EX VIVO AND *IN VITRO* EFFECTS OF OMEGA-3 FATTY ACIDS ON JOINT HEALTH IN SOWS AND GILTS

By

Cara Irene Robison

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ABSTRACT

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Dietary polyunsaturated fatty acids (PUFA) including arachidonic acid (AA), eicosapentaneioc acid (EPA), and docosahexaenoic acid (DHA) alter the production of inflammatory mediators. The objectives of this study were twofold: first, to examine the effects of EPA or DHA alone or in combination on the inflammatory response of stimulated cartilage explants from gilts; second to characterize the effects of dietary PUFA supplementation on bone, cartilage, and synovial fluid in sows and gilts. For the first objective, cartilage was obtained from the humeral-ulnar joints of Yorkshire x Landrace market sized gilts. Explants were harvested from the humeral-ulnar joints within 8 h of slaughter. Explants were allocated to culture plates and cultured in 1 mL of DMEM:F12 medium for 24 h with 10% fetal bovine serum. At 48 and 72 h, 1 mL of treatment media containing 15 ng/ml of recombinant porcine IL-1 was added to each well. At 48, 72, and 96 h after cartilage was allocated to wells, media were removed from each well and reserved for analyses. Media were analyzed for proteoglycan, nitric oxide (NO), interleukin-6 (IL6) and prostaglandin E_2 (PGE_2). In general, when EPA and/or DHA are supplemented to explants in combination with linoleic acid (LA) NO and PGE_2 release is decreased. Explants treated with 25 µg/ml DHA released 53% less NO into the media than explants treated with the same concentration of EPA and 60% less than explants treated with LA alone. These data demonstrate that EPA and DHA are capable of modulating the inflammatory response in porcine articular cartilage *in vitro*. To complete the second objective sows and gilts were fed either a

basal corn/soybean meal based diet (CON), or the basal diet supplemented with PUFA (Gromega 365; JBS United, Sheridan, IN). Sows completed an average of 5.5 parities while gilts reached an average BW of 111 kg at time of slaughter. Cartilage was biopsied from both humeral-ulnar joints of 14 sows (7/trt) and 16 gilts (8/trt) within 30 h of slaughter for fatty acid analysis and explant cultures. Synovial fluid was collected from the carpal joints of each pig post-mortem. The right fused radius/ulna were analyzed using computed tomography (CT). CT scans of the radius/ulna from gilts revealed no differences for cortical width and bone density. Sows fed PUFA had greater cortical width of the proximal ulna ($P<0.05$) and decreased cortical width of the distal radius ($P<0.05$). Sows fed PUFA had increased DHA ($P<0.01$), decreased C20:1 ($P<0.01$), and decreased omega-6 to omega-3 ratio ($P<0.05$) in cartilage. Gilts fed PUFA had increased EPA ($P<0.10$), DHA ($P<0.01$), C22:1 ($P <0.01$), and C22:5 ($P<0.10$) in cartilage. Although the PUFA diet increased omega-3 incorporation into chondrocytes, the biological significance is unclear since concentrations of AA were at least 9-fold higher than EPA or DHA. Bone density was not affected by a PUFA enriched diet. Thus, if omega-3 fatty acids can mitigate inflammation in joints, the benefit may be the result of systemic changes in inflammatory mediators.

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KEY TO ABBREVIATIONS

AA	Arachidonic Acid
COX-2	Cyclooxygenase 2
DHA	Docosahexaenoic Acid
EPA	Eicosapentaenoic Acid
iNOS	Inducible Nitric Oxide Synthase
IL6	Interleukin-6
LA	Linoleic Acid
LNA	Linolenic Acid
NO	Nitric Oxide
NF-κB	Nuclear Factor – Kappa Beta
PG	Proteoglycan
PGE_2	Prostaglandin E_2
PUFA	Polyunsaturated Fatty Acid
rpIL-1β	Recombinant Interleukin-1 Beta

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease resulting from biological and mechanical events that create an imbalance between degradation and synthesis of cartilage and bone. Initiation and progression of OA are not always identical for all human patients because several variables play a role, including, but not limited to, excessive mechanical forces, hereditary factors, injury, and aging. Arthritis is one of the most frequently occurring chronic conditions among adults over 65; only high blood pressure has higher incidence (CDC, 2007). The hip, knee, spine, and hand are most often affected with arthritis (Kuettner and Cole, 2005). In 2003, arthritis, including OA, was estimated to affect 46 million adults in the U.S. This number is expected to increase to 67 million by 2030, with 54% being 65 or older and 25 million having activity-limiting arthritis (Hootman and Helmick, 2006). The direct and indirect costs of OA were estimated at $155 billion in 1994 and are assuredly significantly higher now considering the increase in the percentage of the population affected with OA (Sun et al., 2007). Additionally, arthritic conditions are associated with pain, decreased quality of life, and absences from work (Muchmore et al., 2003). According to one study on the economic impact of arthritis and joint disease (AJD), approximately 3.5% of employees receive an initial medical diagnosis for AJD each year with 24.3% being diagnosed with OA (Muchmore et al., 2003). Therefore, as the average lifespan increases and the population ages, OA will become a problem for an increasing number of people.

Swine also have a high rate of incidence of OA. Joint lesions have been identified as one of the main causes for culling sows in Danish herds, affecting 24% of sows (Kirk et al., 2005), while in the US 15.2% of sows are culled for locomotor problems (USDA, 2007). Research has been conducted to elucidate the impact of OA on lameness leading to culling in swine

production. These losses due to lameness are costly for the producer. Thus, reducing the incidence would be welcomed. A wide variety of nutritional supplements, such as glucosamine sulfate, chondroitin sulfate, and omega-3 fatty acids (FA) have been investigated to determine therapeutic benefits for OA

Omega-3 fatty acids have the potential to be potent modulators of osteoarthritic factors due to their effects on cytokines, growth factors, and eicosanoids (Watkins et al., 2001a). Eisosanoids are produced from fatty acids released from cell membrane phospholipids by phospholipase A_2 and then are modified by a variety of enzymes to form prostaglandins, leukotrienes, or thromboxanes. Mammalian cells are not able to store eicosanoids; therefore production is regulated by the availability of free arachidonic acid (AA) and eicosapentaenoic acid (EPA) obtained through mobilization of phospholipids (Graber et al., 1994). This suggests that the fatty acid composition of the membrane directly influences the type of eicosanoids produced and used for intercellular signaling. Arachidonic acid, EPA, and α-linolenic acid (LNA) can be used as precursors for eicosanoids; however the preferred substrate is AA. Arachidonic acid reacts with cyclooxygenase-2 (COX-2) catalyzing the first committed step in the synthesis of prostaglandins from either omega-6 or omega-3 fatty acids. Cyclooxygenase-2 will oxygenate EPA at 30% the rate of AA with a preference for AA (Wada et al., 2007). When mouse stromal cells were incubated with either 40 μM AA or EPA, AA increased prostaglandin E_2 (PGE_2) production while EPA treated cells did not differ from the control (Wada et al., 2007). In order to achieve an alteration in the type of eicosanoids produced the fatty acid composition of the cell membrane must first be altered.

CHAPTER 1. Omega-3 fatty acids and their effect on bone and cartilage

INTRODUCTION

Normal adult cartilage consists of chondrocytes attached to an extracellular matrix (ECM) comprised of collagen, proteoglycans, and matrix proteins. Chondrocytes only account for 2-5% of the tissue in mature cartilage. The predominant collagens, types II and XI, form the backbone of all cartilage and make up 60% of the dry mass in cartilage (Seibel et al., 1999). Proteoglycans comprise 5-7% of cartilage, and aggrecan, the predominant proteoglycan, forms supramolecular aggregates and gives cartilage its elasticity while contributing to its mechanical properties (Fukui et al., 2001). Chondrocyte and proteoglycan metabolism are controlled primarily by insulin-like growth factor 1 (IGF-1), transforming growth factor-β (TGF-β), interleukin-1β (IL-1), and tumor necrosis factor-α (TNF-α).

Cartilage exists in a relatively steady state until some type of damage or extensive inflammation occurs. When the cartilage attempts to repair itself it enters into a cycle of anabolism and catabolism eventually resulting in cartilage destruction (Fukui et al., 2001). Osteoarthritis (OA) is a degenerative joint disease resulting from biological and mechanical events that create an imbalance between degradation and synthesis of chondrocytes, extracellular matrix, and bone. Initiation and progression of OA is not always identical for all patients because several variables play a role, including, but not limited to, excessive mechanical forces, hereditary factors, injury, and aging. Any of these variables alone or in combination can stimulate chondrocytes and nearby synoviocytes either directly or indirectly through signaling pathways. These pathways induce the synthesis of cytokines, proteolytic enzymes, and reactive oxygen species, which degrade the matrix and release matrix fragments. Eventually, an

imbalance between anabolic and catabolic activity is created, resulting in further destruction of the matrix (Goldring and Goldring, 2007; Loeser, 2006).

JOINT HEALTH IN SWINE

Osteoarthritis and joint disease are significant problems in swine and lead to lameness and ultimately involuntary culling. A considerable amount of research has been conducted to determine the reasons behind involuntary culling of swine from herds around the world over the past 20 years and lameness and/or arthritis have been identified as major reason for culling (Christensen et al., 1995; Kirk et al., 2005; Engblom and Eliasson-Selling, 2008; Ryan et al., 2010; Jensen et al., 2010). Sow longevity is important to producers because when sows complete a higher number of parities, fewer replacement gilts need to be raised on an annual basis. In a retrospective study in Quebec, necropsy reports were examined from 426 sows over 7 years and it was reported that arthritis and osteochondrosis were directly responsible for 5% of the deaths (D'Allaire et al., 1991). A similar study reported that leg weakness was the reason for involuntary culling of 28.5% of sows from 1992 to 1993 (Christensen et al., 1995). Additionally, sow mortality was investigated in Danish pigs from 10 different herds and one of the main causes for culling was identified as arthritis (inflammatory joint lesion), affecting 24% of sows. In 88% of the culled sows, the presence of a non-inflammatory joint lesion was identified in at least one joint (Kirk et al., 2005). A report on a large commercial herd in Sweden indicated that arthritis occurred in 36.5% of culled sows, and half of the sows had arthritis in more than one joint with 77% of those sows being lame and 37% having swollen joints (Engblom and Eliasson-Selling, 2008). Similarly, when the joint surfaces from 36 culled sows were examined it was found that all sows had OA, with lesions on 89% of the humeral heads that became more severe

as parity increased (Ryan et al., 2010). Unfortunately, very little information has been published on sow mortality rates and causes in North America. In 1997, the sow mortality rate in the U.S. was 5.7% based on production records; however there was no explanation provided as to cause of death (Koketsu, 2000).

Retrospective studies such as the ones described above provide important historical data and identify trends for culling in the swine industry. Unfortunately, they do not examine methods to reduce culling. Lameness has been identified as a key problem in the swine industry and the only factor significantly correlated with involuntary culling (Jensen et al., 2010). When examined together these reports from the last 20 years suggest that the incidence of sow lameness, and OA in particular, is increasing.

FATTY ACIDS

There are a variety of nutritional supplements available to alleviate the severity of OA including glucosamine and chondroitin sulfate, omega-3 fatty acids, and avocado-soy unsaponifiables. Specifically, omega-3 fatty acids have the potential to reduce the pain and inflammation associated with OA and joint disease. Fatty acids are characterized based on carbon chain length and level of unsaturation. Long chain polyunsaturated fatty acids (PUFA) are categorized as having 18 or more carbons with 2 or more double bonds. PUFAs are further divided into subcategories, including omega-3 and omega-6, according to the position of the first double bond when counting from the methyl end of the fatty acid. In omega-3 fatty acids the first double bond is located between the third and fourth carbons, when counting from the methyl end of the fatty acid. The most common omega-3 fatty acids are alpha-linolenic acid (LNA,

18:3), eicosapentaenoic acid (EPA, 20:5), docosapentaenoic acid (DPA, 22:5), and docosahexaenoic acid (DHA, 22:6).

Linolenic acid is found mostly in triglycerides and cholesterol esters. EPA is mainly found in cholesterol esters, triglycerides, and phospholipids, while DHA is most abundant in the brain's structural lipids and can also be found in the cerebral cortex, retina, testes, and sperm (Simpoulos, 2008). Linolenic acid is found in high concentrations in seed oil, particularly in canola, flaxseed, walnuts, and soybeans. Eicosapentaenoic acid and DHA are synthesized by marine phytoplankton and are found in high concentrations in marine species, especially salmon, tuna, and menhaden.

Omega-3 fatty acids have a number of beneficial effects when consumed. In animal studies, supplementation with omega-3 fatty acids decreases circulating triglycerides, platelet aggregation, and resting heart rate, and increases erythrocyte deformability (Simopoulos, 2008; O'Connor et al., 2004). In addition, omega-3 fatty acids incorporate into the membranes of erythrocytes and skeletal muscle cells increasing membrane fluidity and altering receptor-substrate interactions. They also impact bone formation, modulate the production of PGE_2, and reduce inflammation associated with arthritis (Watkins et al., 2001b).

INCORPORATION OF FATTY ACIDS INTO JOINT TISSUE

Fatty Acids and Bone

Lipids have an important role in the regulation of skeletal biology and promotion of bone health, which is supported by significant evidence from both animal and human research (Watkins et al., 2001a). Bone metabolism may be influenced by dietary fat through the alteration of prostaglandin biosynthesis (Watkins et al., 1997). Prostaglandins such as PGE_2,

$PGF_{2\alpha}$, and PGI_2 act as local regulators of skeletal metabolism and are produced locally by both osteoblasts and osteoclasts from the conversion of AA and EPA via the COX-2 pathway. Excess PGE_2 results in loss of proteoglycan from cartilage and decreased bone formation rate. However, since high AA concentrations are correlated to high PGE_2 concentrations, if AA concentrations are decreased, a resulting increase in bone formation rate occurs (Watkins et al., 2000). In osteogenic cells prostaglandins are responsible for regulating bone formation and resorption (Watkins et al., 2001a). Moderate concentrations of AA support bone formation; however, very high concentrations of AA decrease bone formation by increasing PGE_2, which alters the action of IGF-1 and inhibits collagen synthesis. Cytokines such as IL-1 and TNF-α can also increase PGE_2 formation in bone (Watkins et al., 1997). Hormones, cytokines, nitric oxide, growth factors, and mechanical forces regulate prostaglandin production in bone through changes in COX-2 (Seibel et al., 1999). Parathyroid hormone, IL-1, TNF-α, FGF, and TGF-β stimulate prostaglandin production while glucocorticoids, retinoic acid, IL-4, IL-13 and a decrease in COX-2 inhibit bone prostaglandin production.

