DISSERTATION

SHORT TERM EFFECTS OF DIETARY FISH OIL ON MATRIX METALLOPROTEINASES IN DOGS WITH ANTERIOR CRUCIATE LIGAMENT DISEASE

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY RODNEY ALAN HANSEN ENTITLED SHORT TERM EFFECTS OF DIETARY FISH OIL ON MATRIX METALLOPROTEINASES IN DOGS WITH ANTERIOR CRUCIATE LIGAMENT DISEASE BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF THE DISSERTATION

SHORT TERM EFFECTS OF DIETARY FISH OIL ON MATRIX METALLOPROTEINASES IN DOGS WITH ANTERIOR CRUCIATE LIGAMENT DISEASE

Matrix metalloproteinases (MMPs) comprise a family of enzymes that degrade extracellular matrix components. Abnormally elevated concentrations of MMPs play key roles in chronic diseases such as cancer and arthritis. Fish oil, containing high amounts of the polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been demonstrated to reduce tissue concentrations of inflammatory mediators tumor necrosis factor (TNF) alpha, urokinase plasminogen activator (uPA), and prostaglandin (PG) E2 that increase with arthritis. These inflammatory mediators are key regulators that increase production of MMPs. Tissue inhibitors of metalloproteinases (TIMPs) down-regulate the activity of MMPs. This study investigated effects of dietary fish oil intake in dogs undergoing surgery for cranial cruciate ligament rupture in a single knee on the response of MMPs to modulation of inflammatory mediators and TIMPs.

Twenty-four mature large breed dogs were fed identical maintenance diets that differed only in PUFA content (90mg/kg/day) as fish oil or beef tallow for 63 days. Plasma and synovial fluid from both surgically and non-surgically affected knees were obtained at the start of diet, on the day of surgery, and at 7, 14, 28, and 56 days following surgery. Plasma was analyzed for arachidonic acid, EPA, DHA and

bicycloPGE2. Synovial fluid was analyzed for uPA, collagenase (MMP-1, 8, and13), and pro and active MMP-2 and MMP-9. The fish oil fed group showed significant increases in total plasma EPA and DHA within one week after start of dietary supplementation and through the duration of the study. The fish oil group also showed significant decreases in uPA in non-surgical synovial fluid at day 28, proMMP-2 at days 7 and 14, and proMMP-9 at day 56. The fish oil fed group showed significant increases in non-surgical synovial fluid TIMP activity at day 7 and 28.

In conclusion, non-surgical synovial fluid concentrations of uPA and MMPs were decreased and TIMP-2 concentrations were increased by dietary supplementation of EPA and DHA.

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List of abbreviations

AA	**arachidonic acid**
COX	**cyclooxygenase**
DHA	**docosahexaenoic acid**
EPA	**eicosapentaenoic acid**
MMP	**matrix metalloproteinase**
PGE_2	**prostaglandin E_2**
TIMP	**tissue inhibitor of metalloproteinases**
uPA	**urokinase plasminogen activator**

CHAPTER 1

INTRODUCTION AND SPECIFIC AIMS

The n-3 long chain polyunsaturated fatty acids EPA and DHA, both significant components of fish oil, have been identified to have beneficial effects in the treatment of arthritis. The molecular mechanisms are unknown. Proinflammatory eicosanoids and cytokines are known to be active in chronic diseases such as osteoarthritis (OA). Dietary supplementation with increased absolute amounts of fish oil has been shown to result in lower concentrations of proinflammatory eicosanoids including Leukotriene B4 (LTB4) and PGE2, and proinflammatory cytokines TNF alpha and IL-1. Proinflammatory eicosanoids of the two and four series, PGE2 and LTB4, are derived from the n-6 fatty acid arachidonic acid. Fish oil contains eicosapentaenoic acid that competes for tissue uptake and enzymatic activation with arachidonic acid, and therefore decreases the synthesis of proinflammatory PGE2 and LTB4.

The inflammatory mediators TNF alpha, PGE2 and urokinase plasminogen activator (uPA) are key regulators in the increased production of the matrix metalloproteinase (MMP) enzymes. MMPs comprise a family of enzymes that are capable of causing the breakdown of the extracellular matrix of tissue. Overproduction of MMPs, notably the collagenases (MMP-1, 8, and 13) and gelatinases (MMP-2 and 9), has been implicated in chronic pathological diseases such as osteoarthritis so that reduction of these enzymes is purported to be beneficial. Suppression of MMPs has been recognized as a key target in the treatment of OA.

Another possible mechanism for the effect of fish oil may involve the increased production of tissue inhibitors of metalloproteinases (TIMPs). The TIMPs are a group of protein inhibitors that regulate many physiological functions. Most notable is the regulation of the deposition and degradation of connective tissue through inhibition of MMPs. The TIMPs are endogenous regulators of MMPs in that they form a non-covalent complex with MMPs and prevent the catalytic action of the enzyme. Current interest in these inhibitory proteins revolves around regulating their function, which in turn could potentially regulate deleterious levels of MMPs in disease.

It has been well established that TIMPs are regulated in part by cytokines. Cytokine regulation has been predominantly investigated in *in vitro* culture studies, but not *in vivo*. Cytokines have been identified that decrease or increase levels of TIMPs. The interleukins, IL-12 and IL-15, increased TIMP-1 levels, as did IL-13. Exogenous IL-11 upregulated TIMP-1. IL-6 enhanced TIMP-1 mRNA abundance, and enhanced expression of TIMP in a dose dependent manner and significantly correlated with increased mRNA abundance of TIMP-1 in lymphoma tissue. The inhibitor TIMP-1 is upregulated by IL-1beta and TNF alpha in brain microvascular endothelial cells and astrocytes. The inflammatory mediator TNF alpha exhibits a bifunctional effect on TIMP-1 expression *in vitro* in that low concentrations induced stimulation and high concentrations caused suppression in a dose dependent manner.

Pro-inflammatory eicosanoids have been found to affect TIMP production in culture studies. The eicosanoid PGE_2 inhibited IL-1 beta stimulated TIMP-1 mRNA abundance and secretion in a dose dependent manner and exogenous PGE_2, and cyclooxygenase inhibitors indomethacin and diclofenac suppressed IL-1 beta induced

production of TIMP-1. Lipoxins (LXA_4) have been demonstrated to affect TIMP production by inducing a 1 to 3 fold increase in TIMP-1 and TIMP-2 respectively. It is unknown if anti-inflammatory effects of eicosanoids derived from fish oil have an affect on TIMP expression.

Fish oil has been demonstrated to have an effect on inflammatory cytokines. In culture studies, cells exposed to eicosapentaenoic acid (EPA) or arachidonic acid (AA) showed that EPA increased collagen synthesis and AA reduced collagen production, while the levels of IL-6 and collagen production showed a significant linear correlation. Investigations of n-3 PUFA supplementation in human clinical arthritis showed that plasma TNF alpha and IL-1 beta synthesis were significantly reduced. Fish oil supplementation has been demonstrated to decrease plasma IL-1 cytokine levels in dogs.

The effect of n-3 PUFAs on MMP levels has not been investigated in humans or in other species with OA. In addition, no studies have addressed the effects of fish oil on TIMPs in an *in vivo* clinical model in humans or in other species with OA.

Study 1 Specific Aims: The objective of this study was to determine if increased dietary intake of fish oil in dogs undergoing surgery for cranial cruciate ligament rupture in a single knee would affect MMP activities in synovial fluid from both surgical and non-surgical arthritic stifle joints.

Hypothesis: A supplemented diet with high n-3 long chain fatty acids (fish oil) content would decrease the production of matrix metalloproteinases in both surgical and non-

surgical synovial fluid from stifle joints in arthritic dogs who have undergone surgery for correction of a torn cranial cruciate ligament.

Study 2 Specific Aims: The objective of this study was to determine if dietary supplementation of fish oil in arthritic dogs would affect TIMP concentrations and uPA activities in synovial fluid from the stifle joint.

Hypothesis: A supplemented diet with high n-3 long chain fatty acids (fish oil) content would increase the production of TIMP-2 and decrease uPA in synovial fluid from the non-surgical and surgical stifle joints from dogs with arthritis.

CHAPTER 2

LITERATURE REVIEW AND REFERENCES

Matrix metalloproteinases

The MMPs comprise a family of enzymes that can degrade various tissues in the body such as bone and cartilage. Specifically, their enzymatic activity cleaves components of the extracellular matrix. They contain common sequences of amino acids making distinct domains, and contain zinc at the active center (Cawston, 1996). The MMP family can be divided into at least five groups that differ by size and substrate acted upon. These five families include the stromelysins (MMP-3, 10,and 11), the collagenases (MMP-1, 8, and 13), the matrilysins (MMP-7, and 12), the gelatinases (MMP-2, and 9), and the membrane-type MMPs (MMP-14, and 15)(Cawston, 1996).

These enzymes are produced in the inactive proenzyme form prior to being cleaved to the biologically active product. The active enzymes act at neutral pH and require calcium for activity. The activation is accompanied by a decrease in molecular weight (Cawston, 1996). Activation can occur proteolytically or by treatment with mercurial compounds *in vitro* (Cawston, 1996). A predominant activation agent is plasmin that is generated by uPA (Leyland et al, 1996).

Three control points have been identified for regulation of MMPs. The initial regulatory point occurs through stimulation by cytokines and growth factors that promote synthesis and secretion of proMMPs. Secondary to stimulation is activation of proMMP

to an active form. Finally, binding of inhibitory agents terminates activity of the active MMP. Binding by inhibitory agents to the proMMP can also occur (Cawston, 1996).

MMPs are inactivated by endogenous TIMPs and alpha2 macroglobulin. The activated MMPs are important in normal metabolic processes, but are also believed to be central in the pathogenesis of osteoarthritis (OA) where there is an imbalance of MMP and TIMP (Cawston, 1996).

Tissue Inhibitors of Metalloproteinases

The TIMPs comprise an endogenously produced protein family that inhibits the activities of MMPs (Gomez et al, 1997). The TIMPs are present in both tissues and fluid compartments, and are produced by a variety of cell types. Four members of the TIMP family have been characterized and are identified as TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Gomez et al, 1997). These four members share structure similarity, size, and inhibitory effect by bonding with MMPs. The inhibitors TIMP-1 and TIMP-2 are soluble, whereas TIMP-3 is insoluble. The inhibitors TIMP-1 and TIMP-2 have been designated as multifunctional proteins in that they both also exhibit growth factor-like activity (Gomez et al, 1997). Canine TIMP-2 is a 22kDa protein and is highly homologous to both equine and human TIMP-2. Canine synovial fibroblasts produce both TIMP-1 and TIMP-2. The inhibitor TIMP-2 is particularly important for inhibitory control of MMP-2 in many chronic pathological processes in dogs (Bee et al, 2000).

Pathogenesis of canine arthritic knee

Rupture of the cranial cruciate ligament (CCL) of the stifle has two pathogenic origins; rupture from trauma and from degeneration. Of all ruptures, degeneration from OA accounts for 80% of canine cases (Moore & Reed, 1996a). Regardless of the cause, clinical features related to the rupture of the CCL include pain from acute inflammation. After two to three weeks, the inflammation decreases and stifle stability improves because of compensatory thickening of periarticular tissue. A gradual decline in limb use after the inflammation has subsided can be attributed to meniscal damage or complete rupture of the ligament. Lameness results from progressive OA due to continued joint instability (Moore & Reed, 1996b).

Role of MMPs in joint degeneration

The overproduction of MMPs or the underproduction of TIMPs has been identified as a primary component in the development and progression of OA in humans, where MMPs are directly involved in the degradation of cartilage extracellular matrix (Imai et al, 1997). Increased activities of metalloproteinases have been associated with human OA. Woessner & Gunja-Smith (1991) found levels of stromelysin-1 (MMP-3) and acid metalloproteinase to be elevated by 150% compared to normal in extracts of human articular cartilage from patients with OA. Similar results were found by Vaatainen et al (1998) where joint lavage fluid from OA patients was higher in MMP-3 than control nonOA patients. In dogs, MMP-3 concentration was

increased in the knee after tibial valgus osteotomy compared to the contralateral knee that served as a control (Panula et al, 1998). The enzymes MMP-1 and MMP-13 were expressed at higher levels in OA chondrocytes compared to normal chondrocytes and MMP-8 mRNA was abundant in OA cartilage but not normal cartilage by PCR analysis (Shlopov et al, 1997). Increases in MMP-3 activity in humans with temporomandibular joint arthritis were detected in synovial fluid when compared with normal patients (Kubota et al, 1997). Reverse transcriptase polymerase chain reaction analysis revealed a 110 fold increase in MMP-9 mRNA in cartilage from human OA patients over expression in cartilage obtained from non-OA autopsy cases. This study suggested that increased expression of MMP-9 precedes fibrillation of cartilage in the development of OA (Tsuchiya et al, 1997). Similar results were found in an investigation of the loose bone-implant interface of human hip replacements. The MMP-9 activity from interface membranes was increased in comparison to controls and was thought to be a major facilitator of osteoclastic bone reabsorption (Yokohama et al, 1995). Increased concentrations of proMMP-2 were found in human OA cartilage examined by gelatin zymography (Imai et al, 1997). When MMP-2 and 9 were analyzed by zymography in synovial fluid from dogs with rheumatoid arthritis, MMP-9 was significantly higher in synovial fluid from diseased dogs than in synovial fluid from disease free dogs (Coughlan et al, 1998).

Role of TIMPs and arthritis

TIMPs play a key role in maintaining balance between extracellular matrix (ECM) deposition and degradation (Gomez et al, 1997). Cartilage extracts showed levels of the

TIMPs were not as elevated in the arthritic patients suggesting that not only are MMP levels elevated in OA, but an imbalance of MMPs to TIMPs may play a role in OA (Woessner & Gunja-Smith, 1991). Similar conclusions were investigated by Ishiguro et al (1996), where synovial fluid from patients with varying degrees of rheumatoid arthritis was investigated for MMP and TIMP concentrations. Mild to moderate functional disability showed higher TIMP levels than those with severe disability. The regulation of TIMP production was more dependent on the disease state and not the concentration of MMP and furthermore the discrepancies between concentrations of TIMP and MMP may be key in cartilage destruction.

Role of Eicosanoids and TIMPs

Eicosanoids of arachidonic acid have been found to affect TIMP production. In human synovial fibroblasts, PGE2 inhibited IL-1 beta stimulated TIMP-1 mRNA expression and secretion in a dose dependent manner (DiBattista et al, 1995). Similar results were found in human rheumatoid synovial fibroblasts when exogenous PGE2 suppressed IL-1 beta induced production of TIMP-1. Suppression was further enhanced when cyclooxygenase inhibitors indomehacin and diclofenac were added. The inhibition of PGE2 and regulation of cyclooxygenase were thought to regulate production of TIMP-1 (Takahashi et al, 1997). Lipoxins (LX) has been demonstrated to affect TIMP production. Exogenous LXA_4 was to cultures of human synovial fibroblasts that induced a 1 to 3 fold increase in TIMP-1 and TIMP-2 respectively (Sodin-Semrl et al, 2000).

Role of eicosanoids in joint degeneration

Eicosanoids derived from arachidonic acid have been found to induce production of MMPs. It has been established that prostaglandin E_2 (PGE_2) is involved in the synthesis of collagenase (Wahl et al, 1977)(Wahl & Lampel, 1987) and increased concentrations of PGE_2 have been detected in synovial fluid from horses affected by degenerative joint diseases (Gibson et al, 1996). Collagenase-1 (MMP-1) is involved in the proteolysis of insulin-like growth factor binding proteins (IGFBP) in human airway smooth muscle cells and leukotriene D4 was found to induce MMP-1 and decrease levels of IGFBP (Rajah et al, 1996). Suppression of PGE_2 resulted in decreased synthesis of 92 kD collagenase (proMMP-9). Moreover human monocytes cultivated with interleukin 4 (IL-4), an inhibitor of PGE_2 synthesis, had decreased production of PGE_2 and 92k kD collagenase (Corcoran et al, 1992). The addition of PGE_2 was found to reverse the action of suppressive cytokines Interferon gamma and IL-4 in cultures of human monocytes (Wahl & Corcoran, 1993). Other eicosanoid derivative such as PGI2 affects uPA production (Hatane et al, 1998). Production of uPA was enhanced by PGI2 in cultured human fibroblasts through stimulation of the cyclic AMP intracellular pathway.

Role of cytokines in joint degeneration

Cytokines have been shown to induce production of MMPs. Synovial fluid from canine osteoarthritic joints showed increased concentrations of TNF alpha in comparison to control joints (Venn et al, 1993). TNF alpha stimulated the expression of MMP-1,

MMP-8, and MMP-13 in OA cartilage (Shlopov et al, 1997). The cytokines IL-1 and TNF alpha stimulate numerous cell types to produce MMP-2 and MMP-9, as well as MMP-3 (Cawston, 1996). Concentrations of proMMP-2 were highly correlated with membrane type MMP-14 and the cytokines IL-1 and TNF alpha. These data suggest that these cytokines induce production of membrane type MMP-14 that in turn activates proMMP-2 leading to degradation of cartilage (Imai et al, 1997). Increased concentrations of TNF alpha and MMP-3 were present in cartilage from dogs after a surgical transection of the cranial cruciate ligament. Chondrocytes stained more intensely for TNF alpha, TNF receptors, and MMP-3 in cartilage showed increased concentrations with mild osteoarthritic changes and this suggested that they are involved in the development of OA (Kammermann et al, 1996). TNF alpha enhanced MMP-9 production in a dose dependent fashion. Cultures of head and neck squamous cells stimulated with TNF alpha showed significantly enhanced production of MMP-9 as well as MMP-2 (Mann et al, 1995). Similar results were found by Kawashima et al (1994) as co-incubation of metastatic osteosarcoma cell cultures with TNF alpha resulted in increased production of MMP-9.

