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Fish oil decreases matrix metalloproteinases in knee synovia of dogs with inflammatory joint disease

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Abstract

This study was designed to determine whether dietary fish oil affects the expression and activity of matrix metalloproteinases (MMP), tissue inhibitors of MMP-2 (TIMP-2) and urokinase plasminogen activator (uPA) in synovial fluid from dogs with spontaneously occurring stifle (knee) instability in a single hind limb resulting from acute cranial cruciate ligament (CCL) injury. Two groups of 12 dogs were fed diets from 1 week prior to surgery on the affected knee to 56 days post-surgery. The fish oil and control diets provided 90 and 4.5 mg, respectively, of combined eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)/kg body weight per day. Plasma and synovial fluid, from both surgical and nonsurgical knee joints, were obtained at start of the diet (−7), surgery day (0) and 7, 14, 28 and 56 days post-surgery. Plasma total EPA and DHA were significantly increased, and plasma total arachidonic acid (AA) was significantly decreased by the fish oil diet. In synovial fluid from the nonsurgical knee, fish oil treatment significantly decreased proMMP-2 expression at Days 7 and 14, and proMMP-9 expression at Day 56, and uPA activity at 28 days and significantly increased TIMP-2 expression at Days 7 and 28. There were no differences in MMP expression or activity, TIMP-2 expression and uPA activity in the surgical joint synovial fluid at any time throughout the study. These results suggest that dietary fish oil may exert beneficial effects on synovial fluid MMP and TIMP-2 equilibrium in the uninjured stifle of dogs with unilateral CCL injury.

Keywords: Inflammatory joint disease; Dogs; Fish oil; Eicosapentaenoic acid (EPA); Docosahexaenoic acid (DHA); Matrix metalloproteinases

1. Introduction

Osteoarthritis (OA) is a degenerative inflammatory joint disease characterized by breakdown of articular cartilage. The breakdown of articular cartilage involves endogenous enzymes that degrade the constituents of the connective tissue matrix. Matrix metalloproteinases (MMP) are a family of enzymes that mediate the breakdown of the extracellular matrix of connective tissue [1]. Overproduction of MMP or underproduction of tissue inhibitors of metalloproteinases (TIMP) plays a major role in the pathogenesis and progression of joint instability and OA [1].

The regulation of MMPs occurs by three mechanisms: enzyme expression, posttranslational activation and inhibition of enzyme activity by binding to tissue inhibitors [2]. MMP expression is up-regulated by certain mediators including inflammatory eicosanoids [3] and cytokines [4]. MMPs are expressed in inactive proforms, and proteolytic activation of pro MMPs occurs in the extracellular matrix [2]. Urokinase plasminogen activator (uPA) is a key agent in the proteolytic activation of MMP in the articular cartilage of arthritic joints [5]. The process involves proteolytic cleavage of plasminogen to plasmin that subsequently cleaves pro-MMP to active MMP [6].

Tissue inhibitors of metalloproteinases form noncovalent complexes with activated MMP in a 1:1 molar ratio, which inhibits the catalytic activity of the enzyme [1]. TIMPs are

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Table 1
Composition of the diet

Ingredient	g/kg diet
Brewers rice	450.0
Soybean meal	120.0
Canola meal	50.0
Chicken, whole carcass	160.0
Catfish meal	200.0
Yeast	20.0
Fat component[a]	70.0
Vitamin mix	0.75
Vitamin C	0.14
Vitamin E	0.2
β-Carotene	0.05
Mineral mix	3.0
Potassium chloride	5.0
Sodium chloride	2.5
Choline chloride	1.24
Taurine	0.52

[a] Control diet contained 35.0 g beef tallow plus 35.0 g safflower oil/kg diet. Fish oil diet contained 35.0 g beef tallow plus 35.0 g menhaden oil/kg diet.

present in both tissues and fluid compartments and are produced by a variety of cell types including chondrocytes [7]. The suppression of MMP has been recognized as a key target in the treatment of OA [8]. There has been much interest in the development of pharmacologic MMP inhibitors [1]. However, the pharmacological inhibition of MMP with the broad-spectrum MMP inhibitor Marmistat caused tendonitis in its Phase III clinical studies [9].