Considerable research has been conducted to elucidate the link between PGE_2 and the effect of dietary omega-3 and omega-6 fatty acids on bone. In order for the diet to elicit changes that alter the production of PGE_2, there must be a shift in the fatty acid content of the bone tissue. Researchers studied rat pups from omega-3 fatty acid-deficient dams and found that initially they were lower in omega-3, but after 8 wk of repletion the concentration of omega-3 in femur and tibia cortical bone and marrow was equal to or higher than rats sustained on an adequate diet. Additionally, during omega-3 repletion, DHA is more efficiently incorporated

into the bone and bone marrow than LNA. Researchers found no difference in femur length; however, tibia length was longer for animals always fed adequate levels of omega-3 when compared to tibias from rats descended from omega-3-deficient dams. Mechanical testing was completed to examine structural changes in the bone, and the omega-3-adequate rats had higher energy to peak load suggesting an increase in bone strength (Reinwald et al., 2004). Young rats fed diets containing purified DHA exhibited increased DHA concentration in plasma phospholipids, serum triglycerides, red blood cells, and bone marrow while AA and omega-6:omega-3 ratio deceased. Additionally, the DHA treatment led to higher bone marrow cellularity than control, and tended to be higher than the safflower diet (Atkinson et al., 1997). Watkins and coworkers (2000) studied the effects of differing ratios of safflower to fish oil (90:10, 80:20, 50:50, and 30:70) in 3-wk-old male weanling rats for 42 d, at which time the rats were euthanized. Fatty acid composition of femur periosteum polar lipids showed increased EPA and DHA, reduced AA and the highest omega-6:omega-3 ratio in the two diets highest in fish oil. The production of PGE_2 in homogenates of liver, tibia, and femur were lower in the 30:70 diet; however, there was no difference in bone formation rate between treatments (Watkins et al., 2000).

Similarly, quail consuming fish oil had higher concentrations of EPA, DHA, and total saturates in tibia fatty acids when compared to controls. Quail fed fish oil or soybean oil showed higher proximal cortical density and mineral content than control animals (Liu et al., 2003). In contrast, rabbits fed diets containing 10% fish oil had significant reductions in feed consumption resulting in reduced cortical bone area and decreased tensile strength, which were attributed to energy restriction (Judex et al., 2000). Kruger and coworkers supplemented rats with varying levels of PUFA and found that DHA may be more effective than EPA in improving bone mass

(Kruger and Schollum, 2005). Docosahexaenoic acid content of red blood cell membranes was positively correlated with right femur and spine bone mineral density (BMD) and bone mineral content. Additionally, concentration of Ca in bone was also positively correlated with DHA in red blood cell membranes. The tuna oil diet contained the highest level of DHA and resulted in increased Ca absorption and decreased urinary Ca excretion (Kruger and Schollum, 2005). In general, the results of PUFA supplementation on bone vary greatly and seem to depend on the type, quantity, and ratio of the fatty acids in the diet.

Bone health is integral to the growth and development of young people and animals and therefore an area of considerable research interest. Postmenopausal bone health also represents a major health problem in elderly women. The most common way to recreate the postmenopausal condition is to remove the ovaries from mature female animals in order to study the effect of experimental conditions on bone loss. The effects of omega-3 fatty acids have been studied using ovariectomized (OVX) animals to simulate menopause. For example, Poulsen and Kruger (2006) used 6-month-old rats to study dietary EPA at varying concentrations when Ca concentrations were adequate. DEXA scans were completed at baseline before OVX and 4 and 9 wk post-OVX. The high dose of EPA increased bone resorption in lumbar vertebrae and femur; however, at the low dose of EPA BMD was not different from OVX, but lower than sham. EPA was not able to prevent OVX-induced bone loss. All the diets did contain corn oil and it is possible that the diets did not alter the omega-6:omega-3 ratio enough. Similar research assessed the effects of fish oil on BMD using DEXA scans in mice fed fish oil for 2 months before undergoing either sham or OVX operations (Sun et al., 2003). Fish oil-fed mice had higher concentrations of EPA and DHA and 60% lower concentrations of AA, resulting in a lower omega-6:omega-3 ratio, which prevented bone loss due to estrogen deficiency. Fish oil-fed

OVX mice had higher BMD in distal left femur and lumbar vertebrae and overall reduced bone loss when compared to the OVX corn oil-fed mice. Additionally, fish-oil-fed OVX mice had the same BMD for central left femur and lumbar vertebrae as control mice on the same diet (Sun et al., 2003). Omega-3 fatty acids have the potential to alleviate hormone induced bone loss; however, reduction of dietary AA may potentially be a key factor for supporting bone health.

Fatty Acids and Cartilage

Due to the avascularity of articular cartilage, chondrocytes have limited access to fatty acids. Chondrocytes may be deprived of omega-6 fatty acids relative to other tissues (Adkisson et al., 1991). Levels of AA, EPA and DHA in the cell membrane will determine what type and how much eicosanoid synthesis will occur in chondrocytes (Broughton and Wade, 2002). Arkill et al. (2006) demonstrated that lauric acid, a relatively insoluble fatty acid, is transported through the matrix at a low, but measurable, rate. These rates may be different for different fatty acids. Composition of cartilage fatty acids may affect the way anti-arthritis drugs act on the inflammatory process; however, when fatty acid profiles were analyzed in adult arthritic and non-arthritic cartilage, no differences in fatty acid concentrations were elucidated (Cleland et al., 1995).

For generations humans and many animals have been naturally supplementing themselves with omega-3 fatty acids by eating salmon, cod, and other cold-water seafood. Brusch and Johnson (1959) fed arthritic patients a strict diet, and cod liver oil, which is known to be rich in omega-3 fatty acids, and observed a reduction in pain and tissue swelling, and improved range of motion. This was the first of many studies on the effects of omega-3 fatty acids on arthritis. Since then, a variety of research has been conducted to elucidate the mechanisms of omega-3 fatty acids on arthritic cartilage. Lippiello et al. (1990) demonstrated

that the main supply of fatty acids to cartilage is the synovial fluid, and that it is sensitive to dietary manipulation of fatty acids. Dolegowska et al. (2006) analyzed articular cartilage from broiler chicks fed a soybean- and cornstarch-based diet for fatty acid content and found that the profile in articular cartilage was almost the same as that for cortical and trabecular bone. Lipids in articular cartilage primarily consist of oleic acid (C18:1) and palmitic acid (C16:0), but did contain some AA (Dolegowska et al., 2006).

Nagao et al. (1991) measured fatty acid profiles in 4-wk-old rabbit articular cartilage that was cultured for 72 h before C^{14} labeled oleic, linoleic acid (LA) and AA were added at concentrations of 5, 50, 250, or 500 μM for 8 h. The incorporation of the fatty acids into the cell increased as the concentration of the fatty acids in media increased. When bovine cartilage was incubated with 18:3 omega-3 for 8 h, aggrecanase activity was decreased in a dose-dependent fashion (Curtis et al., 2000). Shen and coworkers (2004) incubated human osteoarthritic chondrocytes with LA/EPA and found that this combination resulted in decreased PGE_2 and nitric oxide (NO) production in comparison to controls.

A number of studies have also been performed to examine the effect of omega-3 fatty acids on rheumatoid arthritis. A review by James et al. (2003) summarized the findings from 12 clinical studies examining fish oil supplementation on patients with rheumatoid arthritis and found that the most consistently reported outcomes were an improvement in tender joint count and a reduction in inflammation. Berbert and coworkers (2005) examined the effects of 3 g/d soy oil, 3 g/d fish oil, or 3 g/d fish oil and 9.6 ml/d of olive oil on patients with rheumatoid arthritis. Both fish oil diets resulted in significant improvement in right and left hand grip strength, reduced intensity of joint pain, and delayed onset of fatigue. The researchers concluded that the oleic acid in the olive oil did provide some additive benefits by further decreasing joint

pain (Berbert et al., 2005). Men and women that were part of Baker et al.'s Multicenter Osteoarthritis Study (2012) were examined (knee MRI) to look at the correlation between omega-3 fatty acids and cartilage loss in the knee. Higher concentrations of DHA were associated with reduced severity of patellofemoral cartilage loss, but there was no association between EPA concentration and cartilage loss (Baker et al., 2012). Additionally, in healthy adults without knee pain, higher intakes of omega-6 fatty acids in adults were positively associated with bone marrow lesions, which are predictive of cartilage loss (Wang et al., 2007). Goldberg and Katz (2007) performed a meta-analysis and found that studies that provided higher doses of omega-3 showed greater improvement in morning stiffness and a reduction in the number of painful or tender joints compared to low doses of omega-3.

Omega-3 fatty acids have the potential to be potent modulators of osteoarthritic factors due to their effects on prostaglandin production and inducible nitric oxide sulfate (iNOS). When bovine chondrocytes from 7-d-old calves were allowed to form monolayers and incubated with 10, 50, or 100 μg LNA/ml media for 8 h, LNA concentration increased and AA concentration and aggrecanase activity decreased in a dose-dependent fashion (Curtis et al., 2000). In another study, when chondrocyte monolayers formed from 7-d old calves were incubated with EPA, DHA, AA, ALA at 2.5, 5, 10, 20, 30 μg/ml for 8 h, results indicated that EPA, DHA and ALA at 30 μg/ml reduced COX-2, IL-1-α, and TNF-α mRNA concentrations (Zainal et al., 2009). Additionally, human osteoarthritic chondrocytes incubated with LA/EPA at 10 μM in a 1:1 ratio resulted in increased concentration of EPA in the cells and decreased PGE_2 and NO production in comparison to controls. The LA/AA treatment had the lowest NO production, but highest PGE_2 production (Shen et al., 2004).

OMEGA-3 FATTY ACIDS IN SWINE

Supplementation of sows with omega-3 fatty acids has the potential to reduce inflammation and lameness resulting from arthritis. Sows fed isoenergetic diets containing 0, 3.5 or 7% fish oil displayed both a linear and quadratic elevation in serum EPA concentrations over time, with the overall content of serum omega-3 fatty acids increasing from <4% to 23% of total fatty acids on the highest fish oil diet. Additionally, there was an increase in the concentration of omega-3 fatty acids in the milk composition of fish oil-fed sows. Concentrations of EPA increased over time in piglets suckling from fish oil-fed sows demonstrating the capacity of the piglet to digest and absorb omega-3 fatty acids from milk (Fritsche et al., 1993). Frantz and coworkers (2008) examined the effects of fish oil supplementation on osteochondrosis in gilts fed 3.5% fish oil for 84 d. Cartilage from gilts on the fish oil diet required less energy to shear and the highest compression to shear ratio, suggesting that the cartilage was more brittle and easier to tear into 2 pieces (Frantz et al., 2008). Their research did not include fatty acid analysis of the articular cartilage, so it is unknown as to the degree of unsaturation in the cartilage after 84 d.

Studies in piglets have examined tissue incorporation of fatty acids when piglets suckled from fish-oil-fed sows or were fed fish oil or purified fatty acids (Fritsche et al., 1993; Weiler and Fitzpatrick-Wong, 2002; Sampels et al., 2011). Lactating sows fed diets rich in omega-3 fatty acids produced milk containing increased concentrations of EPA and DHA and a reduced omega-6/omega-3 ratio. Additionally, muscle and small intestine samples from piglets reflected the dietary fatty acid profile of the dam's diet (Gabler et al., 2007). Similarly, fatty acid profiles of diets from sows fed saturated fat or linseed oil were reflected in piglet brain total lipids and liver membrane phospholipids (Sampels et al., 2011). Piglets fed formula containing 0.1% wt/wt

DHA had significantly higher bone mineral content and weighed more on d 15 than suckled piglets (Weiler and Fitzpatrick-Wong, 2002). Piglets farrowed and reared from sows fed diets high omega-3 fatty acids may have reduced incidence of inflammatory disease and differential responses to immune challenges, especially if those piglets remained on omega-3 fatty acid diets post-weaning.

SUMMARY

Osteoarthritis has been shown to be a major reason for culling in commercial sow operations around the world. Inflammatory cytokines, such as NO and PGE_2, stimulate the progression of OA by disrupting the balance between synthesis and degradation in joints. Addition of omega-3 fatty acids to the diet reduces the omega-6 to omega-3 ratio, decreases the production of inflammatory mediators, and elicits positive effects on bone through down-regulation of PGE_2.

The objectives of this dissertation were to examine the *in vitro* effects of EPA and DHA on inflammatory response of cartilage explants and the *ex vivo* effects of dietary PUFA on bone, cartilage, and synovial fluid in sows and gilts. The hypotheses were that EPA and DHA will mitigate the inflammatory response in IL-1β stimulated explants, and dietary PUFA would increase EPA and DHA incorporation into cartilage and synovial fluid, alter bone morphology, and mitigate inflammatory response of *ex vivo* cartilage explants.

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CHAPTER 2. Eicosapentaenoic acid and docosahexaenoic acid modulate inflammation in porcine articular cartilage explants

INTRODUCTION

Lameness is a common clinical condition in livestock usually resulting in culling of the affected animal. In livestock of all breeds this can be a cause of serious financial loss, including replacement cost, and is a source of major welfare concerns. Joint lesions have been identified as one of the main causes for culling sows in Danish herds, affecting 24% of sows (Kirk et al., 2005), while in the U.S. 15.2% of sows are culled for locomotor problems (USDA, 2007). Over a 6-month period, production sows culled for lameness were analyzed postmortem. It was found that 31 of the 45 sows were diagnosed with either osteochondrosis or athrosis (Dewey et al., 1993).

The pathogenesis of osteoarthritis (OA) is strongly mediated by the pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (Goldring and Berenbaum, 2004). The inflammatory response in cartilage is regulated through cell signaling pathways that control gene expression of proteins responsible for the production of nitric oxide (NO), prostaglandin E_2 (PGE_2), and interleukin-6 (IL-6). Nitric oxide production plays a significant role in the development of cartilage degradation through inhibition of proteoglycan and collagen synthesis (Scher et al., 2007). When cartilage is stimulated with IL-1β and/or tumor necrosis factor-α, increased NO production occurs. Mastbergen et al. (2008) demonstrated that increased proteoglycan release from human articular cartilage is positively correlated with an increase in both NO and PGE_2 production.

Omega-3 fatty acids have the potential to be potent modulators of osteoarthritic factors due to their ability to reduce prostaglandin and NO production. Eicosanoids formed from eicosapentaenoic acid (EPA) are 10- to 100-fold less potent than those produced from arachidonic acid (AA) and therefore are associated with a decreased inflammatory response (Alexander, 1998). When mouse stromal cells were incubated with either 40 μM AA or EPA, AA increased PGE_2 production, while EPA treated cells did not differ from the control. Additionally, Razzak et al. (2008) demonstrated that when murine macrophages were incubated with lipopolysaccharide and a cyclooxygenase-2 (COX-2) inhibitor, NO production increased; however, when the same cells were incubated with EPA or a COX-2 inhibitor and EPA, NO production was minimal.

Both EPA and docosahexaenoic acid (DHA) can reduce the inflammatory response and the production of NO and PGE_2 in other tissues. The objective of this study was to perform a series of titration experiments to determine the level of EPA and DHA, both alone and in combination, necessary to reduce inflammatory mediators in porcine articular cartilage explants *ex vivo* relative to either linoleic acid (LA) or AA.

MATERIALS AND METHODS

Explant Cultures

Yorkshire x Landrace cross gilts were slaughtered at market weight following the standard practices of the MSU Meat Laboratory. For experiments 1 and 2, front legs were collected from 8 gilts, and for experiments 3 and 4, front legs were collected from 6 gilts. Front legs were removed within 30 min of slaughter. Each experiment was conducted separately. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

The left and right humeral-ulnar joints were opened aseptically under sterile conditions and 50 cartilage disks were harvested with a 6 mm biopsy punch (Miltex, York, PA) from the weight-bearing region of the articular surface of each gilt within 6 h of slaughter. Only visually normal cartilage was selected for biopsy.