Cytokines and TIMPs

Many cytokines have been identified that affect levels of TIMPs. IL-12 and IL-15 induced the release of TIMP-1 in peripheral blood mononuclear cells (Constantinescu et al, 2001). The cytokine IL-13 increased TIMP-1 activity and gene expression when added to explant cultures of bovine nasal cartilage (Cleaver et al, 2001). Exogenous IL-11 upregulated TIMP-1 in rheumatoid arthritic synovial fluid (Hermann et

al, 1998). The cytokine Il-6 enhanced expression of TIMP in a dose dependent manner in human skin and uterine cervical fibroblasts (Sato et al, 1990). Tissue Il-6 significantly correlated with elevated mRNA levels of TIMP-1 in lymphoma tissues taken from 32 non-Hodgkin's lymphoma patients (Kossakowska et al, 1999). In cultured human lung carcinoma and hepatoma cells, IL-6 as well as oncostatin M and leukemia inhibitory factor enhanced TIMP-1 mRNA levels (Richards et al, 1993). Cytokines may affect different TIMPs differentially. The inhibitor TIMP-1 has been shown to be upregulated, although TIMP-3 expression is blocked, by IL-1beta and TNF alpha in brain microvascular endothelial cells and astrocytes (Bugno et al, 1999). The cytokine TNF alpha was also shown to exhibit a bifunctional effect of TIMP-1 production in human fibroblasts in that low concentrations induced stimulation and high concentrations caused suppression in a dose dependent manner (Ito et al, 1990).

Influence of Fish oil on cytokines

Polyunsaturated fatty acids influence cytokine levels in arthritis (Tidow-Kebritchi & Mobarhan, 2001). Arachidonic acid derived eicosanoids induce over-production of inflammatory cytokines. The n-3 polyunsaturated fatty acids, including fish oil, suppress the production of arachidonic acid derived eicosanoids, as EPA is an alternate substrate for production of eicosanoids that are noninflammatory (Calder, 1997). In an *in vitro* study investigating healing response of medial collateral ligament fibroblasts, cells were exposed to eicosapentaenoic acid (EPA) or arachidonic acid (AA). Eicosapentaenoic acid increased collagen synthesis and AA reduced collagen production. The levels of IL-6 and collagen production showed a significant linear correlation (Hankenson et al, 2000).

In human clinical investigations, 32 rheumatoid arthritis patients were supplemented with 3.6g of n-3 PUFAs. The plasma cytokine IL-1beta were significantly reduced when compared to control values and the beneficial effect of n-3 fatty acids was partly attributed to decreases in cytokine production (Esperson et al, 1992). Fish oil and flaxseed oil decrease cytokine production in healthy humans. Both plasma TNF alpha and IL-1 beta synthesis were significantly reduced after both four and eight weeks of supplementation (Caughey et al, 1996). Fish oil supplementation has been demonstrated to alter cytokine levels in dogs. In a study involving twenty-eight dogs with cardiac disease, patients were randomly given fish oil or placebo. Fish oil supplementation significantly decreased plasma IL-1 concentration after eight weeks (Freeman et al, 1998).

N-3 polyunsaturated fatty acids (PUFAs) have been shown to down regulate IL-1 and TNF alpha. In mice fed corn oil or mice fed high n-3 PUFA diets, there were decreased concentrations of pro-inflammatory IL-1 beta, IL-6 and TNF alpha cytokines (Chandrasekar & Fernandes, 1994). The cytokine IL-1 was significantly reduced in dogs fed a fish oil supplement, when compared to dogs fed a control diet (Freeman et al, 1998). Results from cultures of peritoneal macrophages of mice given n-3 PUFA supplementation showed an alteration of TNF alpha activity. Peritoneal macrophages from the mice fed n-3 PUFA diets showed that clearance, but not maximal production of TNF alpha was altered (Somers & Erickson, 1994). Supplementation with n-3 LC PUFA was effective in suppressing symptoms of rheumatoid arthritis in humans and levels of PGE_2, IL-1, and TNF alpha were decreased in peripheral blood leukocyte and monocyte cells (Fernandes & Venkatraman, 1993). In a separate study, dogs were fed n-6 and n-3 fatty acids at a 5:1 ratio that did not alter cytokine production. The authors suggested that

this ratio of n-6:n-3 fatty acids may not be low enough or the dose of n-3 fatty acids may not be high enough to modify cytokine production (Turek & Havek, 1998). N-3 fatty acid incorporation into articular cartilage reduced TNF alpha production (Curtis et al, 2000). Although the mechanism by which fish oil decreases IL-1 and TNF alpha is not known, it is speculated that fish oil may have an influence at the molecular level. Translocation of nuclear factor-kappa-b (NFkb) from the cytoplasm to the nucleus is decreased by EPA. Eicosapentaenoic acid causes a decrease in phosphorylation of the inhibitor of kappa-b (Ikb) complex, which in turn has less affinity for NFkb. This decreases translocation to the nucleus by NFkb and results in less NFkb binding to the promoter regions of the proinflammatory cytokine genes thereby decreasing transcription of these cytokines (Babcock et al, 2000).

Eicosanoids and Polyunsaturated Fatty Acids

The intake of dietary n-3 and n-6 fatty acids can alter eicosanoid production. The proinflammatory PGE2 and LTB4 derived from the n-6 fatty acid arachidonic acid. Fish oil contains eicosapentaenoic acid that can act in competition with arachidonic acid and therefore decrease the synthesis of proinflammatory PGE2 and LTB4 (James et al, 2000). Peritoneal macrophage PGE2 and LTB4 production was significantly decreased in mice fed fish oil for six weeks, compared to mice fed safflower oil, olive oil, or coconut oil (Wallace et al, 2000).

Effect of fish oil on eicosanoids in arthritis

The n-3 PUFA-induced decreases in the n-6 derived eicosanoids have a beneficial influence on arthritis (Leslie et al, 1988). Decreases in prostaglandins and leukotrienes were suggested as factors in decreasing the severity and incidence, and increasing time-to-onset in collagen-induced arthritis in mice fed n-3 PUFA compared to corn oil (Leslie et al, 1985). Arthritis severity decreased with administration of n-3 PUFA in collagen-induced arthritis in mice (Leslie et al, 1988). Prostaglandin production by macrophages was decreased and was attributed to decreased dietary n-6 fatty acids. Cathcart & Gonnerman (1991) demonstrated decreased concentrations of prostaglandins in mice fed high n-3 PUFA diets that resulted in decreased severity of collagen-induced arthritis in mice fed n-3 PUFA compared to mice fed corn oil. Comparable results were found in a crossover type study of human arthritic patients, in that n-3 PUFA (n-3) ingestion subjectively alleviated symptoms of active rheumatoid arthritis. Production of Leukotriene B_4 (LTB4) also decreased with n-3 PUFA ingestion (Kremer et al, 1987). A low dose n-3 PUFA (n-3) diet (< 1 gram/day) did not produce clinical improvements in human patients with psoriatic arthritis. Concentrations of LTB4 and Thromboxane B2 (TXB2) decreased with high n-3 LC PUFA ingestion. Moreover, n-3 PUFA allowed for a reduction in dose of nonsteroidal anti-inflammatory drugs (NSAIDs) taken by the patients (Veale et al, 1994). Decreased tissue-derived proinflammatory eicosanoids PGE2, LTB4, 11-dehydro thromboxane B2, and thromboxane B2 were associated with increases in serum EPA and DHA in dogs given radiation treatment (Anderson et al, 1997), which were fed a diet containing 1 gram fish oil /kg bodyweight per day. Similar results were

obtained by Freeman et al (1998) who showed that circulating PGE2 decreased in dogs fed fish oil supplements compared to dogs fed control supplements.

In vitro nutrition effects on MMP production

In vitro nutrient intervention studies have demonstrated modulation of MMP concentrations. Administration of 1, 25 dihydroxyvitamin D3 was found to inhibit MMP production in rheumatoid synovial fibroblasts after stimulation with IL-1beta, but enhanced MMP production in human articular chondrocytes after stimulation with IL-1beta (Tetlow & Wooley, 1998). The citrus flavinoid, nobiletin, suppressed IL-1 alpha induced production of proMMP-9, proMMP-1, and proMMP-3 in a dose dependent manner as well as decreased production of PGE2 in synovial cells (Ito et al, 1998). Cis 9 oleic acid inhibited MMP-2 activity in a dose dependent manner in cultures of oncogen-transformed human bronchial epithelial cells (Polette et al, 1999). The Chinese herbal TWHF suppressed production and decreased the messenger RNA of levels of MMP-1 and 3 in cultured human synovial fibroblasts (Lin et al, 2001).

In vitro nutrition effects on TIMP production.

In vitro nutrient intervention studies have been shown to increase TIMP levels. TIMP-1 levels were increased by addition of trans retinoic acid to human fibroblasts (Bigg et al, 1998). The Chinese herbal TWHF increased the messenger RNA of levels of TIMP-1 and TIMP-2 in cultured human synovial fibroblasts (Lin et al, 2001).

Summary

To date, there have been no studies that investigate the effect of dietary fish oil supplementation on MMPs and TIMPs in a clinical osteoarthritic model.

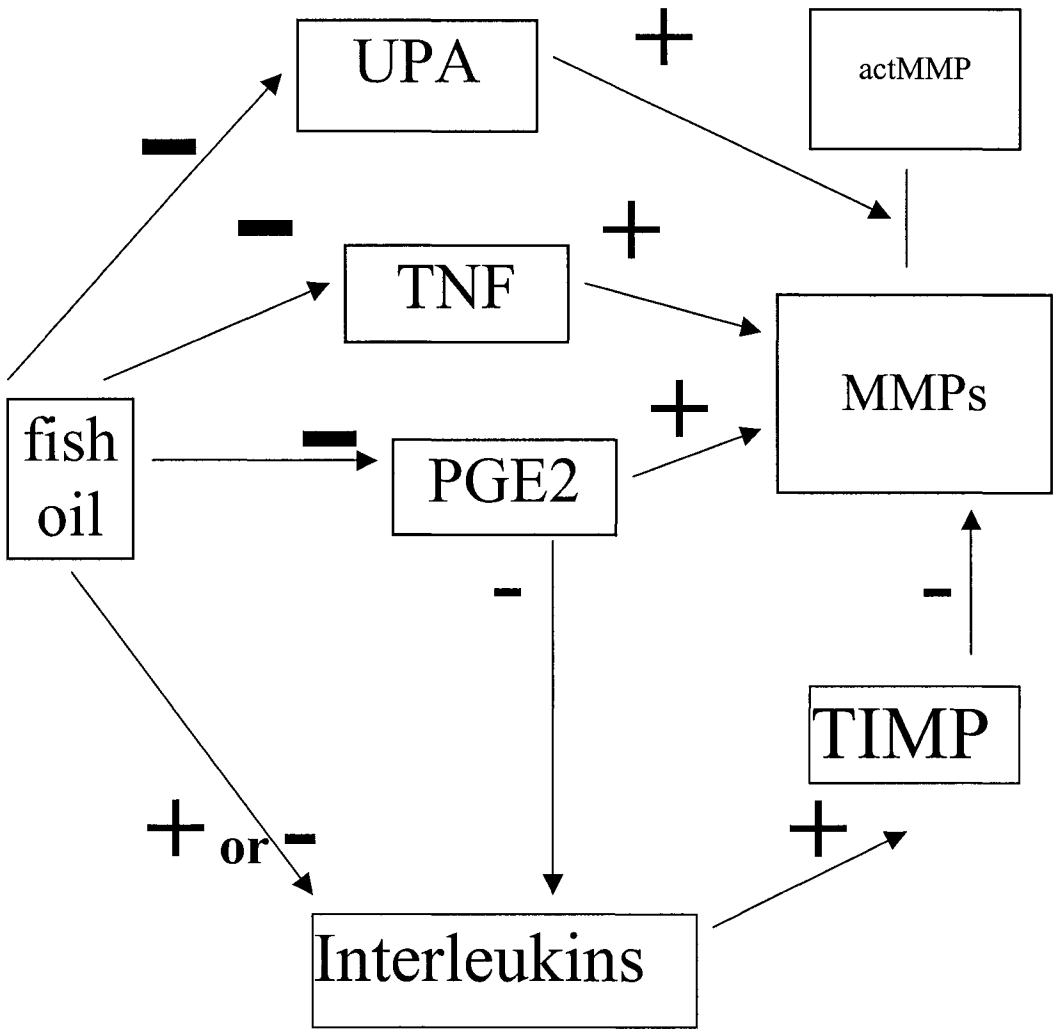

Figure 1. The proposed biochemical pathways where fish oil affects inflammatory agents, MMPs, and TIMP-2

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CHAPTER 3

MATRIX METALLOPROTEINASE RESPONSES IN DOGS FED A FISH OIL-CONTAINING DIET FOLLOWING SURGERY FOR CRANIAL CRUCIATE RUPTURE

Abstract

The n-3 long chain polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both significant components of fish oil, have been shown to reduce the inflammatory mediators tumor necrosis factor (TNF) alpha and prostaglandin (PG) E2. The inflammatory mediators, TNF alpha and PGE2, are key regulators in the increased production of the matrix metalloproteinase (MMP) enzymes. Overproduction of MMPs, notably collagenases (MMP-1, 8, and 13), stromelysin (MMP-3), and gelatinases (MMP-2 and 9), has been implicated in chronic pathological diseases such as arthritis and therefore reduction of these enzymes is beneficial. The objective of this study was to test the hypothesis that increased dietary intake of fish oil in dogs with stifle instability undergoing surgery for cranial cruciate ligament rupture in a single stifle joint would decrease inflammatory mediators and therefore decrease activities of MMPs in both the non-surgical and surgical stifle joints. Twenty-four dogs were fed identical maintenance diets that differed only in the fat composition, 90mg per kg/day, provided as fish oil or beef tallow (control) for a 63 day period. Plasma and synovial fluid from both knees was obtained at the start of the diet, on the day of surgery, and at 7, 14, 28, and 56 days following surgery. Plasma was analyzed for

arachidonic acid (AA), EPA, and DHA. Synovial fluid was analyzed for collagenase (MMP-1, 8, and 13), and pro and active forms of MMP-2 and 9. EPA and DHA were significantly ($p<0.05$) increased in plasma after initiation of diet in the fish oil fed group. The fish oil fed group showed significant ($p<0.05$) decreases in synovial fluid from the non-surgical joint for proMMP-2 at day 7 and 14, and proMMP-9 at day 56. These results suggest that non-surgical synovial concentrations but not surgical concentrations of proMMPs 2 and 9 respond to dietary fish oil supplementation. (Supported by Colorado Agriculture Experiment Station and Nestle-Purina PetCare Grant)

Introduction

Ingestion of dietary n-3 long chain polyunsaturated fatty acids (n-3 PUFAs) C20:5n-3 (EPA) and C22:6n-3 (DHA), or fish oil may have beneficial effects (Fernandes & Venkatraman, 1993). These include modulation of eicosanoids and cytokines that are known to be active in chronic diseases such as osteoarthritis (OA) (Leslie et al, 1988) (Fernandes & Venkatraman, 1993). Dietary supplementation of n-3 PUFAs decrease proinflammatory cytokines and eicosanoids associated with OA (Chandrasekar & Fernandez, 1993) and result in lower concentrations of eicosanoids such as LTB4 and PGE2 (Wahl & Lampel, 1987)(Freeman et al, 1998) and proinflammatory cytokines such as TNF alpha (Cawston et al, 1996) and IL-1 (Freeman et al, 1998). These eicosanoids and cytokines have been shown to increase activities of MMPs, and overproduction of MMPs is a predominant factor in the progression of OA (Tsuchiya et al, 1997).

MMPs are a family of enzymes that as a group are capable of causing the breakdown of the extracellular matrix of bone tissue (Cawston, 1996). Overproduction of MMPs and/or underproduction of tissue inhibitors of metalloproteinases (TIMPs) may play a major role in the progression of OA. Suppression of MMPs has been recognized as a key target in the treatment of OA (Imai et al, 1997).

There has been much interest in the pharmacological development of MMP inhibitors (Cawston, 1996). Inhibition of MMPs may also detrimentally alter the normal structure and function of many tissues. When MMPs are inhibited to the benefit of tissues involved in OA, there may also be detrimental effects to other tissues. This has been evident with the tendonitis associated with the broad-spectrum MMP inhibitor Marmistat in its Phase III clinical studies. In addition, drug therapy can be costly and the potency and bioavailability of drugs may not be adequate to induce a therapeutic response.

The effect of fish oil on MMP levels has not been investigated in humans nor in other species with OA. Nor has the effect of fish oil on MMP production in osteoarthritis been investigated in a post surgical model. Therefore this study was designed to investigate the effect of a diet supplemented with n-3 long chain fatty acids (fish oil) on production of matrix metalloproteinases in arthritic dogs who have undergone surgery for correction of a torn cranial cruciate ligament as a model of the effect of non-surgical and surgical inflammation.