Recent studies in dogs with CCL rupture have shown an increase in synovial fluid MMP-2 and -9 and cathepsin S expression [10], and increased MMP-3 activity and production of sulfated glycosaminoglycans [11]. Immature cross-links between sulfated glycosaminoglycans have been demonstrated in the ruptured ligaments of dogs with CCL rupture, and ligament rupture was associated with increased pro-MMP-2 expression [12]. Increased degradation of collagen in ruptured ligaments has also been demonstrated in dogs with CCL rupture [10]. In dogs with osteoarthritic joints, synovial fluid MMP-2 and MMP-9 activities were dramatically increased compared to dogs with healthy joints [13]. In CCL rupture in dogs and anterior cruciate ligament rupture in humans, inflammatory mediators up-regulate the expression of ligament proteases such as cathepsin [14].

N-3 fatty acids have been shown to have an effect on MMP expression in vitro [15]. Linolenic acid and eicosapentaenoic acid (EPA) long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) suppress expression of MMP-3 (stromelysin) and MMP-13 in human OA cartilage explant cultures [15]. LC n-3 PUFA were postulated to suppress the production of cytokines, inflammatory pathways mediated by cyclooxygenase-2, and MMP expression [15]. EPA inhibits ultraviolet-induced MMP-1 expression in human dermal fibroblasts [16].

The effect of LC n-3 PUFA supplementation on MMP regulation has not been investigated in an in vivo model. Furthermore, no studies have addressed the effect of LC n-3 PUFA on the activators and inhibitors of MMP in vivo. This study was designed to investigate the effects of a diet supplemented with LC n-3 PUFA on the production and activity of canine MMP and TIMP-2 production in a model of joint inflammation secondary to instability in dogs.

Cranial cruciate ligament (CCL) injury is a common cause of hindlimb lameness in dogs typically leading to stifle OA [17] The joint instability is often treated surgically by a tibial plateau leveling osteotomy (TPLO) procedure that limits cranial tibial thrust and slows OA progression [17,18]. Dogs with unilateral CCL injury treated by a TPLO provide an excellent in vivo model to test the efficacy of a fish oil diet in altering MMP expression and activity, TIMP expression and uPA activity in both the injured (surgery) knee and the nonsurgical knee, which may experience increased stresses as a result of decreased weight bearing on the injured limb.

2. Methods and materials

2.1. Animals and diets

Dogs are susceptible to CCL injury, which results in stifle (knee) instability [19,20]. Larger dogs typically undergo a surgical procedure to stabilize the joint, such as TPLO. Although CCL rupture may occur bilaterally, in most cases surgery is performed on a single limb at a time as was the case in this study. This afforded an ideal model to examine the in vivo effect of n-3 fatty acid supplementation on MMP regulation in a severely inflamed, acute (surgical) and a minimally to moderately stressed, chronically affected (nonsurgical) stifle in an individual dog.

Dogs presenting with hindlimb lameness at the Colorado State University Veterinary Teaching Hospital were evaluated for stifle (knee) instability. Twenty-four dogs presenting with stifle instability in a single hindlimb resulting from CCL injury, with or without secondary meniscal injury, were enrolled in this study. The Colorado State University Animal Care and Use Committee approved all procedures. Informed consent was obtained in writing from owners before their dogs were enrolled in the study. All dogs were patients at the Colorado State University Veterinary Teaching Hospital and treated within a 380-day period.

Dogs varied by breed and had a body weight of 34.7±1.8 kg (mean±S.E.M.). Dogs were randomly assigned to one of two diet treatment groups of 12 dogs: an experimental fish oil-based maintenance diet with high levels of EPA and DHA, and a control safflower oil-based

Table 2
Diet analysis, g/kg as fed

Protein	278.5
Carbohydrate	429.0
Fat	126.5
Ash	82.6
Moisture	83.4
kcal/kg	3968.5

Table 3
Fatty acid composition of diet (% total fatty acids)

Fatty acid	Control diet	Fish oil diet
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 ratio	30.80	1.685

maintenance diet with low EPA and DHA content. The investigators working with the dogs or the specimens collected and the owners were blinded to the diet treatment.

The diets were formulated by Nestle Purina Petcare (St. Louis, MO, USA). Components, proximate composition and fatty acid analysis are shown in Tables 1–3. Both diets were made of products typically used in the manufacture of dog foods to provide a complete and balanced canine diet for adult life stages. Both control and fish oil diets contained 4 kcal/g feed. Dogs were fed at 40 kcal/kg body weight per day. The fish oil diet provided 90 mg combined EPA and DHA/kg body weight per day and the control diet provided 4.5 mg combined EPA and DHA/kg body weight per day, providing 2.0 energy percent and 0.1 energy percent, respectively, from combined EPA and DHA. Diet treatments were started 7 days prior to surgery, maintained during hospitalization for surgery and continued through 56 days post-surgery. Clients were instructed to feed nothing, including snacks and treats, other than the diets provided. Compliance was assessed by owner interview and confirmed by gas chromatography of plasma fatty acids.