Cartilage discs were washed twice in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (Ham) (DMEM:F12; Invitrogen, Carlsbad, CA) containing 100 units/mL penicillin-streptomycin (Life Technologies, Carlsbad, CA). Explant discs were randomly placed into the wells of a 24-well culture plate until each well contained two discs. Explants were conditioned for 24 h in 1 mL of base media, containing DMEM:F12 supplemented with amino acids (Rosselot et al., 1992), 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA), 50 μg/mL ascorbate, and 100 units/mL penicillin-streptomycin (Invitrogen, Carlsbad, CA), in a humidified incubator at 37ºC with 7% CO_2.

After 24 h of conditioning media explants were washed twice with 1 mL of sterile phosphate buffered saline to remove FBS from the wells. Saline was completely removed from each well after each wash. Then, 1 mL of treatment media consisting of FBS free base media plus 1 μL/mL insulin-transferrin-sodium selenite supplement (Roche Applied Science, Mannheim, Germany), 0.02 μg/mL thyroxine, and long chain polyunsaturated fatty acids (LCPUFA; Camen Chemical, Ann Arbor, MI) were added to each well (Table 1). At 48 and 72 h 15 ng/mL of recombinant porcine IL-1β (R & D Systems, Minneapolis, MN) were added to the treatment media (**Error! Reference source not found.**). Between 24 and 48 h the explants were ot exposed to IL-1β to serve as the unstimulated control. At 48, 72, and 96 h media were removed from each well and separated into two tubes for analysis. The first tube contained 10 μg/ml indomethacin to prevent further metabolism of PGE_2 and was stored at -20ºC until

analysis. The second tube did not contain any additives and was stored at 4ºC for NO and proteoglycan analysis. Media were analyzed for proteoglycans (PG), NO, interleukin-6 (IL-6), and PGE_2 concentrations.

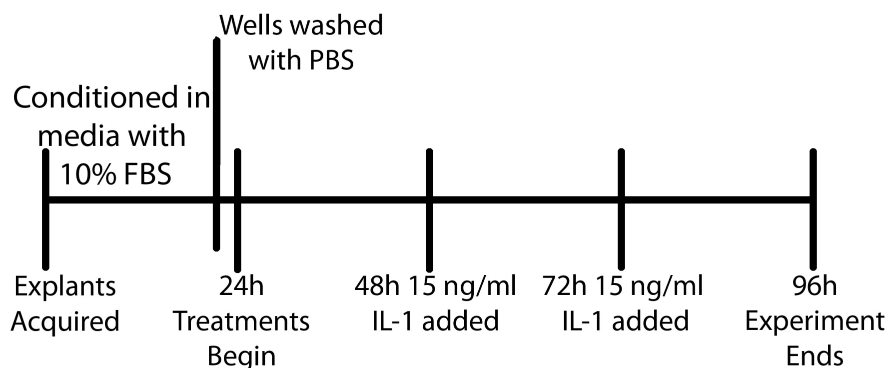

Figure 1. Diagram of explant culture process over 96 h.

Proteoglycan Analysis

Proteoglycan release into media was measured using the dimethylmethylene blue assay (Chandrasekhar et al., 1987). Proteoglycan content was determined by measuring sulfated glycosaminoglycan content using a chondroitin sulfate standard and expressed as µg PG/well. Absorbance at 530 nm with a correction at 590 nm was determined using a Spectramax 300 plate reader (Molecular Devices, Sunnyvale, CA).

Nitric Oxide Analysis

Nitric oxide was measured indirectly by quantifying nitrite, a stable end-product of nitric oxide metabolism, in the media by using the Greiss reaction and a sodium nitrite standard

(Blanco et al., 1995). Absorbance at 540 nm was determined using a Spectramax 300 plate reader (Molecular Devices, Sunnyvale, CA). Results are expressed as μM of NO/well.

PGE_2 Analysis

Prostaglandin E_2 was measured in the media using a commercially available ELISA kit (EHPGE2; Thermo-Fisher Scientific, Pittsburgh, PA) following the manufacturer's instructions. Media samples were diluted as needed in the provided assay buffer and analyzed. Absorbance at 405 nm with a correction at 580 nm was determined using a Spectramax 300 plate reader (Molecular Devices, Sunnyvale, CA)

IL-6 Analysis

Interleukin-6 was measured in the media using a porcine-specific, commercially available ELISA kit (Porcine IL-6 Duoset; DY686, R & D Systems, Minneapolis, MN) following the manufacturer's instructions. Samples were diluted as needed in the reagent diluent and analyzed in duplicate. Absorbance at 450 nm with a correction at 570 nm was determined using a Spectramax 300 plate reader (Molecular Devices, Sunnyvale, CA).

Statistical analysis

Data were analyzed using the mixed procedure of SAS (Version 9.2; SAS Inst. Inc., Cary, NC) with animal and treatment as the fixed effects. The random statement animal (treatment) was used. All data were analyzed as a cumulative response following stimulation with IL-1, such that the response from media collected at 72 h was added to the response from media collected at 96 h. All data will be presented as lsmeans ± SEM. P-values < 0.05 will be discussed as significant while P-values < 0.10 will be discussed as trends.

Table 1. Detailed list of all experiments conducted, the concentration of IL-1β, the control fatty acids: linoleic acid (LA) and arachidonic acid (AA) as well as the treatment fatty acids: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for each treatment.

Experiment No.	Treatment	IL-1β, ng/mL	LA, μg/mL	EPA, μg/mL	DHA, μg/mL	AA μg/mL
1	100 LA	15	100	0		
	6.25 EPA	15	93.75	6.25		
	12.5 EPA	15	87.5	12.5		
	18.75 EPA	15	81.25	18.75		
	25 EPA	15	75	25		
2	100 LA	15	100		0	
	6.25 DHA	15	93.75		6.25	
	12.5 DHA	15	87.5		12.5	
	18.75 DHA	15	81.25		18.75	
	25 DHA	15	75		25	
3	100 LA	15	100	0	0	
	25 EPA	15	75	25	0	
	18 EPA, 6 DHA	15	75	18.75	6.25	
	12.5 EPA, 12.5 DHA	15	75	12.5	12.5	
	6 EPA, 18 DHA	15	75	6.25	18.75	
	25 DHA	15	75	0	25	
4	100 AA	15		0	0	100
	12.5 EPA	15		12.5	0	87.5
	25 EPA	15		25	0	75
	12.5 DHA	15		0	12.5	87.5
	25 DHA	15		0	25	75

RESULTS

Prior to IL-1β stimulation, release of proteoglycans, and production of nitric oxide and IL-6 did not exhibit a treatment effect for any of the 4 experiments. Additionally, experiments 1 and 3 did not exhibit a treatment effect for PGE_2 production prior to IL-1β stimulation. In experiment 2, explants treated with 12.5 and 25 μg/mL of DHA produced more PGE_2 prior to stimulation than those treated with 100 μg/mL of LNA, 6.25 μg/mL of DHA and 18.75 μg/mL of DHA ($P < 0.01$; Table 3). In experiment 4, where AA was used as the control fatty acid, prior to IL-1β stimulation the control produced 1.7 times more PGE_2 than the next highest treatment while the 25 μg/mL of DHA produced the least amount of PGE_2 ($P < 0.01$; Table 5).

Cartilage explants treated with 0 to 25 μg/mL of EPA and stimulated with IL-1β exhibited a trend for an overall effect of treatment ($P = 0.09$) in which the 6.25 and 12.5 EPA treatments released less proteoglycans than 100 LA or 18.75 EPA (Table 2; $P < 0.05$, $P = 0.07$). Proteoglycan release from cartilage explants treated with 0 to 25 μg/mL of DHA or 0 to 25 μg/mL of EPA and DHA and stimulated with IL-1β displayed no difference in cumulative release over 48 h (Table 3 and Table 4). Post IL-1β stimulation, explants with AA as the control fatty acid and treated with 12.5 or 25 μg/mL of EPA released more proteoglycans than those treated with 12.5 and 25 μg/mL of DHA (Table 5).

There was no difference in nitric oxide release from explants treated with varying concentrations of EPA and stimulated with IL-1β ($P < 0.05$). Explants treated with DHA displayed an overall effect of treatment ($P < 0.01$) and at 72 h the 12.5, 18.75 and 25 DHA treatments released less NO than the 100 LA or the 6.25 DHA (Table 3). In explants treated with EPA and DHA alone or in combination NO release exhibited an overall effect of treatment (P <

0.01) and all fatty acid treatments released less NO than the 100 LA treatment ($P < 0.05$; Table 4 and Figure 4). Additionally, the 25 DHA treatment in experiment 3 released less NO than any other treatment ($P < 0.01$). Explants with AA as the control fatty acid exhibited a treatment effect for NO release with the 25 µg/mL of EPA treatment releasing the most NO ($P < 0.01$; Table 5) while there was no difference between the control, 12.5 DHA and 25 DHA treatments.

Prostaglandin E_2 release was not affected by treatment with EPA and stimulation with IL-1β in experiment 1 (Table 2). In experiments 2 and 3, PGE_2 release exhibited an overall effect of treatment ($P < 0.01$) in which all EPA and/or DHA treatments decreased release when compared to the control ($P < 0.05$; Tables 3 and Figure 3) with no difference between the fatty acid treatments. Cumulative release of PGE_2 in experiment 4 was also affected by treatment ($P < 0.01$) with the 25 EPA treatment producing the least amount of PGE_2 and the AA control treatment producing the most ($P < 0.01$; Table 5).

Experiments 1, 2, and 3 did not have any effect on IL-6 release into the media regardless of fatty acid or concentration. However, experiment 4 demonstrated that cumulative release of IL-6 was affected by treatment ($P < 0.01$) such that the 25 µg/mL of EPA treatment produced 50% more than the 12.5 EPA and DHA treatments and 86% more than the 100 AA or 25 DHA treatments ($P < 0.01$; Table 5).

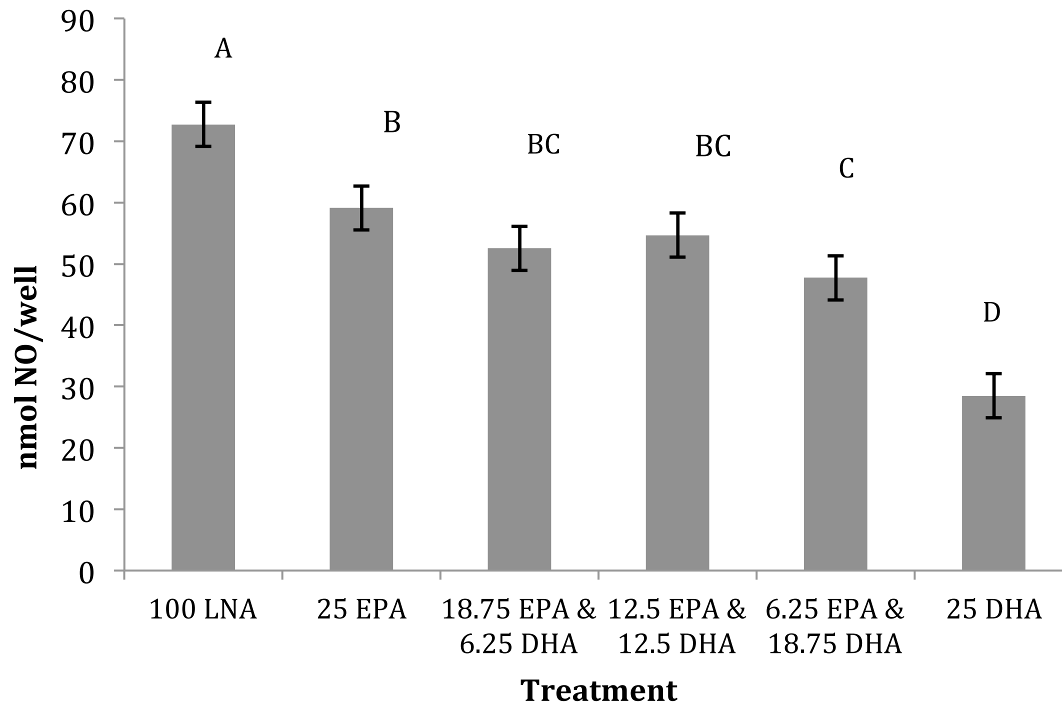

Figure 2. Cumulative NO release from cartilage into media for Experiment 3 when explants were treated with 0 to 25 µg/mL of EPA and DHA alone or in combination (Table 1). NO exhibited a treatment effect ($P < 0.01$). Bars with different letters differ at $P < 0.05$.

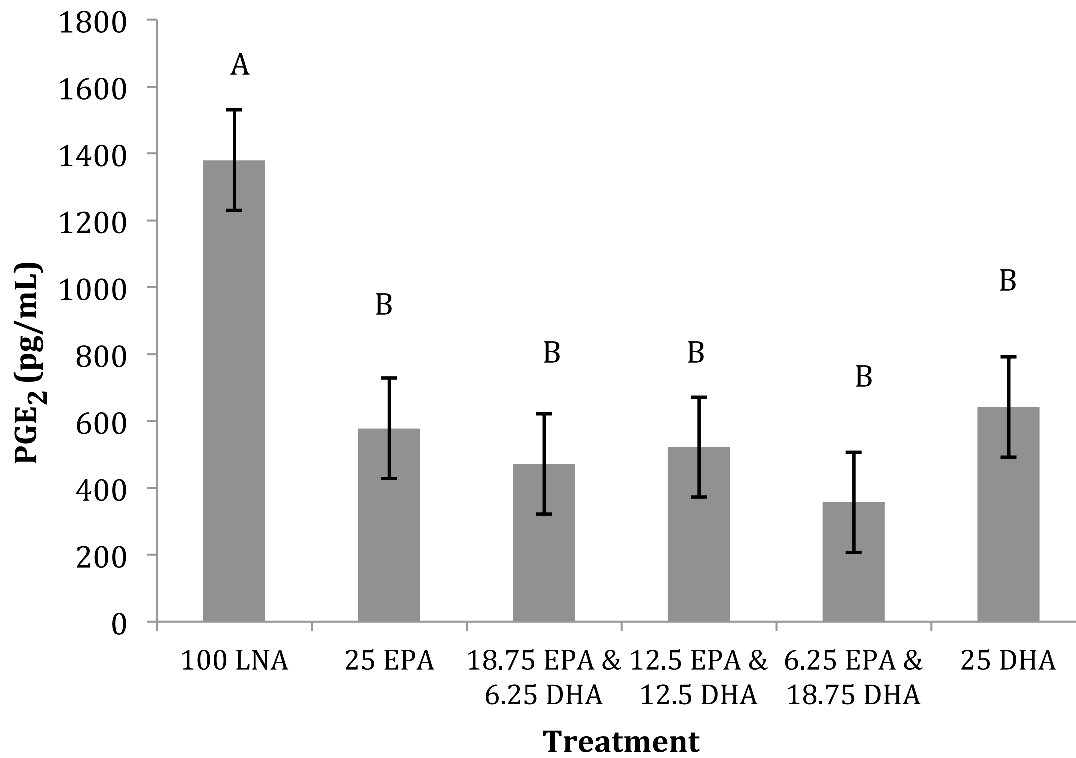

Figure 3. Cumulative PGE_2 release from cartilage into media for Experiment 3 when explants were treated with 0 to 25 µg/mL of EPA and DHA alone or in combination (Table 1). PGE_2 exhibited a treatment effect ($P < 0.01$). Bars with different letters differ at $P < 0.05$.

Table 2. Cumulative proteoglycan, NO, PGE_2, and IL-6 release from cartilage into media for experiment 1 when explants were treated with 0 to 25 µg/mL of EPA (Table 1). Proteoglycan release exhibited trend for a treatment effect (P = 0.087). Means with different letters differ (P < 0.05) for a given analyte.

