medical history, a physical examination can determine if the clinical signs are due to OA or a neurologic problem. The physical exam helps to decide which joints are affected by OA and establishes a degree of severity, even though it is a bit subjective and may vary from veterinarian to veterinarian. An exam also determines if swelling, pain, crepitus, or effusion of

the joint are present. Goniometry has been demonstrated to be a reliable method to measure the ROM of a joint in dogs (Jaegger et al., 2002). A goniometer is a device that is used to measure angles of flexion and extension of a joint. It is likely that a dog experiencing pain will have decreased ROM of the respective joint (Jaegger et al., 2002). Unfortunately, because of the variability due to breed, age, genetics, joint health, etc., standards for normal ROM of a joint are difficult to establish. The study conducted by Jaegger et al. (2002) established normal ROM for healthy Labrador Retrievers, but no other breeds. When evaluating the efficacy of a treatment, an increase of 5 to 10 degrees of ROM is a sign of improvement in a clinical setting. Over a long-term situation, the lack of ROM loss may be a positive outcome (personal communication with Kim Knap, CVT with 12+ years of experience).

Radiography

Radiography is a standard method used by veterinarians in diagnosing OA. Although joint space narrowing may be a better indicator of disease progression in humans (Spector et al., 1993), osteophyte formation is the most common feature used to assess OA presence in dogs (Innes et al., 2004). Radiographs also show subluxation, effusion, subchondral sclerosis, joint space narrowing, and bone remodeling as well as rule out any irregularities that are not related to OA (DeLuke et al., 2012; MacPhail, 2000; Rychel, 2010). Veterinarians use a subjective scoring system to rank the disease progression based on joint effusion, osteophytosis, subchondral sclerosis, intra-articular mineralization, and overall disease severity (Innes et al., 2004).

Gait Analysis

Gait analysis is the process by which quantitative variables are measured and recorded to assess limb functions, gait abnormalities, and to determine the efficacy of treatment interventions (Brown et al., 2013; Kim et al., 2011). In most canine studies, force plate platforms embedded flush with a walkway are used to measure forces between the walkway and foot that are generated when paws contact the floor while walking (McLaughlin, 2001). Peak vertical force (PVF) is the maximal force an animal exerts on a force plate per limb. Vertical impulse (VI) is the amount of time the animal is exerting pressure on the limb. An increase in both PVF and VI compared to baseline would indicate an improvement in joint function and, possibly, reduced pain. Gait analysis has been shown to be an effective method of analyzing the gait of an animal and determining the effectiveness of a variety of treatments (Gupta et al., 2012; Marshall et al., 2010; Mlacnik et al., 2006; Rialland et al., 2013).

Biomarkers

Given the time, cost, special equipment, and clinical expertise required for current OA diagnostic techniques, a need for discovering biomarkers in biological fluids including blood, urine, or saliva exists. Reliable biomarkers would not only reduce cost and time involved with diagnostics, but early detection of the disease would allow for the use of treatments that slow disease progression and extend life span (Mobasheri and Henrotin, 2011; Rorvik and Grondahl, 1995). The measurement of biomarkers using minimally invasive methods also allows sampling over time to monitor disease progression and adjust treatments accordingly (Hegemann et al., 2002; Lohmander, 1997). Although many candidate biomarkers have been suggested and studied, none have been validated to be pre-radiographic biomarkers specific to OA (Mobasheri

and Henrotin, 2011). Matrix and bone components, inflammatory cytokines, proteases, and protease inhibitors are good OA biomarker candidates (Lohmander, 1997; Patra and Sandell, 2011; Tseng et al., 2009). Type II collagen epitopes, such as C2C, are considered strong biomarkers because they are abundant in cartilage, are relatively specific to articular cartilage, and the breakdown of cartilage increases their concentration in synovial fluid and serum (Mobasheri and Henrotin, 2011). Matrix metalloproteases (MMPs) are common biomarkers, as they are known to break down collagen and proteoglycans in the extracellular matrix (Garner et al., 2011; Hegemann et al., 2002). Cartilage oligomeric matrix protein (COMP) interacts with collagen and fibrils and is thought to play a role in the structural integrity of the extracellular matrix (Tseng et al., 2009). C-reactive protein (CRP) is a positive acute phase protein whose concentration increases $\geq$ 25% in response to pro-inflammatory cytokines (Bennett et al., 2013). These, and other biomarkers, have been thought to be good biomarkers for OA in humans and/or canines as their concentrations increase with disease progression (Bennett et al., 2013; Lohmander, 1997; Mobasheri and Henrotin, 2011). As stated earlier, however, they have been used to monitor the severity of disease or response to treatment rather than validated as a pre-radiographic marker of OA. Because a multitude of biological processes occur in the development of OA, a combination of multiple biomarkers may be best to identify early progression of the disease (Mobasheri and Henrotin, 2011; Rousseau and Delmas, 2007).

CURRENT TREATMENTS

Surgery

The objective of surgery is to relieve pain, re-establish limb function and use, eradicate pathologic changes, and prevent the development of, or postpone progression of, pre-existing OA (Cook and Payne, 1997; Dahlberg et al., 2005; Eskelinen et al., 2012). Surgery is generally

recommended and performed on animals when alternative therapies have been unsuccessful. Total joint replacement, most commonly performed on the hip joint, is a biomechanical surgical option ideally suited for severely impacted, middle aged patients of medium to large breeds (Conzemius and Vandervoort, 2005). Excision arthroplasty is also common in hip joints, but has been successful in other joints including the elbow (Conzemius et al., 2003). The procedure involves the removal of a piece of the joint that is limiting the function and causing pain, and leaving a space that allows the joint to move without impairment (Cook and Payne, 1997). Common in unstable joints, athrodesis is the fusion of bones of a joint using plates and screws, essentially eliminating the joint itself, and removing the remaining cartilage (Dyce, 1996). This procedure can be done in almost any joint, besides the hip joint, and is used to decrease pain and ideally improve QOL of the pet.

Pharmacological Agents

Corticosteroids are steroid hormones made by the adrenal cortex, such as hydrocortisone and cortisone. Synthetic mimetics of those hormones are commonly prescribed late in the disease course and if the animal is resistant to other treatments. The mechanism is not fully understood, but they are likely effective because of their anti-inflammatory properties (Pelletier et al., 1994). Limited studies testing the efficacy of corticosteroids have been conducted on dogs, but positive results have been reported. One study treated cranial-cruciate ligament sectioned dogs (n = 24) with oral prednisone (0.25 mg/kg BW/day) or intra-articular injections of triamcinolone hexacetonide (5 mg) at the time of surgery and 4 weeks later, with both treatments resulting in a reduction of osteophyte size ($P < 0.006$ and $P < 0.04$, respectively) compared to the untreated dogs (Pelletier and Martel-Pelletier, 1989). Another study, conducted by the same researchers,

tested the effects of intra-articular injections of methylprednisolone acetate (20 mg) at time of anterior cruciate ligament sectioning and 4 weeks after surgery, with injections resulting in a reduction in incidence ($P < 0.004$) and size ($P < 0.0001$) of osteophytes compared with untreated dogs (Pelletier et al., 1994).

The most widely used analgesics in veterinary medicine are nonsteroidal anti-inflammatory drugs (NSAIDs) (Lascelles et al., 2005) and are a key element in the treatment of OA (Aragon et al., 2007; Sanderson et al., 2009). Six NSAIDs have been approved by the US Food and Drug Administration for use in dogs: carprofen, meloxicam, tepoxalin, firocoxib, deracoxib, and etodolac (Innes et al., 2010). They function by inhibiting the enzyme cyclooxygenase (COX) forms 1, 2, or both. COX-1 converts arachidonic acid to prostaglandins and thromboxane that regulate normal cell homeostasis, such as vasodilation, nociceptor sensitization, renal blood flow maintenance, platelet aggregation, and gastrointestinal (GI) mucosal cell turnover (KuKanich et al., 2012). COX-2 synthesizes prostaglandins from arachidonic acid that are mediators of pain and inflammation (Cho et al., 2015; Lascelles et al., 2005; MacPhail, 2000). Aspirin is an over-the-counter NSAID not licensed for dogs, but the veterinary prescribed NSAIDs are superior as they cause less GI side effects (Rychel, 2010).

Owners should be educated on clinical signs associated with the GI-, renal-, or hepatic-related adverse reactions associated with pharmacological agents (Lascelles et al., 2005; Rychel, 2010). Dogs with pre-existing kidney, heart, or liver problems, or dehydrated pets, are at highest risk for adverse reactions. The most common adverse reactions are GI toxicity resulting in ulceration, prolonged bleeding time, renal damage, and liver damage. Clinical signs of NSAID toxicity include vomiting, diarrhea, blackened/tarry or bloody feces, and anorexia (Monteiro-Steagall et al., 2013). A review of 64 NSAID studies reported that adverse reactions were more

common in clinical trials (62%) compared to research studies (38%), and the two most common signs were vomiting and diarrhea (Monteiro-Steagall et al., 2013). It is hypothesized that NSAIDs with COX-2 selectivity, but having a COX-1 sparing effect (termed the coxibs), decrease the incidence of adverse effects by preserving the GI mucosa. However, this has not been proven in veterinary medicine (Bombardier, 2002; Monteiro-Steagall et al., 2013). Because of the known adverse reactions to NSAIDs, other treatment therapies with fewer side effects are often sought after.

Weight Loss

According to the 2014 National Pet Obesity Awareness Day Survey, approximately 43.8 million, or 52.7%, of dogs in the United States are overweight or obese (BCS $\geq$ 4 on a 5-point scale) (Association for Pet Obesity, 2014). Because obesity is a risk factor for OA, caloric restriction and/or BW loss may be an effective treatment for overweight OA pets (Kealy et al., 2000). Chronic stress on a joint due to excessive BW has been shown to lead to articular cartilage breakdown (Impellizeri et al., 2000; Joshua, 1970). Although the exact mechanism is unknown, BW loss is thought to decrease the biomechanical stress placed on joints. A 6-month study conducted on OA-stricken, overweight dogs (n = 29; mean age = 8.4 years, BCS $\geq$ 4 on a 5-point scale) reported a decrease ($P < 0.01$) in BW from baseline every 30-day time period for up to 6 months after being fed a restricted calorie diet at 60% of the daily metabolizable energy requirement of a BW that was set at 15% less than their baseline BW (Mlacnik et al., 2006). That study used an objective measure of gait analysis and showed improvements ($P < 0.01$) in PVF and VI on days 60, 120, and 180 compared to baseline in the treatment group fed a restricted calorie diet + 2 physical therapy sessions per week giving transcutaneous electrical nerve

stimulation. Another study performed in overweight dogs (n = 9; ages 6 to 13 years; BW = > 10% ideal BW) suffering from OA were fed 60% less than their normal food intake for up to 19 weeks and had decreased ($P < 0.05$) BW (33.4 kg) and BCS (3) compared to baseline (39.0 and 5, respectively) (Impellizeri et al., 2000). Arguably, the most interesting data for diet restriction in dogs suffering from OA comes from a lifelong study conducted on paired Labrador Retrievers (n = 48) (Smith et al., 2006). In that study, dogs were either allotted to the control group (ad-libitum feeding until 3.25 years, then adjusted to prevent obesity) or the restricted-fed group (25% less food than the controls). A difference ($P < 0.001$) in onset of OA was observed between controls (median = 6 years) and restricted-fed (median = 12 years) (Smith et al., 2006). Also, at 14 years of age, 83% of dogs in the control treatment were diagnosed with hip OA compared to 50% of dogs in the restricted-fed group (Smith et al., 2006). Collectively, these results demonstrate that dietary restriction not only decreases BW and BCS and improves gait analysis measurements such as PFV and VI, but it also delays the onset of OA in dogs prone to developing the disease.