2.2. Surgical procedure, stifle arthrocentesis and venipuncture

Dogs were routinely examined under the direction of a board-certified small animal orthopedic surgeon (GEP), and radiographs were taken prior to and at the conclusion of the study. The degree of arthritis in the stifle was determined radiographically and graded on a scale of 0 to 6 (Table 4). The presence of complete cruciate ligament rupture, with or without secondary meniscal injury, and surgical treatment was required for entry into the study. Each dog received a uniform standard operative procedure, joint exploration followed by a TPLO, to the injured stifle [18].

Plasma from whole blood and synovial fluid from both stifles were obtained at the following time points: Day −7 (initial day of diet), Day 0 (immediately prior to surgery) and Days 7, 14, 28 and 56. The dogs were anesthetized with intravenous propofol (Abbot Laboratories, Chicago, IL, USA) at 3.5 mg/kg. Synovial fluid was aspirated using a 20-gauge, 1.5-in. needle and a 6-ml syringe. Synovial fluid, at least 200 μl, was obtained and transferred to a 2-ml cryovial (Nalgene, Rochester, NY, USA) and stored at −80°C until analysis Three milliliters of venous blood was obtained by syringe fitted with a 20-gauge needle and transferred into an EDTA vacutainer tube (Sherwood Medical, St. Louis, MO, USA). Blood was centrifuged for 10 min at 2000×*g* and the plasma was transferred into 2-ml cryovials. Butylated hydroxytoluene was added (0.1 mg/ml plasma) as an antioxidant, and samples stored at −80°C until analysis.

2.3. Fatty acid analysis

Duplicate samples of plasma fatty acids were measured by gas chromatography [21]. Heptadecaenoic acid was used as an internal standard. Two hundred microliters of plasma was extracted with 2 ml chloroform/methanol (2:1 v/v) and vortexed for 1 min. One milliliter of saline (0.9% w/v NaCl)

Table 4
Dog characteristics

Age (years)	Gender	Weight (kg)	OA grade[a] of surgical knee
2	FS	17.1	0
3.3	FS	30	1
4.75	MC	33	4
9	FS	38	2
4	MC	41	2
2.2	MC	43.6	3
7.25	MC	32.4	4
4	M	23.5	1
3.75	MC	32.7	2
4.5	FS	44	4
1.5	FS	31	3
7.3	FS	22.6	4
3.3	MC	41	4
1.8	FS	31.6	2
6.8	FS	17.9	0
7.75	MC	28	4
4.75	MC	36.4	6
3.8	MC	36.4	3
2.75	MC	43.9	2
5	MC	35	5
4.5	FS	38.4	2
4	FS	51.6	5
2.5	FS	42.3	5
2.75	FS	41	5

FS, female spayed; MC, male castrated; F, female intact; M, male intact.
[a] 0, None; 1, very mild; 2, mild; 3, mild–moderate; 4, moderate; 5, severe; 6, very severe.

was then added, mixed and centrifuged at 2000×*g* for 10 min. The chloroform layer was then aspirated, evaporated to dryness under nitrogen at 30°C and dissolved in 1 ml of hexane. Fatty acid methylesters were synthesized by direct transesterfication with boron trichloride/methanol at 60°C for 2 h. One milliliter of water was then added and the hexane layer was aspirated, the samples transferred to chromatography vials (Agilent, San Fernando, CA, USA) and analyzed by gas chromatography (5890 Gas Chromatograph, Agilent) using a 0.32-mm-I.D. capillary column with 0.5-μm film (Innowax 19091N-213 capillary column, Agilent, San Fernando CA) with 1.5 ml/min constant flow. Arachidonic acid (AA), EPA and docosahexaenoic acid (DHA) were identified against retention times of known standards (fatty acid methyl ester standards, Sigma Aldrich, St. Louis, MO, USA) and quantified using the internal standard area percent ratio method.