	100 LA	6.25 EPA	12.5 EPA	18.75 EPA	25 EPA	SEM	P-Value
PG							
Pre IL-1	101.9	101.0	122.8	96.8	103.9	18.4	0.872
Post IL-1	540.1[a]	423.2[b]	452.8[ab]	526.2[a]	478.7[ab]	32.8	0.087
NO							
Pre IL-1	15.7	15.2	14.9	15.5	14.8	0.58	0.795
Post IL-1	86.7	77.6	74.7	80.9	76.1	3.9	0.228
PGE_2							
Pre IL-1	84.0	92.4	71.06	103.7	112.8	19.7	0.584
Post IL-1	917.5	903.3	486.3	865.1	640.4	177	0.346
IL-6							
Pre Il-1	63.2	55.9	34.39	36.0	44.8	11.5	0.323
Post IL-1	6390.3	6059.0	6475.7	6194.7	6419.5	724	0.993

Table 3. Cumulative proteoglycan, NO, PGE_2, and IL-6 release from cartilage into media for experiment 2 when explants were treated with 0 to 25 μg/mL of DHA (Table 1). NO and PGE_2 release exhibited a treatment effect ($P < 0.01$). Means with different letters differ ($P < 0.05$) for a given analyte.

	100 LA	6.25 DHA	12.5 DHA	18.75 DHA	25 DHA	SEM	P-Value
PG							
Pre IL-1	70.2	71.1	66.6	72.2	75.8	5.3	0.81
Post IL-1	380.7	347.9	282.8	298.0	312.3	34.7	0.285
NO							
Pre IL-1	14.3	14.5	13.9	13.0	12.3	0.8	0.270
Post IL-1	74.7^{a}	66.12^{a}	52.05^{b}	52.75^{b}	46.58^{b}	4.8	0.001
PGE_2							
Pre IL-1	22.3^{a}	39.3^{ab}	153.0^{c}	56.3^{a}	104.7^{bc}	24.3	0.008
Post IL-1	1060.1^{a}	431.2^{b}	523.2^{b}	382.3^{b}	474.9^{b}	102	< 0.001
IL-6							
Pre IL-1	42.7	43.3	19.0	13.4	7.1	21.6	0.66
Post IL-1	3923.3	4380.1	3446.5	3844.1	3642.7	938	0.967

Table 4. Cumulative proteoglycan, NO, PGE_2, and IL-6 release from cartilage into media for experiment 3 when explants were treated with 0 to 25 μg/mL of EPA and DHA alone or in combination (Table 1). NO and PGE_2 release exhibited a treatment effect ($P < 0.01$) post IL-1 stimulation. Means with different letters differ ($P < 0.05$) for a given analyte.

	100 LA	25 EPA	18.75 EPA, 6.25 DHA	12.5 EPA, 12.5 DHA	6.25 EPA, 18.75 DHA	25 DHA	SEM	P-Value
PG								
Pre IL-1	66.1	62.8	68.2	69.6	65.5	65.1	4.92	0.941
Post IL-1	473.7	427.7	383.4	478.5	386.1	352.5	40.7	0.189
NO								
Pre IL-1	13.5	12.6	12.6	12.0	11.8	12.1	1.4	0.963
Post IL-1	72.7 [a]	59.1 [b]	52.54 [b]	54.7 [bc]	47.72 [c]	28.5 [d]	3.6	< 0.001
PGE_2								
Pre IL-1	91.0	222.0	292.7	235.3	218.5	376.8	87.44	0.302
Post IL-1	1379.8 [a]	578.2 [b]	471.7 [b]	521.8 [b]	357.1 [b]	641.9 [b]	149.8	0.001
IL-6								
Pre IL-1	25.5	120.5	28.4	145.4	114.0	38.4	57.95	0.529
Post IL-1	6830.8	9870.4	10172	12468	7255.2	6852.9	1768	0.163

Table 5. Cumulative proteoglycan, NO, PGE_2, and IL-6 release from cartilage into media for experiment 4 when explants were treated with 12.5 or 25 µg/mL of EPA or DHA (Table 1). All variables exhibited a treatment effect post IL-1 stimulation ($P < 0.01$). Means with different letters differ ($P < 0.05$) for a given analyte.

	100 AA	12.5 EPA	25 EPA	12.5 DHA	25 DHA	SEM	P-Value
PG							
Pre IL-1	60.9	64.5	78.8	57.9	61.4	6.0	0.125
Post IL-1	126.6^{ac}	208.3^{b}	230.8^{b}	152.2^{a}	96.6^{c}	16.6	< 0.001
NO							
Pre IL-1	6.9	7.0	4.0	7.0	4.1	1.7	0.471
Post IL-1	9.2^{a}	17.4^{b}	21.8^{c}	10.0^{ad}	8.9^{ad}	1.3	< 0.001
PGE_2							
Pre IL-1	34695^{a}	20240^{b}	11180^{bc}	19880^{b}	8680^{c}	3304	< 0.001
Post IL-1	122110^{a}	76420^{b}	30290^{c}	80135^{b}	81670^{b}	7080	< 0.001
IL-6							
Pre IL-1	1.8	3.7	1.7	2.3	2.4	1.5	0.884
Post IL-1	585.8^{a}	2287.6^{b}	4136.4^{c}	2309.5^{b}	238.7^{a}	601.1	< 0.001

DISCUSSION

This work investigated the anti-inflammatory effects of EPA and DHA by measuring inflammatory mediators and tissue degradation in a porcine explant model of inflammatory joint disease. The *in vitro* system of cartilage explants does mimic physiological conditions. The experiments were designed to determine the potential for omega-3 fatty acids to influence inflammatory molecules in porcine cartilage. Supplementation of EPA and/or DHA into porcine explant cultures consistently caused a reduction in NO and PGE_2 concentrations in the media; however, only EPA alone was able to alter PG release. In previous studies, Il-1β has been successfully used to induce cartilage degradation (Wann et al., 2010; Zainal et al., 2009; Chan et al., 2006). DHA was successful at altering chondrocyte metabolism to inhibit NO and PGE_2 production. The reduction in NO is likely due to a concurrent reduction in PGE_2.

Omega-3 fatty acids are known for their anti-inflammatory properties with a reduction in PGE_2 being a consistent finding regardless of tissue. Arachidonic acid is an omega-6 fatty acid, which generally initiates a pro-inflammatory response via its metabolism to PGE_2 in the cell. Arachidonic acid is the preferred substrate for COX-2; however, EPA can also be used. *In vitro* COX-2 has higher specificity for AA than for EPA and preferentially oxygenates AA when both fatty acids are present even if AA is at a low substrate concentration (Wada et al., 2007). In the current experiments, when only EPA or DHA and LA were present in the media, once all AA was released from the cellular membrane any additional reactions would utilize EPA as a substrate for COX-2, resulting in the production of PGE_3. Since the ELISA used in the current study to measure the concentration of PGE_2 in the media has minimal cross reactivity with PGE_3, the lower concentration of PGE_2 in the EPA-treated media may be due to the production

of PGE_3 and not alterations in COX-2 expression. In experiment 4 when AA was present in the media as the control fatty acid instead of LA the PGE_2 levels were 122 times higher after IL-1β stimulation than in the LA treatment of the other experiments. Addition of EPA or DHA at any level was sufficient to reduce PGE_2 production even with an unlimited supply of AA; however the 25 EPA treatment was able to create the most substantial reduction of approximately 75%. In the experiments using LA as the control, the presence of either EPA or DHA at any concentration or combination reduced PGE_2 concentrations similarly. The data from experiment 4 suggests that EPA and DHA are utilizing different pathways to reduce the production of PGE_2 post-stimulation.

In addition to a reduction in PGE_2, supplementation of EPA and/or DHA resulted in the reduction of NO production. Nitric oxide production plays a significant role in the development of cartilage degradation through inhibition of proteoglycan and collagen synthesis (Scher et al., 2007). Mastbergen et al. (2008) demonstrated that increased proteoglycan release from human articular cartilage is positively correlated with an increase in both NO and PGE_2 production. Cyclooxygenase-2, inducible nitric oxide synthase (iNOS), and fatty acids have a very complicated interrelationship that has been explored by many researchers. For example, when AA was added to osteoblast cell cultures an increase in iNOS gene expression occurred; however, when EPA was added to AA-treated cells, EPA prevented an increase in iNOS expression (Priante et al., 2005). This suggests that EPA may be a more potent regulator of iNOS expression than AA. However, when AA was used as the control fatty acid NO production increased when EPA was added to the wells. Little research has been conducted

using chondrocytes and it is possible that other cell types respond differently under these conditions.

Mouse macrophages treated with 60 μM of LA, LNA, AA, EPA, or DHA and stimulated with lipopolysaccharide and interferon-γ to simulate a bacterial endotoxin demonstrated that both EPA and DHA inhibited NO production, while only DHA inhibited iNOS protein and mRNA expression (Komatsu et al., 2003). This suggests that *in vitro* DHA is a more potent regulator of NO and PGE_2 release than EPA, which agrees with our results. This is likely due to the difference in the mechanisms by which each of these fatty acids regulate the inflammatory mediators at a molecular level. Razzak et al. (2008), demonstrated that when murine macrophages were incubated with lipopolysaccharide (LPS) and a COX-2 inhibitor, NO production increased. However, when the same cells were incubated with EPA or a COX-2 inhibitor and EPA, NO production was minimal. The authors attributed this decrease in NO production to a series of omega-3 fatty acid related, multi-factorial events involving iNOS and COX-2.

When DHA was added in conjunction with LA similar results were found as when EPA was used. When either EPA or DHA was added in conjunction with AA only the 25 DHA treatment was able to reduce NO production; all other treatments produced NO concentrations higher than the control. This suggests that EPA and DHA are not working through the same pathways. EPA, DHA, and LNA are potent inhibitors of COX-2 catalyzed prostaglandin biosynthesis in *in vitro* studies (Ringbom et al., 2001). In activated macrophages the transcription factor NF-κB must be activated for iNOS gene expression. When macrophages were supplemented with DHA the amount of NF-κB binding decreased, thus inhibiting both iNOS expression and NO production (Komatsu et al., 2003). In vascular endothelial cell

cultures, when cells were exposed to 25 µmol/L DHA for 48 h before exposure to 10 ng/mL IL-1α, inhibition of COX-2 increased greater than 50% (Massaro et al., 2006). Following a series of experiments in which endothelial cells were incubated with 25 µmol/L DHA for 48 h the researchers concluded that DHA inhibits COX-2 expression through two mechanisms. First, DHA reduces PKCε activation thereby inhibiting COX-2 and iNOS gene expression. Secondly, DHA scavenges reactive oxygen species preventing the production of H_2O_2, which is necessary for NF-κB activation (Massaro et al., 2006). DHA should interface with IL-1 signaling pathways in a similar manner regardless of tissue type, therefore reduction in NF-κB activation can explain the decrease in both NO and PGE_2 production in DHA treated cell cultures since both iNOS and COX-2 require NF-κB activation.

The ability of EPA and DHA to alter inflammatory mediators in vivo depends highly on the incorporation of these fatty acids into the cell membrane. Dietary omega-3 fatty acids are preferentially incorporated into certain tissues. DHA content in plasma, liver, brain, and other organs is highly correlated to erythrocyte DHA levels (Kuratko and Salem, 2009). A suitable biomarker that correlates with EPA and DHA status in articular tissue has not been identified. At the *in vivo* level the concern would be whether or not sufficient levels of EPA and/or DHA could be obtained in the cartilage to elicit the reduction in inflammatory mediators.

These data provide evidence that both EPA and DHA are able to alter the production of NO and PGE_2 in porcine articular cartilage explants. Further research is necessary to more precisely explain the mechanisms by which EPA is altering PGE_2 production and DHA is altering the production of NO. Additionally, it has yet to be determined if EPA and DHA can be

added to a porcine diet at a concentration that will modulate articular cartilage metabolism without adversely impacting other physiological processes in the animal.

APPENDICES

APPENDIX A. The effect of LNA on inflammatory mediators in cartilage explants

INTRODUCTION

Cartilage explant studies have shown that EPA and DHA can reduce the inflammatory response and the production of NO and PGE_2 when supplemented in a serum free media. It has been shown that LNA is capable of inhibiting COX-2, but it is a less potent inhibitor than EPA and DHA (Ringbom et al., 2001). It is hypothesized that LNA will be able to reduce PGE_2 and NO production, but to a lesser extent than EPA and DHA when supplemented at the same levels. The objectives of this experiment were to determine how the supplementation of LNA to porcine articular cartilage explants *in vitro* would affect the inflammatory response as well as to investigate the mechanisms of action resulting in the reduction of inflammatory mediators present in explant media.

MATERIALS AND METHODS

Explant Cultures - Articular cartilage was isolated from the left and right humeral-ulnar joint of Yorkshire x Landrace cross gilts within 4 h of slaughter. Cartilage disks (6mm in diameter) were biopsied from the articular surface. Only visually normal cartilage was selected for biopsy. Two explant disks were randomly selected and cultured in wells of a 24-well culture plate. Explants were conditioned for 24 h in media containing serum free Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with amino acids, ascorbic acid, and 10% fetal bovine serum (FBS). After 24 h on conditioning media explants were washed twice with phosphate buffered saline (PBS) and then treatment media containing serum free DMEM supplemented with amino acids, ascorbic acid, penicillin-streptocycin, insulin-

transferrin-sodium selenite, thyroxine, and long chain polyunsaturated fatty acids (LCPUFA) was added to each well (Figure 4). At 48 and 72 h 5 ng/mL of recombinant porcine IL-1β (rpIL-1; Invitrogen, Carlsbad, CA) was added to the treatment media (Table 6. Concentrations of fatty acids and rpIL-1 added to treatment media during the LNA explant experiment.). Cartilage explants were maintained in a humidified incubator at 37ºC with 7% CO_2. At 48, 72, and 96 h media were removed from each well and reserved for analysis. Indomethacin (10 µg/ml; Sigma-Aldrich, St. Louis, MO) was added to media samples during collection to prevent metabolism of PGE_2 and stored at -20ºC until analysis. Media were analyzed for proteoglycans, NO, and PGE_2 concentrations.

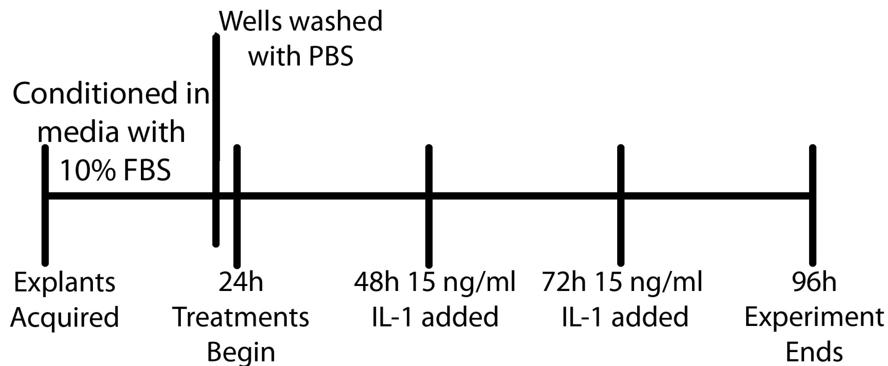

Figure 4. Diagram of explant culture process over 96 h.