Rehabilitation

Osteoarthritis patients are known to have decreased ROM and increased stiffness of joints. Rehabilitation therapies, such as stretching, acupuncture, and hydrotherapy, have been shown to have positive outcomes on symptoms of OA in dogs (Crook et al., 2007; Levine et al., 2010; Levine et al., 2004; Millis and Levine, 1997; Nganvongpanit et al., 2014). An at-home rehabilitation program for Labrador Retrievers (n = 8; mean age = 7.5 years) suffering from OA consisted of stretching the affected joint to full flexion and extension for 10 seconds, 10 times/day for a total of 21 days (Crook et al., 2007). That rehabilitation strategy resulted in an

increase ($P < 0.0005$) in the ROM of the affected joint for all dogs compared to baseline measured by goniometry, with a mean of 14.6° for joint flexion (Crook et al., 2007). Successful treatment using rehabilitation therapies could reduce the need for pharmaceutical products as a treatment of OA.

Acupuncture is an alternative method for pain management of OA. The exact mechanisms by which acupuncture may function are not completely understood, but is believed to be related to the release of endogenous endorphins that change the nociceptive pain, pain arising from the stimulation of nerve cells, as well as the decrease of perceived pain and increase blood circulation and muscle spasm relief (Mittleman and Gaynor, 2000). A study was conducted on 61 OA dogs (117 joints), with acupuncture treatment using 28 gauge, 5 cm long needles for 15 minutes once a week for at least 3 weeks or until a satisfactory result was achieved. Results were scored on a 5-point scale, with 1 being no improvement and 5 being perfect by veterinarians and owners (Janssens, 1986). The results of that study noted that the use of the acupuncture treatment led to scores of 4 or 5 in 62% of the dogs, but the authors did not note whether these scores were from the owner or veterinarian. That study included no placebo group and the treatment was not blinded. Acupuncture as a sole treatment for OA is not effective enough to reduce lameness, but in conjunction with other treatments, it may help to improve QOL with almost immediate improvements in mobility, demeanor, and levels of pain (Kapatkin et al., 2006; Rychel, 2010).

Aquatic therapy is especially good for OA dogs, as buoyancy decreases the weight-bearing force, which allows further flexibility of an affected joint (Levine et al., 2004). A study conducted on healthy dogs (n = 10; mean age = 5.7 years; mean BW = 25.4 kg) tested the vertical ground reaction force (vGRF), the force with the largest magnitude that the ground

imparts on the body in the vertical direction, at 3 water levels (tarsal, stifle, and hip joints) and demonstrated a reduction ($P < 0.001$) of vGRF of 9, 15, and 62%, respectively (Levine et al., 2010). A hydrotherapy study conducted by Nganvongpanit et al. (2014) assigned dogs to three treatments: OA-SW (OA + swimming; n = 22), H-SW (healthy + swimming; n = 18), and H-NSW (healthy + no swimming; n = 15). Dogs in the swimming treatment groups were allowed three 20-minute swim times in an outdoor pool twice a week for a period of 8 weeks. Lameness, joint mobility, pain on palpation, weight-bearing, and overall scores of the OA-SW group were decreased ($P < 0.05$) on week 8 (2.48; 1.48; 1.48; 1.48; 1.19, respectively) compared to baseline (3.00; 1.76; 2.00; 2.05; 1.62, respectively) using a subjective test conducted by two veterinarians 30-minutes apart (Nganvongpanit et al., 2014). Flexion and extension of both hip joints also were improved ($P < 0.05$) at week 8 compared to baseline for the OA-SW group (Nganvongpanit et al., 2014). Hydrotherapy, in the form of underwater treadmill or swimming pools, is an effective form of therapy shown to improve ROM and decrease clinical signs of OA by both subjective and objective measures.

COMMON NUTRACEUTICALS

Glucosamine and Chondroitin

Because glucosamine and chondroitin are components of proteoglycans in articular cartilage, they are logical therapeutic agents to treat OA (Bottegoni et al., 2014; Huskisson, 2008). Glucosamine is made from glucose and is a component of glycosaminoglycan chains, which make up proteoglycans. Because articular damage is associated with OA, it is thought that glucosamine is a substrate that may help repair cartilage through proteoglycan synthesis (Šimáneka et al., 2005). Chondroitin sulfate makes up the majority of glycosaminoglycans,

therefore it is important for the structure of cartilage. It is also known to reduce the concentration of pro-inflammatory cytokines (Bottegoni et al., 2014). The use of CS and glucosamine together have been shown to work additively to help decrease the advancement of OA by favoring matrix synthesis and repairing articular cartilage (Clegg et al., 2006; Johnson et al., 2001).

It is important to note how bioavailable GH and CS are after oral administration of supplements. A study conducted by Adebowale et al. (2002) used healthy beagle dogs (n = 8) to determine the bioavailability of GH and CS after a single dose in a crossover study with the following treatments: (A) IV solution of 500 mg GH and 400 mg low molecular weight CS (LMWCS), (B) 1500 mg GH and 1200 mg LMWCS, and (C) 2000 mg GH and 1600 mg LMWCS. They also conducted a study to test the bioavailability after multiple doses of 1500 mg GH and 1200 mg LMWCS for the first 7 days, before increasing the dose to 3000 mg GH and 2400 mg LMWCS for days 8 to 14 (Adebowale et al., 2002). The results indicated that GH had a mean bioavailability of 12.1 to 12.7% and CS as total disaccharides was 4.8 to 5.0% bioavailable after a single dose (Adebowale et al., 2002).

These two compounds have been shown to reduce the symptoms commonly associated with OA. A double-blind, positive-controlled study reported positive effects of GH/CS in dogs (n = 35) suffering from OA by testing a supplement containing the following active ingredients: GH at 475 mg/g, CS at 350 mg/g, N-acetyl-D-glucosamine at 50 mg/g, ascorbic acid at 50 mg/g, and zinc sulfate at 30 mg/g (McCarthy et al., 2007). For the first 70 days, the supplement was dosed at 1 g active ingredient twice daily for 5 to 19.9 kg dogs, 1.5 g twice daily for 20 to 40 kg dogs, and 2 g active ingredient twice daily for dogs weighing > 40 kg (McCarthy et al., 2007). The dose then was reduced by 1/3 for the next 28 days (McCarthy et al., 2007). In that study, improvements ($P < 0.001$) in overall score of condition, pain on palpation, and weight-bearing

from baseline scores were observed. Another group of OA dogs (n = 7 to 10) showed a reduction ($P < 0.05$) in observational pain overall (51%), after limb manipulation (48%), and after physical exertion (43%) after 150 days of supplementation of 2000 mg GH and 1600 mg CS per day ($P < 0.05$) in a double-blind, placebo-controlled study (Gupta et al., 2012). Although some studies have shown improved pain assessment and joint mobility, other studies that have not shown significant effects of GH and/or CS on symptoms of OA (D'Altilio et al., 2007; Dobenecker et al., 2002; Moreau et al., 2003).

Fish Oil

Fish oil is known to contain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are long-chain omega-3 fatty acids. These fatty acids are associated with inhibiting synthesis and decreasing serum concentrations of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Curtis et al., 2000; Hall et al., 2006; Hielm-Bjorkman et al., 2012). Omega-3 fatty acids also have been shown to decrease the activity of MMPs, a factor in cartilage degradation, and increase tissue inhibitor MMP-2 (Hansen et al., 2008) as well as decrease arachidonic acid concentrations (Calder and Zurier, 2001). A study conducted on mixed-breed OA-stricken dogs (n = 71) tested the supplementation of fish oil to a commercial diet at an average of 110.25 mg/kg BW of EPA and DHA for 16 weeks. No differences in PVF, VI, and use of NSAIDs were reported between treatment groups (Hielm-Bjorkman et al., 2012). There was an increase ($P = 0.021$) of PVF for the fish oil group from baseline to the end of the study and a trend towards improvement for VI ($P = 0.092$) (Hielm-Bjorkman et al., 2012). A 6-month, multicenter study with dogs suffering from OA (n = 127) tested a food supplemented with 3.5% fish oil-based omega-3 fatty acids compared to the control diet (Roush et al., 2010a). In that

study, owners subjectively observed improvements in their dog's ability to rise from a resting position ($P = 0.033$) and reluctance to play ($P = 0.011$) between weeks 0 and 6 for the test food compared to the control. Between weeks 6 to 12 and weeks 12 to 24, dogs fed the test diet had improvements ($P = 0.024$ and $P = 0.003$, respectively) in their ability to walk compared to the control (Roush et al., 2010a). The same study by Roush et al. (2010a) resulted in increased ($P < 0.001$) serum concentrations of omega-3 fatty acids and decreased ($P < 0.001$) serum concentrations of arachidonic acid in the test group vs. the controls, indicating that the fish oil-based omega-3 fatty acids were bioavailable. Another multicenter study conducted on OA dogs ($n = 109$) standardized the dose of carprofen, an NSAID, to 4.4 mg/kg BW/day and assigned dogs to a control or test diet feeding the same diets as in the Roush et al. (2010a) study for 12 weeks (Fritsch et al., 2010). In that study, a more rapid decrease ($P = 0.025$) in carprofen dosage was reported for dogs fed the test diet compared to those fed the control (Fritsch et al., 2010). Roush et al. (2010b) conducted a similar study in OA dogs ($n = 38$) over 90 days using the same diets as described in Roush et al. (2010a). Although they did not observe differences between the test diet and the control for PVF, they observed a mean PFV change (+5.6%; $P = 0.01$) in the test group from day 0 to day 90, indicating that fish oil may improve weight-bearing ability of dogs suffering from OA (Roush et al., 2010b).

