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Immunological, hematological, and serum biochemical effects of high level dietary fish oil and vitamin E supplementation in the dog

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IMMUNOLOGICAL, HEMATOLOGICAL, AND SERUM BIOCHEMICAL EFFECTS OF HIGH LEVEL DIETARY FISH OIL AND VITAMIN E SUPPLEMENTATION IN THE DOG

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Inflammation is a component of the innate immune response. However, severe or prolonged inflammation can be detrimental. Dietary n-3 fatty acid supplementation has proven clinical benefits in chronic inflammatory diseases, most likely due to reduced synthesis of inflammatory mediators and inhibition of lymphocyte proliferation and function. The purposes of this study were to characterize alterations in inflammatory mediator production, and lymphocyte proliferation, in dogs fed a diet consisting of 0.65% n-3 fatty acids (DMB) with an n-6:n-3 fatty acid ratio of 3.4:1. Fifteen dogs were randomly assigned to one of three dietary groups for twelve weeks. Group Sunflower oil received a basal diet supplemented with 12.4g of sunflower oil/day. Groups Fish oil and Fish oil +E each received the same basal diet supplemented with 0.6g of sunflower oil and 7g of menhaden fish oil. Group Fish oil + E also received 0.18g of vitamin E. IL-1, IL-6, TNF-α, PGE_2, and PAF were evaluated both in mononuclear cell culture, and in serum after *in vivo* stimulation with lipopolysaccharide. Lymphocyte proliferation was evaluated by incorporation of tritiated thymidine as well as sequential halving of a fluorochrome dye, CFSE, using flow cytometry. Potential adverse effects of dietary n-3 fatty acid supplementation were assessed through serum vitamin E concentrations, plasma lipid peroxidation, platelet aggregation, and standard hematologic and serum biochemical parameters. Serum levels of IL-6 and PGE_2 as well as mononuclear cell culture levels of PGE_2 were significantly higher among dogs of Group Sunflower oil compared to dogs in Groups Fish oil or Fish oil + E. Lymphocyte proliferation as evaluated by flow cytometry was significantly reduced in Group Fish oil at 12 weeks compared to Groups Sunflower oil and Fish oil + E. There was no significant diet effect on platelet aggregation, lipid peroxidation, or hematologic and biochemical parameters, with the exception of decreased triglycerides in Group Fish oil. These data demonstrate that a significant degree of immunomodulation is possible with a safe dietary intake and ratio of n-3 fatty acids. Future studies should focus on the clinical role of dietary n-3 fatty acid supplementation for the treatment of inflammatory diseases and hyperlipidemia.

CHAPTER I: BACKGROUIND and LITERATURE REVIEW

Immunity

Immunity is defined as the status or quality of being resistant to an infectious disease (Hensyl, 1990). The immune system of humans and animals is divided by function into the innate or natural immunity and acquired or adaptive immunity. Innate immunity is the first line of defense and is present before exposure to potential pathogens. This type of immunity consists of anatomic barriers such as intact skin and mucosal membranes that prevent entry of pathogens into the host. If entry is gained, granulocytes (neutrophils, eosinophils, basophils) and monocytes/macrophages can directly destroy pathogens by extracellular release of lysosomal enzymes and oxygen-derived free radicals, or through engulfment and subsequent nonspecific killing accomplished largely by oxygen-dependent mechanisms (Collins, 1999). Plasma proteins like complement or toxic proteins released from natural killer (NK) cells also play a role in destruction, by the innate immune system, of many pathogens (Abbas et al., 2000) (Calder, 2001).

Acquired immunity involves recognition of specific antigens or peptide components of invading pathogens. Recognition is accomplished either by antigen-specific antibodies produced by B-lymphocytes, termed the humoral response, or by receptors on the surface of T-lymphocytes, called the cell-mediated response. T-lymphocytes are able to recognize foreign peptides only when they are presented by other host cells in conjunction with proteins termed major histocompatibility complex (MHC). The MHC class I complex is present on the surface of all nucleated host cells. It is responsible for presenting peptides that originate from intracellular pathogens, like viruses and certain bacteria (Sharon, 1998). MHC class I is specifically recognized by cytotoxic T-lymphocytes that express the CD8 receptor; CD8+ T-lymphocytes subsequently destroy the infected host cell and prevent further propagation of the pathogen. The MHC class II complex is only found on antigen-presenting cells such as macrophages, dendritic cells, and B-lymphocytes. The peptides presented by this complex are derived from extracellular pathogens that have been engulfed or endocytosed by antigen-presenting cells, and are recognized by helper T-lymphocytes expressing the CD4 receptor. Recognition of foreign antigen in the context of MHC class II stimulates CD4+ T-lymphocytes to produce chemical messengers called cytokines, which activate or inhibit different cell types, depending on the type of pathogen (bacteria, viruses, fungi, or helminthic parasites) (Sharon, 1998; Abbas et al., 2000) . CD4+ helper T-lymphocytes are categorized into two subtypes based on their cytokine production. Type 1 helper (Th1) cells

secrete interferon (IFN)-γ and interleukin (IL)-2 which facilitate cell-mediated immunity by activating macrophages, cytotoxic T-lymphocytes, and NK cells. Type 2 helper (Th2) cells secrete IL-4 and IL-10 which promote B-lymphocytes to produce antibodies. Th2 cells also secrete IL-5 which is an important eosinophil-activating factor.

Communication within the acquired immune system and between the innate and acquired immune systems is accomplished through binding adhesion molecules of adjacent cells and by production and release of chemical messengers (Calder, 2001). The most important of these chemical messengers include synthesized polypeptides called cytokines, and polyunsaturated fatty acid metabolites called eicosanoids. There are a multitude of cytokines, many of which have multiple functions. However, a few of the more important cytokines have been categorized based on their major function(s) or target cell (Collins, 1999). The cytokines most important to the acquired immune response regulate lymphocyte and macrophage activation, proliferation, differentiation and inhibition. These cytokines include IL-2, IL-4, IL-10, IL-12, transforming growth factor (TGF)-β, and interferon (IFN)-γ. The cytokines most involved in natural immunity are tumor necrosis factor (TNF)-α, IL-1, and IL-6. TNF-α, IL-1, and IL-6 are synthesized and released by activated macrophages and promote neutrophil- and macrophage-mediated killing of bacteria and increase expression of adhesion molecules on neutrophils and endothelial cells. IL-1 receptor antagonists have also been shown to inhibit the production of pro-inflammatory eicosanoids LTB_4 (Conti et al., 1993) and PGE2 (Arend et al., 1990). TNF-α, IL-1, and IL-6 stimulate T- and B-lymphocyte proliferation and upregulate MHC, and are therefore an important link between natural and acquired immunity (Calder, 2001). Macrophage secretion of IL-1 also stimulates T-lymphocyte synthesis of IL-2. IL-2, first described as a T-cell growth factor (Morgan et al., 1976), is an important autocrine and paracrine cytokine that promotes the proliferation of T-lymphocytes, B-lymphocytes, and NK cells.

Cytokine receptors consist of one or more transmembrane proteins whose extracellular portions bind cytokines and whose cytoplasmic portions initiate intracellular signaling pathways (Abbas et al., 2000). Activation of these pathways can result in cell proliferation and synthesis of other cytokines, or inhibition of cell activity. Many of the cytokine receptors on the surface of lymphocytes are Type I receptors, including receptors for IL-2, IL-4, IL-6, and IL-12. Type I receptors typically bind cytokines that fold into four α-helical strands. Type II cytokine receptors are also present on lymphocytes. This receptor class includes receptors for IL-10 and IFN-γ.

Inflammation

Inflammation is defined as 'a pathologic process consisting of a dynamic complex of cytologic and histologic reactions that occur in the affected blood vessels and adjacent tissues in response to an injury or abnormal stimulation caused by physical, chemical, or biological agent' (Hensyl, 1990). The "cardinal signs" of inflammation are *rubor*, redness; *calor*, heat; *tumor*, swelling; *dolor*, pain; and *functio laesa*, loss of function. These signs occur as a result of localized increased blood flow, increased vascular permeability which allows complement, antibodies, and cytokines to leave the blood stream, and increased movement of circulating leukocytes into the affected tissue (Collins, 1999). Although inflammation is part of the normal, innate defense against pathogens, it can be harmful. Animals and humans can develop an excessive inflammatory reaction after an inappropriate immune response to benign environmental antigens, leading to food allergies, asthma, and atopy. In other diseases such as rheumatoid arthritis, systemic lupus erythematous and Crohn's disease, inflammatory reactions are directed at 'self' antigens rather than foreign pathogens, and often cause irrepairable tissue damage. Lastly, the immune-mediated inflammatory response to some intracellular bacterial agents and fungi is so intense that it actually causes more host damage than the pathogen, as with *Mycobacterium tuberculosis* infections. Furthermore, chronic, progressive inflammation can be locally destructive to certain tissues as well as detrimental to the body as a whole. Pro-inflammatory cytokines such as TNF-α have been identified as mediators of anorexia, weight loss and anemia associated with chronic infectious diseases (Darling et al., 1990; Argiles et al., 1997). Many cytokines, especially TNF-α and IL-6, have also been implicated in the development of cachexia in people and animals with cancer (Strassmann and Kambayashi, 1995; Tisdale, 1997; Wigmore et al., 1997).

Fatty Acid Nomenclature, Classification and Dietary Sources

Fatty acids are carboxylic acids varying from 2 to 22 carbons in length. The final carbon, opposite the carboxyl group end, is called the "omega" carbon, and is designated by either the letter "ω" or "n". Nomenclature for fatty acids is typically based on the chain length, the number of double bonds within the carbon chain, and the location of the first double bond with respect to the omega carbon (Reinhart, 1996). For example, the polyunsaturated n-3 fatty acid α-linolenic acid is a carboxylic acid consisting of an 18-carbon chain containing 3 double bonds, the first of which is between the 3rd and 4th carbon molecules as counted from the omega. It is designated 18:3n-3 or C18:3n-3 (Figure 1.1). In the same fashion, the saturated fatty acid palmitic acid is simply represented as C16, as it consists of a 16-carbon chain with no double bonds.

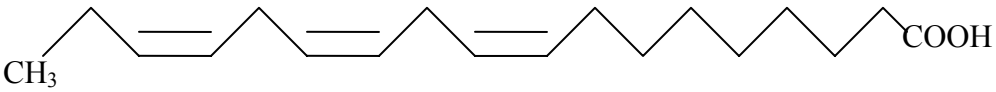

Figure 1.1 α-linolenic acid (18:3n-3). COOH is the carboxyl group on the opposite end from the omega carbon, CH_3. Double bonds are represented by parallel lines.

Animals and humans cannot insert the first double bond in a fatty acid at the third or sixth carbon, nor can they convert one type of fatty acid into another. Therefore, certain essential n-3 and n-6 fatty acids must be provided in the diet (Reinhart, 1996; Calder, 1997; Kelley, 2001). Linoleic acid is the only fatty acid so far proven to be essential in the canine diet, although dogs may have other essential fatty acid requirements (Reinhart, 1996). Animals and humans are capable of elongation and desaturation of fatty acids towards the carboxyl end of the molecules. The n-6 fatty acid linoleic acid (18:2n-6), found in high concentrations within sunflower, safflower, and corn oils, can be desaturated and elongated to produce arachidonic acid (AA; 20:4n-6). Likewise, the n-3 fatty acid α-linolenic acid (18:3n-3) found in flax seed oil can be desaturated and elongated to form eicosapentanoic acid (EPA; 20:5n-3).

Precursor fatty acids such as linoleic and α-linolenic acids compete for the same desaturase enzymes. Therefore, the dietary proportions of n-6 and n-3 fatty acids directly affect the quantity and proportions of AA and EPA that are produced and incorporated into cell membranes. Many marine plants, especially unicellular algae, elongate and desaturate α-linolenic acid to yield the long chain n-3 fatty acids EPA and docosahexaenoic acid (DHA; 22:6n-3). The transfer of these n-3 fatty acids through the food chain is responsible for their abundance in many fish and marine oils (Calder, 1997). Not surprisingly, the consumption of large quantities of marine products was related to higher plasma EPA concentrations in Greenland Inuit Eskimos when compared to age- and sex-matched Danes (Dyerberg et al., 1975).

Eicosanoid and PAF Synthesis and Their Role in Inflammation

Whether derived directly from dietary sources or converted from essential fatty acids like linoleic acid, AA is esterified into leukocyte and other cell membrane phospholipids, particularly in the carbon 2 position of phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine (Collins, 1999; Bochsler and Slauson, 2002). When appropriate leukocyte cell membrane receptors bind extracellular ligands such as cytokines, growth factors, or chemotactic peptides, cytosolic phospholipases are indirectly activated. These phospholipases,

principally phospholipase A_2 (PLA_2), cleave AA from its esterified position within the membrane phospholipids.This also occurs at intracellular membranes such as endoplasmic reticulum and the nuclear membrane (Evans et al., 2001). Freed AA is then metabolized by one of two major pathways to form numerous compounds called eicosanoids (Figure 1.2). Eicosanoids include prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), lipoxins, hydroperoxyeicosatetraenoic acids (HPETE), and hydroxyeicosatetraenoic acids (HETE).

Metabolism of AA by cyclooxygenase (COX) enzymes gives rise to pro-inflammatory eicosanoids, specifically 2-series prostaglandins and thromboxanes. There are two isoforms of COX: COX-1 is a constitutive enzyme that is responsible for day-to-day AA metabolism; COX-2 is transcriptionally regulated and is induced in leukocytes as a result of receptor stimulation during inflammation. Hence, COX-2 induction is responsible for increased synthesis of prostaglandins during an inflammatory response. The proportion of the different prostaglandins produced varies among different leukocytes: macrophages produce large quantities of PGE_2 and PGF_{2a}; neutrophils produce moderate amounts of PGE_2; and, PGD_2 is the predominant prostaglandin produced by mast cells. PGE_2, PGF_{2a}, and PGD_2 are in part responsible for the pain, redness, and swelling of inflamed tissues. In contrast to its local pro-inflammatory effects, PGE_2 also has immunosuppressive and/or anti-inflammatory effects (Tilley et al., 2001). PGE_2 has been found to suppress *in vitro* T-cell proliferation and IL-2 production (Gordon et al., 1976; Goodwin et al., 1977; Baker et al., 1981; Rappaport and Dodge, 1982), as well as NK cell cytotoxicity (Brunda et al., 1980). The inhibitory effect of PGE_2 on lymphocyte proliferation is significantly diminished by the addition of the prostaglandin synthetase inhibitor, indomethacin (Goodwin et al., 1978).

AA may also be metabolized by an alternative pathway involving one of a family of lipoxygenase (LO) enzymes. Of these, the best characterized and most important in the inflammatory response is 5-LO. 5-LO must first interact with 5-lipoxygenase-activating protein (FLAP), and the active enzyme complex then acts on AA to form 5-hydroperoxyeicosatetraenoic acid (5-HPETE) (Collins, 1999; Bochsler and Slauson, 2002). 5-HPETE is the precursor of the leukocyte chemotactic agent hydroxyeicosatetraenoic acid (5-HETE) and the 4-series leukotrienes. Of major importance is leukotriene B_4 (LTB_4), which is a potent pro-inflammatory mediator that acts as a chemotactic agent for neutrophils. LTB_4 also promotes neutrophil chemotaxis and increases their adhesiveness to vessel endothelium, lysosomal enzyme release, and oxygen free radical generation. LTC_4, LTD_4, and LTE_4 collectively produce intense vasoconstriction, increased vascular permeability, and bronchospasm.

Platelet-activating factor (PAF) is actually a family of structurally related bioactive mediators derived from PLA_2-mediated hydrolysis of choline-containing membrane phospholipids (McManus and Pinckard, 2000). The generated lysophosphocholine (lyso-PC) serves as a substrate for PAF acetyltransferase, resulting in the formation of PAF (Figure 1.2). PAF was originally named because it caused aggregation and secretion of rabbit platelets after its release from activated basophils (Benveniste et al., 1972). PAF is now known to have many pro-inflammatory effects including promotion of vascular permeability, leukocyte adhesion to vascular walls, neutrophilic chemotaxis, and production of LTB_4 (Collins, 1999). PAF also stimulates pro-inflammatory activities in target cells including macrophages, eosinophils and vascular endothelial cells.

n-3 Fatty Acid Influence on Eicosanoid and PAF Synthesis

The n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can be derived directly from dietary sources such as fish oil, or they can be synthesized *in vivo* from precursor n-3 fatty acids such as α-linolenic acid. EPA and DHA directly inhibit the metabolism and biological effects of AA by at least four different mechanisms. First, these long chain n-3 fatty acids can be esterified into cell membrane phospholipids, resulting in decreased esterification of AA. Second, phospholipase-induced release of AA from the cell membrane is inhibited by EPA and DHA (Lands et al., 1973). Third, EPA released from the cell membrane competes with AA as substrate for COX and 5-LO enzymes (Reinhart, 1996). Finally, EPA-derived eicosanoids will competitively antagonize the action of AA-derived eicosanoids assuming they bind to and initiate activity through the same target cell receptors (Calder, 2001). Thus EPA, to some extent DHA, and their metabolities, are able to displace AA. This prevents the synthesis and activity of the pro-inflammatory AA metabolites, principally the 2-series prostaglandins and the 4-series leukotrienes.