Table 6. Concentrations of fatty acids and rpIL-1 added to treatment media during the LNA explant experiment.

Treatment	rpIL-1 (ng/mL)	LA (µg/mL)	EPA (µg/mL)	DHA (µg/mL)	LNA (µg/mL)
100 LA	15	100	0	0	0
25 DHA	15	75	0	25	0
25 EPA	15	75	25	0	0
25 LNA	15	75	0	0	25

Proteoglycan Analysis

Proteoglycan release (PG) into media was measured as described previously using the dimethylmethylene blue assay. Proteoglycan content was determined by measuring sulphated glycosaminoglycan content using a chondroitin sulphate standard. Absorbance was then determined using a plate reader (Spectramax 300, Molecular Devices, Sunnyvale, CA) set at 530 with a correction at 590 nm.

Nitric Oxide Analysis

Nitric oxide was measured indirectly by quantifying nitrite in the media by using the Greiss reaction and a sodium nitrite standard. In brief, 75 µL of media was incubated with 75 µL of 1% sulphanilamide and 0.1% N-1-naphthylethylenediamide dihydrochloride in 25% phosphoric acid in duplicate in a 96-well plate for 5 minutes at room temperature. Absorbance was then determined using a plate reader set at 540 nm.

PGE_2 Analysis

Prostaglandin E_2 was measured in media and synovial fluid using a commercially available ELISA kit (EHPGE2; Thermo-Fisher Scientific, Rockford, IL) following the manufacturer's instructions. Media samples were diluted as needed in the provided assay buffer and analyzed.

Statistical analysis

Statistics were analyzed using the mixed procedure of SAS 9.2 with pig and treatment as the fixed effects. The random statement pig (trt) was used. All data was analyzed as a cumulative response following stimulation with IL-1, such that the response from media collected at 72 h was added to the response from media collected at 96 h. All data will be presented as lsmeans ± SEM. P-values < 0.05 will be discussed as significant while p-values < 0.10 will be discussed as trends.

RESULTS

There was no effect of treatment on PG release from the explants ($P = 0.69$; Figure 5) however there was a trend for a treatment effect on NO release ($P = 0.06$; Figure 6). The 25 DHA and 25 LNA treatments released less NO than either the 100 LA or 25 EPA treatment. PGE_2 production was significantly higher ($P < 0.05$) in the 100LA treatment than any other treatment. The 25 EPA treatment tended to produce less PGE_2 than the 25 LNA treatment, but was not different than the 25 DHA treatment (Figure 7).

DISCUSSION AND CONCLUSION

Results from this study show that EPA is slightly better than DHA and LNA at reducing PGE_2 production *in vitro*. This study produced NO levels that were quite comparable to the other explant studies described in this dissertation. There is little information on the effect of LNA on cartilage explants *in vitro*; however, the fatty acid compounds have been tested for their selectivity and inhibitory effect on COX-2. Researchers found that alpha-LNA is a less potent inhibitor of COX-2 than EPA and DHA, but it is more highly selective of COX-2 (Ringbom et al., 2001). Data from this experiment would concur with previous findings as LNA decreased PGE_2 concentrations to the same level as EPA and DHA.

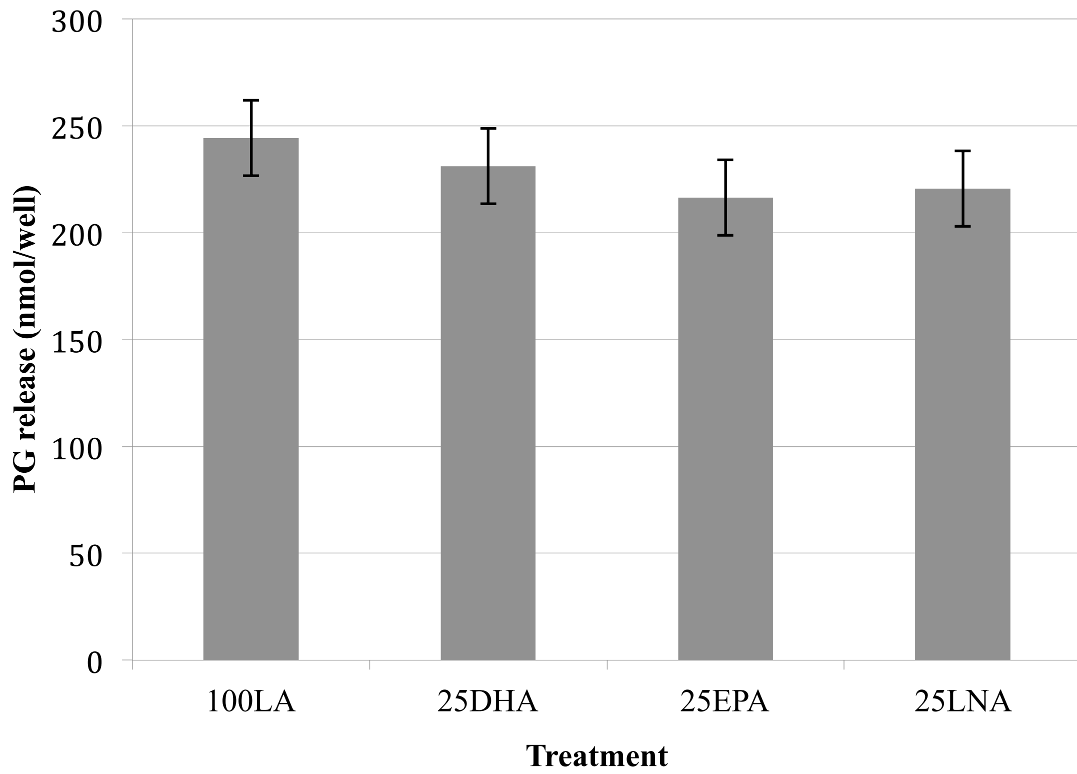

Figure 5. Cumulative PG release from cartilage into media for the LNA experiment when explants were treated with 25 µg/mL of EPA, DHA, or LNA (Table 6). There was no effect of treatment P = 0.69 on PG release.

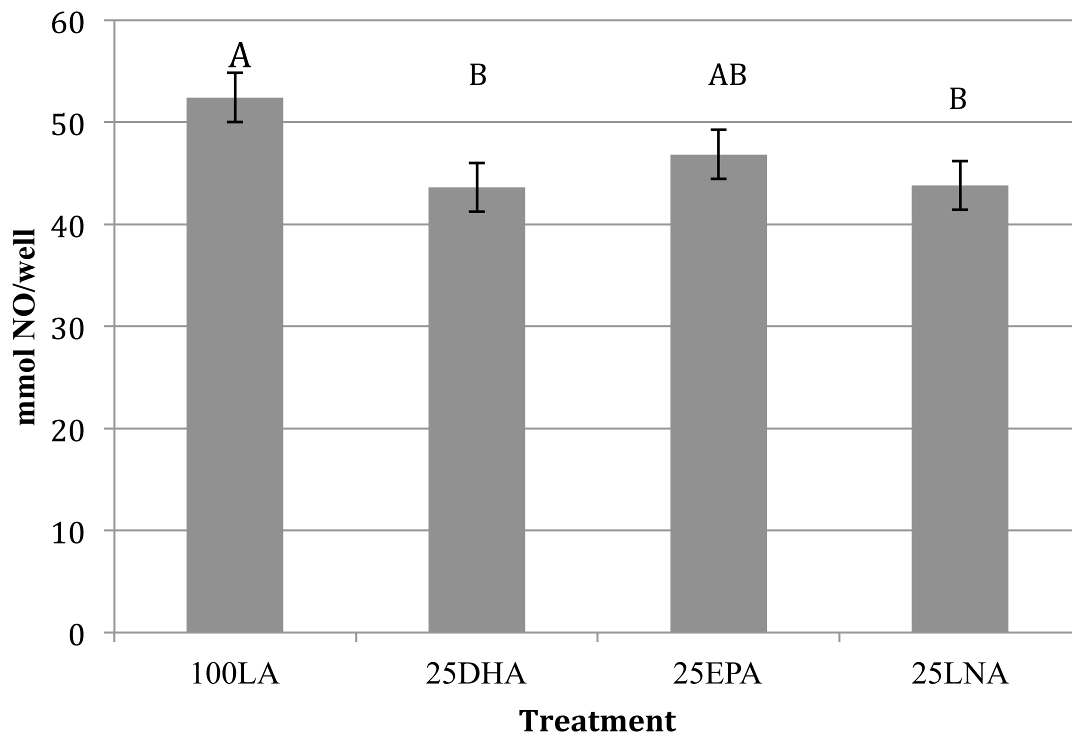

Figure 6. Cumulative NO release from cartilage into media for LNA experiment when explants were treated with 25 µg/mL of EPA, DHA or LNA (Table 6). There was a trend for an overall treatment effect P = 0.06.

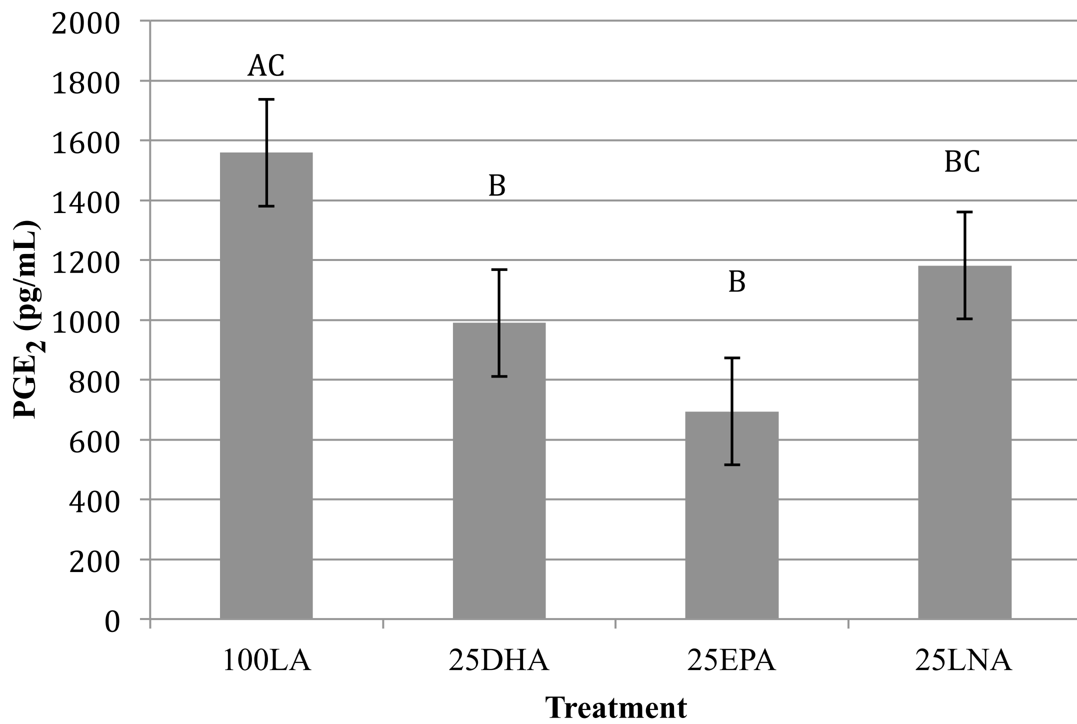

Figure 7. Cumulative PGE_2 release from cartilage into media for LNA experiment when explants were treated with 25 µg/mL of EPA, DHA or LNA (Table 6). There was an overall treatment effect $P < 0.05$.

APPENDIX B. Experimental methods for obtaining and maintaining porcine chondrocyte monolayers in culture

INTRODUCTION

No one has used porcine cartilage to study the effects of PUFA on gene regulation in chondrocytes. The objectives of the present study were 1) to repeatedly obtain confluent porcine chondrocyte monolayers that remain healthy for 10 d, 2) to determine an ideal level of rpIL-1 to stimulate cartilage degradation, and 3) to find and test primers for COX-2 and iNOS in swine, 4) to extract RNA from the chondrocytes and run PCR to determine the expression of COX-2 and iNOS under various PUFA treatments.

MATERIALS AND METHODS

Obtaining Cells

Front legs of market-aged gilts were obtained from the MSU Meat Laboratory. The bulk of the muscle was removed from around the humeral-ulnar joint. The hoof was placed in a plastic bag to prevent contamination in the hood. Then, the leg was moved into the dissection hood and the remaining soft tissue was removed from around the joint and the humeral-ulnar joint was opened to expose the two articular surfaces. Cartilage was carefully harvested from the proximal ulna and radius and the distal humerus using a #10 scalpel. Cartilage was placed into a petri dish containing PBS with pen-strep. Cartilage was washed twice in PBS containing 100 units/mL penicillin-streptomycin (Invitrogen, Carlsbad, CA) and allowed to rest for 30 min. Then, 200 mg of pronase (Roche Diagnostics, Indianapolis, IN) was weighed out and mixed into 20 ml of serum free Dulbecco's modified Eagle's medium: nutrient mixture F-12 (Ham)

(DMEM:F12, Invitrogen, Carlsbad, CA) containing a complete profile of amino acids and sterile filtered. Following the rest period, PBS was drained from the petri dish and the cartilage was added to the pronase solution in a 500 ml sterile bottle. The bottle was placed into an agitating hot water bath at 37°C for 1 h. During the pronase incubation a collagenase (Worthington Biochemical, Lakewood, NJ) solution was prepared containing 50 mg collagenase in 50 ml serum free DMEM-F12 media containing a complete profile of amino acids and sterile filtered. Following the 1 h incubation the cartilage was washed twice in PBS and then added to the collagenase solution and placed into the agitating hot water bath at 37°C for 18 to 24 h.

After the incubation ended the solution was filtered through 20 µm cell strainers into 50 ml conical tubes. Conicals were spun at 2000 rpm for 10 min at 25°C to pellet the cells. Supernatant was discarded and 25 mL of sterile PBS was added and the conical was agitated gently to thoroughly mix the cells. Conicals were spun at 2000 rpm for 10 min at 25°C. This wash was performed twice. Following the second wash 1 mL of PBS was added to one conical to suspend the cells for transfer to the second conical. Then, 2 mL of PBS was added to bring the volume up to 3 ml. Cells were thoroughly mixed to prepare for cell counting.

Counting Cells

In order to visualize the dead cells 40 µL of trypan blue stain (Sigma-Aldrich, St. Louis, MO) was to 10 µL of cells, mixed and allowed to sit for 10 min to allow cells to stain (1:5 dilution factor). Then, 10 µL of cells were pipeted onto one side of a hemocytometer. Cells were counted in 5 of the 1 mm grid boxes and totaled. If the total was less than 100 then additional boxes were counted to obtain total cell count of > 100. The number of dead cells was taken into consideration when preforming cell count calculations. Cell counts were calculated as follows: total of all 5 boxes counted divided by 0.02 equals cells/mm^3. Then, multiply

cells/mm^3 x 1000 mm^3/ml to get number of cells and then multiply by the dilution factor of 5 to get total number of cells in 3 ml of PBS. The cell solution was diluted to a concentration of 12 x 10^6 cells/ml.

Plating Cells

Media was made containing DMEM:F12 media (Invitrogen, Carlsbad, CA) with 100 units/mL penicillin-streptomycin (Invitrogen, Carlsbad, CA), 3% FBS, 1% ascorbic acid, 1% glutamine, 0.3% insulin-transferrin-sodium selenite (Roche Applied Science, Indianapolis, IN), and 0.36% thyroxine (Sigma-Aldrich, St. Louis, MO).