Green-Lipped Mussel

The New Zealand green-lipped mussel (GLM), *Perna canaliculus*, is a newer nutraceutical that contains many nutrients, including vitamins, minerals, omega-3 fatty acids, and glycosaminoglycans patented by Mars Inc. (Bui et al., 2003; Hielm-Bjorkman et al., 2009). Green-lipped mussel is known to have anti-inflammatory properties with active ingredients

including the presence of eicosatetraenoiec acid and a blend of other unique fatty acids (Bierer and Bui, 2002; Bui et al., 2003; Hielm-Bjorkman et al., 2009; Servet et al., 2006). It can be incorporated into food through a freeze-dried powder or as an oil extract (Pollard et al., 2006). One group of researchers conducted a series of 3 clinical studies on dogs (age range: 4 to 13 years; n = 31 to 33/study) with signs of arthritis and evaluated the effects of GLM as a powdered supplement fed on top of a control diet (< 25 kg BW = 450 mg GLM/day; 25 to 34 kg BW = 750 mg GLM/day; > 34 kg BW = 1000 mg GLM/day), or incorporated into a treat or dry main meal diet for a period of 6 weeks (Bierer and Bui, 2002). A veterinarian scored each dog for lameness when walking, trotting, and climbing stairs, resulting in a "visual score". Individual joints were scored for the degree of pain, swelling, crepitus, and mobility reduction and then summed to provide a "manipulation score". Those two scores then were averaged to give a "total arthritic score" (TAS). For all three studies, total arthritic score, joint pain, and joint swelling were reduced ($P < 0.05$) in the GLM treated groups compared to the control (Bierer and Bui, 2002). A multicenter study was conducted over 50 days on OA-stricken dogs (n = 85) evaluating the effects of GLM incorporated into an extruded diet at a dose of 0.3% (Servet et al., 2006). Veterinarians subjectively scored the dogs using the same variables as in the previous study (Bierer and Bui, 2002) for visual score, manipulation score, and TAS. All three scores were reduced ($P < 0.05$) by 36, 33, and 34%, respectively for the GLM group on day 50 compared to baseline (Servet et al., 2006). A study conducted by Pollard et al. (2006) tested a GLM supplement with 125 mg/tablet in dogs with OA over 56 days (n = 81; mean age 8.5 years). In that study, dogs weighing 5 to 15 kg received 3 tablets/day, 16 to 20 kg dogs received 5 tablets/day, 21 to 25 kg dogs received 6 tablets/day, 26 to 45 kg dogs received 8 tablets/day, and 46 to 65 kg dogs received 9 tablets/day. The study showed that 67% of the dogs in the GLM

group had improved clinical assessment scores compared to 41% in the placebo (P = 0.018) after 56 days (Pollard et al., 2006). Finally, a study was conducted on dogs suffering from OA (n = 23), with dogs fed a control diet for 30 days before being switched to a test diet enriched with GLM for 60 days (Rialland et al., 2013). That study tested gait analysis, with GLM treatment resulting in increased (P = 0.0004) PVF at the end of the study compared to baseline (Rialland et al., 2013). Because GLM is heat-sensitive, special processing conditions using cold extraction should be accounted for (Bierer and Bui, 2002; Servet et al., 2006).

Calcium Fructoborate

Calcium fructoborate, patented as FruiteX-B® by VDF FutureCeuticals, Inc., is a manufactured mimetic of a naturally occurring compound found in plants, including various herbs, fruits, and vegetables (Miljkovic, 1999; Miljkovic et al., 2009). Composed of calcium and two fructose molecules bound to boron, CFB is water-soluble and approximately 5% calcium, 92.3% fructose, and 2.7% boron with the linear formula $Ca[(C_6H_{10}O_6)_2B]_2 \bullet 4H_2O$ (Scorei and Rotaru, 2011). Calcium fructoborate has been shown to have no adverse effects and multiple beneficial effects in humans, including antioxidant and anti-inflammatory properties, anti-tumor effects, and ability to reduce pain and improve function of arthritic joints.

In vitro studies, using human keratinocytes, neutrophils, and macrophages, have been conducted to evaluate the antioxidant and anti-inflammatory properties of CFB. It is believed that CFB is a scavenger of superoxide radicals. A study was conducted to test pre-incubation concentrations (0, 45, 90, and 450 nmol) of CFB on human keratinocytes exposed to 100 µmol H_2O_2 for an hour to simulate oxidative stress (Scorei et al., 2005). In that study, there was a reduced concentration of reactive oxygen species (ROS) after pre-incubation with CFB, but it

was not directly proportional to the dose applied because the maximum antioxidant activity was observed at 90 nmol CFB (Scorei et al., 2005). A second study, whereby human polymorphonuclear neutrophils were subjected to 22,500 μM of CFB for 24 hours, observed a 92.9% decrease in ROS levels (Scorei et al., 2007). Another study measured the production of inflammatory markers by lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 cells exposed to 0.2, 0.45, or 1 mM CFB for 24 hours (Scorei et al., 2010). In the same study by Scorei et al. (2010), CFB decreased ($P < 0.01$) IL-1β concentrations by 27% and IL-6 by 90% compared to control cells exposed to LPS without CFB treatment.

Because *in vitro* data were promising, *in vivo* human studies evaluating CFB dose and effectiveness on modulating inflammatory biomarkers were conducted. In a 15-day study conducted in humans (n = 15; ages 59 to 68 years) suffering from primary OA, participants were supplemented with 28.5 mg CFB twice daily (Scorei et al., 2011). In that study, whole blood erythrocyte sedimentation rate, plasma fibrinogen, and serum CRP concentrations were decreased ($P < 0.05$) 10.25, 13.73, and 60.25%, respectively, with CFB treatment compared to baseline. Serum CRP concentrations were shown to be reduced ($P = 0.0102$) by 37% compared to baseline in a study of adult humans (n = 9 to 10; ages 44 to 65 years) afflicted with knee OA after supplementation of 216 mg/day CFB for 14 days (Reyes-Izquierdo et al., 2012). More research testing CFB at a rate of 112 mg/day for 30 days in healthy human adults (n = 26; ages 40 to 60) resulted in a 31.3% reduction ($P < 0.05$) of serum CRP compared to baseline (Rogoveanu et al., 2015). In addition to CRP, the blood markers, low-density lipoprotein, homocysteine, triglycerides, IL-1β, and monocyte chemoattractant protein-1 were reduced ($P < 0.05$) (9.8, 5.5, 9.1, 29.2, and 31%, respectively) compared to baseline, after 30 days of CFB supplementation at a dose of 112 mg/day (Rogoveanu et al., 2015).

Relieving the pain and discomfort associated with OA is a high priority for researchers and pet owners. The Western Ontario and McMaster Universities Arthritis Index (WOMAC) (Bellamy, 1988) and McGill Pain Questionnaire (MPQ) (Melzack, 1975; Melzack, 2005) are questionnaires commonly used to assess physical function and pain of human patients suffering from OA. These surveys may be used to determine the effectiveness of an intervention with the goal of observing decreased scores, representing less pain and physical limitations. After 14 days supplementation with CFB at a dose of 220 mg/day, human adults with self-reported knee discomfort (n = 60; mean age = 49.2 years) showed a 13.73-point reduction in WOMAC score and an 8.9-point reduction in MPQ score ($P < 0.0001$) (Pietrzkowski et al., 2014). The study conducted by Reyes-Izquierdo et al. (2012) resulted in decreased ($P < 0.01$) WOMAC (-22.2 points) and MPQ (-14%) scores after 14 days supplementation with CFB at 216 mg/day. An OA clinical trial tested different doses of CFB in patients (n = 20) suffering from mild to medium OA cases (6 mg CFB/day) and severe OA cases (12 mg CFB/day) (Miljkovic, unpublished data, 2002). Some interesting results were that within eight weeks, CFB was believed to be an effective painkiller in both mildly- and severely- affected patients by allowing 67 and 75% of the patients, respectively, to eliminate or reduce the amount of painkillers needed ($P < 0.05$) (Miljkovic, unpublished data, 2002). Table 2.1 displays the main results of five studies conducted on humans testing CFB. Calcium fructoborate shows promise of being effective at decreasing pain and improving the symptoms associated with OA, but this supplement has not been tested in dogs to see if it would have the same effects.

TABLE

Table 2.1. Human studies evaluating the effects of CFB on blood markers and pain symptoms associated with OA*

Reference	Age (yr)	n	Treatments	CFB doses (mg/day)	Length of treatment	Major outcomes of CFB supplementation (P < 0.05)
Miljkovic, unpublished data, 2012	N/A	20 total	Mild and medium forms	6	8 weeks	CFB for mild to medium forms of OA eliminated the need for other painkillers in 67% of the patients
			Severe forms	12		CFB for severe forms of OA eliminated or decreased the amount of painkillers in 75% of the patients
Scorei et al., 2011	59-68	15	Placebo	0 (fructose)	15 days	CFB 1 decreased whole blood ESR concentrations compared to baseline by -10.25%
		15	CFB-1	57		CFB 1 decreased plasma FBR concentrations compared to baseline by -13.73%
		15	CFB-2	113		CFB 1 decreased serum CRP concentrations compared to baseline by -60.25%
		15	CFB-3	226		
Reyes-Izquierdo et al., 2012	44-65	10	Placebo	0 (fructose)	14 days	Day 7 WOMAC scores decreased by 14.1 points compared to baseline
		9	CFB	216		Day 14 WOMAC scores decreased by 22.2 points compared to baseline
						Day 7 MPQ scores in 8/10 subjects reduced 13% compared to baseline
						Day 14 MPQ scores in 8/10 subjects reduced 14% compared to baseline
						Day 7 serum CRP levels in 7/10 subjects reduced 27% compared to baseline
						Day 14 serum CRP levels in 7/10 subjects reduced 37% compared to baseline
Pietrzowski et al., 2014	35-65	30	Placebo	0 (fructose)	14 days	Day 14 WOMAC scores decreased by 13.73 points compared to baseline
		30	CFB	220		Day 7 MPQ scores decreased by 5.8 points compared to baseline
						Day 14 MPQ scores decreased by 8.9 points compared to baseline
Rogoveanu et al., 2015	40-60	26	Placebo	0 (fructose)	30 days	CFB-1 decreased serum CRP concentrations compared to baseline by -31.3%
		26	CFB-1	112		CFB-1 and CFB-2 decreased serum LDL concentrations compared to baseline by -9.8 and -9.4%, respectively
		26	CFB-2	56		CFB-1 decreased serum homocysteine concentrations compared to baseline by -5.5%
						CFB-1 and CFB-2 decreased serum triglyceride concentrations compared to baseline by -9.1 and -8.8%, respectively
						CFB-1 decreased serum IL-1β concentrations compared to baseline by -29.2%
						CFB-1 and CFB-2 decreased serum MCP-1 concentrations compared to baseline by -31 and -26%, respectively

*OA: osteoarthritis; CFB: calcium fructoborate; N/A: not available; ESR: erythrocyte sedimentation rate; FBR: fibrinogen; CRP: c-reactive protein; WOMAC: Western Ontario and McMaster Universities Arthritis Index; MPQ: McGill Pain Questionnaire; LDL: low-density lipoprotein; IL-1β: interleukin-1β; MCP-1: monocyte chemoattractant protein-1.