2.4. Measurement of collagenase activity

Analysis of stifle joint synovial Type I collagenase activity, representing the sum of MMP-1, -8 and -13 activities, was performed using a Type I Collagenase Activity Assay Kit (Chemicon International, Temecula, CA, USA) in a 96-well microtiter plate. Duplicate 10-μl aliquots of synovial fluid samples from each joint and standards generated with MMP-1 positive controls were incubated with 100 μl of biotinylated collagenase substrate in 96-well microtiter plates. Following incubation, 100 μl of samples and standards was transferred to a separate biotin-binding plate and incubated with streptavidin conjugated with horseradish peroxidase. Optical density following substrate addition was read at 450 nm by a microplate reader system (Labsystems Multiscan MCC/340, Helsinki, Finland).

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

Expression of pro and active forms of MMP-2 and MMP-9 in synovial fluid samples was determined by zymography electrophoresis using gelatin-imbedded polyacrylamide gels originally designed for human MMP analysis [22,23] and adapted for rodent MMP [24] and canine MMP analysis [25] using the NOVEX (Novel Experimental Technology, San Diego, CA, USA) gelatin zymography system. Duplicate samples of 10 μl of stifle synovial fluid mixed with 10 μl buffer containing 0.5 M Tris–HCl (pH 7.0), 20% glycerol, 4% sodium dodecyl sulfate (SDS, w/v) and 0.005% bromophenol blue were loaded into each well. Samples underwent electrophoresis on a 10% Tris-glycine acrylamide gel, with 0.1% gelatin as a substrate, under nonreducing conditions at 125 V for 90 min at room temperature, using 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 running buffer. 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°C in developing buffer [50 mM Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij 35 (w/v), pH 7.6]. Pro and active MMP bands were visualized by staining the gels with 0.5% (w/v) Coumassie blue R250 (Sigma Aldrich) in 40% ethanol/10% glacial acetic acid solution for 3 h. All gels were then destained in deionized water. One nanogram of pro and active human MMP-2 and MMP-9 standards was run on each gel as positive controls (Calbiochem-Oncogene Research Products, Boston, MA, USA). MMPs were quantified by densitometry (Molecular Dynamics, Sunnyvale, CA, USA). For each sample, the density value of each MMP band was compared to the density value of the human MMP standard band. A ratio of the unknown band to human 1-ng standard was then calculated and assigned to each patient sample. MMP values are reported as nanogram of MMP per 10 μl of synovial fluid.

2.6. Measurement of TIMP-2 expression

Joint synovial TIMP-2 expression was measured by ELISA Immunoassay [26] using a TIMP-2 ELISA assay kit (Calbiochem Oncogene Research Products). Duplicate 100-μl samples of synovial fluid and standards were added to precoated 96-well plates and incubated at 23°C for 2 h. Washed plates were then incubated with 100 μl of tetramethylbenzidine/hydrogen peroxide in 20% dimethylformamide at 23°C for 30 min. The absorbance was read at 630 nm on a microplate reader system.

2.7. Urokinase plasminogen activator

Synovial fluid uPA was measured using a uPA activity assay kit (Chemicon International). Duplicate 10-μl samples of synovial fluid and rehydrated uPA-positive control standards were placed in 96-well plates. Following addition of assay buffer and substrate and incubation at 37°C for 2 h, absorbance at 405 nm was read using a plate reader system. One unit of uPA activity is defined as the amount of enzyme equal to an international standard assayed by the fibrinolytic method [27].

2.8. Statistical analysis of data

All data were log transformed to normalize variance. Data were compared by an analysis of variance with repeated measures, where the between-subjects effect was diet and the repeated measures effect was time. Data were analyzed by the Statistical Analysis Systems (SAS Institute Inc., Cary, NC, USA). Differences between treatment groups at each time point were evaluated with *t* tests as tests of effect slices for the response variables plasma AA, plasma EPA, plasma DHA, nonsurgical joint and surgical joint synovial fluid pro and active MMP-2 and MMP-9 expression, collagenase activity (active MMP-1, -8 and -13 activity) and TIMP-2 expression and uPA activity. The significance level was set at $P \leq .05$.

3. Results

Twenty-four dogs completed the study. All dogs had clinical evidence (radiograph or palpation) of joint instabil-

ity in one stifle that was verified at surgery. Twenty-two dogs had radiographic evidence of OA in the injured stifle (Table 4). Although joint instability may occur bilaterally, evidence of OA in the uninjured nonsurgical knee was not assessed radiographically at entry into the study. Increased stress in the nonsurgical knee from shifting weight off of the inflamed surgical knee provided an ideal in vivo model to examine the effects of dietary LC n-3 PUFA on markers of OA in both the inflamed surgical knee and the nonsurgical albeit stressed knee.