Each well in the 24-well plate received 1 mL media and 6 x 10^6 cells were plated in each well of plate 1 (d 0). Media were changed d 4 and media was made as described on d 0 except it only had 2% FBS. Media were changed by carefully removing 1 mL and replacing it with 1 mL of fresh media. On d 7, seven different treatments were made with 0% FBS: + control, - control, 30 µg LA/mL, 30 µg EPA/mL, 20 µg EPA/ml + 20 µg DHA/mL, 30 µg EPA/mL + 30 µg DHA/ml, and 30 µg DHA/ml. Media were changed by removing 1 mL and replacing it with 1 mL of treatment media. On d 10, new treatment media were made without fatty acids that contained 50 ng/mL of rpIL then, 1 mL of media were removed from each well and reserved for NO analysis and 1 mL of treatment media was added. Eight hours later media were removed from each well and saved for NO analysis. The cells were washed twice with sterile PBS and then instructions provided with the Qiagen RNase Protect Mini Kit (Qiagen, Hilden, Germany) were followed to extract RNA directly from the cell cultures. Once RNA was extracted the tubes were frozen at -80. The quantity of RNA was determined by absorbance readings at 260 and 280 nm using a Nanodrop (NanoDrop Technologies, Wilmington, DE).

BSA Conjugated Fatty Acids

Three fatty acids, LA, EPA, and DHA (Nu Chek Prep, Elysian, MN), were conjugated to fatty acid free BSA (Sigma-Aldrich, St. Louis, MO) to make stock solutions for the treatment media (Personal Communications with Jim Leisman). In order to accomplish this 50 mg of the fatty acid was dissolved in 1 ml of a 3:2 hexane:isopropanol solution. Then, 10 mL of 0.1 N NaOH was added to the solution and mixed. The solution was heated until the fatty acid is completely dissolved in the NaOH layer. Then, the hexane:isopropanol was evaporated off with nitrogen gas and heat (70°C). While the fatty acid solution was kept warm it was brought up to a volume of 10 mL with 0.1 N NaOH. Then, 300 mg of fatty acid free BSA were dissolved into 6 mL water and 1 mL of the fatty acid solution was slowly added to the BSA solution to create a 1.67 mg/mL solution of BSA conjugated fatty acid. The fatty acid solutions were then analyzed by gas chromatograph according to methods described in chapter 3. Analysis determined that samples contained 97% of either LA, EPA or DHA .

PCR

Total RNA was extracted using a commercially available kit (Qiagen RNeasy Mini Kit; Qiagen, Hilden, Germany), according to the manufacturer's instructions. The concentration and purity of the RNA was analyzed using the Nanodrop (NanoDrop Technologies, Wilmington, DE). Complementary DNA (cDNA) were obtained by reverse transcription (RT) of 500 ng of total RNA, with the high capacity cDNA reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. For each reaction, a total volume of 15 µL was used, which consisted of 3 µL of diluted cDNA (5 ng/ µL of RNA), 7.5 µL of Power SYBR Green mastermix (Applied Biosystems, Inc., CA, USA), 4.05 µL of dH2O, and 0.45 µL of gene specific primer mix. Custom primers (Invitrogen, Carlsbad, CA, USA) were used to probe for

target mRNA including GAPDH (CGTCCCTGAGACACGATGGT), β-actin (GATCGTGCGGGACATCAAG), iNOS (CAGCGGGATGACTTTCCAA), and COX-2 (CCGACAGCCAAAGACACTCA).

mRNA data were normalized relative to β-actin and GAPDH and then used to calculate expression levels. The comparative Ct method was used to measure the gene transcription in samples. Results are expressed as relative units based on calculation of 2^DDCt, which gives the relative amount of target gene normalized to endogenous control (β-actin and GAPDH) and to the control (untreated) samples with the expression set as 1. Negative controls were either RT without enzyme or PCR without cDNA template.

RESULTS

This experiment was conducted to determine if supplementation of PUFA would alter the expression of COX-2 and iNOS in chondrocyte monolayers when stimulated with 50 ng/mL of rpIL-1. All wells were lysed and processed to obtain RNA, however upon analysis by nanodrop it was discovered that none of the wells yielded enough quality RNA to proceed to cDNA. As mentioned previously, the lysis buffer may not have been strong enough to lyse the chondrocytes.

PCR Results

Seven RNA samples representing three studies were of sufficient quality to advance to PCR. Figure 8 illustrates the results relative fold change for iNOS gene expression. Gene expression was also analyzed for COX-2; however, insufficient expression of the gene was found. Samples for 0 IL, 8 IL, and 33 IL were collected on the same experiment but the IL-1 may have degraded since the time of its purchase. The 25 IL sample was a product of

experiment 6 and new IL-1 was used for that experiment and experiment 8. The DHA + 50 IL-1 and LA + 50 IL-1 were from experiment 8 and although only 2 samples were analyzed at least the treatments vary in their expression of iNOS. These data suggest that further research could yield some interesting results.

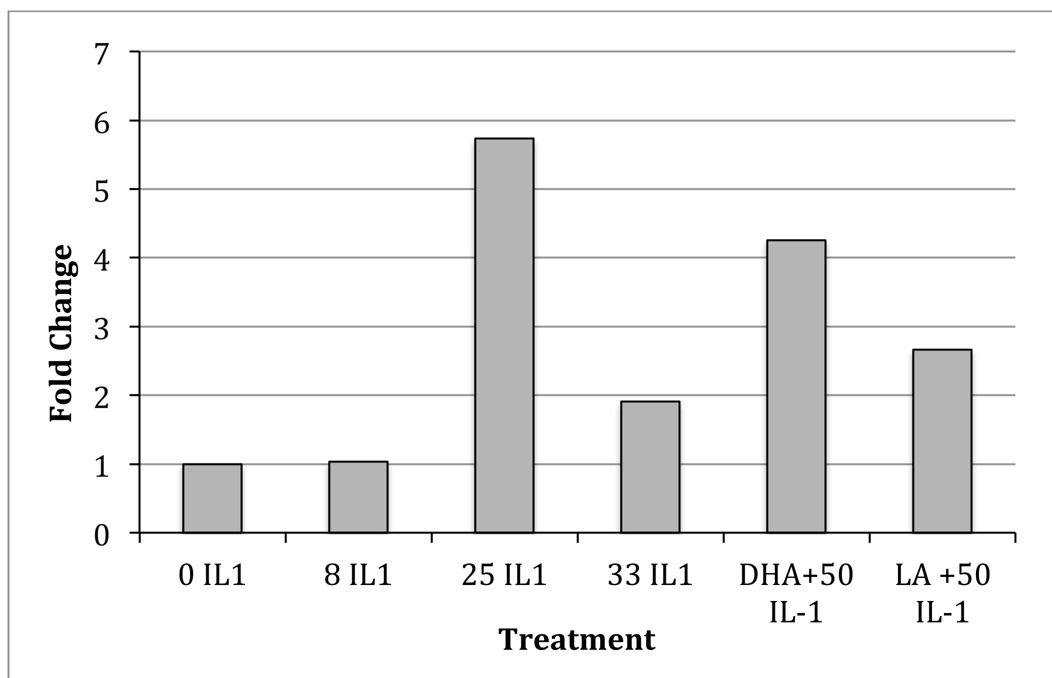

Figure 8. Fold change for iNOS calculated using the -ΔΔCT method with both β-actin and GAPDH as the reference genes.

CONCLUSION

This study involved a substantial amount of troubleshooting; however after a considerable amount of failure and some success a reliable method of obtaining chondrocytes and growing monolayers was developed. With additional time and resources an ideal lysis buffer could be found to guarantee a higher concentration and quality of RNA. cDNA was successfully made from a few tubes of RNA and primers for the pig were identified and tested for GAPDH, beta-actin, iNOS, and COX-2. PCR results demonstrate that iNOS does alter expression in relation to IL-1 concentration and potentially fatty acid concentration. The next step in this line of research would be to determine a reliable lysis buffer for chondrocyte monolayers.

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CHAPTER 3. The impact of dietary long chain polyunsaturated fatty acids on bone and cartilage in gilts and sows

INTRODUCTION

Lameness has been identified as a key problem in the swine industry and joint lesions have been identified as one of the main reasons for culling sows (Kirk et al., 2005). Lame sows are 1.7 times more likely to be removed from the herd within one year, and pain associated with lameness may adversely affect reproductive performance (Anil et al., 2009). Dietary long chain polyunsaturated fatty acids (PUFA) including arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can regulate the production of inflammatory mediators. Lipids also have an important role in the regulation of skeletal biology and promotion of bone health, which is supported by significant evidence from both animal and human research (Watkins et al., 2001). Piglets fed formula containing 0.1% wt/wt DHA had significantly higher bone mineral content and weighed more on d 15 than suckled piglets (Weiler and Fitzpatrick-Wong, 2002). Few studies have looked at how dietary fatty acids can alter the fatty acid profile in cartilage; however, higher dietary concentrations of DHA have been associated with reduced severity of patellofemoral cartilage loss in humans (Baker et al., 2012).

The goal of our study was to determine if dietary PUFA would improve indicators of joint health in swine. The first objective of this study was to characterize the effects of dietary PUFA on cartilage, synovial fluid, and bone density and morphology from multiparous sows and market weight gilts of similar genetics. The second objective was to examine the effects of dietary PUFA on the response of cartilage explants to interleukin-1 (IL-1) stimulation *in vitro.* We hypothesized that dietary PUFA supplementation would increase cortical bone density and

EPA and DHA incorporation into cartilage and synovial fluid. Therefore, we anticipated that cartilage from sows and gilts fed the PUFA supplement would exhibit decreased release of inflammatory mediators into media.

MATERIALS AND METHODS

All animal experiments were conducted at the JBS United research facilities in Frankfort, IN. All procedures were approved by the JBS United Animal Care and Use Committee per corporate policy and adhered to the ethical and humane use of animals for research. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

Experiment 1 - Sows

Fourteen sows were maintained on either a basal corn/soybean diet (no added fat) that served as the control diet (CON; n = 7) and met NRC requirements for all nutrients, or the CON diet supplemented with 1.0% protected fish oil (PUFA; n = 7; Gromega 365; JBS United, Sheridan, IN). Sows were fed their respective diets (Table 7) for an average of 24.5 mo and were slaughtered at an average age of 43 mo of age. The protected fish oil contained 29% total fat, of which 13% was EPA and 15% DHA (Table 8). The remaining ingredients were protein and carbohydrates.

Experiment 2 - Gilts

Sixteen gilts were maintained on either a basal corn/soybean diet (CON; n = 8) or the CON diet supplemented with protected fish oil (PUFA; n = 8; Gromega 365; JBS United, Sheridan, IN). The PUFA diet was formulated to a 10:1 n-6:n-3 fatty acid ratio, which contained 1.5 to 2.3% PUFA and increased as the growth phases progressed. Treatments were matched to constant digestible lysine/metabolizable energy ratios within each growth phase. The protected

fish oil replaced both corn and soybean meal to meet the study ratios of the omega-6:omega-3 fatty acid ratio. Diet composition for the last growth phase is presented in Table 9. Gilts were fed their respective treatments from weaning until slaughter (BW 111 kg).

Experiment 3: Explant Cultures

During the slaughter process both front legs were removed from each animal mid-humerus. The left and right humeral-ulnar joints were opened aseptically under sterile conditions, and 30 cartilage disks were harvested with a 6 mm biopsy punch (Miltex, York, PA) from the weight-bearing region of the articular surface of each pig within 30 h of slaughter for sows and 12 h of slaughter for gilts. Samples from each pig were cultured separately in 12 wells of a 24-well culture plate by placing randomly selected explant disks (2/well) into each of 12 wells. Cartilage explants were cultured in 1 mL of serum-free Dulbecco's modified Eagle's medium: nutrient mixture F-12 (Ham) (DMEM:F12, Invitrogen, Carlsbad, CA) supplemented with amino acids (Rossalot et al., 1992), 50 μg/mL ascorbate, 100 units/mL penicillin-streptomycin (Invitrogen, Carlsbad, CA), 1 μL/mL insulin-transferrin-sodium selenite supplement (Roche Applied Science, Mannheim, Germany), and 0.02 μg/mL thyroxine.

The explants were maintained in culture in a humidified incubator at 37ºC with 7% CO_2. Explants were maintained in media for 24 h prior to the first of 2 d of treatment. Conditioned media were removed and replaced daily. Indomethacin (10 μg/ml) was added to media samples during collection to prevent metabolism of PGE_2 and samples were stored at -20ºC until analyzed for indicators of inflammation. To evaluate the effects of dietary PUFA on cartilage fatty acid profiles, 10 ng/mL recombinant porcine IL-1β (R&D Systems, Minneapolis, MN) was added to induce cartilage degradation during media replacement at 24 and 48 h. At 48 and 72 h of culture, media were collected from each well and reserved for analyses.

Table 7. Ingredients and nutrient composition (as-is basis) of gestation and lactation diets fed to sows throughout Experiment 1 for sows fed either a basal corn/soybean diet (CON) or the basal diet supplemented with protected fish oil (PUFA).

	Gestation		Lactation	
	CON	PUFA	CON	PUFA
Ingredient %				
Corn	75.69	75.69	64.96	64.96
Soybean Meal, 48%	18.66	18.66	27.74	27.74
Premix	4.65[2]	4.65	6.30	6.30
Corn Starch	1.00	--	1.00	--
Gromega365[1]	--	1.00	--	1.00
Total	100	100	100	100
Calculated nutrient content, %				
Crude Fat	3.56	3.78	3.45	3.66
Crude Protein	15.17	15.26	19.09	19.18
Starch	48.86	48.35	42.75	42.25
Lysine	0.75	0.75	1.10	1.10
Phosphorus	0.77	0.77	0.81	0.81
Calcium	0.88	0.88	0.91	0.91
Metabolizable energy, MJ/kg	13.68	13.68	13.56	13.60

[1] Protected EPA plus DHA containing 3.04 g EPA/100 g and 3.32 g DHA/100 g

[2] The premix provided per kg of diet: 662 mg of choline as choline chloride; 3.35 mg of retinyl acetate; 0.06 mg of cholecalciferol; 66 mg of vitamin E as a-tocopherol acetate; 1.4 mg of vitamin K as menadione dimethypyrimidinol bisulfate; 0.44 mg of biotin; 44 mg of niacin; 24 mg of pantothenic acid; 7 mg of riboflavin; 0.03 mg of vitamin B-12; 1.61 mg of folic acid; 0.25 mg of pyridoxine as pyridoxine HCl; 0.48 mg of thiamine; P, 0.43% as monocalcium phosphate; Ca, 0.80% as calcium carbonate; Na, 0.18% as sodium chloride; K, 0.25% as potassium chloride; Mg, 0.02% magnesium; Cu, 10 mg as copper sulfate; Fe, 125 mg as iron sulfate; I, 1.26 mg as potassium iodate; Mn, 60 mg as manganese sulfate; Se, 0.3 mg as sodium selenite; and Zn, 125 mg as zinc sulfate.