Research Design and Methods

Animals and Diets. Twenty-four dogs with stifle instability resulting from clinically confirmed acute cranial cruciate ligament (CCL) injury in a single stifle were enrolled

in the study. All dogs weighed a minimum 20 kgs and varied by breed. All dogs were recruited as patients at the Colorado State University Veterinary Teaching Hospital and treated over a 379 day time span. Dogs were randomly assigned to one of two diet treatments of 12 dogs each: maintenance diet with high fish oil and maintenance diet with low fish oil content.

The diets were formulated by Nestle Purina Petcare (St. Louis, MO) Proximate composition and fatty acid analysis are shown in Tables 1 and 2. Both diets were made of products typically used in the manufacture of pet foods, i.e. rice, whole chicken, catfish meal, canola meal, soybean meal, minerals, vitamins, choline to provide a complete and balanced diet for all life stages. The diets were assessed for palatability and their affect on stool quality prior to being used in the study. There were no problems associated with the use of the experimental diets. Both control and fish oil diets contained 3.5 kcals/gm as a dry kernel feed. Dogs were fed at 40 kcal/kg/day. The high fish oil diet provided 0.75% EPA and DHA by wet weight or 90 mg combined EPA and DHA/kg BW/day for each dog. At entry (day –7) clients were instructed how much to feed their dogs to achieve 90mg/kg/day for the 63 days of the protocol. Diets were maintained during hospital stay for surgery. Clients were firmly instructed to feed nothing other than the diet.

Table 1. Diet composition.

	Control	Fish oil
Protein	27.20%	27.80%
Total Fat	12.60%	12.90%
CHO	43.61%	42.18%
Ash	7.92%	8.02%
Moisture	8.67%	9.10%

Table 2. Fatty acid composition of diet (% total fatty acids).

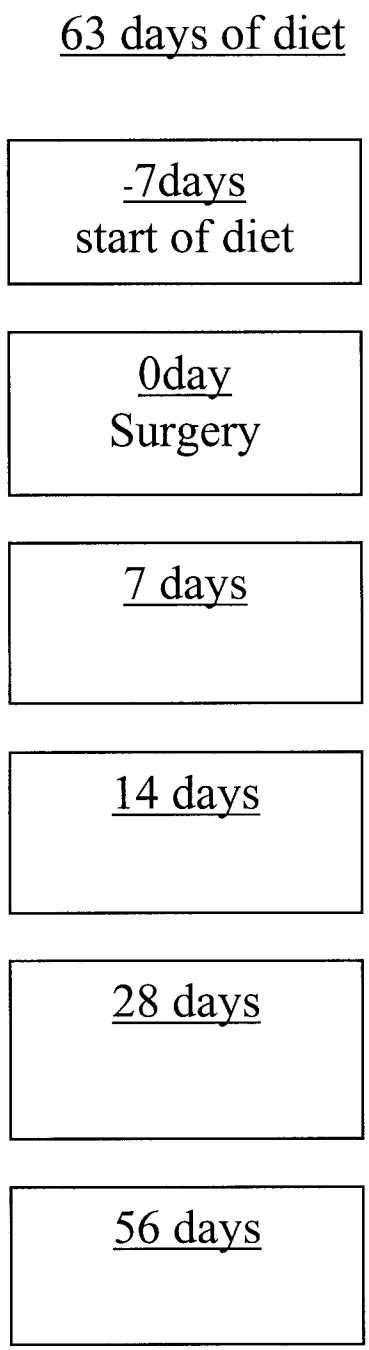
	Control	Fish oil
14:0	1.18%	3.13%
14:1	0.21%	0.22%
15:0	0.18%	0.35%
16:0	18.30%	20.90%
16:1	2.82%	5.63%
17:0	0.47%	0.60%
18:0	7.76%	8.08%
18:1n-9	31.50%	29.80%
18:1n-7	1.51%	2.09%
18:1trans	1.41%	1.87%
18:2n-6	29.10%	11.80%
20:0	0.23%	0.20%
18:3n-6	0.10%	0.18%
20:1	0.40%	0.62%
18:3n-3	0.78%	1.08%
20:2n-6	0.16%	0.25%
22:0	0.11%	<0.10%
20:4n-6	0.43%	0.66%
24:0	0.13%	<0.10%
20:5n-3	<0.10%	3.09%
22:5n-3	<0.10%	0.67%
22:6n-3	0.19%	2.66%
24:1	0.12%	0.13%
total n-6	29.88%	12.64%
total n-3	0.97%	7.50%
n-6/n-3	30.80	1.685

Surgical procedure, stifle arthrocentesis, and venipuncture. Dogs had routine physical examinations under the direction of a board certified small animal orthopedic surgeon and radiographs were taken prior to and at the conclusion of the study. The presence of complete cruciate ligament rupture with or without secondary meniscal injury with subsequent meniscal treatment was required for study entry. Each dog received a uniform standard operative procedure (Tibial Plateau Leveling Osteotomy, US Patent No. 4,677,973).

Synovial fluid was obtained from the affected (CCL ruptured) and non-surgical stifle joints of all dogs was obtained at the following time points: day –7(initial day of diet), day 0 (immediately prior to surgery), day 7, day 14, day 28, and day 56. The dogs were anesthetized with intravenous propofol. A 20-gauge needle with sterile syringe was placed in the joint with negative pressure until synovial fluid appeared in the needle hub. One to two mls of synovial fluid was obtained and transferred to a two ml cryovial for storage at -80°C until analysis.

EDTA plasma from whole blood was obtained at the following time points: day –7 (initial day of diet), day 0 (immediately prior to surgery), day 7, day 14, day 28, and day 56. Three ml blood was obtained with a 20-gauge needle in a sterile six ml syringe and transferred into an EDTA vacutainer tube. The fluid was spun for ten min at 2000g and the plasma was drawn off with a pipette, transferred to a two-ml cryovial, butylated hydroxytoluene (BHT) was added (0.1 mg/ml) as a stabilizer, and stored at -80°C until analysis.

Figure 3-1. Timeline for Research Design Timeline showing 63 total days of diet and time points. Serum, plasma, and synovial fluid were obtained at each of six time points.



EPA and DHA analysis. Gas Chromatography was used to evaluate plasma fatty acid concentrations (Ohto et al, 1990). Plasma samples were allowed to thaw and then were thoroughly vortexed. Each sample was analyzed in duplicate. One hundred µls of isooctane containing 2 mg/ml of heptadecanaenoic acid internal standard was added to a screw top vial. Under a slow stream of Nitrogen gas, the isooctane was evaporated. Two hundred µls of plasma was placed into each vial followed by two mls chloroform and methanol (2:1 v/v) and vortexed. One ml of isotonic saline (0.9% NaCl) was then added, the vial was again vortexed, and then centrifuged at 2,000 x g for ten min.

The chloroform layer was then drawn off and placed in a new vial and evaporated until dry. One ml of hexane reagent was added, followed by three mls of boron trichloride/methanol, and placed in a water bath at 60°C for two hours. One ml of water was then added and the hexane layer was aspirated and placed in a chromatography vial (Agilent, San Fernando, CA) and analyzed by gas chromatography (5890 Gas Chromatograph, Hewlett Packard, San Fernando, CA) using a .32 mm I.D. capillary column with 0.5um film (HP innowax 19091N-213 Capillary Column, Agilent, San Fernando, CA) with 1.5 ml/min constant flow. Concentrations of AA, EPA, and DHA were determined by first determining the area ratio of each sample (ratio of internal standard to peak of interest) and then comparing results to an external set of standards (Sigma, St. Louis, MO) Ratios were then matched to corresponding molar concentrations by means of linear regression.

Measurement of collagenase. Analysis of synovial Type I collagenase was performed using a Type I Collagenase Activity Assay Kit (Chemicon International, Inc., Temecula,

CA). In a 96 well microtiter plate, ten µls of synovial fluid was placed in duplicate. Thirty µls of sample diluent followed by 100 µls of biotinylated collagenase substrate was added to each well. The plate was covered and incubated for two hours at 37°C followed by the addition of ten µls of enhancer. The plate was incubated for an additional 30 min at 37°C.

One hundred µls of the sample or standard/biotinylated collagenase substrate mixture was then transferred to the biotin binding plate and incubated for 30 min at 37°C and washed. One hundred µls of a 1:3000 dilution of Streptavidin-Enzyme Conjugate was added to each well and the plate was incubated for 30 min at 37°C. The plate was then washed again and 100 µls of substrate solution was added to each well and the plate was incubated at room temperature for 20 min. One hundred µls of 1N sulphuric acid was added per well to stop the reaction. The absorbance was read at 450 nms.

Measurement of pro and active forms of MMP-2 and MMP-9. Pro and active forms of MMP-2 and MMP-9 in synovial samples was measured by zymography electrophoresis using gelatin imbedded polyacrylamide gels originally designed for human MMP analysis (Huessen et al, 1980) (Roeb et al, 2001), and adapted for canine MMP analysis (Coughlan et al, 1998) using the NOVEX (Novel Experimental Technology, San Diego, CA) gelatin zymography system. One half microliter of serum or two µls synovial fluid was mixed with ten µls buffer containing 0.5 M Tris-HCl (pH 7.0), 20% glycerol, 4% SDS (w/v), and 0.005% bromophenol blue and placed into each well. Samples underwent electrophoresis on a 10% Tris-glycine acrylamide gel, with 0.1% gelatin used as a substrate under non-reducing conditions at 125V for 90 min at room temperature. The

gels were then removed and washed in renaturing buffer (Triton X-100, 2.5% v/v in water) for 30 min followed by overnight incubation at 37^0 while in developing buffer (50mM Tris, 0.2M NaCl, 5mM CaCl2, 0.02% Brij 35 (w/v), pH 7.6). Pro and active MMP activity was visualized by staining the gels with 0.5% (w/v) Coumassie blue R250 (Sigma, St. Louis, MO) in 40% ethanol/10%glacial acetic acid solution for three hours. All gels were then destained in deionized water. One ng of pro and active MMP-2 and 9 standards were run on each gel as positive controls (Calbiochem-Oncoogene Research Products, Boston, MA). All gels were analyzed wet using a densitometer (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA), with associated software. To obtain a value for each sample, the optical assessment value of each unknown band was compared to the optical assessment value of the human MMP standard band. A ratio of the unknown band to human one ng standard was then calculated and assigned to each patient sample.

Statistical analysis of data. All response variables were normally distributed by log transformation and were analyzed as log transformed. The data from each response variable were analyzed by an analysis of variance (ANOVA) with repeated measures, where the between subjects effect was diet and the repeated measures effect was date. Data were analyzed by the Statistical Analysis Systems (SAS) mixed procedures software Release 8.1 copyright 1999-2000 (SAS Institute Inc., Cary, NC). Differences between treatment groups at each time point were evaluated as t-tests as Tests of Effect Slices (SAS) for the response variables plasma arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, as well as non-surgical and surgical synovial fluid pro MMP-2 and 9, and collagenase. The significance level was set at $p \leq 0.05$.

Results

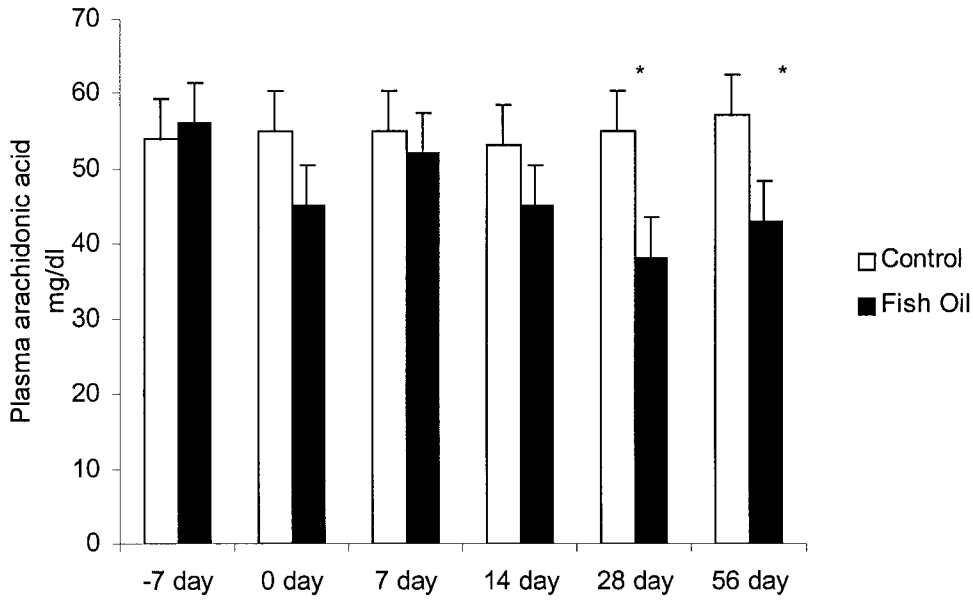
Prior to initiation of specialized diets, there were no significant between group differences in total plasma EPA or DHA concentrations. Dietary supplementation with fish oil resulted in significant increases in both EPA and DHA within one week and remained significantly different ($p<0.0001$) throughout the remainder of the study (Figure 3-1). Total plasma AA was significantly lower in the dogs treated with fish oil supplementation at day 35 after start of feeding and remained significantly lower through the remainder of the study (Figure 3-2).

No significant between or within group differences were found in the activated forms of MMP-2 or MMP-9 in either the non-surgical or surgical synovial fluid. No significant between or within group differences were found in the collagenase analysis in either the surgical or non-surgical synovial fluid.

Significant differences in proMMP-9 in the non-surgical synovial fluid were evident at day 56 (Figure 3-3). There were no other significant between or within group differences in proMMP-9 in the non-surgical synovial fluid. There were no significant between or within differences in proMMP-9 in the surgical synovial fluid.

Significant differences in proMMP-2 were evident at day 7 and day 14 in the non-surgical synovial fluid (Figure 3-4). There were no significant differences between or within groups in proMMP-2 in the surgical synovial fluid.

Total plasma arachidonic, eicosapentaenoic and docosahexaenoic acid concentrations



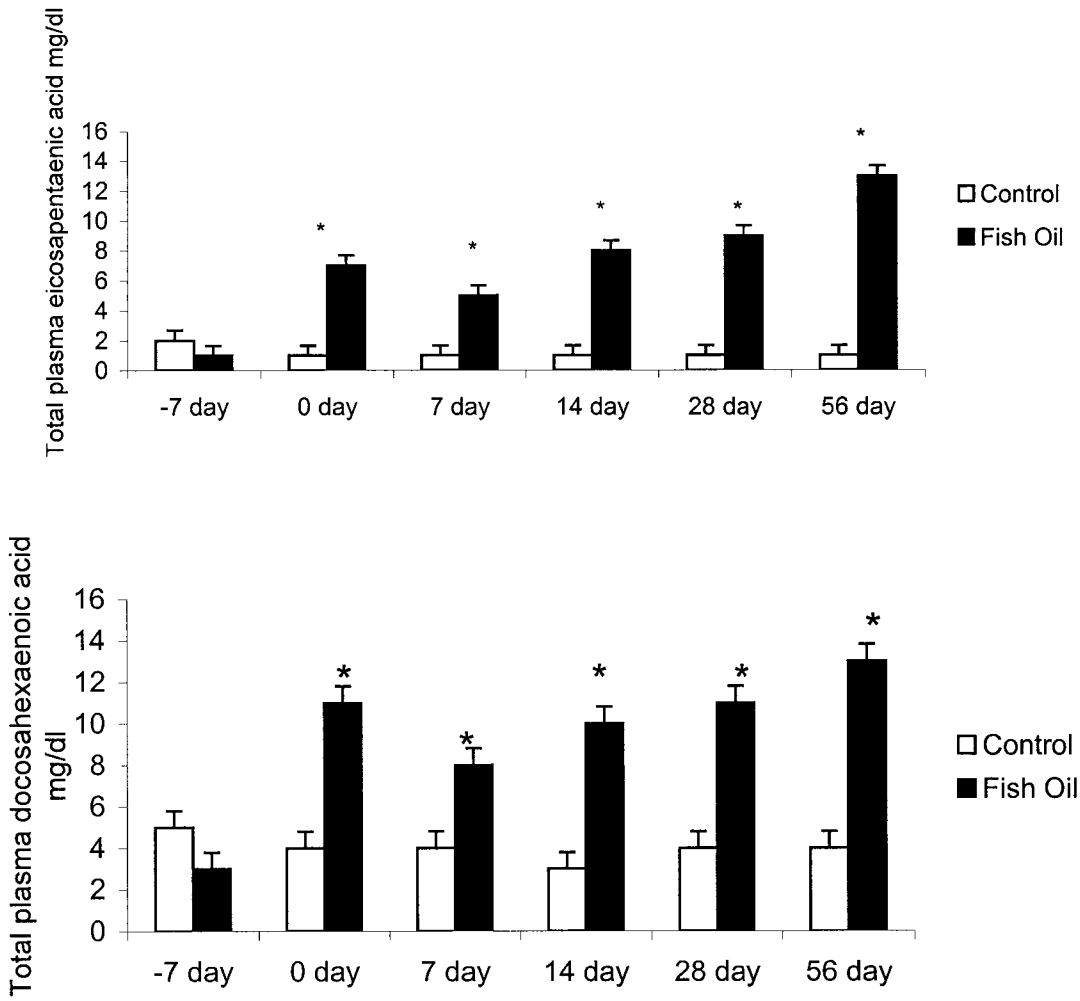


FIGURE 3-2. Total plasma arachidonic, eicosapentaenoic and docosahexaenoic acid concentrations in dogs fed fish oil or control (beef tallow) diet. X axis shows days prior to or post TPLO surgery. Feeding of diets started at –7 days. Values are mean ± SEM. Significant differences (*) are $p \leq 0.05$.