Prior to feeding the experimental diets, there were no significant differences between groups in total plasma AA, EPA or DHA concentrations. Dietary supplementation with fish oil resulted in significant sevenfold increases in EPA and threefold increases in DHA in plasma within 1 week. These increases persisted throughout the remainder of the study (Fig. 1). Total plasma AA was significantly lowered by 30% in the dogs treated with fish oil supplementation by Day 28 (35 days after start of feeding) and remained significantly lower throughout the remainder of the study (Fig. 1).

No significant time or treatment group differences were found in the amounts of activated forms of MMP-2 or MMP-9, or between collagenase activity (active MMP-1, -8 and -13 activity) in either the surgical or nonsurgical stifle synovial fluid (data not shown).

Significant, 20% decreases in pro-MMP-2 were evident at Days 7 and 14 in the nonsurgical joint synovial fluid of the fish oil group as compared to the control group (Fig. 2). The decrease in pro-MMP-2 expression was only observed in the nonsurgical joint synovial fluid and not in the surgical joint synovial fluid. The fish oil treatment affected pro-MMP-9 expression on Day 56 alone. In comparison to the control group, fish oil treatment resulted in a significant 37% decrease in pro-MMP-9 expression in

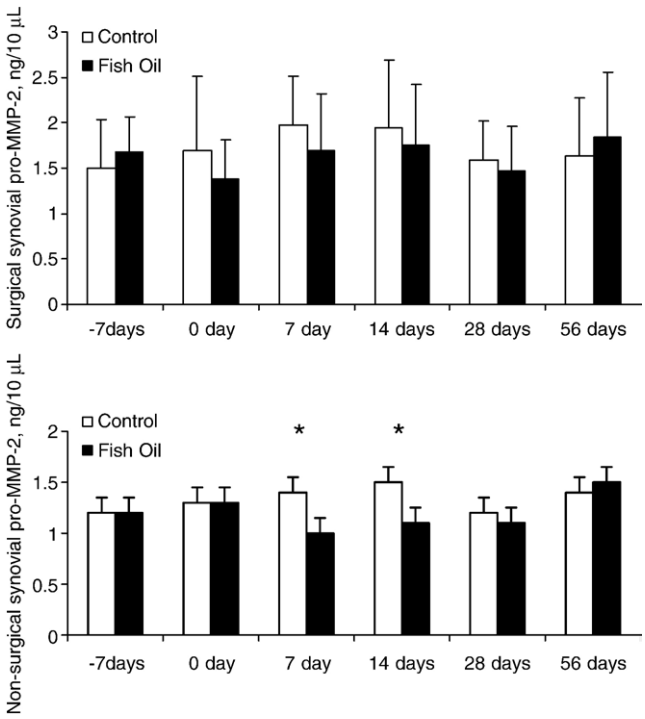

Fig. 2. Pro-MMP-2 expression in the surgical and nonsurgical knee synovial fluid of dogs fed fish oil or control diet. Values are mean±S.E.M., *$P<.05$.

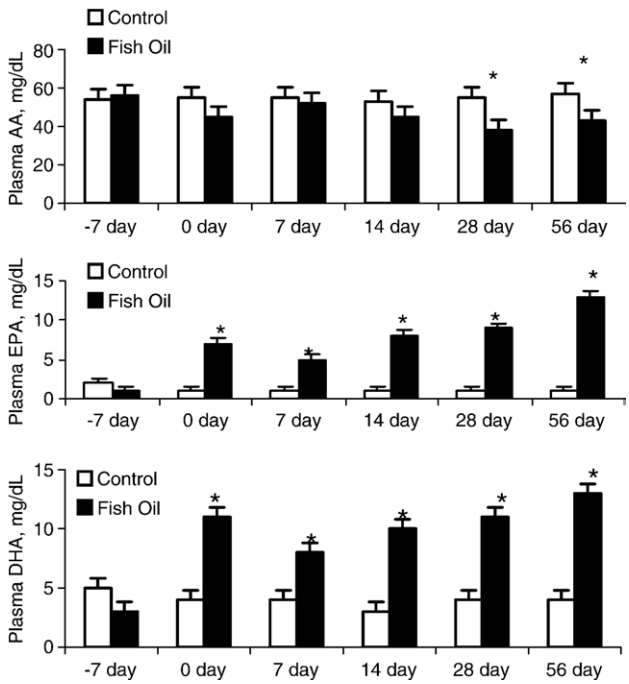

Fig. 1. Total plasma arachidonic, eicosapentaenoic and docosahexaenoic acid concentrations in dogs fed fish oil or control diet. Values are mean±S.E.M., *$P\leq.05$.