EPA metabolism by COX and 5-LO enzymes gives rise to 3-series prostaglandins and thromboxanes and 5-series leukotrienes, respectively. In contrast to their AA-derived counterparts, these EPA-derived eicosanoids are less inflammatory, cause vasodilation, decrease platelet aggregation, and are less immunosuppressive (Reinhart, 1996). For example, thromboxane A_3 (TXA_3) derived from EPA has attenuated proaggregatory action on human platelets when compared to TXA_2 (Whitaker et al., 1979). EPA-derived PGE_3 appears to be a less potent inhibitor of lymphocyte proliferation than PGE_2 (Calder et al., 1992). Lastly, leukotriene B_5 (LTB_5) has markedly reduced potency in chemotactic and vasoconstriction activity when compared to the

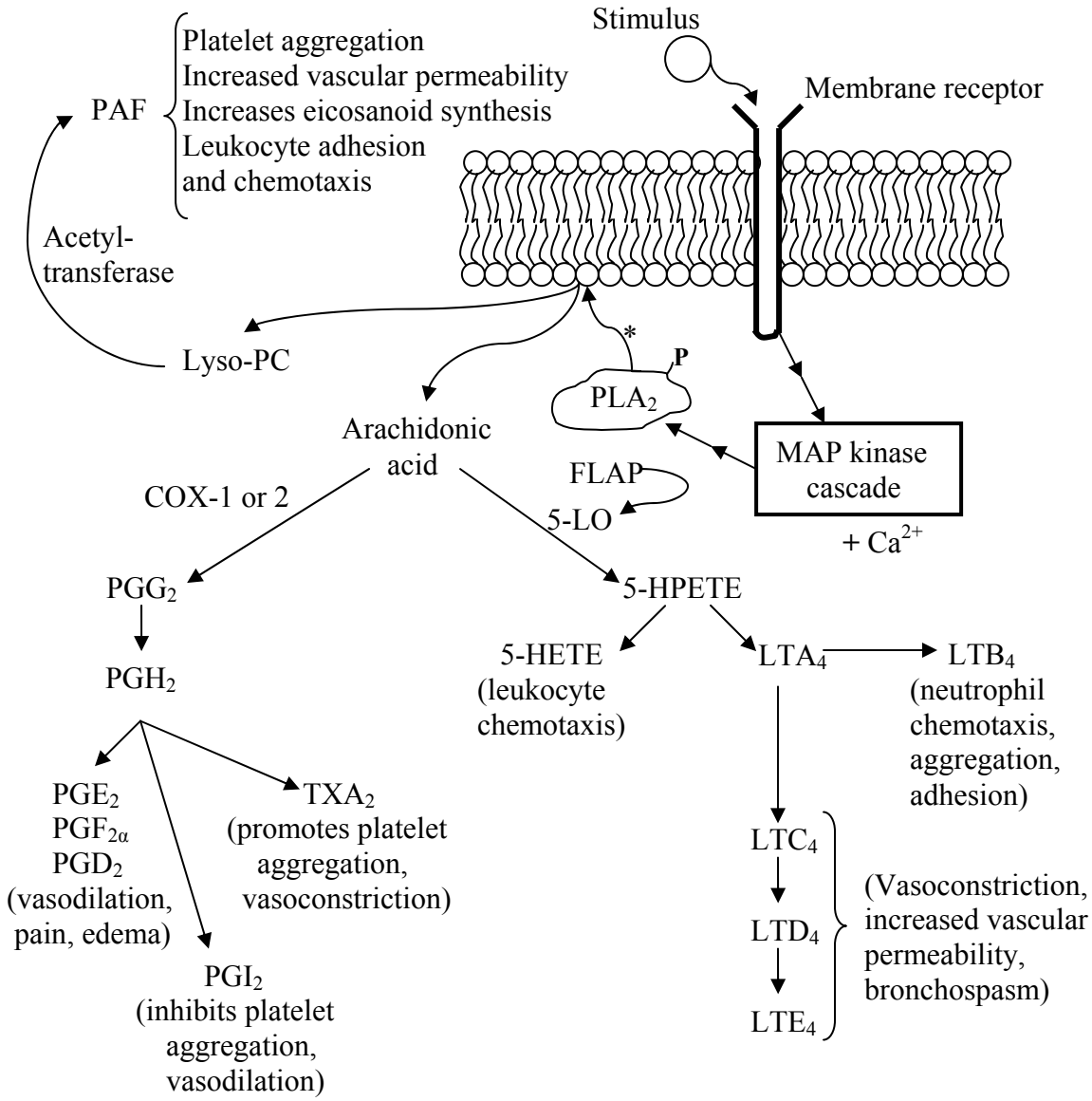

Figure 1.2 Eicosanoid and PAF synthesis. Upon stimulation of the cell surface receptor by cytokines or other mediators, the mitogen-activated protein (MAP) kinase cascade is activated. This leads to the phosphorylation (P = phosphorus) and activation of phospholipase A_2 (PLA_2) in the presence of increased cytosolic calcium (Ca^{2+}). Activated PLA_2 cleaves arachidonic acid (AA) from membrane phospholipids *(including intracellular membranes) such as phosphatidylcholine. Lysophosphocholine (Lyso-PC), also released by PLA_2, is acetylated by PAF acetyltransferase to form platelet-activating factor (PAF). Cytosolic AA enters one of two enzymatic pathways to form numerous proinflammatory eicosanoids. Cyclooxygenase (COX) metabolism of AA leads to the formation of 2-series prostaglandins such as PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (TXA_2). AA may alternatively be metabolized by 5-lipoxygenase (5-LO) after this enzyme is activated by 5-lipoxygenase-activating protein (FLAP), which results in the synthesis of 5-HPETE. 5-HPETE is further metabolized into 5-HETE and the 4-series leukotrienes LTA_4, LTB_4, LTC_4, LTD_4, and LTE_4.

AA-derived LTB_4 (Lee et al., 1988). Therefore, EPA is anti-inflammatory not only due to its competitive inhibition of AA metabolism: more importantly, EPA-derived eicosanoids are less potent promoters of the inflammatory response and are less immunosuppressive.

Unlike other EPA-derived eicosanoids, PGI_3 retains the biologic properties of its AA-derived counterpart PGI_2. Both of these prostaglandins inhibit platelet aggregation and relax vascular smooth muscle *in vitro* (Fischer and Weber, 1984) In addition, PGI_2 production is maintained during periods of EPA dietary supplementation (Fischer and Weber, 1984; von Schacky et al., 1985). At least partial inhibition of platelet aggregation would therefore be expected in animals and humans supplemented with n-3 fatty acids due to the combination of increased PGI_3 production, maintained PGI_2 production, and decreased production of pro-aggregatory TXA_2 (Lee and Austen, 1986).

Due to the apparent similarities between eicosanoid metabolism and PAF synthesis, it would seem plausible that manipulation of membrane fatty acid composition would also effect the production of PAF. EPA is thought to decrease PAF synthesis by replacing the preferred substrate, the arachidonate moiety of the choline-containing phospholipid (Prescott, 1992). Direct supplementation of EPA to a cultured monocyte-like cell line resulted in a markedly diminished capacity to synthesize PAF when compared to cells supplemented with AA (Martin-Chouly et al., 2000).

Effects of n-3 Fatty Acid Dietary Supplementation

The amount of dietary n-3 fatty acid supplementation or the ratio of n-6:n-3 fatty acids needed to maximize benefits without negative effects has not been established. It is likely that the absolute intake of n-3 fatty acids and the dietary n-6:n-3 fatty acid ratio are interdependent. When diets low in polyunsaturated fatty acid are fed, the influence of the n-6:n-3 fatty acid ratio on factors such as lymphocyte proliferation is increased (Jeffery et al., 1997). Another study by the same investigators further supports the importance of the dietary n-6:n-3 fatty acid ratio (Jeffery et al., 1996). Lymphocyte proliferation, serum cholesterol and triglycerides decreased as the n-6:n-3 fatty acid ratio decreased and the total amount of fat remained constant in a diet fed to weanling male rats.

Many authors have investigated the immunologic and hematologic effects of dietary n-3 fatty acid supplementation in humans and rodents. Several canine studies have also been published. Regardless of species most reports have focused on effects on lymphocyte function, platelet aggregation, and chemical mediator synthesis, i.e. cytokines, eicosanoids, and PAF.

Lymphocyte Function

Most reports show that dietary supplementation with polyunsaturated fatty acids (PUFA) reduces *in vitro* proliferation of human (Meydani et al., 1991b; Khalfoun et al., 1996; Terada et al., 2001) and animal lymphocytes (Baker et al., 1981; Calder et al., 1992; Yaqoob et al., 1994; Kuratko, 2000). An early report suggested this suppression was prostaglandin, specifically PGE_2, dependent (Webb and Jamieson, 1976). This implies that n-6 fatty acids such as linoleic and arachidonic acid may cause more suppression than other fatty acids since their metabolism results in the production of PGE_2. However, most reports demonstrate that n-3 fatty acids are more potent suppressors of lymphocyte proliferation (Calder and Newsholme, 1992). This suggests polyunsaturated fatty acid (PUFA)-induced suppression occurs through a prostaglandin-independent mechanism(s), a hypothesis that is supported by the finding that prostaglandin synthesis inhibitors have little to no effect on the fatty acid-mediated inhibition (Santoli and Zurier, 1989; Calder et al., 1992).

Many authors implicate suppressed synthesis and release of IL-2 by T- lymphocytes as an important component in the n-3 fatty acid-induced decrease in lymphocyte proliferation (Santoli and Zurier, 1989; Calder and Newsholme, 1992; Endres et al., 1993; Jolly et al., 1997; McMurray et al., 2000). Jolly and coworkers associated this diminished synthesis of IL-2 with reduced production of the intracellular second messengers, diacylglycerol (DAG) and ceramide (Jolly et al., 1997). These authors also found that mice supplemented with dietary EPA or DHA had reduced IL-2 receptor-α gene transcription within stimulated splenic lymphocytes (Jolly et al., 1998). Decreased cell membrane expression of the IL-2 receptor α-chain (CD25) has been reported on lymphocytes of people with psoriasis and atopic dermatitis receiving supplemental EPA and DHA as well (Soyland et al., 1994). In contrast, however, other researchers found no reduction in the mitogen-induced lymphocyte expression of activation markers such as CD25 and the transferrin receptor CD71 (Khalfoun et al., 1996). Other mechanisms by which n-3 fatty acids could suppress lymphocyte proliferation include inhibition of phospholipase C (PLC)-γ activation (Sanderson and Calder, 1998) and reduced expression of interferon (IFN)-γ receptor (Feng et al., 1999). PLC-γ activation results in downstream events such as induction of IL-2 synthesis and expression of the IL-2 receptor, which are important promoters of lymphocyte proliferaion. Another important consideration is that as the fatty acid composition of lymphocytes changes, so does the fluidity of their cell membranes (Anel et al., 1990). This change in membrane fluidity may affect lymphocyte activation and proliferation by altering expression of cell surface receptors.

While manipulation of dietary PUFA concentration does not appear to affect the proportions of lymphocyte subtypes, including $CD4^+$ T-lymphocytes, $CD8^+$ T-lymphocytes, and $CD16^+$ NK cells (Yaqoob et al., 1994; Jeffery et al., 1997), it does alter cytokine production by certain lymphocyte sybsets. Supplementation of n-3 and n-6 fatty acids is reported to reduce the production of the Th1-type cytokines IL-2 and IFN-γ, without affecting the Th2-type cytokines IL-4 and IL-10 (Nishiyama et al., 2000; Wallace et al., 2001). Other investigators have found that dietary n-3 fatty acid supplementation actually enhances the proliferation of IL-4 driven Th2 $CD4^+$ lymphocytes, with an associated decrease in proliferation of IL-2 driven Th1 $CD4^+$ lymphocytes and $CD8^+$ lymphocytes (Arrington et al., 2001). Regardless, n-3 fatty acids seem to suppress the Th-1 type immune response, which would explain some of the benefits observed following the administration of fish oil to patients with chronic inflammatory diseases (Wallace et al., 2001).

Fewer studies have evaluated lymphocyte proliferation and function among dogs receiving diets supplemented with n-3 fatty acids, and these reports vary with respect to methodology and conclusions. The only study to evaluate *in vitro* lymphocyte proliferation showed that lymphocytes from young dogs eating a ration consisting of 1.65% fish oil on a dry matter basis (DMB), and having an n-6:n-3 fatty acid ratio of 5:1 had an enhanced response to concanavalin A (Con A), phytohemagglutinin-P (PHA), and pokeweed mitogen (PWM) (Kearns et al., 1999). Older dogs also had an enhanced lymphoproliferative response, although the increase was not significant. The discrepancy between these findings and previous reports in other species was attributed to maintenance of a normal plasma vitamin E concentration in the dogs throughout the dietary trial. In contrast, a study evaluating aged dogs fed a ration containing a mixture of fish and corn oils consisting of 4.4% of the diet (DMB) and having an n-6:n-3 fatty acid ratio of 1.4:1, showed significant suppression of the T-lymphocyte function based on results of a delayed-type hypersensitivity (DTH) skin test (Wander et al., 1997). This n-3 fatty acid-induced effect was attributed to increased lipid peroxidation, which has also been implicated as the cause of decreased lymphoproliferative responses by other investigators (Meydani et al., 1993). The only study so far investigating lymphocyte subtypes found that dogs eating a diet with an n-6:n-3 fatty acid ratio of 1.4:1 had a decrease in the percentage of $CD4^+$ lymphocytes after a vaccination challenge compared to controls (Hall et al., 1999).

Platelet Aggregation

Reports describing the influence of dietary n-3 fatty acid supplementation on platelet aggregation are numerous, involve many species, and vary considerably in their results. In most

human studies, adenosine diphosphate (ADP)- and collagen-induced aggregation are inhibited after consumption of n-3 fatty acids (von Schacky et al., 1985; Kristensen et al., 1989; Freese and Mutanen, 1997; Wensing et al., 1999). Two studies reported no n-3 fatty acid-induced change in *in vitro* platelet aggregation in elder human test subjects, but *ex vivo* platelet aggregation was significantly decreased (Salonen et al., 1987; Wensing et al., 1999). The Greenlandic Inuit Eskimo diet of whale, seal, and fish products has been implicated as the reason for the impaired platelet aggregation (Dyerberg and Bang, 1979) and low incidence of acute myocardial infarction observed in this population (Kromann and Green, 1980). Human platelet adhesion to fibrinogen-coated plates is also reduced by fish oil supplementation (Andrioli et al., 1999).

The effects of dietary n-3 fatty acid supplementation on platelet function appear less consistent in animal studies. ADP- and collagen-induced aggregation, platelet production of TXA_2, and aorta production of PGI_2 was inhibited when EPA and DHA were supplemented in rats (Adan et al., 1999). Cats eating an n-3 fatty acid enriched diet with an n-6:n-3 ratio of 1.3:1 for 16 weeks had reduced ADP-induced platelet aggregation and prolonged toenail bleeding time compared to cats consuming 25:1 and 12:1 ratio diets (Saker et al., 1998). However, no significant differences in buccal mucosal bleeding time or collagen-induced platelet aggregation were found in another study that compared control cats to cats that received 1.1g of EPA and 0.6g of DHA daily for 4 weeks (Bright et al., 1994). Horses had no alteration in ADP-induced platelet aggregation after 16 weeks of supplementation with 10% flaxseed oil, a source the of n-3 fatty acid α-linolenic acid (Hansen et al., 2002). In contrast, however, a significant reduction in platelet accumulations within myocutaneous flaps was demostrated in a porcine model of ischemia and reperfusion injury when pigs were supplemented with 7g of n-3 fatty acids daily for 3 weeks (Thorwest et al., 2000).