Chapter 3

Effects of Dietary Calcium Fructoborate Supplementation on Joint Comfort and Flexibility and Serum Inflammatory Markers in Dogs with Osteoarthritis

ABSTRACT

Symptoms of osteoarthritis (OA) afflict approximately 20% of adult dogs in North America. Clinical signs consistent with OA include decreased range of motion of a joint, reduced physical activity, difficulty climbing stairs or onto furniture, and a reduced ability to rise from a lying position. A safe and effective nutraceutical supplement may benefit dogs suffering from OA. Calcium fructoborate (CFB), a mimetic of a naturally occurring molecule, has previously been reported to be safe and effective in humans with joint problems. The objective of this randomized, double-blinded, placebo-controlled study was to evaluate the short-term effects of CFB alone, or in combination with a blend of glucosamine hydrochloride (GH) and chondroitin sulfate (CS), on gait analysis, goniometry, serum inflammatory markers, and owner perception of pain in client-owned dogs. Sixty-four dogs with joint discomfort were recruited and 59 dogs (mean age = 8.42 ± 0.37 yr.; mean BW = 31.11 ± 1.28 kg) completed the study. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee, and pet owners signed an informed consent prior to study initiation. Dogs were randomly assigned to one of four treatments: placebo (60 mg fructose; n = 15), low dose (69 mg CFB; n = 14), high dose (127 mg CFB; n = 14), or combination (69 mg CFB, 500 mg GH and 200 mg CS; n = 16). Treatments were provided once daily as dietary supplements. Small dogs weighing up to 22.9 kg received 1 capsule/day, while large dogs weighing 23 to 50 kg received 2 capsules/day for 28 days. A physical examination, radiographs, goniometry measurements, gait analysis, blood sample collection, and the canine brief pain inventory (CBPI) questionnaire were performed and

administered on days 0 and 28. As expected, a majority (69%) of the dogs were overweight or obese, with a body condition score (BCS) > 6 on a 9-point scale. Dogs fed the low dose (-2.93) and high dose (-2.21) of CFB were shown to improve ($P < 0.05$) in their ability to rise from a lying position from day 0 to day 28 compared to dogs fed the placebo (0.00), but no difference was observed for dogs fed the combination treatment. Dogs assigned the low dose of CFB also tended to have an improved pain severity score (PSS; -1.46; $P = 0.08$) and pain at its worst score (-2.14; $P = 0.06$) from day 0 to day 28 compared to dogs fed the placebo (0.05 and 0.00, respectively). Dogs fed the high dose of CFB had a greater increase ($P = 0.05$) in serum concentration of soluble receptor for advanced glycation end products (sRAGE) from day 0 to day 28 (7.88 ng/mL) compared to dogs fed the placebo (0.83 ng/mL). All blood metabolites were within reference range except total alkaline phosphatase and corticosteroid-induced alkaline phosphatase, which started and ended at concentrations greater than the upper reference range. Dogs assigned the high dose of CFB tended to have a greater reduction ($P = 0.07$) in serum chloride from day 0 to day 28 (-1.64 mmol/L) compared to dogs fed the low dose of CFB (0.08 mmol/L). Given the low number of small dogs recruited and the increased variability noted as a result of their inclusion, a sub-analysis of large dogs only was performed. Large dogs fed the low dose were shown to have decreased ($P < 0.05$) scores for PSS (-1.77) and pain at its worst (-2.45) from day 0 to day 28 compared to the placebo group (0.19 and 0.42, respectively). Large dogs assigned the low dose of CFB tended to have improved scores for pain at its least (-1.27; $P = 0.08$) and pain on average (-1.82; $P = 0.07$) from day 0 to day 28 compared to dogs fed the placebo (0.25 and -0.08, respectively), but no difference was observed for dogs fed the high dose or combination groups. Large dogs fed the low dose also were shown to improve ($P < 0.05$) in their ability to rise from a lying position (-3.09) compared to the placebo treatment (0.25) from

day 0 to day 28. Overall, supplementation of CFB alone was well-tolerated and appeared to have potential for joint discomfort mitigation in canines.

INTRODUCTION

Classifying pets as members of the family is a growing trend in the United States. In 2011, 63.2% of owners viewed their pets as members of the family (American Veterinary Medical Association, 2012). With that trend, a pet's health, lifespan, and quality of life (QOL) have become increasingly important to owners. The American Pet Products Association reported that 65% of the households in the United States own a pet, including a population of over 77 million dogs (American Pet Products Association, 2015). As man's best friend, owners closely monitor their dogs and will go to great lengths to maintain health and avoid pain.

Osteoarthritis, also known as degenerative joint disease or osteoarthrosis, is a debilitating, irreversible, lifelong disease that has no cure. In North America, over 20% of dogs over the age of 1 suffer from OA (Johnston, 1997). Based on the results of a lifelong study conducted on Labrador Retrievers that noted a linear development of OA with age, the incidence in aged dogs is much greater (Smith et al., 2006). Therefore, the prevalence of OA is even higher in geriatric dogs. Osteoarthritis affects mainly the hips, elbows, and stifles of dogs, but also can cause problems in vertebral, carpal, and tarsal joints. Common clinical signs include pain, tenderness, decreased range of motion (ROM), swelling, stiffness, muscle atrophy, crepitus, and effusion. Due to the pain and other symptoms, an affected dog's behavior may change and lead to aggression, decreased activity, limping, problems rising from a lying position, and difficulty climbing stairs or onto furniture. Age is the main risk factor of OA, but other predisposing factors include genetics, size of the breed, obesity, joint deformity, trauma and fractures, surgery, and elbow or hip dysplasia (MacPhail, 2000; Rychel, 2010; Sandell, 2012).

Although there is no cure for OA, modalities exist such that the disease may be managed, resulting in slowed progression and decreased pain and signs associated with the disease. Treatment of OA aims to improve QOL of the animal by relieving pain, decreasing inflammation, increasing activity level, and increasing ROM of the joint (MacPhail, 2000). Nutraceuticals, food or dietary supplements that offer a health or medical benefit have become popular treatments for OA. Some treatments already exist, but novel nutraceuticals are being sought by the industry. Calcium fructoborate is a naturally-occurring plant-mineral complex found in certain fruits, nuts, and legumes. It is commercially manufactured from a proprietary reaction of fructose, calcium, and boric acid that produces a nature-identical molecule composed of calcium bound to mono- and di-fructoborate complexes. Calcium fructoborate has been shown to positively affect humans suffering from symptoms of OA in several clinical studies, but has yet to be tested in dogs. Therefore, the objective of this study was to evaluate the short-term effects of CFB alone, or in combination with a blend of GH and CS, on serum inflammatory markers, goniometry, gait analysis, and owner perception of pain in client-owned dogs. It was hypothesized that the CFB treatment would improve joint mobility, decrease pain, and decrease inflammatory markers.

MATERIALS AND METHODS

Animals and Study Design

Sixty-four adult dogs of various breeds were recruited and fifty-nine dogs (mean age = 8.42 ± 0.37 yr; mean BW = 31.11 ± 1.28 kg; mean BCS = 6.41 ± 0.15) completed a double-blinded, placebo-controlled study in a completely randomized design. Dogs were selected based on clinical signs, history, and orthopedic exams consistent with osteoarthritis. The University of Illinois Animal Care and Use Committee approved all procedures, and pet owners signed an

informed consent form prior to study initiation. The current exercise regimen and diet information were collected for each dog (Figures 3.1 to 3.3). A 14-day washout period prior to enrollment was required for animals being treated with interfering medications including corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and nutraceuticals. Prior to initiation, dogs underwent physical, neurological, and orthopedic examinations conducted by Dr. Tisha Harper, DVM, board-certified small animal surgeon, followed by a radiograph examination performed by Dr. Stephen Joslyn, BVMS, board-certified radiologist, to determine the most affected joint and clinically infer the dogs with OA at the University of Illinois Veterinary Teaching Hospital. For the dogs that completed the study, 8.5, 52.5, and 39.0% were diagnosed with elbow, stifle, and hip OA, respectively. The study was 28 days in length, which was twice the length of CFB studies with positive outcomes in humans, and dogs and owners visited the clinic on days 0 and 28.

Exclusion Criteria

Dogs not able to remain off interfering medications during the washout period and during the 28-day study period were excluded from the study. Also excluded from the study were dogs having evidence of a neurological disease that could affect the outcome measures, malignant neoplasia, or acute instability of the joint.

Treatments

Treatments were provided as dietary supplements distributed in capsules of the same size, color, and shape provided by FutureCeuticals, Inc. (Momence, IL). Each capsule contained the following active ingredients: placebo = none (60 mg fructose); low dose = 69 mg CFB; high

dose = 127 mg CFB; and combination = 69 mg CFB, 500 mg GH, and 200 mg CS (769 mg total). Dosages were based on allometric conversions from dosages tested in humans. Dogs were assigned to treatments through a continuous enrollment over a period of six months and stratified based on BW. A balanced but random allotment was performed so that an equal number of dogs within each BW range were placed on all treatments. Small dogs (BW range: 10 to 22.9 kg) received one capsule/day while large dogs (BW range: 23 to 50 kg) received two capsules/day of their respective treatments throughout the study. Ingestion of the supplements was facilitated by use of Pill Pockets® (The Nutro Company, Franklin, TN).

Goniometry

Angles of flexion and extension were analyzed to determine the range of motion of the most affected joint (hip, elbow, or stifle) of each dog on days 0 and 28 as described previously (Jaegger et al., 2002). To summarize, the center of the goniometer was positioned over the isometric center of the joint with the long axes placed over the proximal and distal long bones. Limbs were flexed or extended fully and measurements then were recorded.

Gait Analysis

Gait analysis was measured on days 0 and 28 by use of a Tekscan walkway (7100 QL Virtual Sensor 4 Mat System, Tekscan, Boston, MA). Dogs walked at a consistent pace of ± 1.50 m/s and acceleration of ± 0.50 m/s^2. A valid walk consisted of the dogs walking within the parameter of the mat and without sidestepping or shaking its head. Multiple walks were recorded per dog per visit to ensure that 5 footfalls could be averaged per visit to use for analysis. Vertical

impulse (VI) and peak vertical force (PVF), expressed as a percentage of BW, of the most affected joint were used for analysis (McLaughlin, 2001).