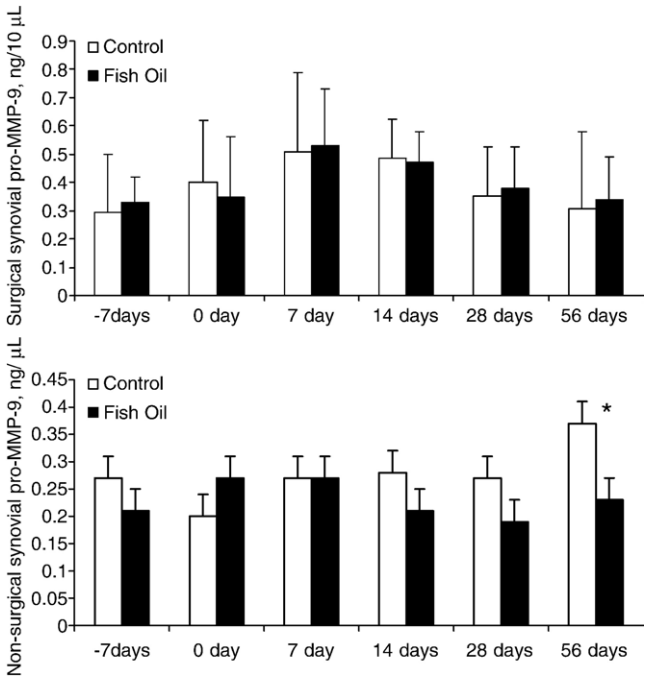

Fig. 3. Pro-MMP-9 expression in the surgical and nonsurgical knee synovial fluid of dogs fed fish oil or control diet. Values are mean±S.E.M., *$P<.05$.

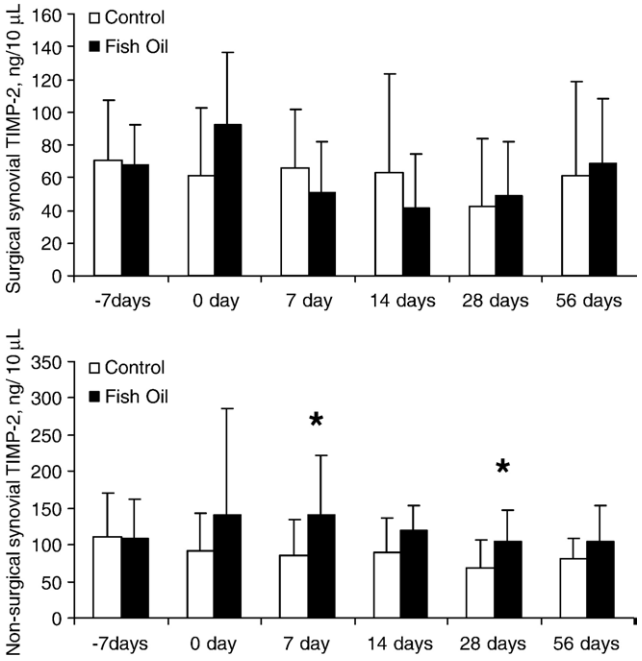

Fig. 4. TIMP-2 expression in the surgical and nonsurgical knee synovial fluid of dogs fed fish oil or control diet. Values are mean±S.E.M., *P<.05.

the nonsurgical joint synovial fluid at Day 56 (Fig. 3). By contrast, fish oil treatment was not associated with any significant decreases in pro-MMP-9 expression in the surgical joint synovial fluid.

Synovial fluid TIMP-2 concentrations in the nonsurgical knee of the fish oil diet group (Fig. 4) were significantly higher at Day 7, 56%, and at Day 28, 48%. No significant differences between groups were found in TIMP-2 in surgical joint synovial fluid.