Healthy dogs consuming diets with an n-6:n-3 fatty acid ratio of 5:1 for 12 weeks did not have significant differences in arachidonic acid-, ADP, or collagen-induced platelet aggregation compared to baseline (Boudreaux et al., 1997). Coagulation screening tests and fibrinogen activities were also unchanged. Dogs receiving treatment for lymphoma or hemangiosarcoma that were concurrently supplemented with menhaden fish oil and arginine at 5.54% of their diet on a dry matter basis had no significant changes in platelet aggregation or coagulation times compared to control patients with the same malignancies (McNiel et al., 1999). However, dogs fed only mackerel fish supplemented with menhaden oil for 10 days had improved patency of small-

diameter arterial prosthetic grafts and prolonged bleeding times, although their collagen- and ADP-induced platelet aggregation was not affected (Casali et al., 1986).

Chemical Mediator Synthesis

Endres and coworkers were the first to show that increased consumption of n-3 fatty acids affects cytokine production. Healthy humans were supplemented with 18g of fish oil a day and their isolated peripheral blood mononuclear cells were found to produce significantly less TNF and IL-1 upon stimulation with bacterial endotoxin (lipopolysaccharide (LPS) from *E. coli*) (Endres et al., 1989). Since then, other human and animal studies have demonstrated similar results. Dietary supplementation of EPA and DHA dramatically suppressed LPS-stimulated mononuclear cell production of IL-1, IL-6, TNF, and PGE_2 in older women and to a lesser extent in young women (Meydani et al., 1991b). However, a later study by the same investigators showed that granulocyte-macrophage-colony stimulating factor (GM-CSF) was not affected by dietary fatty acid manipulation in women or men (Meydani et al., 1993).

Similar results have been obtained in studies investigating the effect of dietary n-3 fatty acid supplementation in rodents. Activated peritoneal macrophages from mice supplemented with fish oil have suppressed production of IL-1, TNF, and PGE_2 (Wallace et al., 2000). Furthermore, macrophages harvested by bronchoalveolar lavage (BAL) from rats supplemented with fish oil have decreased production of LTB_4, TXB_2 (a stable metabolite of TXA_2), and PGE_2 (Mancuso et al., 1997). The only study investigating the *in vivo* effects of fish oil on cytokine production found that fish oil-supplemented mice injected with intraperitoneal *E.coli* LPS had lower peak plasma concentrations of TNF, IL-1, and IL-6 compared to mice supplemented with safflower oil (Sadeghi et al., 1999). Other sources of n-3 fatty acids also appear to effectively reduce the synthesis of inflammatory mediators. A 50% reduction in PAF synthesis was demonstrated in neutrophils from rats supplemented with α-linolenic acid-rich perilla oil compared to the neutrophils from safflower oil supplemented rats (Horii et al., 1991). Dietary n-3 fatty acid-induced inhibition of inflammatory mediator production has also been observed in cells other than immune effector cells. For instance, experimentally-induced hypoxic-ischemic brain tissue of young mice previously supplemented with fish oil showed a significant reduction of endogenously synthesized PAF and LTB_4 (Akisu et al., 2002).

Direct supplementation of EPA and DHA to cell culture media inhibits chemical mediator synthesis. When added to a murine macrophage cell line, these fatty acids and α-linolenic acid suppressed the production of nitric oxide (NO) after activation by LPS (Ohata et al., 1997). In a

similar manner, production of PGE_2, $PGF_{2\alpha}$, and IL-1, but not TNF or IL-6, production by a human decidual cell line (obtained from fetal membranes) was suppressed when cells were cultured with EPA and DHA (Arntzen et al., 1998). PAF production by TNF-stimulated human endothelial cells was also diminished when these cells were incubated with EPA and DHA (Mayer et al., 2002).

Reported production of chemical mediators among dogs supplemented with n-3 fatty acids varies between studies. Maintaining young and older dogs on a n-6:n-3 fatty acid ratio diet of 5:1 for 60 days had no effect on IL-1, IL-6, TNF, or PGE2 production by peritoneal macrophages or peripheral blood mononuclear cells (Kearns et al., 1999). However, dogs fed rations with ratios of 5.3:1 and 10.4:1, where n-3 fatty acids were 3.4 and 1.8 percent of total fatty acids respectively, had significantly less LTB_4 and significantly more LTB_5 synthesized by stimulated neutrophils and skin (Vaughn et al., 1994). These investigators also found that PGE_2:PGE_3 metabolite ratios produced from 4-day-old cutaneous wounds increased as the dietary n-6:n-3 fatty acid ratio increased (Mooney et al., 1998). In contrast to the findings of Kearns and coworkers, however, fish oil supplementation has reduced PGE_2 production by peripheral blood mononuclear cells in dogs consuming a diet with an n-6:n-3 ratio of 1.4:1 (Wander et al., 1997).

Lipid Peroxidation and Vitamin E

Lipid peroxidation is an autocatalytic free radical reaction whereby cell membranes rich in polyunsaturated fatty acids are attacked by oxidizing radicals. The double bonds within the long carbon chains of n-3 fatty acids such as EPA and DHA make cell membranes containing them an easy target for superoxide, hydrogen peroxide, hydroxy radicals and other similar compounds (Muggli, 1989; Mylonas and Kouretas, 1999). These molecules remove a hydrogen atom from a polyunsaturated fatty acid, generating a lipid radical. These lipid radicals then react with molecular oxygen to form lipid peroxy radicals, which can attack surrounding polyunsaturated fatty acids, and initiate a self-perpetuating chain reaction (Mylonas and Kouretas, 1999). The cell membrane damage that results leads to altered immune function, since the cell membrane is important in antigen reception, secretion of cytokines and antibodies, and contact cell lysis. Lipid peroxidation may also result in increased prostaglandin production by neighboring cells (Knight, 2000).

Lipid peroxidation is a process that normally occurs at low levels in all cells (Hubel et al., 1989). Most cells and tissues have a multifaceted defense system to keep this potentially continuous process under control. Antioxidant defense includes enzymes such as superoxide

dismutase, catalase, and glutathione peroxidase; soluble antioxidants like albumin and heme-binding proteins; nutritional antioxidants such as vitamins C and E; and, metal-containing proteins such as cytochromes, ferritin, and lactoferrin (Mylonas and Kouretas, 1999). However, consumption of diets high in polyunsaturated fatty acids could overwhelm these mechanisms and make additional protection necessary. Additional vitamin E (α-tocopherol) supplementation is often recommended in this situation, for two reasons. First, tissue concentrations of vitamin E can be increased by simply increasing dietary intake (Meydani et al., 1990; Duthie et al., 1996; Scott et al., 2001). Second, its lipid-soluble properties allow it to concentrate within the phospholipid membrane, where lipid peroxidation occurs. Vitamin E is the primary defense against lipophilic radicals, whereas vitamin C acts against hydrophilic radicals (Dekkers et al., 1996). Although the dietary requirements for vitamin E for most animal species are estimated to be between 5 to 50 IU/KG of diet (Subcommittee on Vitamin Tolerance, 1987), an equation to calculate the dietary vitamin E requirement has been developed based on the degree of dietary fatty acid unsaturation (Muggli, 1989).

Not all forms of vitamin E are equivalent with respect to biologic activity, and vitamin E nomenclature is confusing. Naturally occurring α-tocopherol, denoted as *d*-α-tocopherol or RRR-α-tocopherol, has the highest potency at 1.49 IU/mg (activity/weight), with its acetate at 1.36 IU/mg (McDowell, 1989). Vitamin E is usually supplemented in the form of the synthetic *dl*-α-tocopheryl acetate, also called all-rac-α-tocopheryl acetate, which has a potency of 1 IU/mg (Subcommittee on Vitamin Tolerance, 1987). The isomers β-, γ-, and δ-tocopherol have very little biological activity. A source of variability between studies is the method of expression of plasma α-tocopherol content. While most authors will simply report plasma α-tocopherol at a concentration per unit of plasma, others report the ratio of plasma α-tocopherol concentration to the amount of circulating lipids, especially in people with widely varying plasma lipid concentrations (Horwitt et al., 1972). Others suggest expressing the α-tocopherol ratio relative to the sum of plasma cholesterol and triacylglycerol concentrations or to cholesterol concentration alone (Thurnham et al., 1986). However, these ratios may be misleading when evalulating experimental populations with diminishing plasma cholesterol concentrations, such as those supplemented with high levels of n-3 fatty acids (Meydani et al., 1991a; Hall et al., 2002).

The *in vivo* relationship between n-3 fatty acid intake, vitamin E intake, and lipid peroxidation has been studied in people and rodents, but is not fully defined. Both young and older women fed fish oil capsules containing 1.68g of EPA, 0.72g of DHA and 6 IU of α-tocopherol for

12 weeks had a significant increase in plasma lipid peroxides at 8 weeks (Meydani et al., 1991a). Interestingly, while plasma concentration of lipid peroxides approached baseline levels at 12 weeks, plasma α-tocopherol levels did not change during this study. Likewise, when postmenopausal women and adult men were supplemented with fish oil or EPA/DHA plus variable quantities of α-tocopherol acetate for 5 to 6 weeks, increased concentrations of lipid peroxidation markers were observed. However, these were independent of the level of α-tocopherol acetate supplementation (Wander et al., 1996; Allard et al., 1997). In the rat, increasing quantities of dietary n-3 fatty acids leads to correspondingly increased concentrations of thiobarbituric acid reacting substances (TBARS), a product of lipid peroxidation, in the urine, heart and liver tissue (L'Abbe et al., 1991). The peripheral blood immune cells from fish oil-fed rats that were injected intraperitoneally with a sterile inflammatory agent, glycogen, also had 45% less α-tocopherol than lard-fed counterparts (Fritsche and McGuire, 1996).

Three studies investigating the role of vitamin E in canine subjects receiving dietary fish oil supplementation are summarized in table 1.1. In contrast to reports in other species as well as the other canine studies listed in this table, decreased concentrations of the oxidation marker malondialdehyde (MDA) and no significant differences in plasma α-topopherol were detected in aged dogs in one study (Kearns et al., 1999). This apparent discrepancy may be due to differences in the dietary n-6:n-3 fatty acid ratios used in these studies. The only canine study population to show an increase in lipid peroxidation also had the only significant decrease in plasma α-tocopherol (Wander et al., 1997). This reduction on plasma α-tocopherol, a promoter of lymphocyte proliferation, may explain why these dogs had depressed cell-mediated immune responses based on a delayed-type hypersensitivity test.

Dietary supplementation of vitamin E inhibits the suppressive effect of n-3 fatty acid supplementation on lymphocyte proliferation (Kramer et al., 1991; Kumar et al., 1992). Presumably this effect is due to reduced lipid peroxidation. However, vitamin E has also been shown to promote lymphocyte function in experimental conditions where oxidative damage to lipids is not present. Proposed mechanisms explaining this promotion of lymphocyte function include a vitamin E-related decrease in macrophage production of lymphocyte-inhibiting PGE_2 (Beharka et al., 1997); inhibition of COX activity through the reduction of peroxynitrite production is a likely cause for the decrease in PGE2 synthesis (Beharka et al., 2002). Vitamin E may also have a more direct enhancing effect on T-cell function, mediated through increased T-cell production of IL-2 (Meydani et al., 1990; Adolfsson et al., 2001). Vitamin E levels in the

thymus may also influence T-lymphocyte differentiation: rats fed a high vitamin E diet had higher percentages of both $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ thymic lymphocytes (Moriguchi et al., 1998).

Table 1.1 Effects of dietary fish oil and vitamin E supplementation in canine studies.

Study	Dietary n-6:n-3 Ratios/ Oil Content	Dietary Vit. E Activity/kg of diet	Subjects	Study Period (weeks)	Effects
Wander et al., 1997	31:1 5.4:1 1.4:1 4.4% Fish and/or Corn oil on DMB	~204 IU	Older Dogs	8	-Plasma αT of 1.4:1 group lower compared to others - Plasma TBARS higher in 1.4:1 group compared to others - Urine TBARS higher in 1.4:1 and 5.4:1 groups compared to 31:1 group
Kearns et al., 1999	25:1 5:1 1.65% Fish oil on DMB	100 IU	Young and Older Dogs	8	-No significant change in plasma αT -Decreased MDA levels of aged dogs fed 5:1 diet
Hall et al., 2002	40:1 1.4:1 4.4% Fish and/or Corn oil on DMB	17 IU 101 IU 447 IU	Older Dogs	~11.5	-No effect of n6:n3 ratio on TBARS -TBARS significantly decreased in 1.4:1 group supplemented with 447 IU vitamin E -Plasma αT of dogs fed 1.4:1 ratio did not increase as much as other group

Plasma α-tocopherol (αT) expressed as simple plasma concentrations. No differences in plasma α-tocopherol were found in any study when concentrations were expressed relative to the plasma concentration of total lipid (cholesterol + triglyceride). TBARS = thiobarbituric acid reacting substances; MDA = malondialdehyde; DMB = dry matter basis.

Hypotheses and Objectives

Hypotheses of this study were:

1. A very low dietary n-6:n-3 fatty acid ratio is needed to significantly affect the production of inflammatory chemical mediators in the dog
2. Polyunsaturated fatty acid supplementation-induced inhibition of lymphocyte proliferation is significantly effected by the n-6:n-3 fatty acid ratio
3. Platelet aggregation is unaffected by manipulation of the dietary n-6:n-3 fatty acid ratio in the dog.
4. Polyunsaturated fatty acid supplementation-induced effects on lipid peroxidation and lymphocyte proliferation will be suppressed by dietary vitamin E supplementation

Specific objectives of this study were:

1. To assess and compare the effects of a moderate and a low n-6:n-3 fatty acid ratio diet on the *in vitro* and *in vivo* production of IL-1, IL-6, TNF, PGE_2, and PAF in the healthy canine;
2. To determine the effect of these diets on *in vitro* lymphocyte proliferation using two methodologies;
3. To assess the effect of these diets on platelet aggregation;
4. To determine the influence of high vitamin E intake and lipid peroxidation in dogs consuming the low n-6:n-3 fatty acid ratio diet using the parameters listed in objectives 1, 2 and 3.

References

Abbas A, Lichtman A, Pober J. 2000. Cellular and Molecular Immunology, 4th ed. Phiadelphia: W. B. Saunders Company.

Adan Y, Shibata K, Sato M, Ikeda I, Imaizumi K. 1999. Effects of docosahexaenoic and eicosapentanoic acid on lipid metabolism, eicosanoid production, platelet aggregation, and atherosclerosis in hypercholesterolemic rats. Biosci Biotechnol Biochem 63:111-119.

Adolfsson O, Huber B, Meydani S. 2001. Vitamin E-enhanced IL-2 production in old mice: Naive but not memory T cells show increased cell division cycling and IL-2-producing capacity. J Immunol 167:3809-3817.

Akisu M, Huseyinov A, Baka M, Yalaz M, Kultursay N. 2002. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the generation of platelet-activating factor and leukotriene B(4) in hypoxic-ischemic brain in young mice. Prostaglandins Leukot Essent Fatty Acids 67:429-433.

Allard J, Kurian R, Aghdassi E, Muggli R, Royall D. 1997. Lipid peroxidation during n-3 fatty acid and vitamin E supplementation in humans. Lipids 32:535-541.

Andrioli G, Carletto A, Guarini P, Galvani S, Biasi D, Bellavite P, Corrocher R. 1999. Differential effects of dietary supplementation with fish oil or soy lecithin on human platelet adhesion. Thromb Haemost 82:1522-1527.

Anel A, Naval J, Gonzalez B, Torres JM, Mishal Z, Uriel J, Pineiro A. 1990. Fatty acid metabolism in human lymphocytes. I. Time-course changes in fatty acid composition and membrane fluidity during blastic transformation of peripheral blood lymphocytes. Biochim Biophys Acta 1044:323-331.

Arend WP, Welgus HG, Thompson RC, Eisenberg SP. 1990. Biological properties of recombinant human monocyte-derived interleukin 1 receptor antagonist. J Clin Invest 85:1694-1697.

Argiles JM, Lopez-Soriano J, Busquets S, Lopez-Soriano FJ. 1997. Journey from cachexia to obesity by TNF. Faseb J 11:743-751.

Arntzen KJ, Brekke OL, Vatten L, Austgulen R. 1998. Reduced production of PGE2 and PGF2 alpha from decidual cell cultures supplemented with N-3 polyunsaturated fatty acids. Prostaglandins Other Lipid Mediat 56:183-195.

Arrington J, Chapkin R, Switzer K, Morris J, McMurray D. 2001. Dietary n-3 polyunsaturated fatty acids modulate purified murine T-cell subset activation. Clin Exp Immunol 125:499-507.