Pro Matrix metalloproteinase 9.

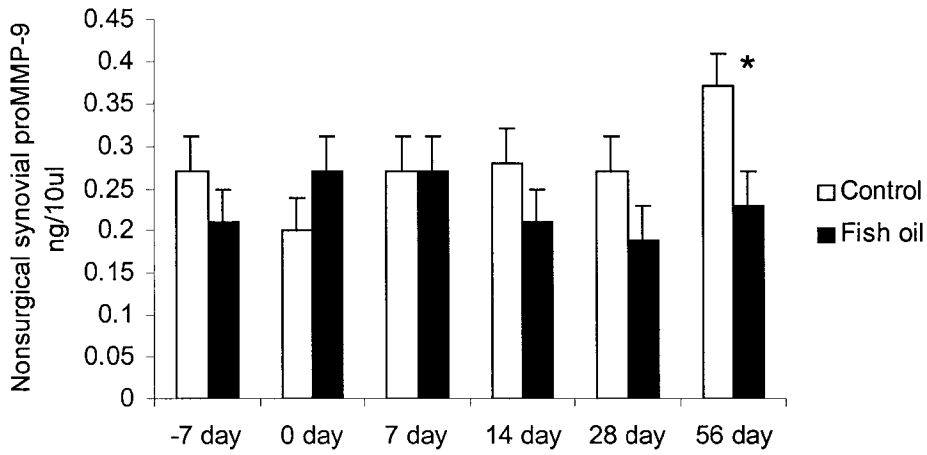


Figure 3-3. ProMMP-9 in the non-surgical synovial fluid in dogs fed fish oil or control (beef tallow) diet. X axis shows days prior to or post TPLO surgery. Feeding of diets started at –7 days. Values are mean ± SEM. Significant differences (*) are $p \leq 0.05$ for between group differences on day 56.

Pro Matrix metalloproteinase 2.

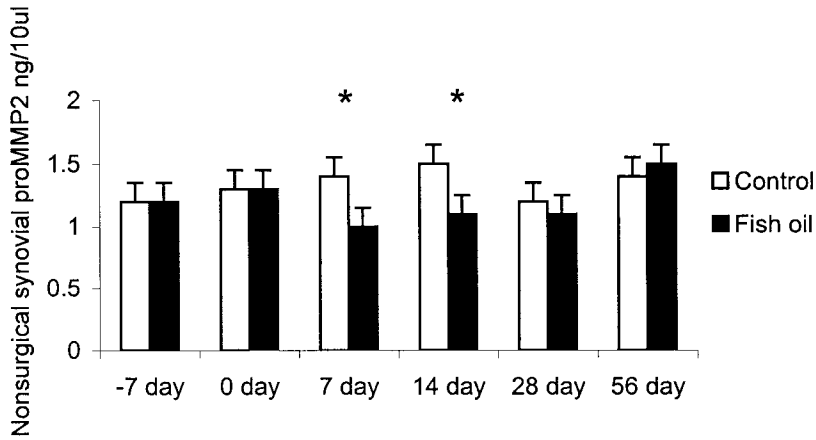


Figure 3-4. ProMMP-2 in the non-surgical synovial fluid in dogs fed fish oil or control (beef tallow) diet. X axis shows days prior to or post TPLO surgery. Feeding of diets started at –7 days. Values are mean ± SEM. Significant differences are indicated with an asterisk (*), $p \leq 0.05$ for a between group mean difference on days 7 and 14.

Discussion

Inflammatory mediators affect matrix metalloproteinase production and are in turn are influenced by n-3 and n-6 polyunsaturated fatty acids. The purpose of this study was to test effect of dietary supplementation with fish oil on production of inflammatory agents and endogenous MMPs in synovial fluid in a model of both surgical and non-surgical inflammation in dogs with osteoarthritis undergoing surgery in a single stifle.

The increase in plasma EPA and DHA after one week of fish oil supplementation which was sustained for the 56 day duration of the study, was consistent with other canine supplementation studies that have shown significant and sustained increases in EPA and DHA concentrations in healthy dogs (Hansen et al, 1998), and in diseased dogs (Ogilvie et al, 2000). In the present study, increased total plasma EPA and DHA were achieved with approximately one-third lower dietary fish oil intake than used in previous canine studies.

Total plasma arachidonic acid was significantly decreased in the fish oil fed group when compared to the control group at 35 days after start of specialized diet (Figure 3-1). This is likely due to competitive inhibition of incorporation of AA into plasma components, similar to results of Hansen et al (1998). These changes were not as marked as Hansen et al (1998) or Ogilvie et al (2000) where total plasma AA levels were decreased sooner and for an increased duration. One explanation could be that

because fish oil level was only about a third of these studies, it may not have been high enough to achieve the same degree of competitive incorporation

The synovial fluid activity of proMMP-2 was significantly decreased in the fish oil fed group at 7 days and 14 days post surgery (14 days and 21 days after start of diet) in the non-surgical stifle. No significant differences were found in the synovial fluid from the surgical knee. These data suggest an early effect of the diet on MMP-2 that diminishes after one month. In addition, these data suggest that the dietary influence of fish oil is only effective in decreasing MMPs in chronically associated inflammation and is not effective in decreasing MMPs after an acute inflammatory stimulus such as surgery.

Differences in the non-surgical synovial proMMP-9 were evident at 56 days post surgery (63 days of diet). Dogs fed fish oil showed lower mean proMMP-9 values, whereas no significant differences were found in the surgical synovial fluid. These results may indicate that dietary supplementation with fish oil only affects proMMP-9 in OA after an extended period of time (two month feeding) and only affects long term MMP production and not short term MMP production such as in the events following surgery. These results are similar to results of Liu & Rose (1995). Decreased activities of tumor homogenate proMMP-9 was associated with inhibition of metastasis of lung cancer in nude mice fed 4% EPA. *In vitro* cultured MDA-MB-435 cells cultured with 0.25- 1.0 μg/ml EPA caused a concentration related decrease in proMMP-9 in mRNA expression (Liu & Rose, 1995).

Comparison of proMMP-2 and proMMP-9 in the non-surgical synovial fluid may suggest dietary intervention predominantly effects the production of MMP-2 in

synovial cells after onset of specialized fish oil diet in OA. Later changes in MMP-9 that occur after further saturation with fish oil may take up to two months following dietary change. These results may suggest that an effect of fish oil on MMP-9 may be slower than MMP-2 or may occur after more thorough compartment and membrane saturation and incorporation.

In this study, significant decreases in the gelatinases but not the collagenases may be attributable to the fibronectin–like domain that is unique to gelatinases. Long chain 18 carbon polyunsaturated fatty acids were found to inhibit gelatinases but not collagenases in micromolar concentrations in fluorometric substrate analyses. An interaction by direct binding of the fatty acid with this fibronectin-like domain was suggested as the mechanism to inhibit the action of these MMPs (Berton et al, 2000). This might suggest that other longer chain PUFAs serve the same function *in vivo* and would explain why an affect of dietary fish oil was significant with proMMP-2 and proMMP-9, but not the collagenases.

More likely the effect of fish oil in this study is due to control factors exerted by n-3 fatty acids in gene expression of cytokines and inflammatory factors as well as MMPs. N-3 fatty acids were found to have a direct effect on expression of cartilage aggrecanases, cytokines IL-1 alpha and TNF alpha, and COX-1 and 2 *in vitro* (Curtis et al, 2000). Furthermore, n-3 fatty acids suppressed expression of COX-2, lipoxygenases-5, TNF alpha, IL-1alpha, and IL-1beta as well as expression of MMP-3 and MMP-13 in human OA cartilage explant cultures. N-3 PUFAs were cited in suppressing the expression of these cytokines, inflammatory factors, and MMPs (Curtis, 2002).

Suppression of the expression of MMPs may explain why no significant between group differences in activated MMPs were found in this study. Suppression of expression would have the greatest impact on the pro form of MMP concentrations and not the active form as MMPs are expressed in the pro form.

Dietary fish oil may affect permeability of compartment membranes that in turn would affect concentrations of MMPs. Activated MMP concentrations were not significantly affected in this study whereas the pro forms of MMPs were significantly affected. Since both forms were not affected, this again might suggest that that the dietary affect of fish oil is on suppression of expression and not on membrane permeability.

These results also suggest that supplementation is only effective on mild inflammatory events with chronic, steady state production of proMMP-2 and 9, and supplementation has no effect on MMP production in an acute, severe inflammatory event such as in post inflammation following surgery or injury.

Further investigations of higher total dose of dietary fish oil might show larger changes in proMMP-2, or faster changes in proMMP-9 production. Furthermore, a higher total dose of fish oil could also produce an effect on active forms of MMP-2 and 9, and collagenases.

In conclusion, dietary supplementation with fish oil can decrease proMMP-2 and 9 in a chronic or mild steady state inflammatory OA model, but fish oil does not have an effect on MMPs after acute or severe inflammatory events in OA.

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CHAPTER 4

RESPONSES OF ACTIVATORS AND INHIBITORS OF OSTEOARTHRITIS IN DOGS FED A FISH OIL CONTAINING DIET

Abstract

Overproduction and overactivation of MMPs, namely the collagenases and MMP-2 and MMP-9 have been implicated in chronic pathological diseases such as arthritis. Tissue inhibitors of metalloproteinases (TIMPs) are protein inhibitors that regulate activities of MMPs. Urokinase plasminogen activator (uPA) is a key agent in the conversion of proMMPs to active forms. The n-3 long chain polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both significant components of fish oil, have been identified to have beneficial effects in treatment of arthritis although the molecular mechanism is unknown. Possible mechanism of fish oil may include the downregulation of MMPs *in vivo* by increase in TIMP production or by a decrease in uPA concentration. The objective of this study was to determine if the effect of intake of fish oil in dogs with arthritic stifles undergoing surgery for cruciate rupture in a single knee would increase TIMP concentrations and decrease uPA activities in the non-surgically treated stifle synovial fluid.

Twenty-four dogs, recruited for surgery of a torn cranial cruciate ligament in a single knee, were fed identical maintenance diets that differed only in fat content (90mg/kg/day) as fish oil or beef tallow (control) for a 63 day period. Plasma and synovial fluid from the non-surgical stifle was obtained at start of diet, on the day of surgery, and at 7, 14, 28, and 56 days following surgery. Plasma was analyzed for

arachidonic acid (AA), EPA, and DHA and synovial fluid was analyzed for TIMP-2 and uPA. Synovial fluid EPA and DHA increased significantly ($p<0.05$) after initiation of diet. The fish oil fed group showed significant ($p<0.05$) increases in synovial TIMP-2 at day 7 and 28, and significant ($p< 0.05$) decreases in uPA in synovial fluid at day 28. These results suggest that dietary supplementation of fish oil decreases synovial uPA activities and increase synovial TIMP-2 following chronic injury. (Supported by Colorado Agriculture Experiment Station and Nestle-Purina PetCare Grant)

Introduction

Tissue inhibitors of metalloproteinases (TIMPs) are a group of protein inhibitors that regulate many physiologic functions. Most notable is their function in regulating the deposition and degradation of connective tissue by their inhibition of matrix metalloproteinases (MMPs). The MMPs are endogenous enzymes that degrade connective tissue and have received considerable attention because they are overproduced in a variety of chronic diseases including arthritis and therefore they have been targets for therapeutic research. Urokinase plasminogen activator (uPA) is a key agent in the activation of MMPs to active forms. The TIMPs are endogenous regulators of MMPs in that they form a non-covalent complex with MMPs and prevent the catalytic action of the enzyme (Cawston et al, 1996). Current interest in these inhibitory proteins and activators revolves around regulating their function, which in turn could potentially regulate deleterious levels of MMPs in disease.

Urokinase plasminogen activator (uPA) is involved in MMP regulation by its role in the cleaving process that converts the pro MMP enzyme to and the active enzyme. It is a predominant activation agent of circulating plasminogen to plasmin. Plasmin is a broad-spectrum proteinase that acts directly on the cleavage of proMMPs to active MMPs (Leyland, 1996).

The TIMPs are present in both tissues and fluid compartments and are produced by a variety of cell types (Gomez et al, 1997). Four different members of the TIMP family have been identified and are sequentially numbered. These four TIMPs share similarity in structure, size, and inhibitory effects by bonding with MMPs.

It has been well established that TIMPs are regulated in part by cytokines and uPA could indirectly be regulated by cytokines. Cytokine regulation has been predominantly investigated in in vitro culture studies and not *in vivo*. Cytokines IL-12, IL-13, and IL-15 increased TIMP-1 concentrations (Constantinescu et al, 2001; Cleaver et al, 2001). Exogenous IL-11 upregulated TIMP-1 (Hermann et al, 1998). The cytokine IL-6 enhanced TIMP-1 mRNA levels (Richards et al, 1993), and enhanced expression of TIMP in a dose dependent manner (Sato et al, 1990) and significantly correlated with elevated mRNA levels of TIMP-1 in lymphoma tissue (Kossakowska et al, 1999). TIMP-1 is upregulated by IL-1beta (Gomez et al, 1997) and TNF alpha in brain microvascular endothelial cells and astrocytes (Bugno et al, 1999). The cytokine TNF alpha exhibits a bifunctional effect of TIMP-1 production in vitro in that low concentrations induced stimulation and high concentrations caused suppression in a dose dependent manner (Ito et al, 1990).

TNF alpha affects uPA secretion in human cell cultures (Bechtel et al, 1996). Culture supernatant showed an increase in uPA as well as uPA receptors on epidermal keritinocytes after addition of IL-1 and TNF alpha. Modulation of TNF alpha levels also directly affect uPA levels (Egawa et al, 1994). Intracellular oxidation states in cultured cells were altered, markedly increasing TNF alpha concentrations that in turn increased the steady state level of uPA.

Of increasing interest is the regulatory influence polyunsaturated fatty acid derived eicosanoids have on TIMP and uPA production. Studies of eicosanoids of arachidonic acid have been found to affect TIMP production in *in vivo* culture studies. Prostaglandin E_2 inhibited IL-1 beta stimulated TIMP-1 mRNA expression and secretion in a dose dependent manner (DiBattista et al, 1995) and exogenous PGE_2, and cyclooxygenase inhibitors indomethacin and diclofenac suppressed IL-1 beta induced production of TIMP-1 (Takahashi et al, 1997). Lipoxins (LXA_4) have been demonstrated to affect TIMP production by inducing a 1 to 3 fold increase in TIMP-1 and TIMP-2 respectively (Sodin-Semrl et al, 2000).

It has been well documented that polyunsaturated fatty acids affect cytokine concentrations in arthritis (Tidow-Kebritchi & Mobarhan, 2001). Central to the development of this mechanism is that arachidonic acid derived eicosanoids induce overproduction of inflammatory cytokines. In culture studies, cells exposed to eicosapentaenoic acid (EPA) or arachidonic acid (AA) showed that EPA increased collagen synthesis and AA reduced collagen production while the levels of IL-6 and collagen production showed a significant linear correlation (Hankenson et al, 2000). Human clinical trials of n-3 PUFA supplementation in patients with rheumatoid

arthritis showed that plasma cytokine IL-1beta was significantly reduced (Esperson et al, 1992) and plasma TNF alpha and IL-1 beta synthesis were significantly reduced (Caughey et al, 1996). Fish oil supplementation has been demonstrated to decreased plasma IL-1 cytokine levels in dogs (Freeman et al, 1998).

It is well recognized that intake of polyunsaturated fatty acids influences metabolism of eicosanoids in mammals. Proinflammatory eicosanoids of the two series PGs (PGE2) and four series LTs (LTB4) are derived from the n-6 fatty acid arachidonic acid. Fish oil contains eicosapentaenoic acid and competes with arachidonic acid and therefore decreases the synthesis of proinflammatory PGE2 and LTB4 (James et al, 2000)(Wallace et al, 2000)(Calder, 1997) and results in synthesis of less inflammatory three series PGs and five series LTs.

Currently, no studies have addressed the effects of fish oil on TIMPs in a clinical model in humans or in an outbred species with OA. No studies have addressed the effects of fish oil on uPA. Therefore the present study was designed to examine if the effect of a diet supplemented with n-3 long chain fatty acids (fish oil) would increase the production of TIMP-2 and decrease the production of uPA in arthritic dogs who have undergone surgery for correction of a torn anterior cruciate ligament.

Research Design and Methods

Animals and Diets. Twenty-four dogs with joint instability resulting from clinically confirmed acute cranial cruciate ligament (CCL) injury in a single stifle were enrolled in the study. All dogs weighed a minimum 20 kgs and varied by breed. All dogs were recruited as clients at the Colorado State University Veterinary Teaching Hospital and

treated over a 379 day time span. Dogs were randomly assigned to one of two diet treatments of twelve dogs each: maintenance diet with high fish oil and maintenance diet with low fish oil content.