Radiographic Assessment

Radiographic examination was performed on the most affected limb on days 0 and 28. For proper positioning of radiographs, dogs were sedated intramuscularly or intravenously with dexmedetomidine hydrochloride (3.3 to 4.4 μg/kg BW) and butorphanol (0.2 mg/kg BW). Dogs were fasted overnight prior to sedation. Different protocols were used depending on the most affected joint: hip: a lateral and an extended ventrodorsal pelvis projection as per the British Veterinary Association/Kennel Club hip scoring guidelines (Dennis, 2012); elbow: one craniocaudal and one medio-lateral projection taken of the elbow with the radio-humeral angle positioned at 45° following the international elbow working group protocol (http://www.vet-iewg.org/joomla/index.php/archive/23-2001-international-elbow-protocol-vancouver); or stifle: a caudo-cranial and medio-lateral projection of the stifle assessed according to Innes et al. (2004).

Serum Chemistry and Inflammatory Marker Analyses

On days 0 and 28, up to 20 mL of blood was collected via radial or jugular venipuncture, after fasting overnight, while the animal was still under sedation for radiographic assessment. Blood was collected into BD Vacutainer® SST™ (Becton, Dickinson and Company, Franklin Lakes, NJ) and Monoject™ tubes (Covidien, Mansfield, MA). Blood was allowed to clot at room temperature before it was centrifuged at 1210 g for 15 minutes at 4°C. Serum from the Monoject™ tube was used for serum chemistry analyses using a Hitachi 911 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN). The remaining serum was pipetted into

cryogenic vials and stored at -80°C until it was shipped to Applied BioClinical, Inc. (Irvine, CA) on dry ice for the analysis of inflammatory and OA markers. Canine-specific, commercialized, enzyme-linked immunosorbent assay kits were used to measure cartilage oligomeric matrix protein (COMP; MBS733921; MyBioSource, San Diego, CA), C-reactive protein (CRP; ab157698; Abcam, Cambridge, MA), matrix metalloproteinase 3 (MMP3; SEA101Ca; Cloud-Clone Corp., Houston, TX), follistatin-like protein-1 (FSTL; ABIN1053764; Antibodies-online, Atlanta, GA), c-terminal cross-linked telopeptide type II collagen (CTX-II; MBS744225; MyBioSource, San Diego, CA), hyaluronan (DHYAL0; R&D Systems, Minneapolis, MN), Col2-3/4C (long mono) (C2C; MBS755913; MyBioSource, San Diego, CA), soluble receptor for advanced glycation end products (sRAGE; CS0290; NeoBioLab, Cambridge, MA), chitinase 3-like protein 1 (CHI3L; E08C1675; American Research Products, Inc., Waltham, MA), and interleukin 6 (IL-6; CA600; R&D Systems, Minneapolis, MN).

Canine Brief Pain Inventory

The same owner of each dog completed the canine brief pain inventory (CBPI) survey on days 0 and 28. The CBPI is a validated survey for OA and bone cancer developed by researchers at the University of Pennsylvania (Brown et al., 2008). The survey consists of a series of ten questions rating the dog's pain and how it interferes with the animal's normal daily routine on a numerical scale from 0 to 10, with "0" representing no pain/interference and "10" representing extreme pain/interference. Four questions pertaining to the dog's pain in the previous 7 days were averaged to create the PSS and six questions concerning how much the dog's pain interferes with their normal activity were averaged to create the pain interference score (PIS).

One additional qualitative question asked the owner to rate the QOL of their animal using descriptors from poor to excellent.

Statistical Analysis

All data were analyzed using the Mixed Models procedure of SAS® (version 9.4; SAS Institute, Inc., Cary, NC). Because day 0 data were highly variable, the differences between day 28 and day 0 were evaluated statistically. Normality was evaluated using Proc Univariate. For non-normally distributed data, the observed values were reported after the data had been transformed using the log function prior to statistical analysis. To control for experiment-wise error, means were separated using a Fisher-protected least significant difference with Tukey's adjustment. Probabilities of $P \leq 0.05$ were accepted as significant, and $P \leq 0.10$ were considered trends. Because the majority of the dogs were large and there was a great deal of variability in the data, a sub-analysis of data from dogs over 23 kg also was conducted.

RESULTS

Analysis of All Dogs

The results for the CBPI data from all dogs are summarized in Table 3.1. Dogs fed the low dose (-2.93) and high dose (-2.21) of CFB were shown to improve ($P < 0.05$) in their ability to rise from a lying position from day 0 to day 28 compared to dogs fed the placebo (0.00), but no difference was observed for dogs fed the combination treatment. Dogs fed the low dose of CFB also tended to have an improved PSS (-1.46; $P = 0.06$) and pain at its worst score (-2.14; $P = 0.08$) from day 0 to day 28 compared to dogs fed the placebo (0.05 and 0.00, respectively). All other CBPI scores were unaffected by treatments. Gait analysis data, namely PVF and VI, were not affected by treatments (Table 3.2).

Table 3.3 presents the inflammatory marker data for all dogs. Dogs fed the high dose of CFB had a greater (P = 0.05) increase in sRAGE concentration between day 0 and 28 (7.88 ng/mL) compared to dogs fed the placebo (0.83 ng/mL). There were no treatment differences for the other inflammatory markers measured.

Serum chemistry data are presented in Table 3.4. All metabolites were within reference range except total alkaline phosphatase and corticosteroid-induced alkaline phosphatase, which started and ended at higher concentrations than the upper reference range values. Dogs fed the high dose of CFB tended to have a greater (P = 0.07) reduction in serum chloride from day 0 to 28 (-1.64 mmol/L) compared to dogs fed the low dose of CFB (0.08 mmol/L). No other treatment differences were observed for serum chemistry analysis.

No significant differences were found for goniometry and radiograph scores after 28 days of treatment.

Sub-Analysis of Data Collected with Large Dogs (> 23 kg)

When only large dogs were evaluated, several CBPI scores were altered in dogs fed the low dose of CFB compared to dogs fed the placebo (Table 3.5). Dogs fed the low dose were shown to have decreased (P < 0.05) scores for the PSS (-1.77) and pain at its worst (-2.45) from day 0 to 28 compared to the placebo group (0.19 and 0.42, respectively). Dogs fed the low dose of CFB tended to have improved scores for pain at its least (-1.27; P = 0.08) and pain on average (-1.82; P = 0.07) compared to dogs fed the placebo (0.25 and -0.08, respectively), but no difference was observed for dogs fed the high dose or combination groups. From the pain interference questions, dogs fed the low dose (-3.09) were shown to improve (P < 0.05) in their

ability to rise from a lying position compared to the placebo treatment (0.25). The rest of the CBPI scores were unchanged by treatment.

Gait analysis (Table 3.6), serum inflammatory markers (Table 3.7), and serum chemistry profiles (Table 3.8) in large dogs were not altered by treatment.

DISCUSSION

As OA is an incurable disease, a combination of treatments can be used to manage the disease. Management strategies aim to alleviate symptoms and pain, improve joint mobility, and slow progression of the disease. Successful treatments depend on the severity of OA in each patient as well as cooperation of owners. Common strategies to alleviate symptoms include pharmacological intervention, administration of nutraceuticals, diet modification or weight loss, rehabilitation, surgery, or alternative treatments such as acupuncture or laser treatments (MacPhail, 2000; Rychel, 2010; Sanderson et al., 2009). With obesity being a leading risk factor of OA, weight management has been reported to be beneficial in alleviating some of the symptoms of OA, with benefits observed in dogs that reduced BW by only 6.1% (Marshall et al., 2010). Simple rehabilitation in the form of stretching has been shown to have significant effects ($P < 0.05$) for improvement of joint range of motion (7 to 23% increase in range) in dogs affected by OA (Crook et al., 2007).

Pharmacological interventions, such as NSAIDs, are the main treatment used (MacPhail, 2000), but side effects are a concern to owners and veterinarians (Comblain et al., 2015; Rychel, 2010). Dietary supplements or nutraceuticals are common alternatives to drug therapy. Chondroitin sulfate and GH are the most popular nutraceuticals for management of OA, but conflicting results exist for dogs. A negative-controlled study conducted by Johnson and others (2001) tested the effects of a supplement containing 200 mg CS, 250 mg GH, and 5 mg

manganese ascorbate per capsule in dogs who underwent cranial cruciate ligament transection (n = 16). Dogs were given 3 capsules every 12 hours for 30 days before the dose was reduced to 2 capsules every 12 hours for the following 4 months (Johnson et al., 2001). Compared to the controls, dogs given the supplement had elevated concentrations of CS epitopes in synovial fluid, suggesting modulation of articular cartilage matrix metabolism (Johnson et al., 2001). A double-blind, placebo-controlled study conducted using arthritic dogs (n = 7 to 10/treatment group) noted a reduction in observational pain overall (51%), after limb manipulation (48%), and after physical exertion (43%) after 150 days of supplementation of 2000 mg GH and 1600 mg CS ($P < 0.05$) (Gupta et al., 2012). Another double-blind, positive-controlled study reporting positive effects of GH/CS in dogs (n = 35) suffering from OA tested a supplement containing the following active ingredients: GH (475 mg/g), CS (350 mg/g), N-acetyl-D-glucosamine (50 mg/g), ascorbic acid (50 mg/g), and zinc sulfate (30 mg/g) (McCarthy et al., 2007). For the first 70 days, the supplement was dosed at 1 g of active ingredients twice daily for 5 to 19.9 kg dogs, 1.5 g of active ingredients twice daily for 20 to 40 kg dogs, and 2 g of active ingredients twice daily for dogs weighing > 40 kg (McCarthy et al., 2007). The dose then was reduced by 1/3 for the next 28 days (McCarthy et al., 2007). In the study conducted by McCarthy et al. (2007), improvements ($P < 0.001$) in overall condition score, pain on palpation, and weight-bearing from baseline scores were observed.

Not all studies have shown positive effects, however. A double-blind, placebo-controlled study testing 22 mg CS/kg BW or 11 mg mussel extract/kg BW in 58 dogs suffering from OA showed no significant improvement in symptoms (Dobenecker et al., 2002). Similarly, research conducted on 71 OA dogs resulted in no differences in gait analysis or subjective assessment when treated with 3 to 4 capsules (number depended on BW; each capsule contained 500 mg

GH, 400 mg CS, and 75 mg manganese ascorbate) for 60 days in a placebo-controlled, double-blind study (Moreau et al., 2003). Variation in experimental procedures among studies may explain the variation in results. Prior studies researching canine OA have been conducted using a variety of experimental designs, lengths of treatment, and animal populations and numbers. Along with those factors, treatments often involve multiple active ingredients with different doses, making it difficult to assess the efficacy of each ingredient alone. The sources and quality of CS and GH also may be different and contribute to the discrepancies reported in the literature (Calamia et al., 2012; Martel-Pelletier et al., 2015). For example, CS may be extracted from various animal sources, including bovine, porcine, chicken, or marine cartilage, and may differ in molecular composition, purity, and production processes.