Baker PE, Fahey JV, Munck A. 1981. Prostaglandin inhibition of T-cell proliferation is mediated at two levels. Cell Immunol 61:52-61.

Beharka A, Wu D, Han S, Meydani S. 1997. Macrophage prostaglandin production contributes to the age-associated decrease in T cell function which is reversed by the dietary antioxidant vitamin E. Mech Ageing Dev 93:59-77.

Beharka AA, Wu D, Serafini M, Meydani SN. 2002. Mechanism of vitamin E inhibition of cyclooxygenase activity in macrophages from old mice: role of peroxynitrite. Free Radic Biol Med 32:503-511.

Benveniste J, Henson PM, Cochrane CG. 1972. Leukocyte-dependent histamine release from rabbit platelets. The role of IgE, basophils, and a platelet-activating factor. J Exp Med 136:1356-1377.

Bochsler P, Slauson D. 2002. Inflammation and Repair of Tissue. In: Slauson D, Cooper B, editors. Mechanisms of Disease; A textbook of comparative general pathology, 3rd ed. St. Louis: Mosby, Inc. p 140-245.

Boudreaux M, Reinhart G, Vaughn D, Spano J, Mooney M. 1997. The effects of varying dietary n-6 to n-3 fatty acid ratios on platelet reactivity, coagulation screening assays, and antithrombin III activity in dogs. J Am Anim Hosp Assoc 33:235-243.

Bright J, Sullivan P, Melton S, Schneider J, McDonald T. 1994. The effects of n-3 fatty acid supplementation on bleeding time, plasma fatty acid composition, and in vitro platelet aggregation in cats. J Vet Intern Med 8:247-252.

Brunda MJ, Herberman RB, Holden HT. 1980. Inhibition of murine natural killer cell activity by prostaglandins. J Immunol 124:2682-2687.

Calder P. 2001. Polyunsaturated fatty acids, inflammation, and immunity. Lipids 36:1007-1024.

Calder P, Bevan S, Newsholme E. 1992. The inhibition of T-lymphocyte proliferation by fatty acids is via an eicosanoid-independent mechanism. Immunology 75:108-115.

Calder P, Newsholme E. 1992. Polyunsaturated fatty acids suppress human peripheral lymphocyte proliferation and interleukin-2 production. Clin Sci 82:695-700.

Calder PC. 1997. n-3 polyunsaturated fatty acids and cytokine production in health and disease. Ann Nutr Metab 41:203-234.

Casali RE, Hale JA, LeNarz L, Faas F, Morris MD. 1986. Improved graft patency associated with altered platelet function induced by marine fatty acids in dogs. J Surg Res 40:6-12.

Collins T. 1999. Acute and chronic inflammation. In: Cotran R, Kumar V, Collins T, editors. Robbins Pathologic basis of disease, 6th ed. Philadelphia: WB Saunders. p 70-74.

Conti P, Panara MR, Barbacane RC, Bongrazio M, Dempsey RA, Reale M. 1993. Human recombinant IL-1 receptor antagonist (IL-1Ra) inhibits leukotriene B4 generation from human monocyte suspensions stimulated by lipopolysaccharide (LPS). Clin Exp Immunol 91:526-531.

Darling G, Fraker DL, Jensen JC, Gorschboth CM, Norton JA. 1990. Cachectic effects of recombinant human tumor necrosis factor in rats. Cancer Res 50:4008-4013.

Dekkers JC, van Doornen LJ, Kemper HC. 1996. The role of antioxidant vitamins and enzymes in the prevention of exercise-induced muscle damage. Sports Med 21:213-238.

Duthie S, Ma A, Ross M, Collins A. 1996. Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. Cancer Res 56:1291-1295.

Dyerberg J, Bang HO. 1979. Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. Lancet 2:433-435.

Dyerberg J, Bang HO, Hjorne N. 1975. Fatty acid composition of the plasma lipids in Greenland Eskimos. Am J Clin Nutr 28:958-966.

Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, van der Meer JW, Cannon JG, Rogers TS, Klempner MS, Weber PC, et al. 1989. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med 320:265-271.

Endres S, Meydani S, Ghorbani R, Schindler R, Dinarello C. 1993. Dietary supplementation with n-3 fatty acids suppresses interleukin-2 production and mononuclear cell proliferation. J Leukocyte Biol 54:599-603.

Evans JH, Spencer DM, Zweifach A, Leslie CC. 2001. Intracellular calcium signals regulating cytosolic phospholipase A2 translocation to internal membranes. J Biol Chem 276:30150-30160.

Feng C, Keisler DH, Fritsche KL. 1999. Dietary omega-3 polyunsaturated fatty acids reduce IFN-gamma receptor expression in mice. J Interferon Cytokine Res 19:41-48.

Fischer S, Weber PC. 1984. Prostaglandin I3 is formed in vivo in man after dietary eicosapentaenoic acid. Nature 307:165-168.

Freese R, Mutanen M. 1997. α-Linolenic acid and marine long-chain n-3 fatty acids differ only slightly in their effects on hemostatic factors in healthy subjects. Am J Clin Nutr 66:591-598.

Fritsche K, McGuire S. 1996. The adverse effects of an in vivo inflammatory challenge on the vitamin E status of rats is accentuated by fish oil feeding. J Nutr Biochem 7:623-631.

Goodwin JS, Bankhurst AD, Messner RP. 1977. Suppression of human T-cell mitogenesis by prostaglandin. Existence of a prostaglandin-producing suppressor cell. J Exp Med 146:1719-1734.

Goodwin JS, Messner RP, Peake GT. 1978. Prostaglandin suppression of mitogen-stimulated lymphocytes in vitro. Changes with mitogen dose and preincubation. J Clin Invest 62:753-760.

Gordon D, Bray MA, Morley J. 1976. Control of lymphokine secretion by prostaglandins. Nature 262:401-402.

Hall J, Tooley K, Gradin J, Jewell D, Wander R. 2002. Influence of dietary long-chain n-3 fatty acids from Menhaden fish oil on plasma concentrations of alpha-tocopherol in geriatric Beagles. Am J Vet Res 63:104-110.

Hall J, Wander R, Gradin J, Du S, Jewell D. 1999. Effect of dietary n-6-to-n-3 fatty acid ratio on complete blood and total white blood cell counts, and T-cell subpopulations in aged dogs. Am J Vet Res 60:319-327.

Hansen R, Savage C, Reidlinger K, Traub-Dargatz J, Ogilvie G, Mitchell D, Fettman M. 2002. Effects of dietary flaxseed oil supplementation on equine plasma fatty acie concentrations and whole blood platelet aggregation. J Vet Intern Med 16:457-463.

Hensyl W, editor. 1990. Stedman's Medical Dictionary, 25th ed. Baltimore: Williams & Wilkins.

Horii T, Satouchi K, Kobayashi Y, Saito K, Watanabe S, Yoshida Y, Okuyama H. 1991. Effect of dietary alpha-linolenate on platelet-activating factor production in rat peritoneal polymorphonuclear leukocytes. J Immunol 147:1607-1613.

Horwitt MK, Harvey CC, Dahm CH, Jr., Searcy MT. 1972. Relationship between tocopherol and serum lipid levels for determination of nutritional adequacy. Ann N Y Acad Sci 203:223-236.

Hubel CA, Roberts JM, Taylor RN, Musci TJ, Rogers GM, McLaughlin MK. 1989. Lipid peroxidation in pregnancy: new perspectives on preeclampsia. Am J Obstet Gynecol 161:1025-1034.

Jeffery N, Newsholme E, Calder P. 1997. Level of polyunsaturated fatty acids and the n-6 to n-3 polyunsaturated fatty acid ratio in the rat diet alter serum lipid levels and lymphocyte functions. Prostaglandins, Leukotrienes, and Ess. Fatty Acids 57:149-160.

Jeffery N, Sanderson P, Sherrington E, Newsholme E, Calder P. 1996. The ratio of n-6 to n-3 polyunsaturated fatty acids in the rat diet alters serum lipid levels and lymphocyte functions. Lipids 31:737-745.

Jolly C, Jiang Y-H, Chapkin R, McMurray D. 1997. Dietary (n-3) polyunsaturated fatty acids suppress murine lymphoproliferation, interleukin-2 secretion, and the formation of diacylglycerol and ceramide. J Nutr 127:37-43.

Jolly C, McMurray D, Chapkin R. 1998. Effect of dietary n-3 fatty acids on interleukin-2 and interleukin-2 receptor alpha expression in activated murine lymphocytes. Prostaglandins, Leukotrienes, and Ess. Fatty Acids 58:289-293.

Kearns R, Hayek M, Turek J, Meydani M, Burr J, Greene R, Marshall C, Adams S, Borgert R, Reinhart G. 1999. Effect of age, breed, and dietary omega-6 (n-6) : omega-3 (n-3) fatty acid ratio on immune function, eicosanoid production, and lipid peroxidation in young and aged dogs. Vet Immuno Immunopathol 69:165-183.

Kelley D. 2001. Modulation of human immune and inflammatory responses by dietary fatty acids. Nutrition 17:669-673.

Khalfoun B, Thibault G, Lacord M, Gruel Y, Bardos P, Lebranchu Y. 1996. Docosahexaenoic and eicosapentaenoic acids inhibit human lymphoproliferative responses in vitro but not the expression of T cell surface activation markers. Scand J Immunol 43:248-256.

Knight J. 2000. Review: free radicals, antioxidants, and the immnume system. Ann Clin Lab Sci 30:145-158.

Kramer T, Schoene N, Douglass L, Judd J, Ballard-Barbash R, Taylor P, Bhagavan H, Nair P. 1991. Increased vitamin E intake restores fish oil-induced suppressed blastogenesis of mitogen-stimulated T lymphocytes. Am J Clin Nutr 54:896-902.

Kristensen S, Schmidt E, Dyerberg J. 1989. Dietary supplementation with n-3 polyunsaturated fatty acids and human platelet function: a review with particular emphasis on implications for cardiovascular disease. J Int Med 225:141-150.

Kromann N, Green A. 1980. Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974. Acta Med Scand 208:401-406.

Kumar G, Das U, Kumar K, Madhavi N, Das N, Tan B. 1992. Effect of n-6 and n-3 fatty acids on the proliferation of human lymphocytes and their secretion of TNF-alpha and IL-2 in vitro. Nutr Research 12:815-823.

Kuratko C. 2000. Proliferation of colonic lymphocytes in response to inflammatory cytokines is lower in mice fed fish oil than in mice fed corn oil. Cancer Letters 148:27-32.

L'Abbe MR, Trick KD, Beare-Rogers JL. 1991. Dietary (n-3) fatty acids affect rat heart, liver and aorta protective enzyme activities and lipid peroxidation. J Nutr 121:1331-1340.

Lands W, LeTillier P, Rome L, Vanderhoek J. 1973. Inhibition of prostaglandin biosynthesis. Adv Biosci 9:15-17.

Lee T, Austen K. 1986. Arachidonic acid metabolism by the 5-lipoxygenase pathway, and the effects of alternative dietary fatty acids. Advan Immunol 39:145-147.

Lee TH, Sethi T, Crea AE, Peters W, Arm JP, Horton CE, Walport MJ, Spur BW. 1988. Characterization of leukotriene B3: comparison of its biological activities with leukotriene B4 and leukotriene B5 in complement receptor enhancement, lysozyme release and chemotaxis of human neutrophils. Clin Sci (Lond) 74:467-475.

Mancuso P, Whelan J, DeMichele SJ, Snider CC, Guszcza JA, Claycombe KJ, Smith GT, Gregory TJ, Karlstad MD. 1997. Effects of eicosapentaenoic and gamma-linolenic acid on lung permeability and alveolar macrophage eicosanoid synthesis in endotoxic rats. Crit Care Med 25:523-532.

Martin-Chouly CA, Menier V, Hichami A, Youmine H, Noel F, Pedrono F, Legrand AB. 2000. Modulation of PAF production by incorporation of arachidonic acid and eicosapentaenoic acid in phospholipids of human leukemic monocyte-like cells THP-1. Prostaglandins Other Lipid Mediat 60:127-135.

Mayer K, Merfels M, Muhly-Reinholz M, al e. 2002. ω-3 fatty acids suppress monocyte adhesion to human endothelial cells: role of endothelial PAF generation. Am J Physiol Heart Circ Physiol 283:H811-H818.

McDowell L. 1989. Vitamin E. In: Vitamins in Animal Nutrition. San Diego: Academic Press. p 93-131.

McManus LM, Pinckard RN. 2000. PAF, a putative mediator of oral inflammation. Crit Rev Oral Biol Med 11:240-258.

McMurray D, Jolly C, Chapkin R. 2000. Effects of dietary n-3 fatty acids on T cell activation and T cell receptor-mediated signaling in a murine model. J Infect Dis 182:S103-S107.

McNiel E, Ogilvie G, Mallinckrodt C, Richardson K, Fettman M. 1999. Platelet function in dogs treated for lymphoma and hemangiosarcoma and supplemented with dietary n-3 fatty acids. J Vet Intern Med 13:574-580.

Meydani M, Natiello F, Goldin B, Free N, Woods M, Schaefer E, Blumberg J, Gorbach S. 1991a. Effect of long-term fish oil supplementation on vitamin E status and lipid peroxidation in women. J Nutr 121:484-491.

Meydani S, Barklund M, Liu S, Meydani M, Miller R, Cannon J, Morrow F, Rocklin R, Blumberg J. 1990. Vitamin E supplementation enhances cell-mediated immunity in healthy elderly subjects. Am J Clin Nutr 52:557-563.

Meydani S, Endres S, Woods M, Goldin B, Soo C, Morrill-Labrode A, Dinarello C, Gorbach S. 1991b. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. J Nutr 121:547-555.

Meydani S, Lichtenstein A, Cornwall S, Meydani M, Goldin B, Rasmussen H, Dinarello C, Schaefer E. 1993. Immunologic effects of national cholesterol education panel step-2 diets with and without fish-derived acid enrichment. J Clin Invest 92:105-113.

Mooney MA, Vaughn DM, Reinhart GA, Powers RD, Wright JC, Hoffman CE, Swaim SF, Baker HJ. 1998. Evaluation of the effects of omega-3 fatty acid-containing diets on the inflammatory stage of wound healing in dogs. Am J Vet Res 59:859-863.

Morgan DA, Ruscetti FW, Gallo R. 1976. Selective in vitro growth of T lymphocytes from normal human bone marrows. Science 193:1007-1008.

Moriguchi S, Hamada M, Yamauchi K, Sakai K, Yamamoto S. 1998. The role of vitamin E in T-cell differentiation and the decrease of cellular immunity with aging. J Med Invest 45:1-8.

Muggli R. 1989. Dietary fish oils increase the requirement for vitamin E in humans. In: Chandra R, editor. Health effects of fish and fish oils. St. John's, Newfoundland: ARTS Biomedical Publishers and Distributors. p 201-210.

Mylonas C, Kouretas D. 1999. Lipid peroxidation and tissue damage. in vivo 13:295-310.

Nishiyama A, Cavaglieri C, Curi R, Calder P. 2000. Arachidonic acid-containing phosphatidylcholine inhibits lymphocyte proliferation and decreases interleukin-2 and interferon-γ production from concanavalin A-stimulated rat lymphocytes. Biochimica et Biophysica Acta 1487:50-60.

Ohata T, Fukuda K, Takahashi M, Sugimura T, Wakabayashi K. 1997. Suppression of nitric oxide production in lipopolysaccharide-stimulated macrophage cells by omega 3 polyunsaturated fatty acids. Jpn J Cancer Res 88:234-237.

Prescott S. 1992. What are the effects of dietary fatty acid modification on platelet eicosanoid metabolism, platelet-activating factor, and platelet function? How might these alterations influence thrombosis? Am J Clin Nutr 56:801S-802S.

Rappaport RS, Dodge GR. 1982. Prostaglandin E inhibits the production of human interleukin 2. J Exp Med 155:943-948.

Reinhart G. 1996. Review of omega-3 fatty acids and dietary influences on tissue concentrations. In: Carey D, Norton S, Bolser S, editors. Recent advances in canine and feline nutritional research: Proceedings of the 1996 Iams international nutrition symposium. Wilingtion: Orange Frazer Press. p 235-242.

Sadeghi S, Wallace F, Calder P. 1999. Dietary lipids modify the cytokine response to bacterial lipopolysaccharide in mice. Immunology 96:404-410.

Saker K, Eddy A, Thatcher C, Kalnitsky J. 1998. Manipulation of dietary (n-6) and (n-3) fatty acids alters platelet function in cats. J Nutr 128:2645S-2647S.