The diets were formulated by Nestle Purina Petcare (St. Louis, MO) (Tables 1 and 2). Both diets were made of products typically used in the manufacture of pet foods, i.e. rice, whole chicken, catfish meal, canola meal, soybean meal, minerals, vitamins, choline to provide a complete and balanced diet for all life stages. The diets were assessed for palatability and their affect on stool quality prior to being used in the study. There were no problems associated with the use of the experimental diets. Both control and fish oil diets contained 3.5 kcals/gm as a dry kernel feed. Dogs were fed at 40 kcal/kg. The high fish oil diet provided 0.75% EPA and DHA by wet weight or 90 mg combined EPA and DHA/kg BW/day for each dog. At entry (day –7) clients were instructed how much to feed their dogs to achieve 90mg/kg/day for the 63 days of the protocol. Diets were maintained during hospital stay for surgery. Clients were firmly instructed to feed nothing other than the diet.

Surgical procedure, stifle arthrocentesis, and venipuncture. Dogs had routine physical examinations under the direction of a board certified small animal orthopedic surgeon and radiographs were taken prior to and at the conclusion of the study. The presence of complete cruciate ligament rupture with or without secondary meniscal injury with subsequent meniscal treatment was required for study entry. Each dog received a uniform standard operative procedure (Tibial Plateau Leveling Osteotomy, US Patent No. 4,677,973).

Table 1. Diet composition.

	Control	Fish oil
Protein	27.20%	27.80%
Total Fat	12.60%	12.90%
CHO	43.61%	42.18%
Ash	7.92%	8.02%
Moisture	8.67%	9.10%

Table 2. Fatty acid composition of diet (% total fatty acids).

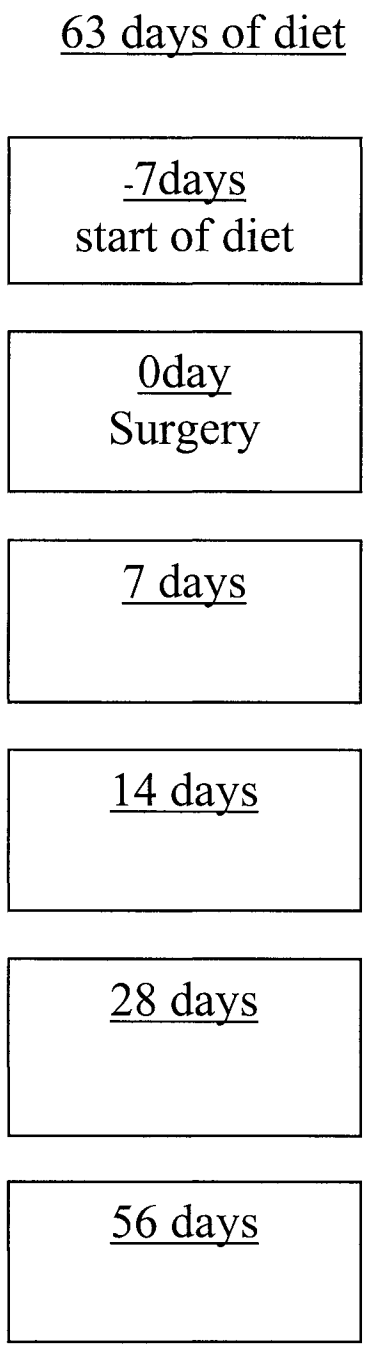
	Control	Fish oil
14:0	1.18%	3.13%
14:1	0.21%	0.22%
15:0	0.18%	0.35%
16:0	18.30%	20.90%
16:1	2.82%	5.63%
17:0	0.47%	0.60%
18:0	7.76%	8.08%
18:1n-9	31.50%	29.80%
18:1n-7	1.51%	2.09%
18:1trans	1.41%	1.87%
18:2n-6	29.10%	11.80%
20:0	0.23%	0.20%
18:3n-6	0.10%	0.18%
20:1	0.40%	0.62%
18:3n-3	0.78%	1.08%
20:2n-6	0.16%	0.25%
22:0	0.11%	<0.10%
20:4n-6	0.43%	0.66%
24:0	0.13%	<0.10%
20:5n-3	<0.10%	3.09%
22:5n-3	<0.10%	0.67%
22:6n-3	0.19%	2.66%
24:1	0.12%	0.13%
total n-6	29.88%	12.64%
total n-3	0.97%	7.50%
n-6/n-3	30.80	1.685

Synovial fluid from the non-surgery knee was obtained at the following time points: day –7(initial day of diet), day 0 (immediately prior to surgery), day 7, day 14, day 28, and day 56. The dogs were anesthetized with intravenous propofol. A 20-gauge needle with sterile syringe was placed in the joint with negative pressure until synovial fluid appeared in the needle hub. One to two mls of synovial fluid was obtained and transferred to a two ml cryovial for storage at -80°C until analysis.

EDTA plasma from whole blood was obtained at the following time points: day –7 (initial day of diet, day 0 (immediately prior to surgery), day 7, day 14, day 28, and day 56. Three ml blood was obtained with a 20-gauge needle in a sterile, empty (no chelating agents) six ml syringe and transferred into an EDTA vacutainer tube. The fluid was spun for ten min at 2000g and the plasma was drawn off with a pipette, transferred to a two-ml cryovial, butylated hydroxytoluene (BHT) was added (0.1 mg/ml) as a stabilizer, and stored at -80°C until analysis.

AA, EPA and DHA analysis. Gas Chromatography was used to evaluate plasma fatty acid concentrations (Ohta et al, 1990). Plasma samples were allowed to thaw and then were thoroughly vortexed. Each sample was analyzed in duplicate. One hundred μls of isooctane containing two mg/ml of heptadecanaenoic acid internal standard was added to a screw top vial. Under a slow stream of Nitrogen gas, the isooctane was evaporated. Two hundred μls of plasma was placed into each vial followed by two mls of a 2:1 mixture of chloroform and methanol and vortexed. One ml of isotonic saline (0.9% NaCl) was then added, the vial was again vortexed, and then centrifuged at 2,000 x g for ten min.

Figure 5-1. Timeline for Research Design Timeline showing 63 total days of diet and time points. Serum, plasma, and synovial fluid were obtained at each of six time points.



The chloroform layer was then drawn off and placed in a new vial and evaporated until dry. One ml of hexane reagent was added, followed by three mls of boron trichloride/methanol, and placed in a water bath at 60°C for two hours. One ml of water was then added and the hexane layer was aspirated and placed in a chromatography vial (Agilent, San Fernando, CA) and analyzed by gas chromatography (5890 Gas Chromatograph, Hewlett Packard, San Fernando, CA) using a .32 mm I.D. capillary column with 0.5um film (HP innowax 19091N-213 Capillary Column, Agilent, San Fernando, CA) with 1.5 ml/min constant flow.. Concentrations of AA, EPA and DHA were determined by first determining the area ratio of each sample (ratio of internal standard to peak of interest) and then comparing results to an external set of standards (Sigma, St. Louis, MO) Ratios were then matched to corresponding molar concentrations by means of linear regression.

Measurement of urokinase plasminogen activator. Analysis synovial urokinase plasminogen activator was done with the uPA Activity Assay Kit (Chemicon International, Inc., Temecula, CA). To a 96 well plate, ten µls of synovial fluid as well as rehydrated uPA positive control for use as standards was added in duplicate. To each sample and standard, 160 µls of deionized water was added, followed by 20 µls of assay buffer provided in the kit. Twenty µls of rehydrated chromogenic substrate was then added and the plate was incubated at 37°C for exactly two hours. The plate was assayed at emission of 405 nms.

Measurement of TIMP-2. Synovial TIMP-2 analysis was conducted by ELISA Immunoassay (Fujimoto et al, 1995) using commercial available TIMP-2 ELISA assay

kit available from Oncogene Research Products (Boston, MA). One hundred µls of synovial fluid was well vortexed with 100 µls of peroxidase conjugate. One hundred µls of sample or standard was then placed in duplicate in a precoated 96 well plate. The plate was covered and allowed to incubate at 23°C for exactly two hours. The plate was then washed and one hundred µls of tetra-methylbenzidine/hydrogen peroxide in 20% dimethylformamide was added to each well and allowed to incubate at 23°C for exactly 30 min. The absorbance was read at 630 nms.

Statistical analysis of data. All response variables were normally distributed by log transformation and were analyzed as log transformed. The data from each response variable were analyzed by an analysis of variance (ANOVA) with repeated measures, where the between subjects effect was diet and the repeated measures effect was date. Data were analyzed by the Statistical Analysis Systems (SAS) mixed procedures software Release 8.1 copyright 1999-2000 (SAS Institute Inc., Cary, NC). Differences between treatment groups at each time point were evaluated as t-tests as Tests of Effect Slices (SAS) for the response variables plasma arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, as well as non-surgical synovial fluid TIMP-2 and uPA. The significance level was set at $p \leq 0.05$.

Results

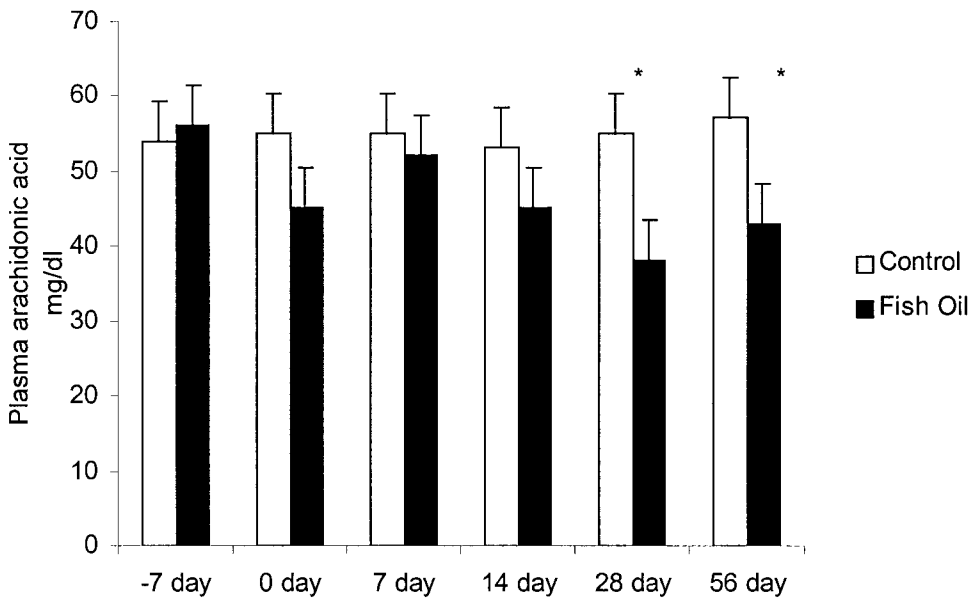
Prior to initiation of specialized diets, there were no significant between group differences in total plasma EPA or DHA. Dietary supplementation with fish oil resulted in significant increases in both EPA and DHA within one week and remained

significantly different ($p < 0.0001$) throughout the remainder of the study (Figure 4-2). Total plasma AA was significantly lower in the dogs treated with fish oil supplementation at day 35 after start of feeding and remained significantly lower through the remainder of the study (Figure 4-2).

Prior to initiation of specialized diets, there were no significant between group differences in non-surgical synovial TIMP-2 concentration. Log means and SEMs from Figure 4-3 show that there was a trend throughout the study after initiation of specialized diets for control subjects to exhibit lower TIMP-2 values. Concentrations of TIMP-2 were significantly different at day 7 ($p = 0.04$) and day 28 ($p = 0.01$) where fish oil fed dogs had higher TIMP-2 concentrations than control dogs.

At initiation of study before feeding of diets, there were no significant between group differences in non-surgical synovial uPA activities. Log means and SEMs from Figure 4-4 show that there was a trend throughout the study after initiation of specialized diets for control subjects to exhibit higher uPA values. Significant differences ($p = 0.05$) between groups occurred at day 28 where control values were higher than fish oil fed values.

Total plasma arachidonic, eicosapentaenoic and docosahexaenoic acid concentrations.



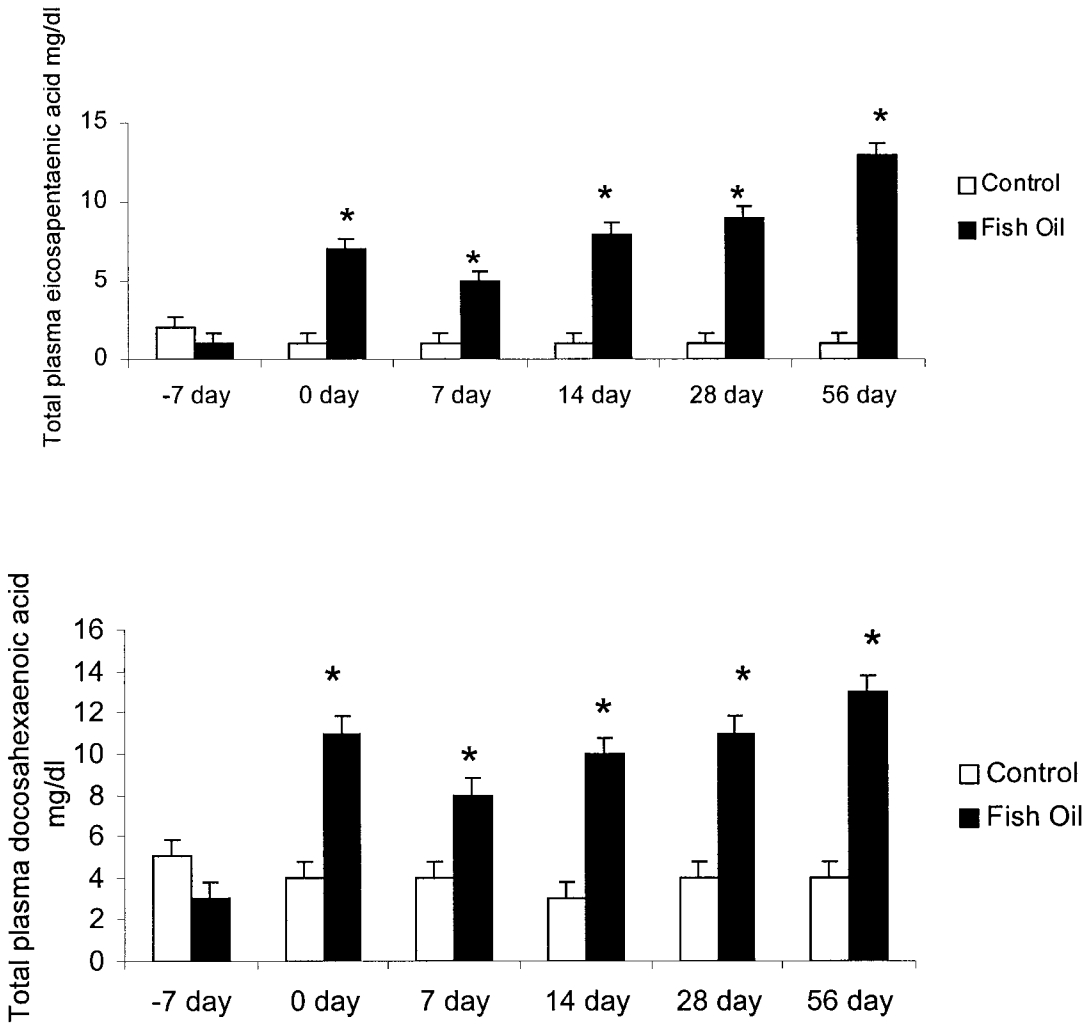


Figure 4-2 . Total plasma arachidonic, eicosapentaenoic and docosahexaenoic acid concentrations in dogs fed fish oil or control (beef tallow) diet. X axis shows days prior to or post TPLO surgery. Feeding of diets started at –7 days. Values are mean ± SEM. Significant differences (*) are $p \leq 0.05$.

TIMP-2 levels.

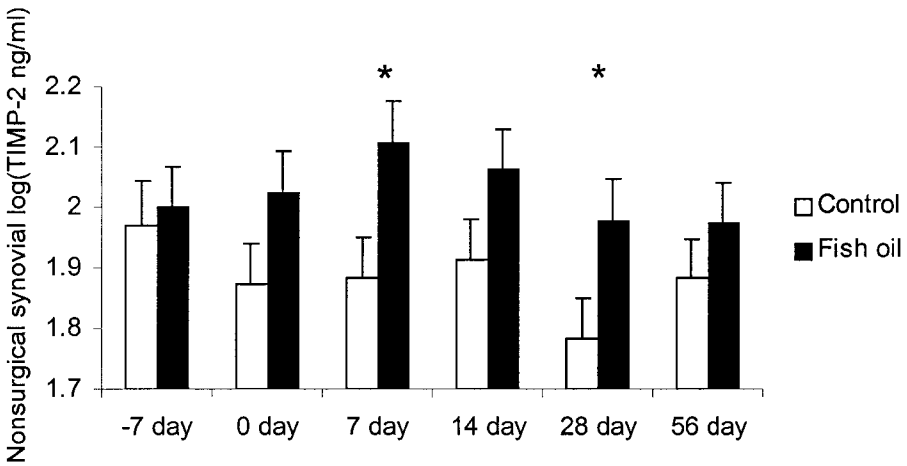


Figure 4-3. TIMP-2 levels in synovial fluid from nonsurgical knee in dogs fed fish oil or control (beef tallow) diet. X axis shows days prior to or post TPLO surgery. Feeding of diets started at –7 days. Values are mean ± SEM. Significant differences are indicated with an asterisk (*), $p \leq 0.05$ for a between group mean difference on days 7 and 28.