[3] Premix provided per kg of diet: 662 mg of choline as choline chloride; 3.35 mg of retinyl acetate; 0.06 mg of cholecalciferol; 66 mg of vitamin E as a-tocopherol acetate; 1.4 mg of vitamin K as menadione dimethypyrimidinol bisulfate; 0.44 mg of biotin; 44 mg of niacin; 24 mg of pantothenic acid; 7 mg of riboflavin; 0.03 mg of vitamin B-12; 1.59 mg of folic acid; 0.25 mg of pyridoxine as pyridoxine HCl; 0.48 mg of thiamine; P, 0.43% as monocalcium phosphate; Ca, 1.0% as calcium carbonate; Na, 0.21% as sodium chloride; K, 0.37% as potassium chloride; Mg, 0.06% magnesium; Cu, 10 mg as copper sulfate; Fe, 136 mg as iron sulfate; I, 1.26 mg as potassium iodate; Mn, 60 mg as manganese sulfate; Se, 0.3 mg as sodium selenite; Zn, 125 mg as zinc sulfate; and 0.08% Lys as lysine HCL.

Table 8. Fatty acid composition of the PUFA supplement as a percentage of the lipid.

Fatty Acid	PUFA[1] Supplement (% of total lipid)
14:0	6.77
16:0	16.93
16:1	9.3
18:0	3.35
18:1	10.68
18:2 (n-6)	4.91
18:3 (n-3)	2.12
20:0	0.13
20:1	0.94
20:4 (n-6)	1.22
20:5 (n-3)	13.46
22:5 (n-3)	2.95
22:6 (n-3)	14.72
Total n-3	33.24
Total n-6	6.13
(n-6)/(n-3)	0.18

[1]Supplement contained 22.3% fat

Table 9. Ingredients and nutrient composition (as-is basis) for the final growth phase fed to gilts during Experiment 2 for gilts fed either a basal corn/soybean diet (CON) or the basal diet supplemented with protected fish oil (PUFA).

	CON	PUFA
Ingredient %		
Corn	84.28	82.90
Soybean Meal, 48%	13.57	12.67
Premix	2.15	2.15
Gromega365[1]	--	2.28
Total	100	100
Calculated nutrient content, %		
Crude Fat	3.72	3.68
Crude Protein	13.74	13.50
Lysine	0.73	0.72
Phosphorus	0.38	0.37
Calcium	0.44	0.45
Metabolizable energy, MJ/kg		
n-6 to n-3 PUFA Ratio	10	10

[1] Premix provided per kg of diet: 882 mg of choline as choline chloride; 40 KIU of retinyl acetate; 0.9 KIU of cholecalciferol; 41 mg of vitamin E as a-tocopherol acetate; 0.5 mg of vitamin K as menadione dimethypyrimidinol bisulfate; 0.1 mg of biotin; 55 mg of niacin; 20 mg of pantothenic acid; 6 mg of riboflavin; 0..02 mg of vitamin B-12; 0.35 mg of folic acid; P, 0.37% as monocalcium phosphate; Ca, 0.45% as calcium carbonate; Na, 0.19% as sodium chloride; K, 0.63% as potassium chloride; Mg, 0.16% magnesium; Cu, 14 mg as copper sulfate; Fe, 164 mg as iron sulfate; I, 0.92 mg as potassium iodate; Mn, 48 mg as manganese sulfate; Se, 0.3 mg as sodium selenite; Zn, 109 mg as zinc sulfate; and 0.72% Lys as lysine HCL.

[2] Protected EPA plus DHA containing 3.04 g EPA/100 g and 3.32 g DHA/100 g

Synovial Fluid

Synovial fluid was collected post-mortem from the right carpal joint of each sow and from the right and left carpal joints of each gilt using a sterile 20 G, 1.5 in needle on a 5 cc syringe prior to opening the joint. Indomethacin (10 µg/ml) was added to the synovial fluid from each joint to prevent metabolism of PGE_2 and stored at -20ºC until analysis.

Fatty Acid Analysis

Articular cartilage was isolated from the left and right humeral-ulnar joints of sows within 30 h of slaughter and gilts within 12 h of slaughter. Cartilage was stored at -20ºC until analyzed. Approximately 0.5 g of cartilage was pulverized to a fine powder in an SPEX 6750 freezer mill (Sample Prep, Metuchen, NJ) and extracted for fatty acid analysis. Synovial fluid was obtained from the humeral-ulnar joints post-mortem as described above. The extraction method was a modification of the Folch method (Folch et al., 1957). Cartilage or 1 mL of synovial fluid was extracted with chloroform (EM Science, Gibbstown, NJ), methanol, and water (2:1:1, v/v), and vortexed for 15 min. The chloroform layer was removed and then evaporated with the use of a RapidVac (7910010; Labconco, Kansas City, KS). The lipid was reconstituted with 2 ml hexane (Sigma-Aldrich, St. Louis, MO) and 1 ml of internal standard (C19:0; Nu Chek Prep, Elysian, MN) in hexane and methylated. Samples were analyzed on a Clarus 500 gas chromatograph (Perkin and Elmer, Waltham, MA) with a 100 m column with sp2560 wax coating (Supelco, St. Louis, MO) and analyzed using Empower software (Waters, Milford, MA).

Bone Morphology

After cartilage was removed from the humeral-ulnar joint, the right fused radius/ulna from each pig was cleaned of muscle and connective tissue and stored at -20ºC until analysis via computed tomography (CT; GE Healthcare, Waukesha, WI). Prior to analysis each bone was

placed onto a QCT phantom (Image Analysis, Inc., Columbia, KY) that would serve as the standard curve for calculating the density of the bone. Cortical density was determined at the proximal ulna, central radius, and distal radius using Mimics software (Materialise, Plymouth, MI). Trabecular density was determined at the distal radius. Cortical width was measured at the proximal ulna and central and distal radius using Mimics software (Materialise, Plymouth, MI).

Proteoglycan Analysis

Proteoglycan (PG) release into media was measured using the dimethylmethylene blue assay (Chandrasekhar et al., 1987). Proteoglycan content was determined by measuring sulfated glycosaminoglycan content using a chondroitin sulfate standard and expressed as μg PG/well. Absorbance at 530 nm with a correction at 590 nm was determined using a Spectramax 300 plate reader (Molecular Devices, Sunnyvale, CA). Sample concentration of PG was determined using a linear standard curve.

Nitric Oxide Analysis

Nitric oxide was measured indirectly by quantifying nitrite, a stable end-product of nitric oxide metabolism, in the media using the Greiss reaction and a sodium nitrite standard (Fenton et al., 2000). Absorbance at 540 nm was determined using a Spectramax 300 plate reader (Molecular Devices, Sunnyvale, CA). Results are expressed as μM of NO/well.

Prostaglandin E_2 Analysis

Prostaglandin E_2 was measured in media and synovial fluid using a commercially available ELISA kit (EHPGE2; Thermo-Fisher Scientific, Rockford, IL). Synovial fluid was digested with 50 U/mL of hyaluronidase from bovine testes at 37°C for 40 min to reduce the viscosity of the sample. Absorbance at 405 nm with a correction at 580 nm was determined using a Spectramax 300 plate reader (Molecular Devices, Sunnyvale, CA).

Interleukin-6 Analysis

Interleukin-6 was measured using a porcine-specific, commercially available ELISA kit (Porcine IL-6 Duoset; DY686, R & D Systems, Minneapolis, MN) following the manufacturer's instructions. Absorbance at 450 nm with a correction at 570 nm was determined using a Spectramax 300 plate reader (Molecular Devices, Sunnyvale, CA).

Statistical Analysis

Data for the *in vitro* experiments were analyzed as a cumulative response following stimulation with IL-1, such that the response from media collected at 48 h was added to the response from media collected at 72 h. Data were analyzed using the mixed procedure of SAS (Version 9.2; SAS Inst. Inc., Cary, NC) with pig, treatment, and diet in the model. Two random statements were used: pig*diet, and pig*diet*treatment. Data from the *in vitro* experiments are presented as lsmeans ± SEM.

Data for cartilage and synovial fluid fatty acid analysis and bone morphology were also analyzed using the mixed procedure of SAS. Each age group was analyzed separately with diet in the model. A random statement was included with pig nested in diet. Data are presented as lsmeans ± SEM. Differences are considered significant at $P < 0.05$ and tendencies at $P < 0.10$.

RESULTS

Cartilage Fatty Acids

Dietary fatty acids did not greatly alter the fatty acid composition of the cartilage (Table 10). The DHA concentration increased in response to increasing dietary supply ($P < 0.01$) in both the sows and gilts. Additionally, sows fed the PUFA supplement had a decrease in C20:1 ($P = 0.065$) and the omega-6:omega-3 ratio ($P < 0.05$). Gilts fed the PUFA supplement had

increased DHA ($P < 0.01$) and C22:5 (DPA; $P < 0.01$). Additionally, C22:1 ($P = 0.057$), and EPA ($P < 0.10$) tended to increase in the cartilage of PUFA-supplemented gilts.

Synovial Fluid Fatty Acids

Dietary fatty acids did not greatly alter the fatty acid composition of the synovial fluid (Table 11). Gilts fed the PUFA supplement had the same concentration of fatty acids in their synovial fluid as gilts fed the CON diet, with a tendency for an increase in C22:5 ($P < 0.10$). PUFA-supplemented sows also had increased concentrations of EPA ($P < 0.05$), C22:5 ($P < 0.05$) and DHA ($P < 0.01$). This resulted in an increase in the percentage of total omega-3 ($P < 0.01$) and a trend for a decrease in the ratio of omega-6 to omega-3 ($P < 0.10$).

Synovial Fluid

Synovial fluid from the right carpal joint of PUFA supplemented sows tended to have higher concentrations of PGE_2 ($P < 0.10$; Figure 9) than CON sows. Synovial fluid from the right humeral-ulnar joints of gilts showed no effect of diet on PGE_2 concentration ($P = 0.61$; Figure 10).

Bone

The CT scans of the radius/ulna from gilts revealed no differences for cortical width and bone density. Sows fed the PUFA supplement had greater cortical width of the proximal ulna (2.0 vs 1.5 ± 0.13 mm; $P < 0.05$) and decreased cortical width of the distal radius (2.2 vs 2.7 ± 0.13 mm; $P < 0.05$) than CON sows. However, no differences in cortical or trabecular bone density were detected for the sows (Table 12).

In vitro Experiments

Cartilage explants from sows treated with IL-1 increased the release of proteoglycans, NO, PGE_2, and IL-6 into the media ($P < 0.05$; Table 13) when compared with the control

Table 10. Percentages of fatty acids in the cartilage of sows and gilts fed either a basal corn/soybean diet (CON) or the basal diet supplemented with protected fish oil (PUFA). The corresponding p-values for the effects of diet within age are reported.

	Sow		Gilt	
Fatty Acid, %	CON	PUFA	CON	PUFA
C14:0	2.15 ± 0.16	1.80 ± 0.18	1.91 ± 0.13†	2.34 ± 0.17
C14:1	3.12 ± 0.66	3.13 ± 0.71	1.62 ± 0.20	1.26 ± 0.25
C16:0	25.30 ± 0.73	26.47 ± 0.79	20.58 ± 0.35	20.25 ± 0.45
C16:1	2.58 ± 0.21	2.55 ± 0.22	4.98 ± 0.30	4.84 ± 0.39
C18:0	11.47 ± 0.82	12.75 ± 0.88	12.93 ± 0.21	12.97 ± 0.26
C18:1	25.96 ± 2.0	23.71 ± 2.2	30.04 ± 0.71	29.53 ± 0.92
C18:2 n6	4.06 ± 0.39	3.71 ± 0.42	3.31 ± 0.16	3.13 ± 0.21
C18:3 n3	0.51 ± 0.12	0.63 ± 0.13	0.34 ± 0.03	0.29 ± 0.03
C18:3 n6	3.70 ± 0.35	3.33 ± 0.33	1.47 ± 0.09	1.38 ± 0.11
C20:4 AA	3.11 ± 0.19	2.88 ± 0.20	4.54 ± 0.19	4.71 ± 0.24
C20:5 EPA	0.30 ± 0.08	0.32 ± 0.09	0.26 ± 0.04†	0.36 ± 0.05
C22:5	0.21 ± 0.03	0.27 ± 0.03	0.33 ± 0.04**	0.55 ± 0.05
C22:6 DHA	0.09 ± 0.02**	0.22 ± 0.03	0.17 ± 0.03**	0.36 ± 0.03
Total n3	2.88 ± 0.31	3.54 ± 0.34	2.78 ± 0.25	2.93 ± 0.33
Total n6	13.86 ± 0.61	12.82 ± 0.66	14.18 ± 0.37	14.06 ± 0.47
n6:n3 ratio	5.07 ± 0.40*	3.76 ± 0.43	5.49 ± 0.46	5.06 ± 0.60

** Difference between treatments for that age $P < 0.01$.

*Difference between treatments for that age $P < 0.05$.

†Difference between treatments for that age $P < 0.10$.

Table 11. Percentages of fatty acids in the synovial fluid of sows and gilts fed either a basal corn/soybean diet (CON) or the basal diet supplemented with protected fish oil (PUFA). The ccorresponding p-values for the effects of age within diet are reported.

	Sow		Gilt	
Fatty Acid, %	CON	PUFA	CON	PUFA
C14:0	1.32 ± 0.09†	0.97 ± 0.13	1.50 ± 0.20	1.16 ± 0.28
C14:1	8.73 ± 0.98	8.80 ± 1.4	6.84 ± 1.6	8.63 ± 2.4
C16:0	17.2 ± 0.80	15.1 ± 1.1	18.7 ± 0.70	19.0 ± 1.1
C16:1	1.25 ± 0.21	1.76 ± 0.29	1.05 ± 0.11	1.14 ± 0.16
C18:0	11.3 ± 0.48	11.3 ± 0.68	12.3 ± 0.36	11.6 ± 0.55
C18:1	28.4 ± 2.2	23.6 ± 3.2	20.6 ± 1.2	21.7 ± 1.9
C18:2 n6	10.7 ± 0.41	10.8 ± 0.58	12.7 ± 1.2	12.2 ± 1.8
C18:3 n3	0.06 ± 0.02	0.14 ± 0.04	0.05 ± 0.04	0.17 ± 0.06
C18:3 n6	1.54 ± 0.23	1.94 ± 0.33	1.90 ± 0.21	1.28 ± 0.32
C20:4 AA	4.70 ± 0.63	6.17 ± 0.89	4.97 ± 0.56	3.46 ± 0.86
C20:5 EPA	0 ± 0.03*	0.19 ± 0.05	0.10 ± 0.10	0.29 ± 0.15
C22:5	0.75 ± 0.15*	1.53 ± 0.22	1.16 ± 0.20†	1.92 ± 0.31
C22:6 DHA	0.21 ± 0.07**	0.72 ± 0.11	1.07 ± 0.25	1.30 ± 0.40
Total n3	1.50 ± 0.20**	3.11 ± 0.28	3.05 ± 0.49	4.28 ± 0.75
Total n6	18.3 ± 0.90†	21.3 ± 1.3	21.1 ± 1.7	17.8 ± 2.7
n6:n3 ratio	13.8 ± 1.8†	6.86 ± 2.6	8.25 ± 1.4	4.2 ± 2.1

** Difference between treatments for that age P < 0.01.
*Difference between treatments for that age P < 0.05.
†Difference between treatments for that age P < 0.10.

treatment. No effect of diet or diet by treatment was found for these variables in cartilage from the sow ($P > 0.50$, $P > 0.50$).

Treatment of cartilage explants from gilts with rpIL-1 increased proteoglycan, NO, PGE_2, and IL-6 released into the media ($P < 0.05$) compared with the control treatment (Table 14). However, no effect of diet or diet by treatment was found for these variables in ($P > 0.50$).