Salonen R, Nikkari T, Seppanen K, et a. 1987. Effect of omega-3 fatty acid supplementation on platelet aggregability and platelet produced thromboxane. Thromb Haemost 57:269-272.

Sanderson P, Calder P. 1998. Dietary fish oil appears to prevent the activation of phospholipase C-γ in lymphocytes. Biochimica et Biophysica Acta 1392:300-308.

Santoli D, Zurier R. 1989. Prostaglandin E procursor fatty acids inhibit human IL-2 production by a prostaglandin E-independent mechanism. J Immunol 143:1303-1309.

Scott K, Hill R, Lewis D, Boning A, Sundstrom B. 2001. Effect of α-tocopherol acetate supplementation on vitamin E concentrations in Greyhounds before and after a race. Am J Vet Res 62:1118-1120.

Sharon J. 1998. Basic Immunology. Baltimore: Williams and Wilkins.

Soyland E, Lea T, Sandstad B, Drevon A. 1994. Dietary supplementation with very long-chain n-3 fatty acids in man decreases expression of the interleukin-2 receptor (CD25) on mitogen-stimulated lymphocytes from patients with inflammatory skin diseases. European J Clin Investigation 24:236-242.

Strassmann G, Kambayashi T. 1995. Inhibition of experimental cancer cachexia by anti-cytokine and anti-cytokine-receptor therapy. Cytokines Mol Ther 1:107-113.

Subcommittee on Vitamin Tolerance. 1987. Vitamin E. In: Tolerance SoV, editor. Vitamin Tolerance of Animals. Washington DC: National Academy Press. p 23-30.

Terada S, Takizawa M, Yamamoto S, Ezaki O, Itakura H, Akagawa K. 2001. Suppressive mechanisms of EPA on human T cell proliferation. Microbiol Immunol 45:473-481.

Thorwest M, Balling E, Kristensen S, Aagaard S, Hakami A, Husted S, Marqversen J, Hjortdal V. 2000. Dietary fish oil reduces microvascular thrombosis in a porcine experimental model. Thromb Res 99:203-208.

Thurnham DI, Davies JA, Crump BJ, Situnayake RD, Davis M. 1986. The use of different lipids to express serum tocopherol: lipid ratios for the measurement of vitamin E status. Ann Clin Biochem 23 (Pt 5):514-520.

Tilley S, Coffman T, Koller B. 2001. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. J Clin Invest 108:15-23.

Tisdale M. 1997. Biology of cachexia. JNCI 89:1763-1773.

Vaughn D, Reinhart G, Swaim S, Lauten S, Garner C, Boudreaux M, Spano J, Hoffman C, Conner B. 1994. Evaluation of effects of dietary n-6 to n-3 fatty acid ratios on leukotriene B synthesis in dog skin and neutrophils. Vet Dermatol 5:163-173.

von Schacky C, Fischer S, Weber PC. 1985. Long-term effects of dietary marine omega-3 fatty acids upon plasma and cellular lipids, platelet function, and eicosanoid formation in humans. J Clin Invest 76:1626-1631.

Wallace F, Miles E, Calder P. 2000. Activation state alters the effect of dietary fatty acids on pro-inflammatory mediator production by murine macrophages. Cytokine 12:1374-1379.

Wallace F, Miles E, Evans C, Stock T, Yaqoob P, Calder P. 2001. Dietary fatty acids influence the production of Th1- but not Th2-type cytokines. J Leuko Biol 69:449-457.

Wander R, Du S, Ketchum S, Rowe K. 1996. Effects of interaction of RRR-α-tocopheryl acetate and fish oil on low-density-lipoprotein oxidation in postmenopausal women with and without hormone-replacement therapy. Am J Clin Nutr 63:184-193.

Wander R, Hall J, Gradin J, Du S, Jewell D. 1997. The ratio of dietary (n-6) to (n-3) fatty acids influences immune system function, eicosanoid metabolism, lipid peroxidation and vitamin E status in aged dogs. J Nutr 127.

Webb DR, Jr., Jamieson T. 1976. Control of mitogen-induced transformation: characterization of a splenic suppressor cell and its mode of action. Cell Immunol 24:45-57.

Wensing AG, Mensink RP, Hornstra G. 1999. Effects of dietary n-3 polyunsaturated fatty acids from plant and marine origin on platelet aggregation in healthy elderly subjects. Br J Nutr 82:183-191.

Whitaker MO, Wyche A, Fitzpatrick F, Sprecher H, Needleman P. 1979. Triene prostaglandins: prostaglandin D3 and icosapentaenoic acid as potential antithrombotic substances. Proc Natl Acad Sci U S A 76:5919-5923.

Wigmore SJ, Fearon KC, Maingay JP, Ross JA. 1997. Down-regulation of the acute-phase response in patients with pancreatic cancer cachexia receiving oral eicosapentaenoic acid is mediated via suppression of interleukin-6. Clin Sci (Lond) 92:215-221.

Yaqoob P, Newsholme E, Calder P. 1994. The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation. Immunology 82:603-610.

CHAPTER II: HEMATOLOGICAL AND SERUM BIOCHEMICAL EFFECTS OF DIETARY FISH OIL AND VITAMIN E SUPPLEMENTATION IN THE DOG

Introduction

The n-3 fatty acids, particularly eicosapentaenoic acid (EPA), have been extensively studied with respect to not only their effects on the immune response, but also in their ability to influence platelet aggregation. The low incidence of heart disease in Greenlandic Inuit Eskimos (Kromann and Green, 1980) has been attributed to impaired platelet aggregation secondary to a diet high in n-3 fatty acid-rich marine life. (Dyerberg and Bang, 1979). The effects of n-3 fatty acids on platelet aggregation are thought to be largely due to their impact on eicosanoid metabolism. The EPA-derived thromboxane A_3 (TXA_3) has attenuated proaggregatory activity compared to thromboxane A_2 (TXA_2) which is derived from the n-6 fatty acid arachidonic acid (AA) (Whitaker et al., 1979). The EPA-derived prostaglandin I_3 (PGI_3) retains the anti-aggregatory activity of its AA-derived counterpart, prostaglandin I_2 (PGI_2) (Fischer and Weber, 1984). Finally, EPA reduces the production of another proaggregatory lipid mediator, platelet-activating factor (PAF) (Martin-Chouly et al., 2000). Through these mechanisms and possibly others, dietary n-3 fatty acid supplementation appears to have the potential to reduce platelet aggregation, thereby reducing the incidence of blood clot formation or thrombosis. However, the results of studies investigating this effect in a variety of species have been mixed. While many human (Kristensen et al., 1989; Freese and Mutanen, 1997; Wensing et al., 1999) and animal (Saker et al., 1998; Adan et al., 1999) studies have found diminished platelet aggregation in subjects supplemented with n-3 fatty acids, other studies have failed to document significant changes in platelet function after manipulation of dietary fatty acids (Salonen et al., 1987; Bright et al., 1994; Hansen et al., 2002). A diet high in n-3 fatty acids does not appear to significantly attenuate *in vitro* platelet aggregation in healthy dogs (Boudreaux et al., 1989; Vaughn and Reinhart, 1996) or canine cancer patients (McNiel et al., 1999).

The only other hematologic or serum biochemical effect associated with dietary n-3 fatty acid supplementation is decreased serum triglyceride concentrations. Greenlandic Inuit Eskimos have lower serum triglycerides as well as reduced platelet aggregation, and this may also contribute to their low incidence of atherosclerosis and secondary heart disease (Bang et al., 1971). Dietary n-3 fatty acid supplementation reduces serum triglycerides in people with primary hyperlipoproteinemia (Illingworth et al., 1989; Harris, 1996) as well as normal test subjects (Sanders et al., 1989; Harris, 1997), with no apparent effect on total cholesterol. Although dietary

n-3 fatty acid supplementation in normal dogs has either had no effect (Hall et al., 1999) or has been associated with increased serum triglycerides (Kearns et al., 1999), it is still recommended by some authors for the treatment of canine idiopathic hyperlipidemia (Bauer, 1995). Although statistically significant increases in the serum concentrations of the hepatocellular-specific enzyme alanine transferase (ALT) and cholesterol have been reported in dogs fed high levels of dietary n-3 fatty acids, concentrations of these analytes were not outside the reference range for healthy dogs (Hall et al., 1999).

While reduced platelet aggregation could be a beneficial or an adverse side effect of dietary n-3 fatty acid supplementation, depending on the circumstances, n-3 fatty acid-induced tissue lipid peroxidation can only be detrimental to cells throughout the body. The numerous double bonds within long chain n-3 fatty acids makes them prone to attack by oxygen free radicals, initiating a self-perpetuating chain reaction that damages host tissue. Lipid peroxidation has been implicated in the pathogenesis of a number of diseases (Mylonas and Kouretas, 1999), and impairs cell-mediated immune responses as well (Meydani et al., 1993; Wander et al., 1997). Vitamin E is probably one of the more important antioxidants in the defense against lipid peroxidation because of its lipid solubility and concentration within phospholipid cell membranes. Accordingly, increased intake of vitamin E has been suggested when higher levels of polyunsaturated fatty acids (PUFA) are consumed (National Research Council, 1985; Muggli, 1989). Many human and animal studies report increased concentrations of lipid peroxidation markers in subjects consuming increased quantities of n-3 fatty acid (L'Abbe et al., 1991; Meydani et al., 1991; Wander et al., 1996; Allard et al., 1997; Wander et al., 1997). Evidence of lipid peroxidation as well as a significant reduction in plasma α-tocopherol (vitamin E) has been documented in dogs consuming rations high in n-3 fatty acids (Wander et al., 1997).

The purpose of this study was to determine the effect of an n-3 fatty acid enriched diet with a low n-6:n-3 fatty acid ratio, with and without vitamin E supplementation, on platelet aggregation, select hematology and serum biochemistry parameters, and lipid peroxidation in young, healthy Walkerhound-cross dogs. Dietary induced-changes in each dog was confirmed by evaluating serum vitamin E concentrations and the fatty acid proportions of plasma phospholipids.

Materials and Methods

Experimental Animals

Fifteen healthy Walker Hound-cross dogs were obtained for the study from a colony. The study population consisted of eight intact females, six intact males and one castrated male ranging

in age from one to four years. Body weights ranged from 46 to 59 pounds. During the study, dogs were housed individually in covered outdoor runs and fed once daily. Water was available ad libitum. The experimental protocol was approved by the Louisiana State University Institutional Animal Care and Use Committee.

Diet Formulations

Previous to the experiment, each dog received approximately 2832 kcal of Laboratory Canine Diet 5006 (Lab Diet, Purina Mills, LLC, Gray Summit, MO) per day (800g fed x 3.54 kcal/g ME). Experimental diets were formulated to meet energy requirements based on the equation from the 1974 National Research Council report, ME = 132 x $BW_{kg}^{0.75}$ (National Research Council, 1985). The experimental population was randomly assigned to three dietary treatment groups for the duration of the twelve week study. The first group (Sunflower oil) received approximately 500g of Laboratory Canine Diet, top dressed with 12.4g of sunflower oil and a 3-5g compressed portion of Hill's canned canine r/d® (Science Diet, Hill's Pet Nutrition, Inc., Topeka, KS). The second group (Fish oil) received 0.6g of sunflower oil, 7g of menhaden fish oil (Omega Protein, Inc., Reedville, VA), and a 3-5g compressed portion of Hill's canned canine r/d® top dressed on approximately 500g of Laboratory Canine Diet. The third group (Fish oil + E) received the same diet as Group B with 0.17g of α-tocopherol acetate injected into the compressed portion of r/d® canned food. The Laboratory Canine Diet kibble, the sunflower oil, and the menhaden fish oil were analyzed by Woodson-Tenent Laboratories (Divison of Eurofins Scientific, Inc., Memphis, TN) (Table 2.1), (Appendix I).

Experimental Design

Dogs were fed their assigned diets for 12 weeks. One week previous to the trial and at weeks 6 and 12 of the dietary treatment, blood was obtained from each dog for evaluation of plasma phospholipid concentrations, serum vitamin E concentrations, platelet aggregation, complete blood count, serum biochemical analysis and plasma lipid peroxide concentrations.

Plasma Phospholipid Analysis

Serum was isolated from blood samples on the day of collection and aliquots were frozen at -80^{o}C until shipped overnight on dry ice to the Comparative Nutrition Research Laboratory, Texas A&M University, College Station, TX. Total lipid extraction was performed using the Folch method. Briefly, 9ml of a 2:1 (chloroform: methanol) solvent solution was added to 500µl of serum. The sample was shaken for 15 minutes, 2ml of distilled water was added to each tube, and the sample was shaken for an additional 10 minutes. The sample tubes were then centrifuged

Table 2.1 Diet fatty acid and vitamin E concentrations (as fed basis).

	Pre-study Diet	Group Sunflower oil	Group Fish oil	Group Fish oil + E
Fatty acid (g/day)				
Total Saturated Fat	22.08	15.2	15.7	15.7
Total MUFA	26.96	19.7	18.4	18.4
Total PUFA	16.32	17.2	13.4	13.4
Total Unsat. Fat	43.28	37	31.8	31.8
Total Fat	65.36	52.2	47.5	47.5
16:1 n7	1.92	1.2	1.9	1.9
18:1 n9	24.56	18.2	16.1	16.1
18:2 n6	15.04	16.4	9.8	9.8
20:4 n6	0.16	0.1	0.15	0.15
22:4 n6	<0.1	<0.1	<0.1	<0.1
18:3 n3	0.8	0.5	0.6	0.6
18:4 n3	0.16	0.1	0.3	0.3
20:4 n3	<0.1	<0.1	0.1	0.1
20:5 n3	<0.1	<0.1	0.75	0.75
22:5 n3	<0.1	<0.1	0.15	0.15
22:6 n3	0.08	<0.1	1.0	1.0
Total n6	9.55	16.6	10.2	10.2
Total n3	0.65	0.69	2.98	2.98
n6:n3 ratio	14.69	23.98	3.4	3.4
Vitamin E (IU/day)	39.2	33.0	25.1	196.6

for 15 minutes at 1200xg. The bottom layer of each sample was transferred into clean test tubes followed by the addition of 5ml of 3:48:47 (chloroform: methanol: water). After an additional 15 minute vortex and centrifugation, the bottom layer of each sample was again transferred into a clean tube and evaporated to dryness under a stream of nitrogen and frozen at -20^{o}C for storage.

After the residue of each sample tube was dissolved in 300µl of chloroform, the lipids were subfractionated by thin layer chromatography (TLC) on 20 x 20cm silica gel G coated 250µm thick glass plates (Fisher Scientific, Pittsburgh, PA). The TLC plates were cleaned in 2:1 (chloroform: methanol) and heated to 100^{o}C for 40 minutes prior to the fractionation. After the plates cooled for 5 minutes, 150µl of each sample was applied in a narrow band about 1cm from the bottom of the plate. TLC standard mixture 18-5-A (Nu-Check Prep, Elysian, MN) was applied to the last lane of each TLC plate. The plates were developed in a filter paper lined TLC tank

equilibrated with 80:20:1 (hexane: diethyl ether: glacial acetic acid) mobile phase until the solvent front reached 1cm from the top of the plate. After visualization in an iodine vapor chamber, the phospholipid subfraction placement was identified based on established standards and scraped into clean Teflon capped test tubes.

Recovery of the phospholipids was performed by transmethylation. Briefly, 2ml of 4% H_2SO_4 in methanol was added to each tube followed by 2 minutes of vortexing and 1 hour of incubation in a 90^{o}C water bath. After the tubes were allowed to cool to room temperature, 3ml of hexane was added, followed by centrifugation for 15 minutes at 1200xg. The top layer containing the fatty acids was transferred to clean test tubes and evaporated to dryness under a stream of nitrogen. Dried samples were frozen at -20^{o}C for storage until gas chromatography was performed.

The resultant fatty acid methyl esters of the phospholipid subfractions were resuspended in 30µl of hexane; one µl of each sample was injected onto an Omegawax 320 fused silica capillary column (0.25µm thickness, 30m long, 0.32mm ID) (Supleco, Inc., Bellafonte, PA) using a Hewlett Packard Series II 5890 Gas Chromatograph (Hewlett Packard Co, Palo Alto, CA) with a split ratio of 50:1. Helium was used as the carrier gas at a flow rate of 1ml/minute, and the final oven temperature was 230^{o}C. A flame ionization detector was used and results were generated with a Hewlett Packard HP Chemstation software package. Fatty acid methyl ester standards (68-B, 20-A, Nu-Check Prep, Elysian, MN) were used to identify the individual fatty acids by retention time comparisons.