Urokinase plasminogen activitor activity.

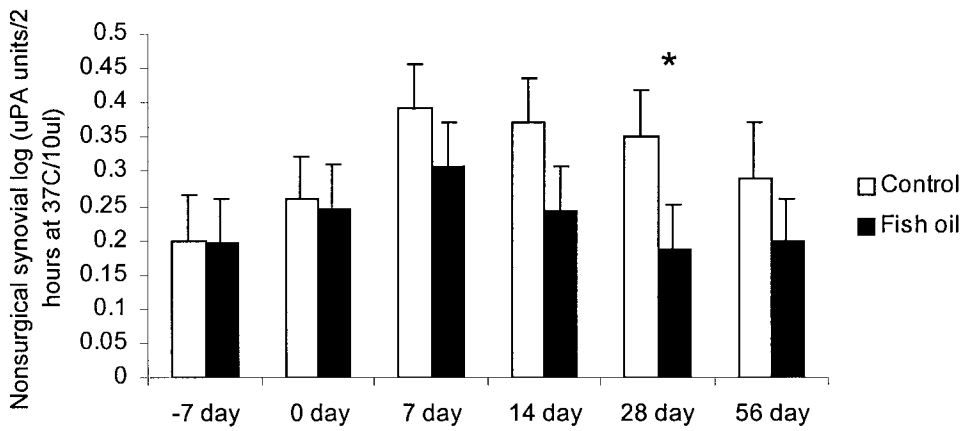


Figure 4-4. Urokinase plasminogen activator activity in the non-surgical synovial fluid in dogs fed fish oil or control (beef tallow) diet. X axis shows days prior to or post TPLO surgery. Feeding of diets started at –7 days. Values are mean ± SEM. Significant differences are indicated with an asterisk (*), $p \leq 0.05$ for a between group difference on day 28.

Discussion

Feeding fish oil increased concentrations of TIMP-2 and decreased uPA activity in synovial fluid from chronically inflamed arthritic joints. It has been established that fish oil affects inflammatory agents such as IL-6, TNF alpha, and PGE2. It has also been shown that these inflammatory agents may affect production of TIMPs and may indirectly effect uPA production. This is the first study to investigate the effect of fish oil on TIMP and uPA production. Furthermore, this is the first *in vivo* study to investigate the effect of fish oil on TIMP and uPA production in a clinical model.

This study showed significant increases in total plasma EPA and DHA after one week of fish oil supplementation that was sustained for the 56 day duration of the study. This is consistent with other canine supplementation studies that have sustained significantly elevated EPA and DHA in normal dogs (Hansen et al, 1998), and in disease dogs (Ogilvie et al, 2000). This study demonstrated that significantly increased total plasma EPA and DHA were achieved with approximately one-third less dietary fish oil intake than used in previous canine studies.

Total plasma arachidonic acid was decreased and significantly lower in the fish oil fed group when compared to the control group 35 days and 63 days after the start of the specialized diet or 28 days and 56 days post surgery (Figure 4-2). This is likely due to competitive inhibition of incorporation of AA into plasma components, similar to results of Hansen et al (1998). These results were not as marked as reported by Hansen et al (1998) or Ogilvie et al (2000). Total plasma AA levels were depressed sooner and

for a longer duration in both of these studies. One explanation could be that because fish oil supplementation was only about a third as in these studies, it may not have been high enough to achieve the same degree of competitive incorporation.

The TIMP-2 is a soluble protein produced by many different cells. It is found in both tissues and fluid compartments (Gomez et al, 1997). In this study, TIMP-2 was readily found in synovial fluid. This study employed the use of an enzyme linked immunosorbant assay by Oncogene Research Products (Boston, MA). The assay has been validated to detect TIMP-2 from human, mouse, rat, guinea pig, rabbit and bovine and detects the free and complexed TIMP-2 with active forms of MMP-1, 2, 3, 7, 8, and 9 (Fujimoto et al, 1995).

Synovial fluid from the non-surgical knee showed significant between group differences at day 7 and day 28 post surgery or day 14 and day 35 of diet feeding in that the group fed fish oil had higher mean values of TIMP-2. Conversely, the synovial fluid demonstrated a trend for the control group to have higher uPA values as evidenced by the significant between group differences at day 28 where the control group had higher uPA activities than the fish oil group. Because the non-surgical knee reflects a steady state response to agonists of MMP and TIMP production, the differences in uPA and TIMP-2 between the fish oil and control groups is most likely attributable to feeding fish oil. In this study, the effect of the specialized diets may not be active in the latter days post surgery at 56 days when no significant between group differences were found for either uPA or TIMP-2.

The effect of dietary intervention was shown to attenuate inflammatory mediators during mild chronic inflammatory events such as those associated with

chronic OA in Chapter 3, but not severe, acute inflammatory events such as those associated with surgery or trauma. This might explain transient effects on TIMP-2 concentrations. Because the surgical knee is recovering, the dog is favoring the surgical knee and a limping gait might employ additional and abnormal use of the non-surgical knee aggravating any arthritic condition still existing, resulting in a mechanism driven trauma driving more severe type inflammation. Severe inflammation resulting from limping might attenuate any dietary effect on mild inflammation from feeding of specialized diets. A limping gait of the non-surgical knee would aggravate the chronic condition resulting in increased inflammation. Any steady state TIMP-2 or uPA production that could be affected by fish oil nutrition might be attenuated, as dietary fish oil supplementation is only affective in mild inflammatory events.

Several factors may have influenced levels of TIMP-2 in this study. The cytokines IL-12, IL-15, IL-13 and IL-11 increase TIMP-1 levels (Constantinescu et al, 2001) (Cleaver et al, 2001) (Hermann et al, 1998). Although fish oil has been shown to affect production of cytokines and interleukins such as IL-6, the effect of fish oil increasing production of these interleukins is not known. If the anti-inflammatory effect of n-3 fatty acids had an impact on inflammatory cytokines such as TNF alpha (Chandrasekar & Fernandes, 1994)(Somers & Erickson, 1994)(Fernandez & Venkatraman, 1993)(Curtis et al, 2000) the decrease of these inflammatory agents may have influenced production of TIMPs.

The eicosanoid derivatives of AA include PGE2 and LTB4 that have been shown to increase TNF alpha (Calder, 1997). Eicosanoids such as PGE2 have been identified as key in increased production of MMPs (Corcoran et al, 1992)(Wahl et al,

1977, Wahl & Lample 1987). Feeding with fish oil should decrease any 2 series PGs or four series LTs derived from AA by competitive incorporation. This synergistic effect of fish oil reducing TNF alpha directly and through eicosanoid metabolism may suggest a mechanism that could have increased TIMP-2 in the fish oil fed group at seven and 14 days after the start of specialized diets.

There are other eicosanoids of the lipoxygenase pathway that are derived from AA and may affect cytokines, eicosanoid metabolism, and TIMP concentrations. These derivatives include the lipoxins that are formed from AA acted upon by lipoxygenase-15. Lipoxin A4 inhibits IL-1 beta-induced IL-6 and matrix metalloproteinase-3 production in cell culture (Sodin-Semrl et al, 2000). This interaction of eicosanoids on cytokines involved in TIMP production may serve as an additional pathway of regulation of TIMPs by fish oil. If lipoxins such as LXA4 affect MMP production, this would increase free TIMP as less would be bound to MMPs. The effect of other AA derivatives on TIMPs remains to be investigated.

Other factors should be considered that might influence concentrations of both uPA and TIMP-2 in this study. Dietary fish oil can affect compartment membrane permeability that in turn would affect concentrations of TIMP-2 and uPA. The effect of dietary fish oil may be directly on the expression of TIMP-2 and uPA and not through intermediate inflammatory agents. The effect of dietary fish oil directly on TIMP-2 expression *in vivo* is not known but Curtis et al (2002) found that n-3 or n-6 PUFAs had no effect on expression of TIMP mRNA abundance in human cartilage explants.

This study investigated only one activator (uPA) and one inhibitor (TIMP-2). The effects of fish oil supplementation may be related to its action on other activators

and inhibitors of MMPs. In addition, fish oil may have an unknown action directly on the production of TIMPs.

Urokinase plasminogen activator activity was significantly decreased at 28 days post surgery or 35 days after start of diet in the non-surgical synovial fluid. Subchondral bone osteoblasts secrete uPA that are involved with bone remodeling. Carprofen, a cyclooxygenase-1 (COX-1) and COX-2 inhibitor, has been shown to decrease uPA activity in culture media of osteoblasts from OA dogs (Pelletier et al, 2000). Because products of the COX enzyme include PGE2 from AA, this might suggest an association between AA products and uPA. The same mechanism might decrease uPA from AA products if AA was reduced by competitive incorporation from EPA and DHA into cell membranes.

The effect of fish oil on TNF alpha may have directly affected uPA activities because TNF alpha has been shown to affect uPA secretion in human cell cultures (Bechtel et al, 1996), and modulation of TNF alpha levels also directly affects uPA levels (Egawa et al, 1994).

This study investigated TIMP-2 by ELISA methodology available primarily for human TIMP investigation. Because ELISA technology for TIMP-1 analysis for species such as dog become available, further studies of the effect of nutrient intervention by fish oil on arthritis should be conducted.

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CHAPTER 5

Overall Conclusions

Increased concentrations of matrix metalloproteinase (MMP) enzymes are key in the development of osteoarthritis (OA). Activities of MMPs are affected by the activation by urokinase plasminogen activator (uPA) and inhibited by tissue inhibitors of metalloproteinases (TIMPs). Fish oil can affect certain cytokines such as interleukins and TNF alpha, and eicosanoids such as prostaglandin E2. These in turn can influence the production of MMPs, TIMPs, and uPA.

It was the intent of this study to determine if the addition of dietary fish oil could benefit the disease state of OA by decreasing concentrations of the MMP enzyme, and affect the agents uPA and TIMP-2 involved in the regulation of the action of this enzyme. The studies described here were designed to evaluate the effect of fish oil on MMPs, TIMPs, and uPA.

Study 1 Specific Aims: The objective of this study was to determine if increased dietary intake of fish oil in dogs undergoing surgery for cranial cruciate ligament rupture in a single knee would affect MMP activities in synovial fluid from both surgical and non-surgical osteoarthritic stifle joints.

Hypothesis: A supplemented diet with high n-3 long chain fatty acids (fish oil) content would decrease the production of matrix metalloproteinases in both surgical and non-surgical synovial fluid from stifle joints in osteoarthritic dogs who have undergone surgery for correction of a torn cranial cruciate ligament.

Primary findings:

The results of this study suggest that dietary fish oil supplementation did decrease proMMP-2 and proMMP-9 in the stifle joint subject to long term joint instability, but not following surgery induced injury. These findings suggest that the influence of fish oil is only on the proMMP form and not the active form, and that the effects of fish oil are found only in a chronic, steady state environment.

Future directions:

The results of this study showed an effect of fish oil on MMPs in the synovial fluid from log term joint instability and not the synovial fluid from surgically induced injury. It would be of particular interest to design an experimental project that directly investigates the effect of fish oil on MMPs over an extended duration in a chronic arthritic condition only such as degenerative joint disease in dogs. In addition, this study was only able to investigate the gelatinases and collagenases involved with joint instability. It would be of interest to also examine the matrilysins, membrane-type MMPs, and stromelysins as these groups have also been identified as key enzymes involved with arthritic conditions.

Study 2 Specific Aims: The objective of this study was to determine if increased intake of fish oil in arthritic dogs would affect TIMP concentrations and uPA activities in synovial fluid from the stifle joint.

Hypothesis: A supplemented diet with high n-3 long chain fatty acids (fish oil) content would increase the production of tissue inhibitors of metalloproteinases 2 (TIMP-2) and

decrease urokinase plasminogen activator (uPA) in synovial fluid from the non-surgical and surgical stifle joints from dogs with arthritis.

Primary findings:

The results of this study suggest that dietary fish oil supplementation did increase TIMP-2 and decrease uPA in the non-surgical stifle joint, but had no effect on TIMP-2 or uPA in the surgical stifle joint. These findings suggest that the influence of fish oil is only in a chronic, steady state environment.

Future directions:

The results of this study showed an effect of fish oil on TIMPs and uPA in the synovial fluid from long term joint instability and not the synovial fluid from surgically induced injury. It would be of particular interest to design an experimental project that directly investigates the effect of fish oil on TIMPs and uPA over an extended duration in a chronic arthritic condition only such as degenerative joint disease in dogs. In addition, this study was only able to investigate TIMP-2 involved with joint instability. It would be of interest to also examine TIMP-1 as this inhibitor interacts with MMPs in arthritic conditions.

CHAPTER 6

APPENDICES

Appendix A:

Materials and Methods

Protocol for surgical procedure. Dogs had routine physical examinations under the direction of a board certified small animal orthopedic surgeon and radiographs were taken prior to and at the conclusion of the study. The presence of complete cruciate ligament rupture and secondary meniscal injury with subsequent meniscal treatment was required for study entry. Each dog received a uniform standard operative procedure (Tibial Plateau Leveling Osteotomy, US Patent No. 4,677,973). Postoperative analgesia was provided as subcutaneous administration of morphine sulfate (0.5- 1.0 mg/kg) every 8 hours as needed. Suture was removed two weeks post surgery.

Protocol for stifle arthrocentesis.

Synovial fluid was obtained at the following time points: day –7(initial day of diet), day 0 (immediately prior to surgery), day 7, day 14, day 28, and day 56. Synovial fluid was obtained from the affected (CCL ruptured) and non-surgical joints of all dogs. The dogs were anesthetized with intravenous propofol (6 mg/kg). The area around the stifle joints were clipped and cleaned with chlorhexidine solution. The stifle was flexed and a 1.5-in 20-gauge needle was introduced into the joint just lateral to the patellar ligament mid-way between the femoral condyles and the tibial plateau. The needle was directed caudomedially until synovial fluid appeared in the needle hub. A sterile 6-cc syringe was used to apply negative pressure to aspirate fluid from the joint. One to two mls of synovial fluid was obtained and transferred to a two ml cryovial for storage at -80˚C until analysis.

Protocol for venipuncture.

EDTA plasma, heparin plasma, and serum from whole blood was obtained at the following time points: day -7(initial day of diet, day 0 (immediately preoperative to surgery), day 7, day 14, day 28, and day 56. Six ml blood was obtained with a 20 gauge needle in a sterile, empty (no chelating agents) in a six ml syringe and transferred into vacutainer tubes with additives for collection of plasma (EDTA and heparin) and serum. The filled tubes were allowed to sit at room temperature (approx 22° C) for 20 min. The fluid was spun for ten min at 2000g after which the serum or plasma was drawn off with a pipette, transferred to a two-milliter cryovial and stored at -80°C until analysis. Butylated hydroxytoluene (BHT) was added (0.1 mg/ml) to the EDTA plasma as a stabilizer. Vacutainers were obtained from Sherwood Medical (St. Louis, MO).

Polyunsaturated fatty acid analysis.

Gas Chromatography was used to evaluate plasma fatty acid concentrations (Ohto et al, 1990). EDTA plasma samples were allowed to thaw and then were thoroughly vortexed. Each sample was analyzed in duplicate. In a four-ml screw top vial with a Teflon (PTFE) lined cap (Supelco, Bellefonte, PA) 100 µls of isooctane containing two mg/ml of heptadecanaenoic acid internal standard was added. Under a slow stream of Nitrogen gas, the isooctane was evaporated. Two hundred µls of plasma was measured with a Hamilton gas tight syringe and placed into each vial followed by two mls of a 2:1 mixture of chloroform and methanol. The vial was vortexed on high for one minute. One ml of isotonic saline (0.9% NaCl) was then added and the vial was again vortexed for one minute and then centrifuged at 2,000 x g for ten min.

The resulting solution was composed of an aqueous layer on top, a proteinaceous layer in the middle, and a chloroform layer at the bottom. The aqueous layer was drawn off and discarded. The chloroform layer was then drawn off and placed in a new 15 ml vial with a Teflon cap (Supelco, Bellefonte, PA). This chloroform layer was then evaporated until dry with a slow stream of nitrogen gas in a 30°C water bath. After the sample was dried, one ml of hexane reagent was added, followed by three mls of boron trichloride/methanol. The vial was capped and placed in a water bath at 60°C for two hours. It was removed from the water bath and cooled to room temperature (approx 22° C), one ml of water was added and the sample was vortexed to quench the reaction. The sample was cooled to 4°C for 15 min to separate layers. The hexane layer was aspirated and placed in a chromatography vial (Agilent, San Fernando, CA) and head space flushed with nitrogen gas. Prepared samples were analyzed within one week.

Samples were analyzed by gas chromatography (5890 Gas Chromatograph, Hewlett Packard, San Fernando, CA) using a capillary column (HP innowax 19091N-213 Capillary Column, Agilent, San Fernando, CA) with initial temperature set at 150°C (1 minute), to 200°C at 15C/min, and to 250°C(5min) at 2°C/min. Injector and detector temperatures were set to 255°C. Three µls of each sample were analyzed. Linoleic acid, linolenic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid concentrations were determined by first determining the area ratio of each sample (ratio of internal standard to peak of interest) and then comparing results to an external set of standards (Sigma, St. Louis, MO) Ratios were then matched to corresponding molar concentrations by means of linear regression.