DISCUSSION

Dietary PUFA treatment increased the concentration of DHA in the cartilage of sows and gilts. In the sows, DHA increased 2.4 fold, but DHA only accounted for 0.22% of the total fatty acids. Due to their low turnover rate, articular chondrocytes may have a decreased rate of incorporation of fatty acids into their phospholipids. Research using fluorescently-labeled lauric acid has shown that fatty acids are transported through the cartilage matrix at a low, but measurable, rate (Arkill and Winlove, 2006). Little research has been conducted to examine the effects of omega-3 fatty acid supplementation on cartilage. Rats fed diets containing 10% menhaden fish oil (much higher than in our study) demonstrated a 70% decrease in the concentration of LA and AA in articular cartilage and a 32% inhibition of proteoglycan synthesis (Lippiello et al., 1990). In other studies where omega-3 fatty acids were supplemented, significant amounts of EPA and DHA were found to be incorporated into skeletal muscle (Ayre and Hulbert, 1996; Stubbs and Kisielewski, 1990), as well as epidermal (Fischer and Black, 1991), liver (Calviello et al., 1997; Garg et al., 1990), kidney (Calviello et al., 1997), and erythrocyte phospholipids (Christensen et al., 1999) in proportion to dietary content.

Although the PUFA-supplemented diet did increase omega-3 incorporation into cartilage, the biological significance is unclear since concentrations of AA were at least 9-fold higher than

Table 12. Bone density and cortical width of the right radius and ulna in sow and gilts for sows fed either a basal corn/soybean diet (CON) or the basal diet supplemented with protected fish oil (PUFA).

	Sow		Gilt	
Measurement	CON	PUFA	CON	PUFA
Proximal Ulna Cortical Density, mg/cc	2150 ± 28	2130 ± 30	1610 ± 58	1650 ± 78
Central Radius Cortical Density, mg/cc	2230 ± 31	2250 ± 49	2060 ± 121	2180 ± 34
Distal Radius Cortical Density, mm/cc	2130 ± 44	2020 ± 50	1710 ± 104	1780 ± 72
Distal Radius Trabecular Density, mm/cc	170 ± 43[†]	270 ± 39	180 ± 28	150 ± 19
Proximal Ulna Cortical Width, mm	1.5 ± 0.1*	2.0 ± 0.15	1.5 ± 0.05	1.3 ± 0.1
Central Radius Cortical Width, mm	4.1 ± 0.3	4.0 ± 0.2	2.9 ± 0.1	3.0 ± 0.2
Distal Radius Cortical Width, mm	2.7 ± 0.1*	2.2 ± 0.1	2.0 ± 0.1	2.0 ± 0.1

* Difference between treatments for that age $P < 0.05$.
† Difference between treatments for that age $P < 0.10$.

Table 13. Cumulative release of PG, NO, PGE_2, and IL-6 presented as lsmeans from sow cartilage into media for sows fed either a basal corn/soybean diet (CON) or the basal diet supplemented with protected fish oil (PUFA). There was no effect of diet or treatment x diet interactions for any variable measured (P > 0.05).

Measurement	IL-1[1]	CON	PUFA	SEM
PG, µg	-	141.2	145.2	55.1
PG, µg	+	636.8	598.4	55.1
NO, µM	-	6.3	5.8	3.3
NO, µM	+	32.9	31.7	3.3
PGE_2, pg/mL	-	139.2	362.6	160.8
PGE, pg/mL	+	426.3	522.3	163.1
IL-6, pg/mL	-	1512.8	2194.7	955.0
IL-6, pg/mL	+	6383.4	7312.7	960.3

[1] A + designates the addition of recombinant porcine IL-1β at 10ng/mL while a – designates the control.

Table 14. Cumulative release of PG, NO, PGE_2, and IL-6 presented as lsmeans from gilt cartilage into media for sows fed either a basal corn/soybean diet (CON) or the basal diet supplemented with protected fish oil (PUFA). There was no effect of diet or treatment x diet interactions for any variable measured ($P > 0.05$).

Measurement	IL-1[1]	CON	PUFA	SEM
PG, µg	-	141.2	145.2	55.1
PG, µg	+	636.8	598.4	54.9
NO, µM	-	6.3	5.8	3.3
NO, µM	+	32.9	31.7	3.3
PGE_2, pg/mL	-	132.5	167.6	330.3
PGE_2, pg/mL	+	951.9	1539.3	330.3
IL-6, pg/mL	-	264.0	85.8	128.1
IL-6, pg/mL	+	9059.7	10025	1297.4

[1] A + designates the addition of recombinant porcine IL-1β at 10 ng/mL while a – designates the control.

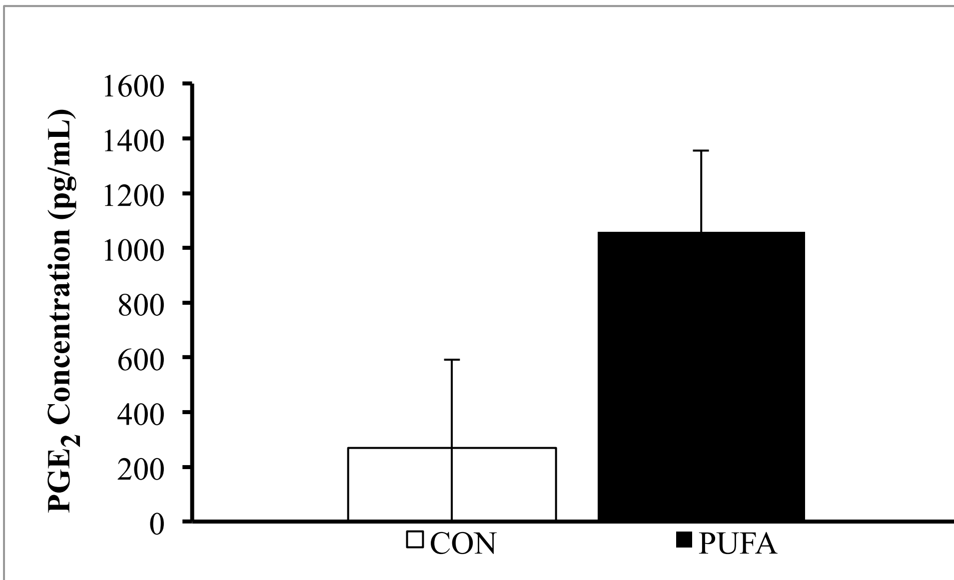

Figure 9. PGE_2 release from right carpal joint synovial fluid of sows fed either a basal corn/soybean diet (CON) or the basal diet supplemented with protected fish oil (PUFA). There was a tendency for sows on the PUFA diet to have greater PGE_2 release (P = 0.099).

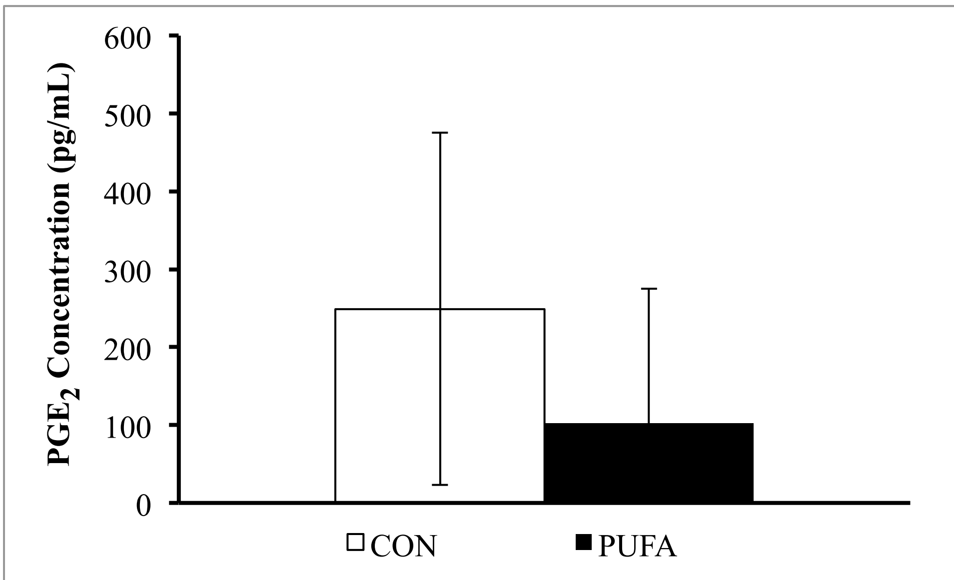

Figure 10. PGE_2 release from right humeral-ulnar joint synovial fluid of gilts fed either a basal corn/soybean diet (CON) or the basal diet supplemented with protected fish oil (PUFA). There was no effect of diet (P = 0.61).

either EPA or DHA. The dietary concentration of EPA and DHA in the total diet may not have been high enough to elicit measurable metabolic changes in our explant cultures.

Animals in the present study were supplemented with PUFA at 1% or 2.3% of their diet. Research with miniature pigs fed menhaden oil at 15% of their diet for 23 wks showed a significant increase in both EPA and DHA concentration in the erythrocyte phospholipids compared to corn oil-fed controls (Berlin et al., 1998). In a review, Kim et al. (2010) suggest that 1% purified PUFA ethyl esters would provide an equivalent intake to human consumption.

In our studies, diet also altered fatty acid concentrations in the synovial fluid of the sows. The PUFA supplemented diet fed to sows increased EPA, DPA, and DHA concentrations and the omega-6 to omega-3 ratio was reduced by half compared to the CON. There was a tendency for DPA to be higher in gilts fed the PUFA supplement. This increase in omega-3 fatty acids did not change the PGE_2 concentrations in the synovial fluid, which is likely a reflection of AA concentrations in the cartilage and synovium. With the exception of PGE_2, the other variables showed no effect due to dietary treatment. PGE_2 release tended to be higher in sows fed the PUFA supplement. There was no diet-induced reduction of AA in either the cartilage or synovial fluid. Research has shown that AA does not decrease IL-1 mediated levels of COX-2; however, EPA decreased mRNA and protein levels of COX-2 (Hurst et al., 2009). In the present study, AA was present in the synovial fluid at concentrations 8.5 times higher than DHA and 32 times higher than EPA. When EPA is present, it can compete with AA as a substrate for some of the inflammatory enzymes, such as COX-2, thereby reducing the production inflammatory mediators. Eicosanoids formed from EPA are 10- to 100-fold less potent than those produced from AA and therefore are associated with a decreased inflammatory response (Alexander,

1998). Omega-3 fatty acids may also mitigate inflammation in joints as the result of systemic changes in inflammatory mediators (James et al., 2003). Dogs fed 90 mg combined EPA and DHA/kg BW/d had increased plasma EPA and DHA after 7 d; however, EPA and DHA only suppressed markers of osteoarthritis development in the synovial fluid sporadically over the 56 d study (Hansen et al., 2008).

Rabinowitz et al. (1979) analyzed the fatty acid composition of synovial fluid samples from human male knee joints and the composite of C20, C22 and C24 fatty acids was 4.0 ± 1.3%. In our study, the composite of C20, C22, and C24 fatty acids was 5.77% in CON sows. This might suggest that the combined amounts of the PUFA are conserved in the synovial fluid.

Bone is a dynamic tissue that is continuously remodeled, and the role of lipids in bone metabolism has been documented (Watkins et al., 2003). Current research has examined the role of dietary omega-3 fatty acids on the bone modeling and remodeling process, but results from these studies are varied. Piglets fed either a 4.5:1 or 9:1 ratio of omega-6:omega-3 fatty acids plus either 30 or 60 g/L fat for 21 d were scanned to determine bone mineral content. No dietary alterations in femur, lumbar spine or whole-body bone mineral content were found (Weiler and Fitzpatrick-Wong, 2002). Judex and coworkers (2000) fed 28-d-old rabbits diets containing 10% fish oil for 40 d and found that tibial cortical bone area was decreased by 17% while longitudinal tibial growth was reduced by 4%. However, there were no differences in cross-sectional bone quality. Also, this study reported that the fish oil treatment reduced energy intake resulting in a lower body mass. In the present study bone for the most part was not impacted by the dietary treatment. Cortical width at the distal radius was less in the PUFA-fed sows, while no differences in bone were observed in the gilts. Rats fed 17 g of omega-3 fatty acids from

menhaden oil for 42 d tended to have improved bone remodeling and reduced PGE_2 production by as much as 55% as the ratio of omega-6:omega-3 fatty acids decreased (Watkins et al., 2000).

IMPLICATIONS

While the PUFA supplement did increase omega-3 incorporation into chondrocytes, the biological significance is unclear. Perhaps if the dietary concentrations of EPA and DHA in the PUFA supplement were higher, metabolic changes in our explant cultures may have occurred. Future research should include a dose response study to determine if increased levels of PUFA supplementation would benefit gilts and sows. Also, systemic markers of inflammation should be evaluated.

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CONCLUSION AND IMPLICATIONS

The *in vitro* studies demonstrated that DHA does a better job decreasing NO levels in recombinant porcine IL-1 stimulated cartilage explants, but both EPA and DHA are equally capable of decreasing PGE_2 levels. It is likely that EPA is reducing PGE_2 through competition with AA for binding sites on COX-2, resulting in the production of PGE_3, a less inflammatory eicosanoid. Additionally, no additive or synergistic effects of combining EPA and DHA were found in the rpIL-1 stimulated explants. Previous research has found that DHA is capable of inhibiting iNOS gene transcription and decreasing the binding of NF-κβ to the nucleus. NF-κβ translocation activates target genes, such as iNOS, in response to cytokine stimulation. The purpose of the chondrocyte monolayer experiments was to determine how fatty acid supplementation to the monolayers altered gene expression in response to stimulation with IL-1. Although only a small amount of data was collected, differential expression of iNOS was found suggesting that obtaining a complete data set would provide substantial evidence for how EPA and DHA work to reduce the inflammatory response.

When sows and gilts were supplemented with dietary PUFA changes in the fatty acid profile of cartilage and synovial fluid were evident. Sows exhibited increased concentrations of omega-3 fatty acids and a decrease in the omega-6:omega-3 ratio in synovial fluid. DHA concentration increased in the cartilage of both sows and gilts; however, it was only 0.22% of total fatty acids. This leads to question as to whether these fatty acid changes in tissue are physiologically significant.

Cartilage from these pigs was also used in a simple explant study to determine if diet would affect the response of the cartilage to IL-1 stimulation, but no effect of diet was found. Bone density and cortical width were also measured; however no differences were found

between diets for the gilts and only minor changes in cortical width were determined for the sows. Due to the heavy musculature of the pig, alterations in bone by dietary changes would be difficult to induce.

Additionally, while both sow gestation and lactation diets contained the same percentage of PUFA supplement, the feed consumption during lactation increased at least 3-fold, thus increasing the total amount of PUFA consumed during lactation. In other species changes plasma fatty acid concentrations in response to omega-3 supplementation can be seen after 7 d. In future studies it would be ideal to keep the total fatty acid levels constant. In the future it would be interesting to conduct a dose-response trial utilizing varying levels of dietary omega-3 fatty acids starting at weaning and following the same group of pigs through 3-5 parities with analysis of joints and tissue fatty acids at a variety of time points. Thus, it would provide information on the changes in milk fatty acids and responses in a second generation.

In conclusion, dietary omega-3 fatty acids are capable of changing the fatty acid composition of cartilage in synovial fluid in pigs. Additionally, EPA and DHA act as inflammatory mediators at the joint level through the reduction in NO and PGE_2.