Serum Vitamin E Analysis

Serum was isolated from blood samples on the day of collection and aliquots were frozen at -80^{o}C until shipment overnight on dry ice to the Texas Veterinary Medical Diagnostic Laboratory (College of Veterinary Medicine, Texas A & M University, College Station, TX). Samples were protected from light at all times using aluminum foil and amber glassware. After thawing, 500µl of serum and 500µl of ethanol/.01% butylhydroxytoluene (BHT) were combined in a culture tube and placed on a rotary extractor for 20 minutes. The samples were extracted by adding 1ml of hexane to each tube, followed by rotary extraction for an addition 20 minutes. The sample tubes were then centrifuged for 16 minutes at 800xg to separate the hexane layer from the polar components. The hexane layer was removed and passed through a hydrophobic Teflon filter to remove particulate material and any remaining water.

The concentrations of serum vitamin E were determined by reverse-phase high performance liquid chromatography (HPLC) using 95% methanol / 5% deionized water at a flow rate of 2.5 ml/minute and a μBondapak C18 column (Waters Corporation, Milford, MA). Eluted α-tocopherol and α-tocopherol acetate peaks were identified at an ultraviolet absorbance of 280nm by a Waters 484 UV-Vis Detector (Waters Corporation, Milford, MA). The total serum vitamin E concentration was determined by summation of the α-tocopherol and α-tocopherol acetate peaks.

Platelet Aggregation Assay

Nine milliliters of whole blood was drawn from the jugular vein of each dog and placed in a vacutainer tube containing 1ml of 3.8% sodium citrate. The samples were centrifuged at 150xg for 12 minutes. The platelet rich plasma (PRP) was removed and the original sample was recentrifuged at 3000xg for 5 minutes to isolate platelet poor plasma (PPP). The platelet concentration of the PRP was determined using the Unopette Blood Diluting Pipette System (Becton Dickinson and Co, Frankin Lakes, NJ) and a hemacytometer (American Optical Corp, Buffalo, NY). The PRP was adjusted to a platelet concentration of approximately 200,000/uL using autologous PPP.

Platelet aggregation was measured turbidometrically in a dual channel lumi-aggregometer (Model 550, Chrono-log Corp, Havertown, PA). Briefly, a cuvet containing a stir bar and 500uL of PPP was placed in one of two chambers; the light transmittance through the PPP represents 100% aggregation. Well-mixed PRP was placed in the second chamber. Collagen at a concentration of 20μg/ml, or adenosine 5'-diphosphate (ADP) at a concentration of 20μM, was added by forceful infusion to the bottom of the PRP tube to stimulate aggregation. The voltage representing the difference in light transmittance between the PRP and PPP was automatically determined and applied to a strip chart recorder. The maximal change in light transmittance that occurred within 3 minutes was determined using the point of maximal shape change as baseline (Boudreaux et al., 1989).

Hematology and Serum Biochemistry Analysis

Calculated red blood cell hematocrit, platelet concentration, and white blood cell concentration were obtained from whole blood in EDTA using a standard impedance hematology analyzer (Baker System 9110 plus, Biochem Immunosystems Inc, Allentown, PA). Serum triglyceride concentrations were obtained using a 'dry' chemistry analyzer system, in which biochemical reactions occur on film and are then measured optically (Vet Test 8008, Idexx Laboratories Inc, Westbrook, MA). All other biochemical analytes were measured using a

standard spectrophotometric analyzer (Olympus AU 600, Olympus America Inc, Melville, NY), and reagents manufactured by the same company.

Plasma Lipid Peroxide Analysis

Plasma from each dog was isolated from heparinized blood by centrifugation and stored at -80^{o}C until time of analysis. The concentration of plasma lipid hydroperoxides was determined using a colorimetric, quantitative assay according to the manufacturer's instructions (Bioxytech LPO-560, Oxis International, Inc., Portland, OR). The assay is based on the oxidation of ferrous ions to ferric ions by hydroperoxides. The newly-formed ferric ions bind with xylenol orange to produce a stable, colored complex that can be measured spectrophotometrically. Briefly, 90µl of each plasma sample was pretreated with 10µl of catalase to eliminate H_2O_2 interference with the lipid hydroperoxide measurement. A corresponding blank sample was made to obtain the net absorbance for each test sample. Ten µl of reducing agent 20mM *tris* (2-carboxyethyl) phosphine (TCEP) in deionized water was added to each blank sample. Ten µl of deionized water was also added to each test sample. After 30 minutes of incubation at room temperature, 900µl of newly made working reagent was added to all tubes. The working reagent was prepared the day of each assay run and consisted of 1 volume of color developer (25mM ferrous ammonium sulfate in 2.5M H_2SO_4) to 100 volumes of chromogen (125µM xylenol orange in methanol). Following incubation for 60 minutes at room temperature, all tubes were centrifuged at 10,000xg for 10 minutes to remove all flocculated material. After the spectrophotometer was zeroed with deionized water, the absorbance of all supernatants was measured at 560nm.

The net absorbance was obtained by subtracting the absorbance value of each sample blank from the absorbance value of its corresponding sample. The concentration of hydroperoxides in each plasma sample was calculated from the net absorbance and the hydroperoxide apparent molar extinction coefficient of 4.31×10^4 M^{-1} cm^{-1} using the following equation:

$$[LOOH] = (\text{Net } A_{560}/\varepsilon) \times \delta \quad \text{where:}$$

[LOOH] = concentration of lipid hydroperoxides in the sample (µM)

Net A_{560} = Net absorbance at 560nm

$\varepsilon = 0.0431$ M^{-1} cm^{-1}

δ = dilution factor = 11.2 (1.010ml / 0.090ml)

Statistical Analysis

The effect of group and time on the concentration of plasma fatty acids was evaluated using a multivariate model that accounted for the random variance of dog. Fatty acids with the

same degree of unsaturation were evaluated together. Statistical significance of the interaction of group and time was determined using Wilk's lambda with $p \leq 0.05$. Where there was a significant interaction of group and time, contrasts were made for each plasma fatty acid using Tukey's test to determine where the difference occurred. An experiment-wide type I error of 0.05 was maintained.

All other data were considered continuous and found to follow a normal distribution with rejection of the null hypothesis of normality at $p \leq 0.05$ using the Shapiro-Wilk test. The effect of diet and time was evaluated using a mixed effect linear model that accounted for the random variance of each dog, and the repeated measurements in each dog. Where there was significant interaction of diet and time, least squares means comparisons were made between and within diet groups to determine where the significance effects occurred. If the groups behaved differently then they were considered to be different. If the groups showed similar significant changes over time, then between group comparisons were made at the significant time points. Type I error was maintained at 0.05 for all between and within group comparisons. All data were summarized as mean +/- SEM. PROC NPAR1WAY (SAS v8.0, SAS Institute, Cary, NC) was used for the analysis.

Results

All 15 dogs consumed between 75 and 100% of the amount of food calculated to be required to meet individual maintenance energy requirements each day throughout the 12 week period of study. All dogs maintained optimal body condition without significant weight loss or gain (Appendix II).

Consumption of the test diets was confirmed by observing the dogs at the time of feeding, and by the significant increase in plasma phospholipid n-3 fatty acids (Table 2.2). Previous to the study, the plasma fatty acid profile of dogs in each of the three diet groups was not significantly different. After eating the experimental diet for 12 weeks, the concentration of plasma saturated and polyunsaturated fatty acids (PUFA) still did not differ significantly among dogs in the three groups. However, there were significant but inconsistent changes in the total monounsaturated fatty acids (MUFA) among the three groups. The total MUFA concentration steadily increased in Group Sunflower. Dogs in Group Fish oil +E had increased total plasma MUFA concentrations at 6 weeks, but these returned close to baseline by 12 weeks. Variable patterns were found in the concentrations of the individual MUFAs in both fish oil groups over the course of the experiment, i.e. 16:1 n7, 18:1 n7, and 18:1 n9.

Table 2.2 Effect of diets on plasma phospholipid fatty acids.

	Group Sunflower oil			Group Fish oil			Group Fish oil + Vit E		
Fatty Acid	**Week 0**	**Week 6**	**Week 12**	**Week 0**	**Week 6**	**Week 12**	**Week 0**	**Week 6**	**Week 12**
18:2 n6	14.9±1.7	15.9±1.1	17.8±0.77	17.5±0.9	16.6±2.3	14.3±1.1	15.4±1.2	15.5±1.5	16.7±1.5
20:4 n6	16.9±1.2*	15±1.6*†	12.1±1.4†	15.0±0.9*	9.4±1.5†	10.4±0.3*†	16.4±1.2*	11.1±1.0†	9.5±0.8*†
22:4 n6	1.8±0.2	0.9±0.25	1.0±0.2	1.6±0.1*	1.0±0.3*	0.4±0.1†	1.6±0.1	1.0±0.3	1.2±0.6
18:3 n3	0.31±0.03	0.37±0.1	0.38±0.12	0.25±0.03	0.2±0.05	0.32±0.03	0.29±0.07	0.35±0.1	0.36±0.09
20:5 n3	0.3±0.1*	1.1±0.2*†	1.6±0.5†a	0.4±0.1*	1.2±0.4*	2.8±0.6†b	0.3±0*	1.6±0.1†	3.3±0.4‡b
22:5 n3	1.4 ±0.1	1.2 ±0.1a	1.3 ±0.16a	1.1 ±0.1*	2.2 ±0.4*ab	3.6 ±0.8†b	1.4 ±0.1*	2.4±0.4*b	3.5±0.5†b
22:6 n3	0.3 ±0.0*	0.4 ±0.1*	0.78 ±0.1†	0.3 ±0*	0.6 ±0†	0.9 ±0.1‡	0.3 ±0*	0.4 ±0.1*	0.7 ±0.1†
Total n6	35.3±2.0	32.8±2.3	31.9±1.5	36.5±1.3*	27.9±3.9†	25.9±1.0†	35.1±0.9	28.6±2.7	28.2±1.4
Total n3	2.3±0.1	3.0±0.22	4.0±0.4a	2.1±0.1*	4.1±0.8†	7.6±1.6‡b	2.3±0.2*	4.8±0.4†	7.9±0.8‡b
n6:n3 Ratio	15.6±0.9*	10.8±.4†	8±0.5†a	17.6±0.4*	7.8±2.1†	4.4±1.2†ab	15.7±1.4*	6.0±0.3†	3.7±0.3†b
16:1 n7	0.7±0.1	1.1±0.3	1.2±0.4	0.5±0*	1.4±0.1†	0.7±0.1*	0.8±0.1*	1.5±0.3†	0.6±0.2*
18:1 n7	4.8±0.5	6.2±1.3	7.7±1.3	4.6±0.4*	7.1±1.5*†	7.8±0.4†	5.6±0.5	7.0±1.7	7.9±0.8
18:1 n9	6.4±0.6	7.2±0.4a	6.8±0.6	7.6±0.5	7.1±1.1a	5.2±0.6	6.5±0.5*	10.5±2.5†b	7.6±1.1*†
Total SFA	45.7±2.3	48.3±5.0	44.6±2.1	43.7±0.8	48.2±4.6	46.1±2.1	44.9±1.5	44.2±2.5	42.4±3.2
Total MUFA	12.8±0.7*	15.2±1.3†a	16.5±1.4†	13.6±0.6	16.4±0.5a	14.2±0.5	13.6±0.7*	20.1±1.9†b	17.0±1.7*†
Total PUFA	37.6±2.1	33.0±4.8	35.9±1.8	38.5±1.3	32.0±4.3	33.5±1.8	37.4±0.9	33.4±3.0	36.2 ±1.9

Values are percent of specified fatty acid within plasma phospholipid fraction reported as means +/- SEM, n = 5. Significant difference within a group over time is indicated by different symbols (*,†,‡). Significant differences between dietary groups within a time point are identified by lower case letters (a,b). SFA = saturated fatty acids. MUFA = monounsaturated fatty acids. PUFA = polyunsaturated fatty acids.

As expected, there was a significant increase in total n-3 fatty acids and a decrease in total n-6 fatty acids in both groups supplemented with fish oil from week 0 to weeks 6 and 12. This fish oil-induced effect was also reflected in the significantly decreased n-6:n-3 fatty acid ratios of both groups. No significant difference in individual or total n-3 or n-6 fatty acids was detected between the two groups supplemented with fish oil. Surprisingly, Group Sunflower oil also had a significantly decreased n-6:n-3 fatty acid ratio (48.7% reduction), although not to the same magnitude as Groups Fish oil and Fish oil + E (75% and 76.4%, respectively). This sunflower oil-induced reduction of the n-6:n-3 fatty acid ratio was predominantly the result of a significant decline in arachidonic acid (20:4 n-6) combined with a significant elevation in eicosapentaenoic acid (20:5 n-3).

The combined concentration of serum α-tocopherol and α-tocopherol acetate (serum vitamin E) did not differ significantly among the three groups previous to the study (Figure 2.1), (Appendix III). After consumption of the diets for 6 and 12 weeks, the total concentrations of serum vitamin E of Group Fish oil + E significantly increased when compared to the week 0 concentration. Comparison between groups also found the serum vitamin E concentrations of dogs in Group Fish oil + E at weeks 6 and 12 to be significantly higher than those of the Groups Sunflower oil and Fish oil. The serum vitamin E concentrations of the latter two groups did not change significantly over the course of the study. When vitamin E was expressed as a ratio relative to serum triglyceride, cholesterol, or total lipid (triglyceride + cholesterol) concentrations, the differences within and between dietary groups were the same with two exceptions. First, the serum vitamin E concentration of Group Fish oil + E significantly increased from week 6 to 12 when expressed relative to serum triglycerides alone. Secondly, when expressed relative to serum triglyceride and total lipid concentrations, Group Fish oil had a significantly increased serum concentration of vitamin E from week 0 to week 12.

Platelet aggregation in platelet rich plasma as induced by ADP was not significantly different within or between dietary groups at any time point (Figure 2.2). Collagen-induced platelet aggregation among dogs in Group Fish oil +E was significantly reduced at week 6 compared to baseline, but rebounded to a value not significantly different than baseline by week 12. There was no significant difference between the three dietary groups at any time point when evaluating collagen-induced aggregation (Figure 2.3), (Appendix IV).

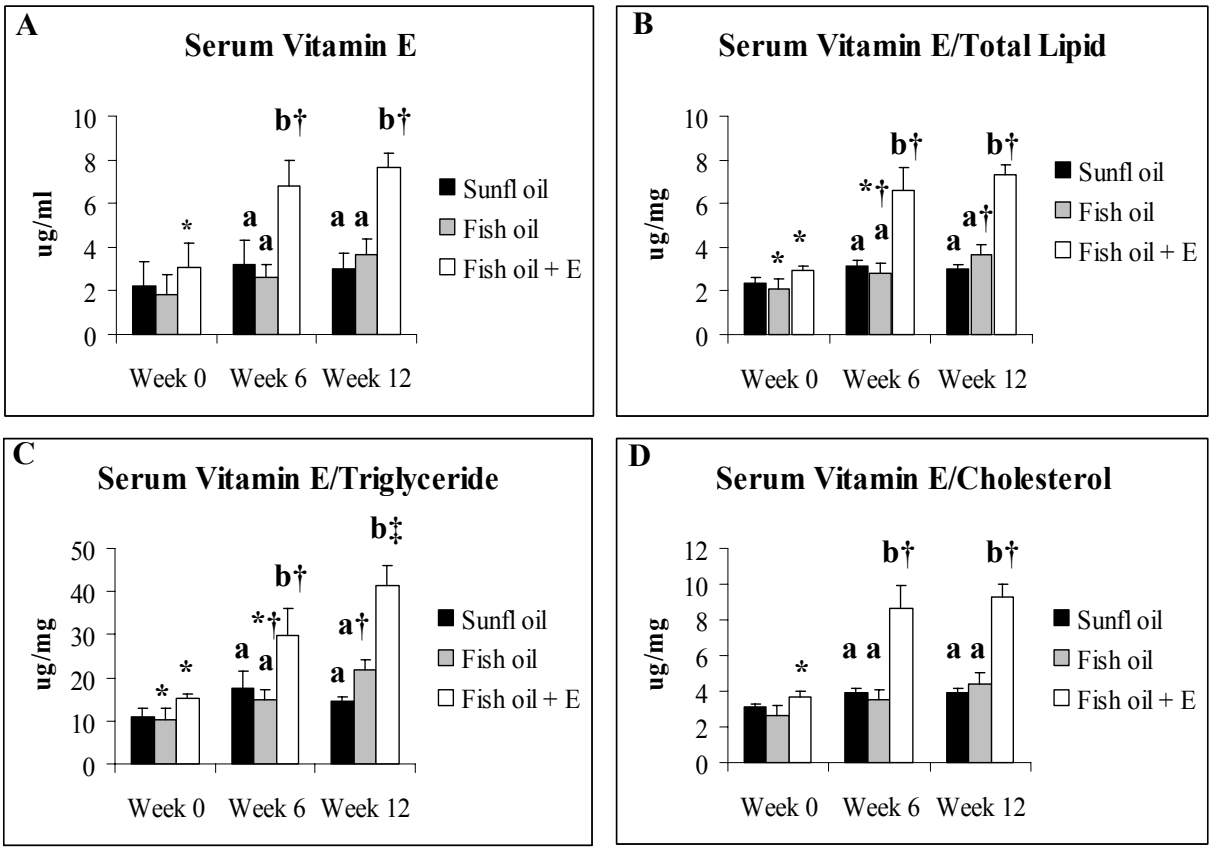

Figure 2.1 Effect of diet on serum vitamin E. Serum vitamin E levels are expressed as a concentration per unit of plasma (A), and as ratios per unit of total lipid (cholesterol + triglyceride) (B), triglyceride (C), and cholesterol (D). Data is expressed as means +/- SEM, n=5. Significant difference within a group over time is indicated by different symbols (***,†,‡**). Significant differences between dietary groups within a time point are identified by lower case letters (a,b).