Even though nutraceuticals with the ability to alleviate symptoms of OA in the dog already exist, the search for other novel chondroprotective agents is of interest. This was the first canine clinical trial to evaluate the efficacy of CFB in alleviating clinical signs, improving joint motion, and decreasing inflammation associated with OA. Calcium fructoborate, a nature-identical compound consisting of calcium, fructose, and boron, is available as a stable, bioavailable, water-soluble white powder developed and patented by FutureCeuticals, Inc. (Miljkovic, 1999).

Previous studies researching CFB in humans have used the Western Ontario and McMaster Universities Arthritis Index (WOMAC) (Bellamy, 1988) and McGill Pain Questionnaire (MPQ) (Melzack, 1975; Melzack, 2005) to assess physical function and pain of human patients suffering from OA. Those surveys determine the effectiveness of an intervention by asking participants to answer a series of questions about their pain and hardships with physical movements and then scoring the questionnaires, with decreased scores representing less

pain and physical limitations. One study showed a 29% reduction ($P < 0.01$) in WOMAC score and a 14% reduction ($P < 0.01$) in MPQ score after knee OA-afflicted adult humans (n = 19; ages 45 to 64 years; mean BMI = 28.7 kg/m^2) who consumed 108 mg CFB twice a day over a 14-day period (Reyes-Izquierdo et al., 2012). Another 14-day study in humans with self-reported joint discomfort (n = 60; mean age = 49.2 years) tested CFB at a dose of 110 mg twice daily and showed decreased ($P < 0.0001$) WOMAC (-13.73) and MPQ (-8.9) scores on day 14 compared to baseline (Pietrzkowski et al., 2014).

Pet owners in the current study completed the CBPI survey to assess the pain and QOL of their pet. In this study, including both the analysis of all dogs and the sub-analysis of large dogs, we observed some improvement in pain and lameness scores with CFB supplementation at the low dose. The reduction in pain and improved functionality reported by dog owners is in agreement with previous human CFB data. As dogs are unable to provide an assessment of pain, owners were required to complete the questionnaire for them. Even though significant improvements were found for individual questions, the PIS and PSS scores carry the most weight because the CBPI has been validated for those scores. The current study reported significant decreases for PSS in both the analysis of all dogs and sub-analysis of large dogs. The authors acknowledge that the CBPI scoring system is subjective, but believe it is a good representation because the same owner completed the questionnaire on each day. Decisions in regards to management and/or treatments will ultimately come from the owners. Therefore, improvements observed and reported by owners within 28 days are viewed as positive results.

Blood inflammatory biomarkers continue to be measured as potential indicators for disease progression of OA. They also are targets for treatment options, as it is known that OA is characterized by inflammation of the joints. C-reactive protein is a common marker of

inflammation used to evaluate the severity of joint diseases in humans (Ohno et al., 2006). Previous studies in humans testing the effects of CFB on blood CRP concentrations have shown positive results. A 30-day study testing 112 mg CFB/day in healthy adult humans (n = 78; ages 40 to 60 years; BMI 24 to 27 kg/m^2) demonstrated a 31.3% reduction in serum CRP concentrations (Rogoveanu et al., 2015). The experiment conducted by Reyes-Izquierdo et al. (2012) on adult humans suffering from knee OA (n = 19; ages 45 to 64 years; mean BMI = 28.7 kg/m^2) reported a 37% reduction in serum CRP concentrations after 14 days of CFB supplementation of 216 mg/day. Also, a 15-day study in adult humans afflicted with primary OA (n = 60; ages 59 to 68 years) testing a treatment of 57 mg CFB/day reported decreased serum CRP concentrations by 60.25% (Scorei et al., 2011). In this study, no differences were reported in CRP concentrations among treatments. Despite its use in humans, CRP has not been validated as an inflammatory marker in the dog, and our results seem to support this observation (Bennett et al., 2013; Hurter et al., 2005).

In the current study, inflammatory markers had fewer differences than expected. The only difference observed in these markers was an increased sRAGE concentration in dogs fed the high CFB dose compared to dogs fed the placebo. The receptor for advanced glycation end products (RAGE) generates reactive oxygen species and is involved in a cascade of events leading to inflammation and cartilage damage (Chayanupatkul and Honsawek, 2010). Serving in a protective role, sRAGE is a competitor of RAGE and functions by binding to similar ligands such as HMGB-1 and S100/calgranulins (Chayanupatkul and Honsawek, 2010; Santilli et al., 2009). Therefore, an increase in sRAGE after treatment suggests increased binding and interference with RAGE, potentially leading to decreased inflammation and reduction of cartilage damage.

Goniometry and gait analysis were not different in the current study, but have been shown to improve in past OA studies. A study conducted on osteoarthritic Labrador Retrievers (n = 10; mean age = 7.5 years) resulted in a 14.6° improvement ($P < 0.0005$) of joint flexion in all dogs after 21 days of stretching (Crook et al., 2007). Previous research in OA dogs (n = 38; ages = 1 to 18 years) noted an increase ($P = 0.01$) in the gait analysis (PVF) after 90 days of feeding a test diet supplemented with fish oil-based omega-3 fatty acids (Roush et al., 2010b). Another 14-day study evaluating the effects of carprofen, an NSAID, in dogs suffering from OA (n = 68; ages = 3 to 14 years) showed decreases for PSS and PIS ($P = 0.002$ and $P = 0.03$, respectively) and increases in PVF and VI ($P = 0.006$ and $P = 0.02$, respectively) compared to the placebo (Brown et al., 2013). Although there were significant differences, they did not show correlation between decreased PSS and PIS scores with an increase in PVF and VI (Brown et al., 2013).

The lack of differences in gait analysis, goniometry, and inflammatory markers among treatments in this study may have been due to several factors including the dosages tested, the timing of dosage in relation to meal times, a short length of study, or a relatively small number of animals recruited and assigned to each treatment group. There was a wide variation in sizes, breeds, and ages of dogs as well as baseline severity of OA. All of these factors may affect the measurements taken and lead to increased variability. At this time, it is unclear why the low dose appeared to be more effective. Further studies testing different doses and/or dosing at multiple times per day would be beneficial. In this study, small and large dogs were given different dosages per day, but future studies could divide the dogs into more weight categories including a medium and/or giant sizes. Owners were not instructed to provide the treatment with or without food, so it is unclear if supplement/meal time had an effect. Human CFB studies have usually followed twice a day dosing protocol compared to the once a day dosing protocol used in this

study. It is unknown whether twice a day CFB supplementation would have led to more beneficial results due to an extended period of CFB exposure in the circulation and/or tissues. Future research on CFB use in dogs could include a dose response study to identify the most effective dose or determine if there is a threshold for minimum severity of OA for CFB to be most beneficial. It would be valuable to conduct a long-term study testing CFB, as it is possible that 28-days of supplementation was not long enough to see improvements in range of motion and gait analysis. Further experimentation exploring more inflammatory markers may identify the best markers for progression of the disease in the dog. Studies that examine the long-term efficacy and safety of CFB for use in canine OA also may also be beneficial.

CONCLUSION

In conclusion, the results of this randomized, double-blinded, placebo-controlled clinical trial showed that CFB is associated with some mitigation of joint pain and improved mobility in the dog over a 28-day period. The CBPI, a validated survey, provided a good indication that owners observed a difference in their dog's behavior with CFB treatment. As this was the first study testing CFB supplementation in dogs, further experimentation with a larger sample size and over a longer time period should be conducted to confirm the effectiveness of CFB on alleviating symptoms associated with OA.

TABLES AND FIGURES

Table 3.1. Canine brief pain inventory scores for all dogs expressed as the difference between data collected on day 28 and day 0

Variable	Placebo[1]	Low Dose[2]	High Dose[3]	Combination[4]	SEM	P-Value
Pain at its worst[*]	0.00[x]	-2.14[y]	-0.43[xy]	-0.53[xy]	0.593	0.08
Pain at its least[*]	0.33	-0.93	-0.64	0.03	0.379	0.08
Pain on average[*]	-0.27	-1.43	-0.57	-0.78	0.427	0.29
Pain as of right now[*]	0.13	-1.36	-1.14	-0.37	0.460	0.09
Interference of general activity[*]	-1.27	-1.79	-1.36	-0.69	0.466	0.42
Interference of enjoyment of life[*]	-0.80	-1.86	-1.00	-1.00	0.568	0.58
Interference with the ability to rise from a lying position[*]	0.00[a]	-2.93[b]	-2.21[b]	-1.38[ab]	0.558	<0.01
Interference with the ability to walk[*]	0.00	-1.43	-1.36	-1.25	0.534	0.20
Interference with the ability to run[*]	-0.87	-1.64	-0.57	-1.31	0.614	0.63
Interference with the ability to climb up[*]	-0.40	-2.21	-1.57	-1.44	0.520	0.12
Overall quality of life[+]	0.20	0.14	0.21	0.47	0.165	0.50
Pain severity score (PSS)[±]	0.05[x]	-1.46[y]	-0.70[xy]	-0.41[xy]	0.388	0.06
Pain interference score (PIS)[†]	-0.56	-1.98	-1.35	-1.18	0.393	0.10

[ab]Mean values in the same row with unlike superscript letters differ ($P < 0.05$).

[xy]Mean values in the same row with unlike superscript letters differ ($P < 0.10$).

[1]Placebo contained 60 mg of fructose.

[2]Low dose contained 69 mg of calcium fructoborate (CFB).

[3]High dose contained 127 mg of CFB.

[4]Combination contained 69 mg CFB, 500 mg glucosamine hydrochloride, and 200 mg chondroitin sulfate.

[*]Questions were scored on a scale of 0 to 10, with 0 representing no pain/interference and 10 representing extreme pain/interference.

[+]Question was answered with qualitative answers ranging from poor to excellent.

[±]PSS is the average of 4 questions rating the dog's pain intensity in the last 7 days.

[†]PIS is the average of 6 questions rating how much the dog's pain interferes with its normal activity.

Table 3.2. Gait analysis values for all dogs expressed as the difference between data collected on day 28 and day 0

Variable	Placebo[1]	Low Dose[2]	High Dose[3]	Combination[4]	SEM	P-Value
Peak vertical force (N/m)[*]	37.94	13.28	-52.68	-4.52	34.50	0.32
Vertical impulse (s)	0.00	0.11	0.04	-0.01	0.160	0.95

[*]N/m as a % of body weight.

[1]Placebo contained 60 mg of fructose.

[2]Low dose contained 69 mg of calcium fructoborate (CFB).