Measurement of BicycloPGE2.

Heparin plasma BicycloPGE2 analysis was conducted by Enzyme Immunoassay (Maclouf et al, 1987) using commercial available enzyme immunosorbant assay (EIA) kits available from Cayman (Ann Arbor, MI). An aliquot of 500 µls was mixed with two mls of ethanol. This mixture was incubated for five min at 4°C and then centrifuged at 3000xg for 10 min. The liquid phase was then decanted into a clean glass test tube and the ethanol was evaporated under a slow stream of nitrogen gas. One ml of EIA buffer provided in the kit was then added to each sample.

The sample was then passed through an activated six ml C-18 SPA cartridge (Supelco, Bellefonte, PA) and eluted with five mls ethyl acetate with 1% methanol. The solution is then evaporated to dryness with a slow stream of nitrogen gas. Five hundred µls of EIA buffer is added and vortexed.

To derivitize the sample, 150 µls of carbonate buffer is added and the sample is incubated at 37°C overnight. Four hundred µls of phosphate buffer and 300 µls of EIA buffer are added the following day.

To the precoated 96 well plate, 50 µls of standard or sample are added to each well. Fifty µls of antibody and 50 µls of tracer are added to each standard or sample and the plate is incubated for 18 hours at room temperature. After incubation the plates are washed and 200 µls Ellman's reagent is pipetted into each well.

The plates are placed in the dark on a rotary shaker and allowed to incubate for 60 min. The absorbance of each well is read at 405 nms. Eicosanoid concentrations for the samples are calculated by linear regression of the %bound /bound (0) and base ten log of each standard.

Measurement of urokinase plasminogen activator.

Analysis of serum and synovial urokinase plasminogen activator was done with the uPA Activity Assay Kit (Chemicon International, Inc., Temecula, CA). To a 96 well plate, ten µls of synovial fluid or serum as well as rehydrated uPA positive control for use as standards was added in duplicate. To each sample and standard, 160 µls of deionized water was added, followed by 20 µls of assay buffer provided in the kit. Twenty µls of rehydrated chromogenic substrate was then added and the plate was incubated at 37°C for exactly two hours. The plate was assayed at emission of 405 nms and sample concentration values were derived from standard values that were calculated by linear regression.

Measurement of collagenase.

Analysis of serum and synovial Type I collagenase was done with the Type I Collagenase Activity Assay Kit (Chemicon International, Inc., Temecula, CA). In a 96 well microtiter plate, ten µls of serum or synovial fluid was placed in duplicate. Thirty µls of sample diluent followed by 100 µls of biotinylated collagenase substrate was added to each well. The plate was covered and incubated for two hours at 37°C. After two hours, ten µls of enhancer was added to all standards and samples and the plate was covered for an additional 30 min at 37°C.

One hundred µls of the sample or standard/biotinylated collagenase substrate mixture was then transferred to the biotin binding plate provide in the kit. The plate was then incubated for 30 min at 37°C and washed. One hundred µls of a 1:3000 dilution of Streptavidin-Enzyme Conjugate was added to each well and the plate was incubated for 30 min at 37°C. The plate was then washed again and 100 µls of substrate

solution was added to each well and the plate was incubated at room temperature for 20 min. One hundred µls of 1N sulphuric acid was added per well to stop the reaction. The absorbance was read at 450 nms. Sample concentration values were derived from standard values that were calculated by linear regression.

Measurement of pro and active forms of MMP-2 and MMP-9.

Analysis of the pro and active form of MMP-2 and MMP-9 in all serum and synovial samples was conducted by zymography electrophoresis using gelatin imbedded gels originally designed for human MMP analysis (Huessen et al, 1980) (Roeb et al, 2001), and adopted for canine MMP analysis (Coughlan et al, 1998) using the NOVEX (Novel Experimental Technology, San Diego, CA) gelatin zymography system. One half microliter of serum or two µls synovial fluid was mixed with ten µls buffer containing 0.5 M Tris-HCl (pH 7.0), 20% glycerol, 4% SDS (w/v), and 0.005% bromophenol blue and placed into each well. Samples underwent electrophoresis on a 10% Tris-glycine acrylamide gel, with 0.1% gelatin used as a substrate under non-reducing conditions at 125V for 90 min at room temperature. The gels were then removed and washed in renaturing buffer (Triton X-100, 2.5% v/v in water) for 30 min followed by overnight incubation at 37^{0}C while in developing buffer (50mM Tris, 0.2M NaCl, 5mM CaCl2, 0.02% Brij 35 (w/v), pH 7.6). Pro and active MMP activity was visualized by staining the gels with 0.5% (w/v) Coumassie blue R250 (Sigma, St. Louis, MO) in 40% ethanol/10%glacial acetic acid solution for three hours. All gels were then destained in deionized water. One ng of pro and active MMP-2 and 9 standards were run on each gel as positive controls (Calbiochem-Oncogene Research Products, Boston, MA). All gels were analyzed wet using a densitometer (Personal

Densitometer, Molecular Dynamics, Sunnyvale, CA), with associated software. To obtain a value for each sample, the optical assessment value of each unknown band was compared to the optical assessment value of the human MMP standard band. A ratio of the unknown band to human one ng standard was then calculated and assigned to each patient sample.

Measurement of TIMP-2. Synovial and serum TIMP-2 analysis was conducted by ELISA Immunoassay (Fujimoto et al, 1995) using commercial available TIMP-2 ELISA assay kit available from Oncogene Research Products (Boston, MA). In two ml siliconized centrifuge tubes, ten µls of serum diluted with 90 mls ELISA buffer or 100 µls of synovial fluid was mixed with 100 µls of peroxidase conjugate and the mixture is well vortexed. One hundred µls of sample or standard was then placed in duplicate in the procoated 96 well plate provide with the kit. The plate was covered and allowed to incubate at 23C for exactly two hours. The plate was then washed and one hundred µls of tetra-methylbenzidine/hydrogen peroxide in 20% dimethylformamide was added to each well. The plate was again covered and allowed to incubate at 23˚C for exactly 30 min. The absorbance was read at 630 nms. Sample concentration values were derived from standard values that were calculated by linear regression.

Appendix B:

Bicyclo Prostaglandin E2 Data

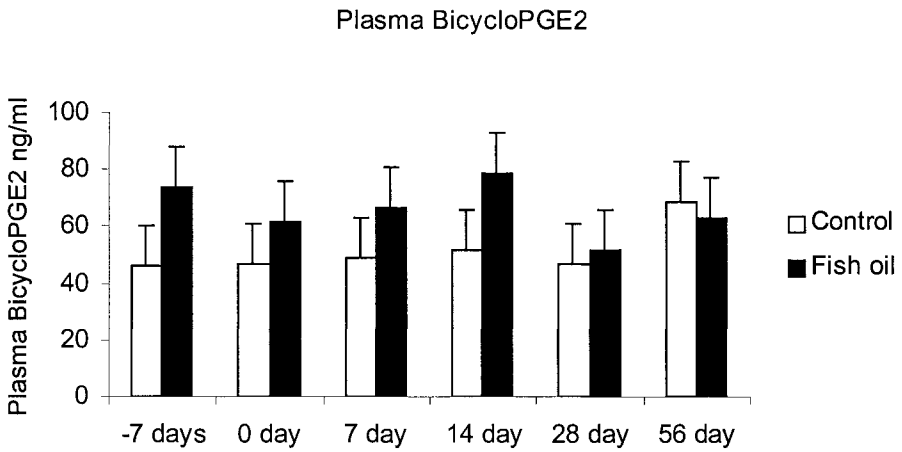

Figure 6-1. BicycloProstaglandin E2 (BicycloPGE2) concentrations in plasma in dogs fed fish oil or control (beef tallow) diet. X axis shows days prior to or post TPLO surgery. Feeding of diets started at –7 days. Values are mean ± SEM. No significant differences were found between or within groups.

BicycloPGE2 did not differ between groups at any time point. BicycloPGE2 is the circulating metabolite of PGE2. Although PGE2 is measurable in canine synovial fluid (Jovanovic et al, 2001), limited quantity of synovial fluid obtained from the synovial joint in this study dictated measuring the metabolite in the plasma.

Appendix C:

Non-surgical Synovial Collagenase

Collagenase concentrations.

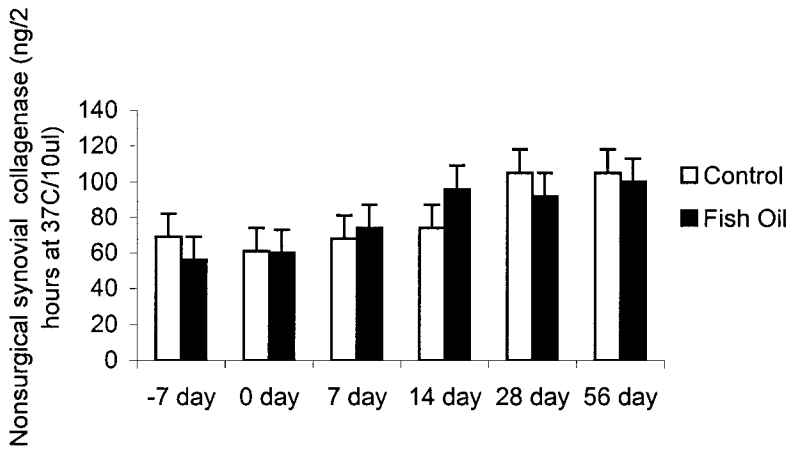

Figure 6-2. Collagenase (MMP-1, 8, and 13) activity in the non-surgical synovial fluid in dogs fed fish oil or control (beef tallow) diet. X axis shows days prior to or post TPLO surgery. Feeding of diets started at –7 days. Values are mean ± SEM. No significant differences were found between or within groups. No significant differences were found in synovial fluid obtained from the surgical knee (data not shown)

Appendix D

Subject Signalment

Table 6-1 Subject Signalment

ID	limb	breed	age (yr)	gender	weight (kg)	duration (mo)	OA grade
1	R	Aust Shep	2	FS	17.1	1	0
2	L	Rott	3.3	FS	30	2	1
3	R	Husky	4.75	MC	33	0.25	4
4	L	mix	9	FS	38	1	2
5	R	mix	4	MC	41	4	2
6	L	Lab	2.2	MC	43.6	8	3
7	R	Lab mix	7.25	MC	32.4	0.75	4
8	R	Eng Bull	4	M	23.5	0.5	1
9	R	Golden	3.75	MC	32.7	2	2
10	L	mix	4.5	FS	44	12	4
11	R	Lab	1.5	FS	31	7	3
12	L	mix	7.3	FS	22.6	4	4
13	R	mix	3.3	MC	41	7	4
14	L	Lab	1.8	FS	31.6	2	2
15	R	mix	6.8	FS	17.9	0	0
16	L	Lab mix	7.75	MC	28	5	4
17	R	Lab	4.75	MC	36.4	24	6
18	L	mix	3.8	MC	36.4	6	3
19	R	Lab	2.75	MC	43.9	1	2
20	L	Lab	5	MC	35	28	5
21	L	Lab	4.5	FS	38.4	3	2
22	L	Newf	4	FS	51.6	18	5
23	R	Pyren	2.5	FS	42.3	1	5
24	L	Pyren	2.75	FS	41	1.25	5

OA grading scale

0 none
1 very mild
2 mild
3 mild-moderate
4 moderate
5 severe
6 very severe

Appendix E

Owner Consent Form

OWNER CONSENT AGREEMENT

I agree to enter my animal into the Ralston-Purina Arthritis clinical study. I understand that my animal will be entered into a randomized clinical trial. The regimen my pet will receive is the standard accepted treatment methods and drugs as well as a specialized experimental diet. The diet is being tested as an anti-arthritis food produced by Ralston-Purina.

I have been informed and understand the potential risks and benefits of the diet and agree to cooperate to the best of my ability with specific requirements. I agree to administer the diet as prescribed and to present my animal for reexamination as required. I also understand that if I or my animal fail for any reason to fulfill the specific requirements, I will forfeit the $500 awarded at the end of the protocol.

Signed_____

Date_____

Witness_____

Date_____

Appendix F

Client Information Sheet

Client Information Sheet

Purpose of Project: When the ligaments of the knee break, this can result in a condition called osteoarthritis. In this condition, the cartilage and bone can begin to wear away resulting in pain, discomfort, and loss of function of the joint. Veterinarians can treat this condition with medication, but there is strong evidence that high amounts of certain dietary fats can also treat this condition. These fats are sometimes referred to as fish oil. They are naturally occurring and even low amounts are needed in a normal diet by both dogs and humans to survive! A major dog food company is funding this project to investigate the addition of these fats to food to help dogs with osteoarthritis.

Procedures to be used: Your dog will receive the usual treatment and medication it needs for the osteoarthritis. You will be given a 56 day supply of food with either the nutriceutical (fatty acid) or a placebo. Unless the CSU clinicians prescribe different, you should only feed your dog the food and **nothing** else-this includes table scraps, treats, caplets, and any other supplements! You will need to follow all instructions for the care of your dog and bring your dog in on the days needed for evaluation.

Do not feed your dog before any treatment or evaluation visit!

Risks: Your dog will get the usual treatment for rupture of the tendons in the knee. The only difference is the diet it will eat for 56 days. **In no way** will the diet compromise the treatment of your dog. This dietary treatment has been approved by two animal care committees to insure the health of your dog.

Some side effects of the diet do occur in some dogs. These can include but is not limited to: burping, diarrhea, loss of appetite, and bad breath. They usually only last a day or two.

All information gathered in the treatment of your dog will be kept confidential. You will have the right to terminate participation in this study at any time, but will forfeit any compensation for the treatment of your dog.

For questions about the study please contact:
Rod Hansen (Project Coordinator)
CSU-VTH 970 491-4511

For questions or concerns about the health of your dog, please contact your CSU attending clinician.
CSU VTH Front Desk 970 221-4535

Appendix G

Animal Care and Use Committee Application and Consent

Colorado State University Animal Care and Use Committee
Animal Research/Teaching Protocol Review Form - FORM A-100

Instructions: Form A-100 must be completed for all new animal research and
Colorado State University Animal Care and Use Committee
Animal Research/Teaching Protocol Review Form - FORM A-100

Instructions: Form A-100 must be completed for all new animal research and teaching protocols involving vertebrate animals. Included in this designation is unfunded research and graduate research. Animal Care and Use Committee (ACUC) approval of this protocol must be obtained before animals can be purchased, boarded or used for research or teaching at Colorado State. Since regulations require continuing review of animal research and animals used in teaching, another Form A-100 must be filed at the end of each three-year period. To obtain approval to continue the research/teaching in years two and three (or five and six, etc.), Form A-101, Animal Research/Teaching Protocol Continuation Review Form, must be submitted before the expiration of Committee approval, shown on the approval form.

The ACUC must decide whether the proposed use of animals is consistent with the PHS "Guide for the Care and Use of Laboratory Animals" and the Animal Welfare Act. Any departure must be justified in writing by the investigator. The following is intended to elicit specific information needed to review your protocol. Continuation pages may be used as necessary. However, specific answers must be provided to each question. DO NOT attach pages from a proposal and insert "see attached" in the space for the answer. If a question does not apply to your research/teaching, type "NA" in provided space. ANSWERS MUST BE TYPED.

Return the original and eleven copies of Form A-100 to: Linda L. Kovar, Coordinator, Office of Regulatory Compliance, 608 University Services Center. The Committee meets on the third Tuesday of the month. The deadline for submission is at noon on the second Tuesday of each month. Allow seven to ten business days after the meeting for a response. Inquiries concerning this form and the review process may be directed to the Coordinator at 0232.

1. Principal Investigator*: Mary Harris 2. Telephone: 491- 7462

3. All investigators (including all individuals involved in implementing research):

Rod Hansen
Ken Allen
Mary Harris
Greg Ogilvie
Martin Fettman
G. Elizabeth Pluhar
Randall Fitch

4. Department:Food Science and Human Nutrition
5. College:Applied Human Sciences

6. Project Title: THE EFFECTS OF N-3 LONG CHAIN FATTY ACIDS ON MATRIX METALLOPROTEINASE LEVELS IN DOGS WITH OSTEOARTHRITIS

Maximum - 90 characters/spaces

7. If this is a continuation filing, insert ACUC protocol number:

8. Funding Agency:Purina Pet Foods Agency deadline: June 15, 1999

9. Type of Project: TEACHING __ RESEARCH X

10. Project Start Date: January 1, 1999 Project End Date: January 1, 2002

11. Why must animals be used for this project? Can tissue cultures, computer models, clinical human studies, or other alternatives to animals be used?
The enzyme of interest must be studied in vivo in the osteoarthritic dog. This enzyme is only found in living mammals and the nutrition-production relationship has never been studied in the dog

*Principal Investigator must be a faculty member.
A-100 (November 1994). Supersedes all previous forms.