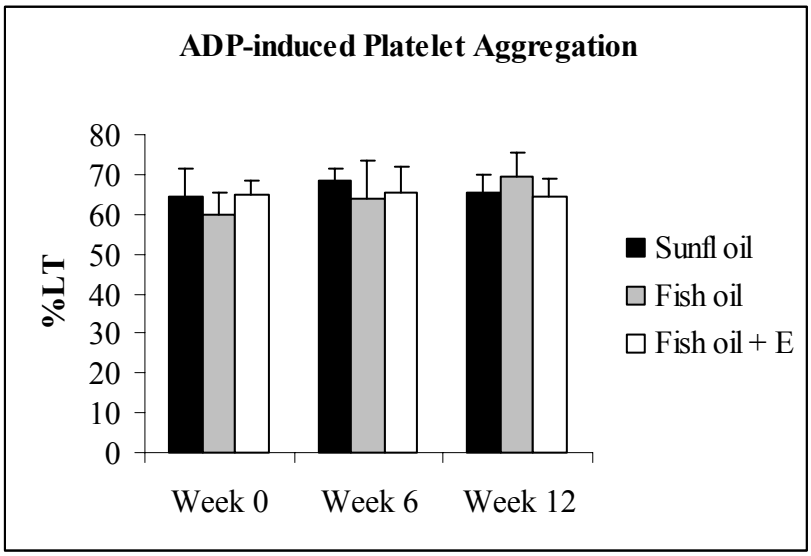

Figure 2.2 Effect of diet on ADP-induced platelet aggregation. Values are percent light transmittance (%LT) reported as means +/- SEM, n=5.

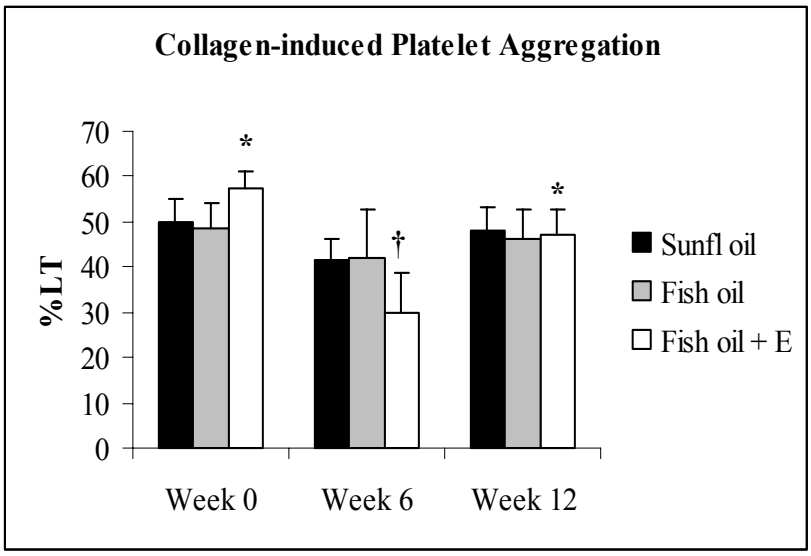

Figure 2.3 Effect of diet on collagen-induced platelet aggregation. Values are percent light transmittance (%LT) reported as means +/- SEM, n=5. Significant difference within a group over time is indicated by different symbols (***,†**).

Aside from the serum cholesterol concentration of Group Fish oil + E, none of the hematologic or serum biochemical parameters examined were outside the laboratory reference interval for any of the three dietary groups at any time point (Table 2.3), (Appendices V, VI, VII). The serum cholesterol concentration of Group Fish oil + E was significantly decreased at weeks 6 and 12 when compared to baseline. However, there were no significant differences in serum cholesterol concentrations between the three dietary groups at weeks 0, 6, or 12. Reduced serum triglyceride concentrations were found in Groups Fish oil and Fish oil + E by week 12, but only the week 12 triglyceride concentration of Group Fish oil was significantly lower when compared to week 0 in this group. Comparison between groups also revealed this parameter to be significantly lower than the concentration of serum triglyceride in Group Sunflower Group at week 12. Although week 12 serum triglyceride concentration in Group Fish oil was within the laboratory reference interval, it was below published reference intervals (Wagner et al., 1999).

There was no evidence of oxidative damage in any of the dietary groups. Plasma lipid hydroperoxide concentrations did not differ significantly from baseline at weeks 6 or 12 for any of the three dietary groups, and there was no significant difference between groups at any of the three time points (Figure 2.4), (Appendix VIII).

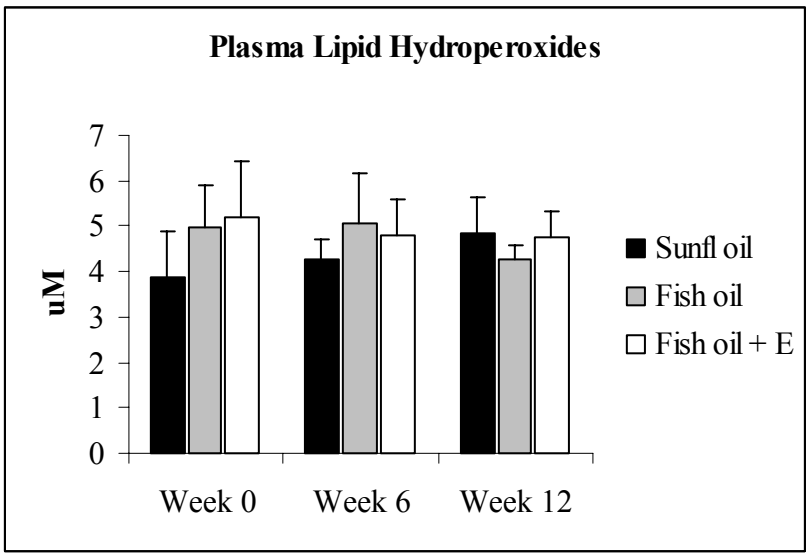

Figure 2.4 Effect of diet on plasma lipid hydroperoxide concentrations. Values are expressed as means +/- SEM, n = 5.

Table 2.3 Effect of diet on selected hematology and serum biochemistry parameters.

	Group Sunflower oil			Group Fish oil			Group Fish oil + Vit E		
Analyte (unit) (RI)	**Week 0**	**Week 6**	**Week 12**	**Week 0**	**Week 6**	**Week 12**	**Week 0**	**Week 6**	**Week 12**
HCT (%) (33-55)	49.2±2.6	48±1.5	50.2±1.6	52.6±1.3	48.6±1.3	48.9±1.9	49.1±2.9	50±1.2	49.8±.6
PLT(x10^3/uL) (200-700)	264±22	271±17	239.4±11	272.6±19	320.8±28	295±22	309±65	292.6±41	317.2±33
WBC (x10^3/uL) (6-17)	15.4±.5	12.9±.6	14.1±1.5	15.6±.9	14.6±1.3	14.5±.6	13.9±1	14±1.1	16.9±1.1
Glucose (mg/dL) (80-115)	87.6±3.5	88.8±1.9	86.8±4.6	89±3.7	87.6±4.1	84.6±3.9	92.4±2.5	93.4±3	83±1.7
ALT (U/L) (0-60)	44.2±8.5	45.6±4.6	37±2.6	53.4±19	47.8±11.6	35.4±3.9	34.6±4.8	42.2±4.3	36.6±4
ALP (U/L) (0-100)	42.8±7.2	43.4±3.5	40.6±7.4	41.6±4.7	38.4±4.2	30.6±1.6	44.6±7.9	36.4±3.8	45.4±13.3
BUN (mg/dL) (8-24)	20±2.2	20.4±2.4	24.4±2.2	23.8±3	24±1	24.2±1.1	21.2±1.7	21±1.5	22.4±2.52
Creatinine (mg/dL) (0.5-1.7)	.94±.06	.96±.05	1±.05	.94±.06	.94±.06	1±.04	.96±.06	1±.03	1±.04
Cholesterol (mg/dL) (150-240)	167±25	178±21	181.2±30	169.2±18	151.8±17.8	147.8±21	191.2±37*	142±16.4†	149.2±15.3†
Triglyceride (mg/dL) (20-110)	51.6±8.6	43.2±5.2	50.8±8.9a	50.4±8.7*	34.8±1.8*†	29.4±5.2†b	42.8±5	43.6±6.7	34±3.6ab

Values are reported as means +/- SEM, n = 5. Significant difference within a group over time is indicated by different symbols. Significant differences between dietary groups within a time point are identified by lower case letters (a,b). HCT = hematocrit. PLT = platelets. WBC = white blood cells. ALT = alanine aminotransferase. ALP = alkaline phosphatase. BUN = blood urea nitrogen.

Discussion

Dietary supplementation with n-3 fatty acids inhibits platelet aggregation in humans and animals (Kristensen et al., 1989; Freese and Mutanen, 1997; Saker et al., 1998; Adan et al., 1999). This effect was first described in Greenlandic Eskimos, who consume a significantly larger quantity of n-3 fatty acids than other human populations (Dyerberg and Bang, 1979). Significant n-3 fatty acid-induced inhibition of platelets was not found in healthy dogs fed a ration with an n-6:n-3 fatty acid ratio diet of 5:1 (Boudreaux et al., 1997), or among canine cancer patients supplemented with menhaden fish oil and arginine at 5.54% on a dry matter basis yielding an n-6:n-3 fatty acid ratio of 0.3:1 (McNiel et al., 1997). A significant decrease in collagen-induced platelet aggregation was noted after 6 weeks in 1 of 2 diet groups in the current study in which the dietary n-3:n-6 fatty acid ratio was 3.4:1 after addition of 0.65% n-3 fatty acids on dry matter basis (DMB). However, these values returned close to baseline after an additional 6 weeks on the same diet (week 12 of study). This temporary effect on collagen-induced platelet aggregation has been documented in other studies (Thorngren and Gustafson, 1981; Boudreaux et al., 1997). Since different glycoproteins mediate ADP- and collagen-induced platelet aggregation (Peerschke, 2002), a significant reduction of *in vitro* aggregation induced by only one agonist implies that other pro-aggregation mechanisms remain in place. Therefore, this temporary reduction of collagen-induced platelet aggregation is thought to be of little clinical significance. The results of platelet aggregation analysis in the current study confirm that young, healthy dogs consuming a ration with a n-6:n-3 fatty acid ratio of 3.4:1(1.65% added oil on dry matter basis) have preserved platelet function.

Another potential effect of diets high in polyunsaturated fatty acids such as n-6 or n-3 fatty acids is increased lipid peroxidation of all cells and tissues (Hall, 1996). The long chain polyunsaturated fatty acids that are incorporated into cell membrane phospholipids are easy targets for oxygen free radicals, due to their numerous double bonds (Mylonas and Kouretas, 1999). Evidence of increased lipid peroxidation has been found in both human (Meydani et al., 1991; Allard et al., 1997) and animal studies (L'Abbe et al., 1991; Wander et al., 1997) in which test subjects were supplemented with n-3 fatty acids. Diets enriched with the lipid-soluble antioxidant vitamin E at levels greater than established requirements have been proposed for humans and dogs eating diets high in PUFAs (National Research Council, 1985; Muggli, 1989). However, given the variability from study to study, it is difficult to specifically

define the vitamin E (α-tocopherol) intake needed to prevent significant lipid peroxidation that may be caused by high n-3 fatty acid diets in the dog. A formula has been proposed that calculates human vitamin E requirements based on the number of double bonds within dietary PUFAs (Muggli, 1989). The National Research Council's Subcommittee on Dog Nutrition recommends an α-tocopherol:PUFA ratio (mg/g) of at least 0.5 be maintained (National Research Council, 1985). All three of the experimental diets used in the current study far exceeded these recommendations. Therefore, it is not surprising that no significant differences in plasma lipid peroxide concentrations were found between any of the dietary groups, even though dogs in Group Fish oil + E had significantly higher serum vitamin E concentrations. Evidence of significantly increased lipid peroxidation has only been reported once in dogs consuming an n-3 fatty acid enriched diet (Table 2.4). This observation may have been related to the lower n-6:n-3 fatty acid ratio and the high percentage of PUFA-rich oil within the diet of the dogs in this study (Wander et al., 1997).

Table 2.4 Dietary comparison of canine studies.

	% Oil Added (DMB)	Dietary n-6:n-3 Fatty Acid Ratio	Selected PUFA Concentration* (g/kg diet DMB)	Vitamin E (IU/kg diet DMB)
Kearns et al.	1.65	5:1	Not given	100
Wander et al.	4.4	1.4:1	42.9	451.6
Hall et al.	4.4	1.4:1	33.85	1870
Current Study	1.65	3.4:1	56.05	427

* Polyunsaturated fatty acid (PUFA) concentration on a dry matter basis (DMB) was determined by the sum of 18:2n-6 + 18:3n-6 + 18:3n-3 + 18:4n-3 + 20:2n-6 + 20:3n-6 + 20:3n-3 + 20:4n-6 + 20:4n-3 + 20:5n-3 + 21:5n-3 + 22:2n-6 + 22:4n-6 + 22:5n-6 + 22:5n-3 + 22:6n-3.

The significant decrease in serum cholesterol noted here in Group Fish oil + E, was observed among n-3 fatty acid supplemented dogs in one earlier study (Hall et al., 1999). Nevertheless, serum cholesterol concentrations as well as all other serum biochemistry and hematology parameters assayed here were within the laboratory reference interval, suggesting

that this level and duration of n-3 fatty acid supplementation is safe in the dogs. There was a significant reduction of serum triglycerides in one of two diet groups supplemented with n-3 fatty acids (Group Fish oil), in agreement with work in human subjects (Sanders et al., 1989; Harris, 1997); this has not been previously reported in the dog. Diets rich in n-3 fatty acids are suggested to reduce serum triglycerides by decreasing the production of VLDL particles, which typically contain a high concentration of triglycerides. Furthermore, fish oil is a poor substrate for enzymes responsible for triglyceride synthesis (Elliott, 2001). Hyperlipidemia is a disturbance of lipid transport characterized by significantly high levels of serum triglycerides. Clinical signs include vomiting, diarrhea, and potentially seizures. The dietary n-3 fatty acid-induced decrease in serum triglycerides observed in this study may prove beneficial in dogs with hyperlipidemia.

The control group in this study was supplemented with sunflower oil to increase the n-6:n-3 fatty acid ratio to a level more consistent with most standard canine diets. Sunflower oil is high in linoleic acid (LA, 18:2 n-6), which is typically desaturated and elongated to arachidonic acid (AA, 20:4 n-6) as its principle end-product. The first and probably primary rate-limiting step in this pathway is regulated by the enzyme Δ6-desaturase (Cook, 1991; Dunbar and Bauer, 2002). Alpha-linolenic (LNA, 18:3 n-3) competes directly for this desaturase enzyme plus others, and is eventually metabolized to eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) (Simopoulos, 1991; Dunbar and Bauer, 2002). The significant decrease in AA with its resultant decrease in the plasma phospholipid n-6:n-3 fatty acid ratio of Group Sunflower oil in the current study was therefore surprising (Table 2.2). EPA and DHA concentrations were also significantly increased, despite undetectable dietary intake (Table 2.1), thus suggesting endogenous synthesis of these fatty acids. These unexpected findings may be the result of several contributing factors. Early reports suggested that LNA is a preferred substrate for Δ6-desaturase compared to LA (Hagve and Christophersen, 1984; Cook, 1991). This may have contributed to the increased EPA and DHA and the decreased AA concentrations in the plasma phospholipids of the dogs studied here. Decreased AA concentrations may also have been related to reduced Δ6-desaturase activity. Fasting for at least 12 hours is thought to diminish Δ6-desaturase activity (Inkpen et al., 1969; De Gomez Dumm and Brenner, 1975), and the dogs in the current study were fed once daily. High dietary LA intake has been associated with depressed desaturase activity as

well (Uchiyama et al., 1967) and specifically with the inhibition of Δ6-desaturase (unpublished data, J.Bauer). Lastly, there is evidence to suggest that low endogenous levels of AA favor the esterification of EPA into phospholipids as opposed to triacylglycerols (Hagve and Christophersen, 1984). Therefore, the decreased AA and increased EPA concentrations found in the plasma phospholipid fraction of dogs in Group Sunflower oil may have reflected changes present only in the lipid fraction that was evaluated, and may not have been representative of the entire lipid profile.