[3]High dose contained 127 mg of CFB.

[4]Combination contained 69 mg CFB, 500 mg glucosamine hydrochloride, and 200 mg chondroitin sulfate.

Table 3.3. Serum inflammatory marker concentrations for all dogs expressed as the difference between data collected on day 28 and day 0

Variable	Placebo[1]	Low Dose[2]	High Dose[3]	Combination[4]	SEM	P-Value
Cartilage oligomeric matrix protein (ng/mL)	-0.68	-2.71	-1.55	-3.66	1.036	0.60
C-reactive protein (ng/mL)	-308.60	-168.38	159.36	904.50	563.708	0.39
Matrix metalloproteinase 3 (ng/mL)	-0.03	-0.15	0.09	0.04	0.228	0.90
Follistatin-like protein-1 (ng/mL)	-12.79	-21.71	-8.28	-16.20	145.391	0.83
C-terminal cross-linked telopeptide type II collagen (ng/mL)	-0.03	0.99	-0.03	0.10	0.463	0.38
Hyaluronan (ng/mL)	3.87	-1.48	-3.29	0.21	6.120	0.86
Col2-3/4C (long mono) (ng/mL)	0.18	-1.18	1.58	1.03	1.329	0.22
Soluble receptor for advanced glycation end products (ng/mL)	0.83^{y}	12.33^{xy}	7.88^{x}	9.92^{xy}	3.429	0.05
Chitinase 3-like protein 1 (ng/mL)	-4.95	-4.50	-9.15	-3.46	2.868	0.24
Interleukin 6 (pg/mL)	-2.16	-3.27	-0.19	-1.20	2.381	0.83

^{xy}Mean values in the same row with unlike superscript letters differ ($P < 0.10$).

[1]Placebo contained 60 mg of fructose.

[2]Low dose contained 69 mg of calcium fructoborate (CFB).

[3]High dose contained 127 mg of CFB.

[4]Combination contained 69 mg CFB, 500 mg glucosamine hydrochloride, and 200 mg chondroitin sulfate.

Table 3.4. Serum chemistry values for all dogs expressed as the difference between data collected on day 28 and day 0

Variable	Placebo[1]	Low Dose[2]	High Dose[3]	Combination[4]	SEM	P-Value
Creatinine (mg/dL)	-0.03	0.01	-0.02	0.02	0.023	0.43
Blood urea nitrogen (mg/dL)	-2.47	-0.43	-0.79	0.44	1.031	0.24
Total protein (g/dL)	-0.05	-0.04	-0.03	0.06	0.054	0.49
Albumin (g/dL)	-0.05	-0.01	-0.06	0.01	0.037	0.56
Globulin (g/dL)	0.00	-0.03	0.03	0.05	0.044	0.60
Albumin: globulin	0.00	0.01	-0.04	0.01	0.023	0.51
Calcium (mg/dL)	-0.01	0.09	-0.04	0.08	0.090	0.69
Phosphorous (mg/dL)	-0.01	0.04	0.16	0.09	0.194	0.94
Sodium (mmol/L)	-0.53	0.14	-1.21	-0.56	0.561	0.43
Potassium (mmol/L)	0.12	0.16	0.03	0.13	0.061	0.50
Sodium: potassium	-0.73	-1.00	-1.29	-0.88	0.520	0.90
Chloride (mmol/L)	-0.67^{xy}	0.08^{x}	-1.64^{y}	-0.94^{xy}	0.435	0.07
Glucose (mg/dL)	0.60	-0.57	-3.14	-1.50	4.064	0.93
Total alkaline phosphatase (ALP) (U/L)	4.07	8.00	-3.50	2.79	3.788	0.21
Corticosteroid-induced ALP (U/L)	1.07	3.31	0.79	-0.36	2.307	0.64
Alanine aminotransferase (U/L)	-0.60	-0.86	3.00	-2.93	3.868	0.45
Gamma-glutamyltransferase (U/L)	-0.13	-0.21	0.00	0.25	0.423	0.87
Total bilirubin (mg/dL)	0.01	-0.01	0.02	-0.01	0.018	0.67
Cholesterol (mg/dL)	-4.38	4.62	2.62	5.60	5.307	0.55
Triglycerides (mg/dL)	8.55	4.64	5.75	2.87	6.369	0.93
Bicarbonate (mmol/L)	0.13	0.14	1.14	1.00	0.508	0.35

^{xy}Mean values in the same row with unlike superscript letters differ ($P < 0.10$).

[1]Placebo contained 60 mg of fructose.

[2]Low dose contained 69 mg of calcium fructoborate (CFB).

[3]High dose contained 127 mg of CFB.

[4]Combination contained 69 mg CFB, 500 mg glucosamine hydrochloride, and 200 mg chondroitin sulfate.

Table 3.5. Canine brief pain inventory scores for large dogs expressed as the difference between data collected on day 28 and day 0

Variable	Placebo[1]	Low Dose[2]	High Dose[3]	Combination[4]	SEM	P-Value
Pain at its worst[*]	0.42[a]	-2.45[b]	-0.09[ab]	-0.62[ab]	0.668	0.02
Pain at its least[*]	0.25[x]	-1.27[y]	-0.36[xy]	0.00[xy]	0.424	0.08
Pain on average[*]	-0.08[x]	-1.82[y]	-0.27[xy]	-0.92[xy]	0.486	0.07
Pain as of right now[*]	0.17	-1.55	-0.82	-0.50	0.508	0.14
Interference of general activity[*]	-1.17	-2.00	-1.36	-0.85	0.552	0.52
Interference of enjoyment of life[*]	-0.75	-2.00	-0.73	-1.31	0.685	0.54
Interference with the ability to rise from a lying position[*]	0.25[a]	-3.09[b]	-2.09[ab]	-1.62[ab]	0.615	<0.01
Interference with the ability to walk[*]	-0.42	-1.45	-1.00	-1.00	0.589	0.68
Interference with the ability to run[*]	-1.17	-1.64	0.00	-1.15	0.689	0.42
Interference with the ability to climb up[*]	-0.58	-2.18	-1.36	-1.46	0.604	0.34
Overall quality of life[+]	0.08	0.18	0.09	0.50	0.180	0.30
Pain severity score (PSS)[±]	0.19[a]	-1.77[b]	-0.39[ab]	-0.50[ab]	0.427	0.02
Pain interference score (PIS)[†]	-0.64	-2.06	-1.09	-1.23	0.442	0.17

[ab]Mean values in the same row with unlike superscript letters differ ($P < 0.05$).

[xy]Mean values in the same row with unlike superscript letters differ ($P < 0.10$).

[1]Placebo contained 60 mg of fructose.

[2]Low dose contained 69 mg of calcium fructoborate (CFB).

[3]High dose contained 127 mg of CFB.

[4]Combination contained 69 mg CFB, 500 mg glucosamine hydrochloride, and 200 mg chondroitin sulfate.

[*]Questions were scored on a scale of 0 to 10, with 0 representing no pain/interference and 10 representing extreme pain/interference.

[+]Question was answered with qualitative answers ranging from poor to excellent.

[±]PSS is the average of 4 questions rating the dog's pain intensity in the last 7 days.

[†]PIS is the average of 6 questions rating how much the dog's pain interferes with its normal activity.

Table 3.6. Gait analysis values for large dogs expressed as the difference between data collected on day 28 and day 0

Variable	Placebo[1]	Low Dose[2]	High Dose[3]	Combination[4]	SEM	P-Value
Peak vertical force (N/m)[*]	61.29	16.49	-45.97	-20.14	3.911	0.24
Vertical impulse (s)	-0.12	0.12	0.00	-0.09	0.175	0.77

[*]N/m as a % of body weight.

[1]Placebo contained 60 mg of fructose.

[2]Low dose contained 69 mg of calcium fructoborate (CFB).

[3]High dose contained 127 mg of CFB.

[4]Combination contained 69 mg CFB, 500 mg glucosamine hydrochloride, and 200 mg chondroitin sulfate.

Table 3.7. Serum inflammatory marker concentrations for large dogs expressed as the difference between data collected on day 28 and day 0

Variable	Placebo[1]	Low Dose[2]	High Dose[3]	Combination[4]	SEM	P-Value
Cartilage oligomeric matrix protein (ng/mL)	-0.54	-3.13	-1.78	-4.68	1.269	0.29
C-reactive protein (ng/mL)	74.17	-239.00	408.55	1130.69	684.483	0.13
Matrix metalloproteinase 3 (ng/mL)	-0.04	-0.24	0.00	-0.02	0.272	0.93
Follistatin-like protein-1 (ng/mL)	-14.28	-25.41	-9.13	-19.29	7.911	0.97
C-terminal cross-linked telopeptide type II collagen (ng/mL)	0.00	0.93	-0.18	-0.48	0.536	0.33
Hyaluronan (ng/mL)	-2.26	-4.22	-2.99	-4.61	6.243	0.99
Col2-3/4C (long mono) (ng/mL)	-0.12	-2.07	1.66	0.14	1.586	0.31
Soluble receptor for advanced glycation end products (ng/mL)	1.85	13.99	8.77	11.99	4.140	0.19
Chitinase 3-like protein 1 (ng/mL)	-5.44	-4.82	-3.72	-3.01	2.154	0.38
Interleukin 6 (pg/mL)	-1.20	-3.07	-0.83	-1.70	1.882	0.87

[1]Placebo contained 60 mg of fructose.

[2]Low dose contained 69 mg of calcium fructoborate (CFB).

[3]High dose contained 127 mg of CFB.

[4]Combination contained 69 mg CFB, 500 mg glucosamine hydrochloride, and 200 mg chondroitin sulfate.