12. Animal Information:

12a. Species: Canine

12b. Strain/Breed: Any canine breed over 30 pounds

12c. Total Number/year: 24 total (clients)

12d. Justification for number of animals. Statistical justification of animal numbers is required for RESEARCH animals. For COURSES, animal numbers may be justified by citing the number of animals per student, procedure, etc. since there can be no statistical justification of the animals in this situation.
Two groups are in the design of the project. Power and sample size computations for analysis of variance were made using previously completed , unpublished data from pilot projects involving n-3 fatty acids and MMPs. Assuming equal group size (n=10) for two nonparametric groups with repeated measures, differences in magnitude of groups MMP concentration was (1-beta) =.768. By increasing group size to 12 (n=12), the power calculation (1-beta) was increased to .865. It would be unwise to use fewer subjects without adverse impact on outcome.

12e. Animal vendor: (Clinical study)
Check if applicable: Virus Antibody Free NA
Non-Virus Antibody Free NA

12f. Why was this species chosen? The animal must be canine as the primary objective is investigating an additive for dog food.

13. Identify attending veterinarian: LAR__ OTHER: Dr. Ogilvie, VTH room C118
If other, insert name of DVM and specify room number and building where medical
records will be kept.

14. Where will the animals be housed[1]? For overnight procedures, the dogs will be housed at Colorado State University Veterinary Teaching Hospital, Fort Collins, CO. Otherwise, as these dogs are client owned, they will be kept at the client's home

15. Where will the procedures involving animals be conducted (Bldg/Room)?

Colorado State University Veterinary Teaching Hospital, Fort Collins, CO

16. Attach a lay summary that describes the objective of this project.
Ingestion of dietary n-3 long chain polyunsaturated fatty acids (N-3 LC PUFAs) C20:5n-3 and C22:6n-3, or fish oil have many beneficial biological effects. These include modulation of eicosanoids and cytokines that are known to be active in chronic diseases such as osteoarthritis (OA). Eicosanoids and cytokines directly influence the production of matrix metalloproteinases (MMPs) involved in the pathologic lesions associated with OA. Therefore, overproduction of MMPs in OA play a major roll in the progression of OA that results in discomfort for millions of dogs world-wide. Suppression of MMPs has been recognized as pivotal in the treatment of OA. Dietary supplementation

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University policy does not allow overnight housing in laboratories without permission from the Animal Care and Use Committee. The Department of Laboratory Animal Resources must be notified each time any animal is housed in such facilities.

of N-3 LC PUFAs may improve the pain and suffering associated with OA by repressing the production of MMPs by the interaction with cytokines and eicosanoid production. Because the effects of dietary N-3 LC PUFAs on OA have not been investigated in the dog, work is needed to determine the effects of this supplementation on MMP production in OA. The results may benefit millions of dogs each year who suffer form OA.

The primary goal of this research project is to document the beneficial effects of high dietary n-3 LC PUFAs on canine OA. Supplementation with increased absolute amounts of N-3 LC PUFAs have been shown to decrease eicosanoids such as LTB4 and PGE2 (Wahl, 1987)(Freeman, 1998) and cytokines such as TNF alpha (Cawston, 1996). Similarly, these eicosanoids and cytokines have been shown to increase levels of MMPs resulting in progression of this debilitating disease. In fact, overproduction of MMPs is the predominant factor in the progression of OA (Tsuchiya, 1997). To our knowledge, the effect of N-3 LC PUFAs on MMP levels has not been investigated in an outbred species with naturally occurring OA. If dietary N-3 LC PUFAs benefit dogs with OA by modulating cytokines, eicosanoids metabolites and MMPs, this approach will likely benefit countless millions of dogs, cats and people with this debilitating disease.

17. Attach a description of all methods and procedures performed on animals. ***Include in this description detailed information about procedures used on animals. For blood sample collection, state method of collection, site of collection, volume collected, and frequency of collection. For injections, state substance injected, volume, route and frequency of injection. The Animal Care and Use Committee must be able to follow the course of an individual animal's stay at CSU from arrival to euthanasia, and know the details of all procedures performed on this animal during the project.***

1. Twenty-four client owned dogs with degenerative osteoarthritis resulting in clinically confirmed acute cruciate ligament (ACL) injury (rupture of the anterior cruciate ligament) will be used in the study. Dogs will be stratified by degree of OA and ligament injury. Dogs will be have routine physical examinations by the surgical clinicians and radiographs will be taken prior to, at four weeks, and at the conclusion of the study. In addition a hemogram, biochemical profile, and urinalysis will be obtained prior to surgery. For these procedures six ml blood will be drawn from the jugular vein using a six cc syringe with a 20 gauge needle. Urine collection will be by collection while animal is voiding.

On the day of surgery, each dog will be anesthetized using Atropine sulfate, acetylpromazine, and Isoflorane. Each dog will have a uniform standard operative procedure to repair the anterior cruciate ligament. The presence of complete versus partial cruciate ligament rupture and secondary meniscal injury and subsequent meniscal treatment will also be recorded.

After general anesthesia has been induced, the skin over the lumbosacral junction will be clipped and prepped with chlorhexidine solution. A 2.5-in 22-g spinal needle will be placed into the

lumbosacral epidural space and 0.5 mg/kg of preservative-free morphine will be injected into the epidural space. The dog will be placed in lateral recumbency with the affected limb down to allow diffusion of the local anesthetic to the ipsilateral spinal nerves.

After the joint had been explored and the joint capsule closed, 0.5 mg/kg of bupivicaine will be injected into the stifle joint for local analgesia using an appropriately sized syringe and a 1-in 22-g needle.

Postoperative analgesia will be provided by subcutaneous administration of morphine sulfate (0.5 1.0 mg/kg) every 8 hours.

Suture removal will be two weeks post surgery.

2. The dogs will be randomly divided into two groups of twelve dogs each:

Group 1- n-3 LC PUFA supplement (7.5% by dry wt of total diet)

Group 2- (control) corn oil supplement (7.5% by drywt of total diet)

Dogs will be fed the supplemented diets for a duration of 63 days (7 days before surgery and 56 days after surgery). The dietary supplementation protocol is patterned after Anderson (1997).

3. Synovial fluid and serum for TNF alpha, PGE2, and MMP analysis will be obtained by needle and syringe via sterile arthocentesis and venipuncture, respectively, at the following time points: day -7, day 0 (preoperative), day 7, day 14, day 28, and day 56 (figure 2). Synovial fluid will be obtained from the affected (ACL ruptured) and contralateral joints of all dogs.

Protocol for stifle arthrocentesis:

An intravenous catheter will be placed in the cephalic vein and secured with tape. The dogs will be anesthetized with intravenous propofol (6 mg/kg) followed by intravenous diazepam (0.5 mg/kg). The skin around the stifle joint will be clipped and prepped with chlorhexidine solution. The stifle will be flexed and a 1.5-in 20-gauge needle will be introduced into the joint just lateral to the patellar ligament mid-way between the femoral condyles and the tibial plateau. The needle will be directed caudomedially until synovial fluid appears in the needle hub. A sterile 6-cc syringe will be used to apply negative pressure to aspirate fluid from the joint.

Two ml synovial fluid will be obtained in a sterile, empty (no chelating agents) syringe or red top (no additives) vacutainer. Four ml blood will be obtained in a sterile, empty (no chelating agents) syringe or red top (no additives) vacutainer.

During performance of the procedure in this study, the severity of arthritis will be assessed by the surgeons at every time point using the Physical Examination Lameness/Pain Score (DeHaan, 1994) (Vasseur, 1995) and documented with videography. The patient will be examined and assessed daily by the owner during all

63 days of the study. The owner will score the appetite, activity level, and lameness of the patient (Bateman, 1994) .

18. Investigator responsibility for personnel training. The ACUC is required by Federal regulation to ensure that all personnel caring for, handling or using animals are adequately trained. This may be accomplished by formal training programs, classes or individual instruction by investigators. Investigators are responsible for ensuring that their personnel are trained, which means that there must be, at the very least, a written description of all procedures(standard operating procedure), and some vehicle for ensuring that employees are competent in implementing animal procedures.

    In addition, all scientists, research technicians, animal technicians and other personnel involved in animal care, treatment and use are required to attend pertinent ACUC training seminars and read the Colorado State University Animal Care Handbook (contact Committee Director for information). By signing this form, you are verifying that all persons involved in this project are adequately trained.

    Describe briefly the personnel training process(es). Personnel will be DVMs, or CVTs

    All personal will adhere to all regulations set forth by the ACUC.

19. Is animal use: ACUTE (less than 24 hrs) _ CHRONIC X

20. If the animal will undergo surgery; procedures that may cause MORE THAN momentary or slight pain, distress or discomfort (bleeding procedures are not considered to cause more than momentary pain, etc.); or procedures requiring an anesthetic, analgesic or tranquilizer, complete section 20. If not, go to Item 21.

20a. Describe surgery and/or procedure(s). On the day of surgery, each dog will be anesthetized using Atropine sulfate, acetylpromazine, and Isoflurane. Each dog will have a uniform standard operative procedure to repair the anterior cruciate ligament. The presence of complete versus partial cruciate ligament rupture and secondary meniscal injury and subsequent meniscal treatment will also be recorded. After general anesthesia has been induced, the skin over the lumbosacral junction will be clipped and prepped with chlorhexidine solution. A 2.5-in 22-g spinal needle will be placed into the

lumbosacral epidural space and 0.5 mg/kg of preservative-free morphine will be injected into the epidural space. The dog will be placed in lateral recumbency with the affected limb down to allow diffusion of the local anesthetic to the ipsilateral spinal nerves. After the joint had been explored and the joint capsule closed, 0.5 mg/kg of bupivicaine will be injected into the stifle joint for local analgesia using an appropriately sized syringe and a 1-in 22-g needle. Postoperative analgesia will be provided by subcutaneous administration of morphine sulfate (0.5 1.0 mg/kg) every 8 hours.

Suture removal will be two weeks post surgery

20b. What methods and specific sources (e.g., Medline, Animal Welfare Information Center, National Agricultural Library, key words, etc.) were used to determine that alternatives to potentially painful procedures (INCLUDING TERMINAL SURGERY) were not available?
Medline (fishoil, MMP, cytokine, n-3)

20c. What anesthetic, analgesic or tranquilizer will be used? Include all drugs, doses and routes. For analgesics and tranquilizers, also INCLUDE FREQUENCY AND DURATION of administration.

After general anesthesia has been induced, the skin over the lumbosacral junction will be clipped and prepped with chlorhexidine solution. A 2.5-in 22-g spinal needle will be placed into the lumbosacral epidural space and 0.5 mg/kg of preservative-free morphine will be injected into the epidural space. The dog will be placed in lateral recumbency with the affected limb down to allow diffusion of the local anesthetic to the ipsilateral spinal nerves. After the joint had been explored and the joint capsule closed, 0.5 mg/kg of bupivicaine will be injected into the stifle joint for local analgesia using an appropriately sized syringe and a 1-in 22-g needle. Postoperative analgesia will be provided by subcutaneous administration of morphine sulfate (0.5 1.0 mg/kg) every 8 hours.

20d. If the animal will recover from surgery/anesthesia, describe post-operative care. Each animal will be observed for apnea and abnormal heart rate. Temperature will be monitored to 99C. Excubation time and Quality of Recovery will be recorded.

20e. What analgesics will be used post-operatively? USDA regulations state that administration of analgesics following a surgical procedure is not optional unless justified as part of the research (e.g., pain research). To comply with this regulation, investigators performing a surgical procedure must SUBMIT A SCHEDULE OF DRUG, DOSE, ROUTE OF ADMINISTRATION, AND FREQUENCY OF ADMINISTRATION THAT WILL BE USED WHETHER OR NOT SIGNIFICANT PAIN IS ANTICIPATED. USDA does not require a specific duration of administration; however, the ACUC currently suggests a minimum of three days administration of analgesics following surgical procedures. Extensive justification is required if pain will not be alleviated.

After general anesthesia has been induced, the skin over the lumbosacral junction will be clipped and prepped with chlorhexidine solution. A 2.5-in 22-g spinal needle will be placed into the lumbosacral epidural space and 0.5 mg/kg of preservative-free morphine will be injected into the epidural space. The dog will be placed in lateral recumbency with the affected limb down to allow diffusion of the local anesthetic to the ipsilateral spinal nerves. After the joint had been explored and the joint capsule closed, 0.5 mg/kg of bupivicaine will be injected into the stifle joint for local analgesia using an appropriately sized syringe and a 1-in 22-g needle. Postoperative analgesia will be provided by subcutaneous administration of morphine sulfate (0.5 1.0 mg/kg) every 8 hours.

If analgesics will not be given, explain why. NA

20f. Describe any expected post-operative complications.

Mild swelling, some inflammation

20g. If the animal will undergo additional surgery after the first surgery, describe procedure, state when and justify.
NA

21. Describe the clinical signs which will determine when euthanasia will be performed, including any anticipated illness. In accordance with the goal of minimizing pain and suffering, the end point should occur when expected objectives have been achieved.
NA

22. If a muscle relaxant or paralytic drug will be used, describe and justify.
NA

23. If the animal will be physically restrained (other than brief restraint involved in holding or transporting the animal), describe and justify.
NA

24. If the animal will be exposed to unusual housing conditions, food or water deprivation for more than 24 hours (48 hours for ruminants), or extreme environmental conditions, describe and justify.
NA

25. What method of euthanasia will be used? ***Include DOSE AND ROUTE of administration for drugs. Euthanasia method should be consistent with the AVMA Panel Report on Euthanasia (JAVMA, V.202, No. 2, pg. 229-249, January 15, 1993 for discussion of methods). According to the AVMA's Report, decapitation should not be conducted without sedation or anesthesia. If decapitation is used without sedation, provide scientific justification as to why sedation or anesthetic cannot be used.***
NA

26. If any animal deaths are expected (besides euthanasia) during the project, describe. No complications are expected with this this procedure.

27. Will the animals or their wastes be biohazardous?
NO x YES
If no, go to signature page. If yes, will they be:

27a. Infectious? NO _ YES _ Agent:

If yes, has this use been approved by the Institutional Biosafety Committee?

NO _ YES _ Date:

27b. Carcinogenic?
NO _ YES _ Agent:

27c. Radioactive?
NO _ YES _ Isotope:

If yes, has this use of radioactivity been reviewed and approved by the Radiation Safety Committee?
NO _ YES

27d. Other (describe)

Describe all biohazards (e.g., identify the specific chemicals, microorganisms, isotopes, etc.). Use additional sheets if necessary.

NOTE: Copies of the PHS Guide for the Care and Use of Laboratory Animals (which includes the U. S. Government Principles) and the Animal Welfare Act are available in the office of the Director, Animal Care and Use Committee.

INVESTIGATOR'S ASSURANCES:

a. I have read the U.S. Government Principles and relevant portions of the PHS Guide and USDA regulations/Animal Welfare Act, and I agree to abide by these policies, guidelines and laws in the conduct of all activities involving animals.

b. I verify that I and my employees have read the Colorado State University Animal Care Handbook and I agree to abide by these policies and guidelines.

c. I will promptly report proposed changes in species, surgical procedures or biopsies, restraint, ascites production, food deprivation, euthanasia, etc. to the ACUC.

d. I agree to furnish the ACUC with any relevant information on animal use it requests.

e. I am aware that deviations from an approved protocol or violations of pertinent policies, guidelines or laws could result in immediate suspension of this project.

f. I assume responsibility for the ethical conduct of this project and for protecting the welfare of the subjects.

g. I assure that I have conducted appropriate literature review and this research will not repeat extant research unless indicated by attached appendix.

_____

Principal Investigator Date

I understand my signature on this form acknowledges that I have reviewed this protocol.

_____

Department Head Date

Dr. Harris,
Thanks for your prompt response to ACUC questions regarding
"The Effects of
N-3 Long Chain Fatty Acids on Matrix Metalloproteinase Levels in Dogs with
Osteoarthritis." Based on your reply, the protocol is approved on 02/22/2000
for 24 client-owned dogs per year. Protocol number is 00-004A-01. Hard copy
of approval will be sent in a few days.

Polly Grant
Regulatory Compliance
Colorado State University
410 University Services Center
Ft, Collins, CO 80523
970-491-0236 (voice)
970-491-2293 (fax)
pgrant@research.colostate.edu

Appendix H

Selected Power Calculations

Reported as the date with the largest diet effect with
the corresponding power computed for that diet difference

Response	Power
Total plasma AA (C20:4)	0.73
Total plasma EPA (C20:5)	0.99
Total plasma DHA (C22:6)	0.99
Collagenase serum	0.19
Collagenase surgical synovial fluid	0.24
Collagenase non-surgical synovial fluid	0.17
TIMP-2 surgical synovial fluid	0.23
TIMP-2 non-surgical synovial fluid	0.58
TIMP-2 serum	0.09
uPA Serum	0.48
uPA surgical synovial fluid	0.29
uPA non-surgical synovial fluid	0.56
actMMP-2 surgical synovial fluid	0.29
actMMP-2 non-surgical synovial fluid	0.25
actMMP-2 serum	0.58
actMMP-9 surgical synovial fluid	0.06
actMMP-9 non-surgical synovial fluid	0.27
actMMP-9 serum	0.45
bicycloPGE2 Plasma	0.54
proMMP-2 surgical synovial fluid	0.23
proMMP-2 non-surgical synovial fluid	0.51
proMMP-2 serum	0.26
proMMP-9 surgical synovial fluid	0.12
proMMP-9 non-surgical synovial fluid	0.61
proMMP-9 serum	0.39