In the nonsurgical knee synovial fluid, there was a significant 63% reduction in uPA activity at 28 days in the fish oil-treated dogs (Fig. 5). There was no effect of fish oil treatment on surgical knee synovial fluid uPA activity.

4. Discussion

N-3 and n-6 polyunsaturated fatty acids affect MMP production associated with joint diseases such as OA [15], but there are few reports on the effects of fish oil on MMP activators and inhibitors. The purpose of this study was to test the effect of dietary fish oil supplementation on expression and activity of MMP, TIMP-2 production and uPA activity. This investigation is unique in that it allowed the examination of the effect of fish oil on these markers of articular cartilage degradation in an in vivo spontaneously occurring joint disease model in moderately stressed (the nonsurgical joint) and more severely inflamed (the surgically treated joint) stifles in individual dogs living with their owners. Stifle damage and CCL injury in dogs have been shown to increase cartilage degradation and MMP expression and activity [10–14], but there are no reports on the effect of LC n-3 PUFA on the mediators of cartilage matrix homeostasis such as MMP, TIMP-2 and uPA in vivo.

This study showed significant increases in total plasma EPA and DHA after 1 week of fish oil supplementation which 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 [28] and diseased dogs [29]. However, in this study, increased total plasma EPA and DHA were achieved with approximately one-third less dietary fish oil than used in the previous canine studies [28,29].

Total plasma AA was significantly lower in the fish oil diet group when compared to the control group 35 days and 63 days after the start of the experimental diet (28 and 56 days post-surgery). This is likely due to competitive inhibition of incorporation of AA into plasma components such as phospholipids. These results were not as marked as those reported previously by Hansen et al. [28] and Ogilvie et al. [29]. Total plasma AA levels were depressed sooner and for a longer duration in both of these studies. A plausible explanation is that the lower dose of fish oil supplementation used in this study may not have been high enough to achieve the same degree of competitive incorporation.

In this study, the effect of fish oil diet on stifle joint synovial fluid MMP expression, TIMP-2 production and uPA activity was evident only at certain times in the stressed nonsurgical knee, but not in the severely inflamed, surgically treated joint. Our interpretation is that the severe inflammation from CCL injury and subsequent surgery is too great to be affected by the 2.0 energy percent LC n-3 PUFA diet. However, for the nonsurgery stifle, which is stressed or mildly inflamed from the dog shifting weight off of the injured leg or from slight instability due to a partial CCL rupture, some of the markers of OA development

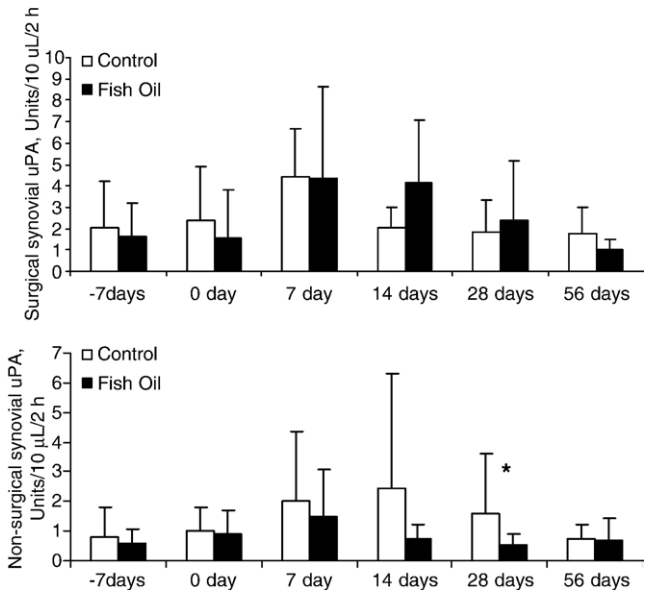

Fig. 5. uPA activity in the surgical and nonsurgical knee synovial fluid of dogs fed fish oil or control diet. Values are mean±S.E.M., *P<.05.

(pro-MMP-2 and -9, TIMP-2, and uPA activity) were suppressed by the fish oil diet. However, this effect was significant at only some time points of the study in the nonsurgical stifle but never evident in the surgical stifle (Figs. 2–5).

The data suggest that the effect of fish oil on synovial fluid MMP may be in part associated with the changes found in plasma AA concentrations, perhaps due to competitive incorporation of EPA and DHA which decreases AA. MMP production may be subsequently decreased through mechanisms involving decreased AA-mediated prostaglandin (PG) E_2 production, as suggested by Perez et al. [24].