Table 3.8. Serum chemistry values for large dogs expressed as the difference between data collected on day 28 and day 0

Variable	Placebo[1]	Low Dose[2]	High Dose[3]	Combination[4]	SEM	P-Value
Creatinine (mg/dL)	-0.03	0.02	-0.03	0.02	0.027	0.38
Blood urea nitrogen (mg/dL)	-2.50	-1.09	-0.73	1.23	1.220	0.18
Total protein (g/dL)	-0.04	-0.03	-0.04	0.07	0.060	0.49
Albumin (g/dL)	-0.04	0.00	-0.07	0.02	0.041	0.43
Globulin (g/dL)	0.00	-0.03	0.04	0.05	0.049	0.65
Albumin: globulin	0.01	0.00	-0.05	0.02	0.024	0.30
Calcium (mg/dL)	0.00	0.14	0.04	0.06	0.072	0.60
Phosphorous (mg/dL)	-0.01	-0.10	0.16	0.14	0.221	0.82
Sodium (mmol/L)	-0.67	-0.09	-1.00	-0.23	0.619	0.73
Potassium (mmol/L)	0.14	0.14	0.06	0.13	0.066	0.83
Sodium: potassium	-1.00	-1.00	-0.91	-0.92	0.537	0.10
Chloride (mmol/L)	-0.83	0.00	-1.73	-0.69	0.479	0.13
Glucose (mg/dL)	1.42	-3.82	-1.45	-4.85	4.404	0.74
Total alkaline phosphatase (ALP) (U/L)	5.00	2.91	-1.18	-1.67	5.825	0.53
Corticosteroid-induced ALP (U/L)	1.17	5.70	1.64	-3.92	3.269	0.57
Alanine aminotransferase (U/L)	0.42	-6.90	-1.90	-4.58	3.362	0.45
Gamma-glutamyltransferase (U/L)	0.08	-0.73	0.09	0.62	0.410	0.16
Total bilirubin (mg/dL)	0.00	0.01	0.00	-0.02	0.018	0.91
Cholesterol (mg/dL)	-5.80	4.10	3.70	9.67	6.204	0.36
Triglycerides (mg/dL)	19.36	-1.55	-15.90	4.77	12.286	0.35
Bicarbonate (mmol/L)	0.00	0.09	1.45	1.08	0.567	0.21

[1]Placebo contained 60 mg of fructose.

[2]Low dose contained 69 mg of calcium fructoborate (CFB).

[3]High dose contained 127 mg of CFB.

[4]Combination contained 69 mg CFB, 500 mg glucosamine hydrochloride, and 200 mg chondroitin sulfate.

Figure 3.1. Classification of diets collected from the 59 dogs completing the study

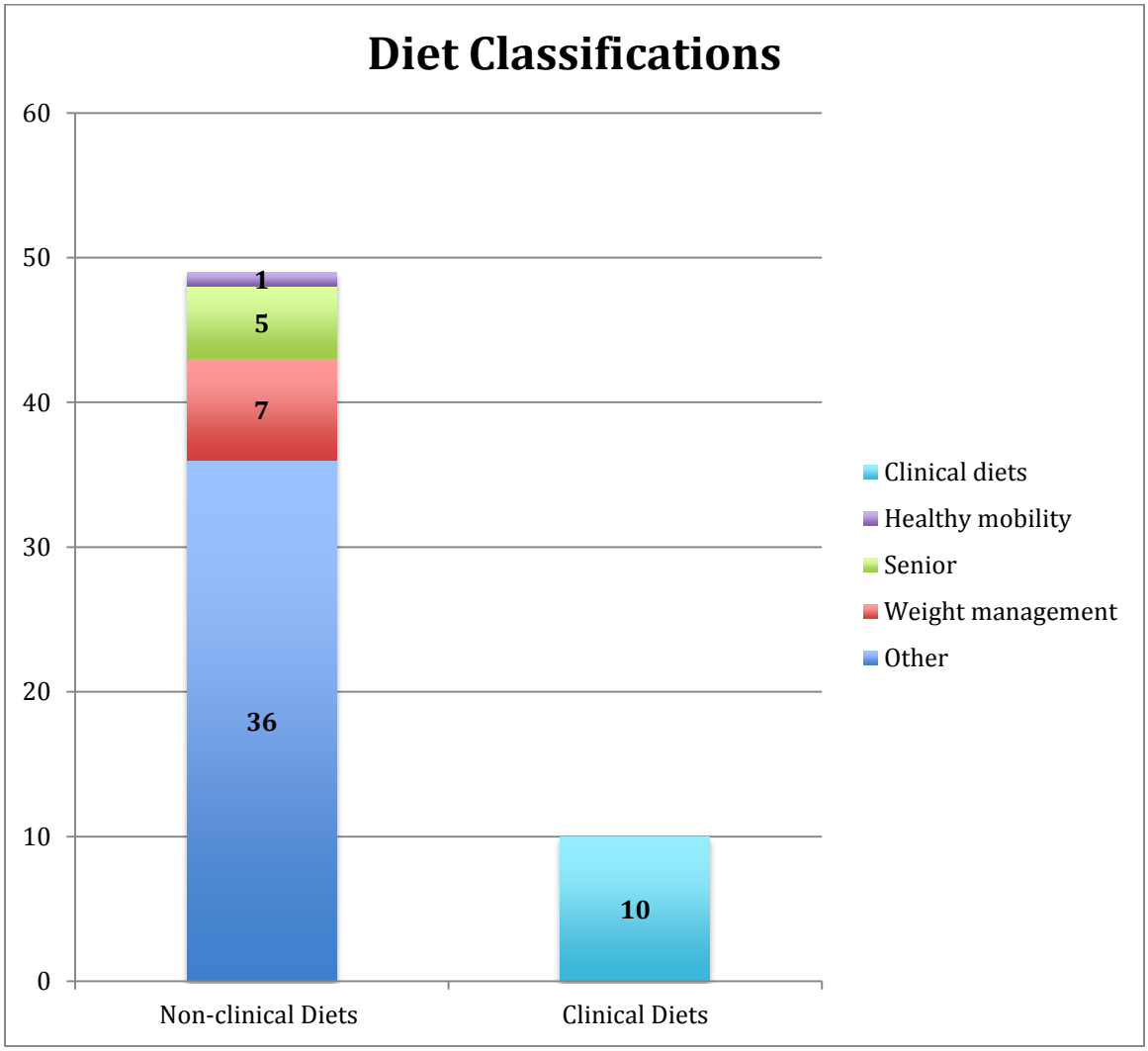


Figure 3.2. Percentage of clinical diet claims of the diets collected from the 59 dogs completing the study

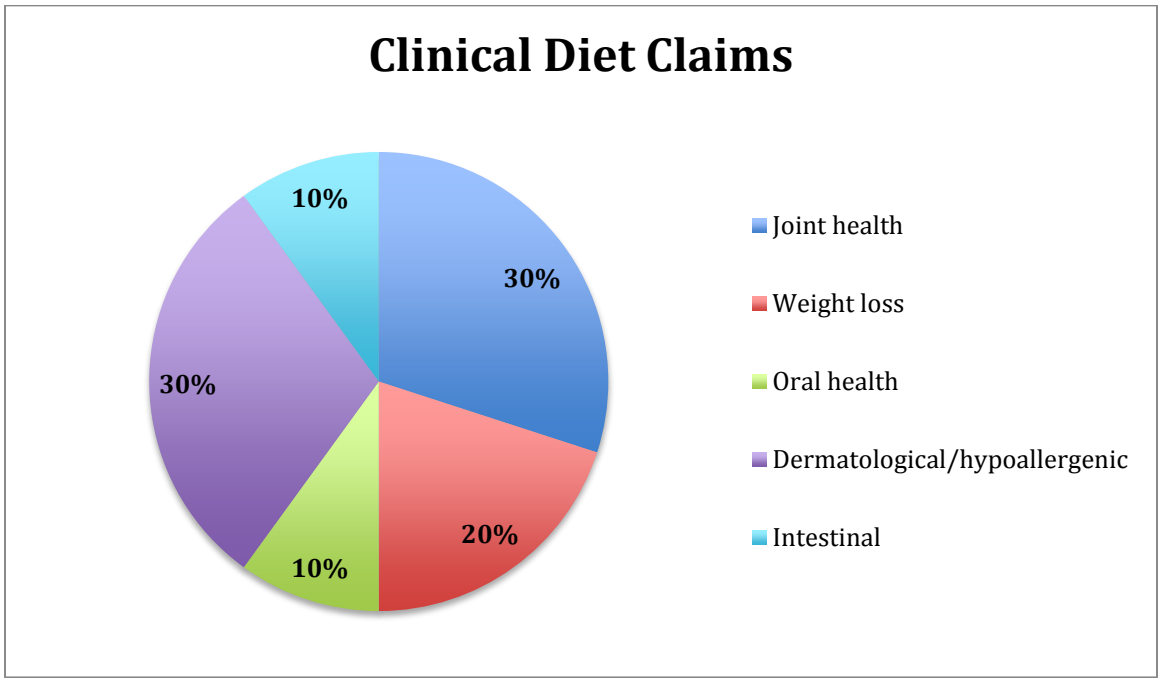
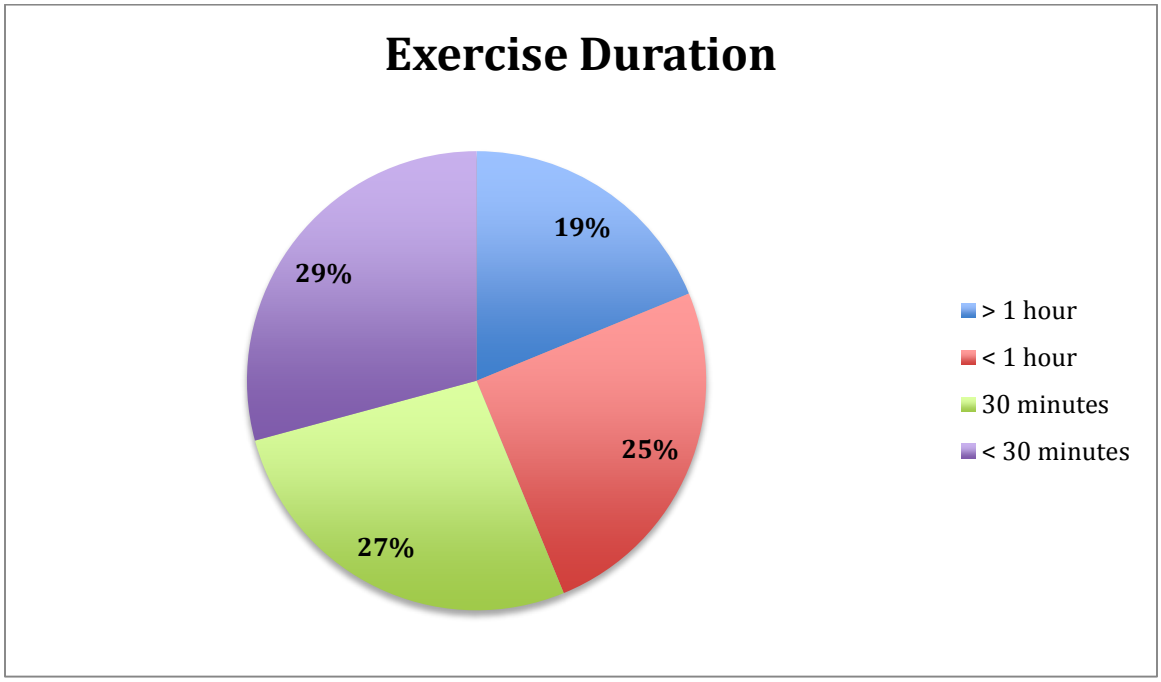


Figure 3.3. Exercise duration data from the 59 dogs completing the study



Chapter 4

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