In both surgical and nonsurgical synovial fluid, the amounts of pro-MMP-2 and proMMP-9 were 2-5-fold higher than those of the activated forms (data not shown). Expression of MMP involves the appearance of the pro-MMP forms with subsequent activation of a portion of this pool to the active MMP forms by post-translational proteolytic cleavage. This activation may be mediated by a variety of proteases such as uPA and other MMPs [1,2,30], or by eicosanoid-mediated increased uPA expression [31]. We found no between-group significant differences in activated MMP-2 and MMP-9 in either surgical or nonsurgical synovial fluid throughout the study. Since activated MMP-2 and MMP-9 constitute a minor fraction of total MMP, with the pro forms predominating, then the data suggest that n-3 PUFA treatments may affect pro-MMP-2 and pro-MMP-9 expression in the nonsurgical knee. However, this effect was only evident at Days 7 and 14 for pro-MMP-2 expression and Day 56 for pro-MMP-9 expression.

TIMP-2 was measured in dog stifle synovial fluid using an enzyme-linked immunosorbant assay which had been previously validated to detect TIMP-2 in human, mouse, rat, guinea pig, rabbit and bovine tissue. The assay detects both free TIMP-2 and TIMP-2 complexed with active forms of MMP-1, -2, -3, -7, -8 and -9 [26]. TIMP-2 concentrations were significantly increased at Days 7 and 28 in the nonsurgical joint synovial fluid, but not at any other time point (Fig. 4).

Although this study did not address the mechanism(s) by which dietary fish oil may affect MMP, TIMP and uPA changes, other studies have suggested mechanisms by which these changes may occur. DHA and EPA suppress two-series PG and four-series leukotriene derived from AA by competitive incorporation, and PGE_2 and LTB_4 have been shown to increase TNF alpha expression [32]. Dietary supplementation with n-3 PUFA decreases expression of proinflammatory cytokines and eicosanoids and results in lower concentrations of eicosanoids such as LTB_4 and PGE_2 [24,33,34] and proinflammatory cytokines such as TNF alpha [1] and IL-1 [34]. These eicosanoids and cytokines have been shown to increase the activity of MMP [35–38]. McCabe et al. [39] concluded that DHA significantly increased TIMP-1 production in vitro in renal carcinoma cells through reduction of two-series PG production. These mechanisms may represent a synergistic action by fish oil of decreasing MMP and increasing TIMP-2 production working through both TNF alpha and PGE_2 reduction [15].

The effect of fish oil on expression of cytokines and inflammatory mediators as well as MMP also resulted in a direct effect on expression of cartilage aggrecanases, cytokines, IL-1 alpha and TNF alpha, and COX- 2 in vitro [40,41]. Furthermore, n-3 fatty acids have been shown to suppress expression of COX-2, 5-lipoxygenases, TNF alpha, IL-1 alpha, IL-1 beta, MMP-3 and MMP-13 in human OA cartilage explant cultures [15,40–42].

An alternative explanation of the effect of dietary fish oil on TIMP concentrations may be its effect on the permeability of compartment membranes that in turn would affect concentrations of MMP and TIMP-2. If a change in permeability was the primary cause of changes in MMP concentrations in synovial fluid, then differences should be noted in both the activated and pro-MMP concentrations within the synovial fluid of the joint. Since activated MMP concentrations were not significantly affected in this study in contrast to the pro forms, this suggests that the effect of dietary fish oil is mediated by suppression of expression and not alterations in membrane permeability.

The results of this study suggest that 2.0 energy percent dietary supplementation of fish oil affects the production or activation of pro-MMP-2 and pro-MMP-9, and the expression of TIMP-2 in joint synovial tissues with mild to moderate inflammation. However, this effect was not robust and consistent since it was apparent only at certain time points. Despite this, fish oil supplementation has no effect on MMP production or activity or TIMP-2 production in the severely inflamed joint as occurs with CCL injury and subsequent surgery. We cannot explain why the fish oil diet affected MMP, TIMP-2 and uPA at only certain time points. Perhaps a dose larger than the 2.0 energy percent combined EPA and DHA would produce more robust and consistent results. Furthermore, all the dogs in this study lived with their owners, and exercise-induced stress to the nonsurgical joint was not controlled. Differences in exercise-induced stresses could affect the inflammatory process, particularly in the nonsurgical joint due to favoritism of the surgically treated joint.

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