In summary, this study shows that consumption of a diet consisting of 0.65% n-3 fatty acids on a DMB with an n-6:n-3 fatty acid ratio of 3.4:1 did not cause a clinically significant or sustained inhibition of platelet aggregation in young adult dogs. Increased lipid peroxidation was not apparent in these animals whether or not they received additional dietary vitamin E supplementation. The routinely recommended intake of dietary vitamin E may therefore be adequate for young dogs with this level of fatty acid consumption. Increased intake of n-3 fatty acids decreases serum triglyceride concentrations in the dog, and may have a role in the treatment of hyperlipidemia. Finally, the metabolism of dietary n-6 and n-3 fatty acids may be much more complex than the simple pathways imply. Further studies are required to fully elucidate these mechanisms.

References

Adan Y, Shibata K, Sato M, Ikeda I, Imaizumi K. 1999. Effects of docosahexaenoic and eicosapentanoic acid on lipid metabolism, eicosanoid production, platelet aggregation, and atherosclerosis in hypercholesterolemic rats. Biosci Biotechnol Biochem 63:111-119.

Allard J, Kurian R, Aghdassi E, Muggli R, Royall D. 1997. Lipid peroxidation during n-3 fatty acid and vitamin E supplementation in humans. Lipids 32:535-541.

Bang HO, Dyerberg J, Nielsen AB. 1971. Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos. Lancet 1:1143-1145.

Bauer J. 1995. Evaluation and dietary considerations in idiopathic hyperlipidemia in dogs. JAVMA 206:1684-1688.

Boudreaux M, Dillon A, Spano J. 1989. Enhanced platelet reactivity in heartworm-infected dogs. Am J Vet Res 50:1544-1547.

Boudreaux M, Reinhart G, Vaughn D, Spano J, Mooney M. 1997. The effects of varying dietary n-6 to n-3 fatty acid ratios on platelet reactivity, coagulation screening assays, and antithrombin III activity in dogs. J Am Anim Hosp Assoc 33:235-243.

Bright J, Sullivan P, Melton S, Schneider J, McDonald T. 1994. The effects of n-3 fatty acid supplementation on bleeding time, plasma fatty acid composition, and in vitro platelet aggregation in cats. J Vet Intern Med 8:247-252.

Cook H. 1991. Fatty acid desaturation and chain elongation in eukaryotes. In: Vance D, Vance J, editors. Biochemistry of lipids, lipoproteins, and membranes. New York: Elsevier. p 154-163.

De Gomez Dumm I, Brenner R. 1975. Oxidative desaturation of alpha-linolenic, linoleic, and stearic acids by human liver microsomes. Lipids 10:315-317.

Dunbar B, Bauer J. 2002. Metabolism of dietary essential fatty acids and their conversion to long-chain polyunsaturated metabolites. JAVMA 220:1621-1626.

Dyerberg J, Bang HO. 1979. Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. Lancet 2:433-435.

Elliott D. 2001. What's the deal with hypertriglyceridemia? In: 19th Annual American College of Veterinary Internal Medicine Forum. Denver. p 530-532.

Fischer S, Weber PC. 1984. Prostaglandin I3 is formed in vivo in man after dietary eicosapentaenoic acid. Nature 307:165-168.

Freese R, Mutanen M. 1997. α-Linolenic acid and marine long-chain n-3 fatty acids differ only slightly in their effects on hemostatic factors in healthy subjects. Am J Clin Nutr 66:591-598.

Hagve T-A, Christophersen B. 1984. Effect of dietary fats on arachidonic acid and eicosapentaenoic acid biosynthesis and conversion to C22 fatty acids in isolated rat liver cells. Biochim Biophys Acta 796:205-217.

Hall J. 1996. Potential adverse effects of long-term comsumption of (n-3) fatty acids. Comp Cont Ed Prac Vet 18:879-895.

Hall J, Wander R, Gradin J, Du S, Jewell D. 1999. Effect of dietary n-6-to-n-3 fatty acid ratio on complete blood and total white blood cell counts, and T-cell subpopulations in aged dogs. Am J Vet Res 60:319-327.

Hansen R, Savage C, Reidlinger K, Traub-Dargatz J, Ogilvie G, Mitchell D, Fettman M. 2002. Effects of dietary flaxseed oil supplementation on equine plasma fatty acie concentrations and whole blood platelet aggregation. J Vet Intern Med 16:457-463.

Harris WS. 1996. n-3 fatty acids and lipoproteins: comparison of results from human and animal studies. Lipids 31:243-252.

Harris WS. 1997. n-3 fatty acids and serum lipoproteins: animal studies. Am J Clin Nutr 65:1611S-1616S.

Illingworth D, Conner W, Hatcher L, al e. 1989. Hypolipidemic effects of n-3 fatty acids in primary hyperlipoproteinaemia. J Intern Med Suppl 225:91-97.

Inkpen C, Harris R, Quackenbush F. 1969. Differential responses to fasting and subsequent feeding by microsomal systems of rat liver: 6- and 9-desaturation of fatty acids. J Lipid Res 10:277-282.

Kearns R, Hayek M, Turek J, Meydani M, Burr J, Greene R, Marshall C, Adams S, Borgert R, Reinhart G. 1999. Effect of age, breed, and dietary omega-6 (n-6) : omega-3 (n-3) fatty acid ratio on immune function, eicosanoid production, and lipid peroxidation in young and aged dogs. Vet Immuno Immunopathol 69:165-183.

Kristensen S, Schmidt E, Dyerberg J. 1989. Dietary supplementation with n-3 polyunsaturated fatty acids and human platelet function: a review with particular emphasis on implications for cardiovascular disease. J Int Med 225:141-150.

Kromann N, Green A. 1980. Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974. Acta Med Scand 208:401-406.

L'Abbe MR, Trick KD, Beare-Rogers JL. 1991. Dietary (n-3) fatty acids affect rat heart, liver and aorta protective enzyme activities and lipid peroxidation. J Nutr 121:1331-1340.

Martin-Chouly CA, Menier V, Hichami A, Youmine H, Noel F, Pedrono F, Legrand AB. 2000. Modulation of PAF production by incorporation of arachidonic acid and eicosapentaenoic acid in phospholipids of human leukemic monocyte-like cells THP-1. Prostaglandins Other Lipid Mediat 60:127-135.

McNiel E, Ogilvie G, Fettman M, Salman M. 1997. Platelet hyperfunction in dogs with malignancies. J Vet Intern Med 11:178-182.

McNiel E, Ogilvie G, Mallinckrodt C, Richardson K, Fettman M. 1999. Platelet function in dogs treated for lymphoma and hemangiosarcoma and supplemented with dietary n-3 fatty acids. J Vet Intern Med 13:574-580.

Meydani M, Natiello F, Goldin B, Free N, Woods M, Schaefer E, Blumberg J, Gorbach S. 1991. Effect of long-term fish oil supplementation on vitamin E status and lipid peroxidation in women. J Nutr 121:484-491.

Meydani S, Lichtenstein A, Cornwall S, Meydani M, Goldin B, Rasmussen H, Dinarello C, Schaefer E. 1993. Immunologic effects of national cholesterol education panel step-2 diets with and without fish-derived acid enrichment. J Clin Invest 92:105-113.

Muggli R. 1989. Dietary fish oils increase the requirement for vitamin E in humans. In: Chandra R, editor. Health effects of fish and fish oils. St. John's, Newfoundland: ARTS Biomedical Publishers and Distributors. p 201-210.

Mylonas C, Kouretas D. 1999. Lipid peroxidation and tissue damage. in vivo 13:295-310.

National Research Council, Subcommittee on Dog Nutrition. 1985. Nutient Requirements of Dogs. Washington, DC: National Academy Press.

Peerschke E. 2002. The laboratory evaluation of platelet dysfunction. Clin Lab Med 22:405-420.

Saker K, Eddy A, Thatcher C, Kalnitsky J. 1998. Manipulation of dietary (n-6) and (n-3) fatty acids alters platelet function in cats. J Nutr 128:2645S-2647S.

Salonen R, Nikkari T, Seppanen K, et a. 1987. Effect of omega-3 fatty acid supplementation on platelet aggregability and platelet produced thromboxane. Thromb Haemost 57:269-272.

Sanders T, Hinds A, Pereira C. 1989. Infuence of n-3 fatty acids on blood lipids in normal subjects. J Intern Med Suppl 225:99-104.

Simopoulos A. 1991. Omega-3 fatty acids in health and disease and in growth and development. Am J Clin Nutr 54:438-463.

Thorngren M, Gustafson A. 1981. Effects of 11-week increases in dietary eicosapentaenoic acid on bleeding time, lipids, and platelet aggregation. Lancet 2:1190-1193.

Uchiyama M, Nakagawa M, Okui S. 1967. Effect of free unsaturated fatty acids on fatty acid desaturation by liver preparations. J Biochem 62:1-6.

Vaughn DM, Reinhart GA. 1996. Influence on dietary fatty acid ratios on tissue eicosanoid production and blood coagulation parameters in dogs. In: Carey D, Norton S, Bolser S, editors. Recent Advances in Canine and Feline Nutritional Research - Proceedings of the1996 Iams Nutrition Symposium. Wilmington: Orange Frazer Press. p 243-255.

Wagner J, Greaves K, Schwenke D, Bauer J. 1999. Lipids and Lipoproteins. In: Loeb W, Quimby F, editors. The Clinical Chemistry of Laboratory Animals, 2nd ed. Philadelphia: Taylor & Francis. p 181-228.

Wander R, Hall J, Gradin J, Du S, Jewell D. 1997. The ratio of dietary (n-6) to (n-3) fatty acids influences immune system function, eicosanoid metabolism, lipid peroxidation and vitamin E status in aged dogs. J Nutr 127.

Wander RC, Du SH, Ketchum SO, Rowe KE. 1996. alpha-Tocopherol influences in vivo indices of lipid peroxidation in postmenopausal women given fish oil. J Nutr 126:643-652.

Wensing AG, Mensink RP, Hornstra G. 1999. Effects of dietary n-3 polyunsaturated fatty acids from plant and marine origin on platelet aggregation in healthy elderly subjects. Br J Nutr 82:183-191.

Whitaker MO, Wyche A, Fitzpatrick F, Sprecher H, Needleman P. 1979. Triene prostaglandins: prostaglandin D3 and icosapentaenoic acid as potential antithrombotic substances. Proc Natl Acad Sci U S A 76:5919-5923.

CHAPTER III: EFFECTS OF DIETARY FISH OIL AND VITAMIN E SUPPLEMENTATION ON CANINE LYMPHOCYTE PROLIFERATION

Introduction

Dietary supplementation with fish oil or its main n-3 fatty acid constituents, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has long been reported to have anti-inflammatory and immunosuppressive effects that are potentially beneficial to animals and humans with chronic inflammatory diseases. Despite variable rates of n-3 fatty acid supplementation and differing n-6:n-3 fatty acid ratios, most human and rodent studies agree that diets supplemented with n-3 polyunsaturated fatty acids suppress lymphocyte proliferation and function (Meydani et al., 1991; Calder and Newsholme, 1992; Endres et al., 1993; Jeffery et al., 1997b; Jolly et al., 1997) . Dietary supplementation with n-3 fatty acid appears to be a more potent suppressor of lymphocyte proliferation than dietary supplementation with n-6 fatty acids (Calder and Newsholme, 1992; Calder, 1998), although prostaglandin E_2 (PGE_2), an eicosanoid derived from the n-6 fatty acid arachidonic acid (AA), is reportedly more immunosuppressive than the corresponding n-3 fatty acid-derived prostaglandin E_3 (PGE_3) (Reinhart, 1996). This suggests a prostaglandin-independent mechanism for the observed decrease in lymphocyte proliferation, even though many of the immunomodulating effects of n-3 fatty acids are thought to be related to their effect on eicosanoid synthesis.

Originally designated T-cell growth factor, interleukin (IL)-2 is an important autocrine and paracrine cytokine that promotes T-cell, B-cell, and natural killer cell proliferation and differentiation (Sharon, 1998). The mechanism behind the effects of fish oil or its n-3 fatty acid constituents on lymphocyte proliferation may be related in part to decreased production of IL-2 (Calder and Newsholme, 1992; Kumar et al., 1992; Endres et al., 1993; Jolly et al., 1997; Terada et al., 2001). Reduced expression of the IL-2 receptor on the lymphocyte surface would have an equivalent effect (Soyland et al., 1994; Terada et al., 2001).

Reduction of lymphocyte proliferation in experimental subjects fed diets supplemented with n-3 fatty acids may also be related to the production of lipid peroxides, with subsequent oxidative damage of cell membranes (Meydani et al., 1991; Kumar et al., 1992). The numerous double bonds within the long carbon chains of n-3 and n-6 fatty acids allow cell membranes containing them to become easy targets for oxygen-derived free radicals (Muggli, 1989). While reduced lymphocyte function may be desirable in patients with chronic inflammatory diseases, widespread oxidative damage of cell membranes would be detrimental. Vitamin E is an important

fat-soluble antioxidant capable of protecting unsaturated fatty acids from oxidative damage, and is reported to restore diminished lymphocyte proliferation secondary to fish oil supplementation (Kramer et al., 1991; Kumar et al., 1992). The promotion of lymphocyte proliferation by vitamin E may be due to inhibition of oxidative damage (Kumar et al., 1992), but could also be related to decreased PGE_2 production or increased IL-2 synthesis (Beharka et al., 1997; Adolfsson et al., 2001).

Few studies have evaluated lymphocyte proliferation and function in dogs eating diets supplemented with n-3 fatty acids. Those that exist have varied in methodology, and report contrasting conclusions. The only study to assess mitogen-stimulated lymphocyte proliferation found an increased mitogenic response in young dogs eating an n-3 fatty acid supplemented diet with an n-6:n-3 ratio of 5:1. Older dogs consuming the same diet had no change in their mitogenic response (Kearns et al., 1999). This study also evaluated mononuclear cell production of PGE_2, serum vitamin E concentration and serum markers of oxidative stress. An increase in one of two serum markers of oxidation in the older dogs was the only other significant finding. In contrast, a report evaluating aged dogs fed a diet with an even lower n-6:n-3 fatty acid ratio of 1.4:1 documented significant suppression of the cell-mediated immune response based on results of a delayed-type hypersensitivity (DTH) skin test (Wander et al., 1997). These dogs also had evidence of increased lipid peroxidation, lower plasma vitamin E concentrations, and decreased mononuclear cell PGE_2 production when compared to aged dogs receiving a ration with an n-6:n-3 fatty acid ratio of 31:1.

The objective of this study was to determine the effect of feeding a low n-6:n-3 ratio diet consisting of 0.65% n-3 fatty acids (DMB) for 12 weeks on lymphocyte activation and proliferation in healthy, young dogs, using the standard tritiated thymidine method as well as flow cytometry techniques. The effect of dietary vitamin E supplementation on any changes induced by the diet was also assessed. A tertiary objective of this study was to evaluate diet-induced changes in plasma vitamin E and lipid peroxide concentrations and mononuclear cell production of PGE_2, and relate these to the results of the lymphoproliferative assays.

Materials and Methods

The *in vitro* experiments reported in this chapter were conducted using the experimental animals and diet formulations already described in Chapter II. The peripheral blood mononuclear cells used for the lymphoproliferative studies and PGE_2 synthesis analysis were collected at week

0 and week 12 of the dietary treatments. Methods and results of serum vitamin E and plasma lipid peroxide level determinations were presented in Chapter II.

Peripheral Blood Mononuclear Cell Isolation

Prior to blood collection in each of the 15 dogs, two 50ml conical tubes were filled with approximately 20ml of a density gradient of 1.077 (Lymphoprep, Nycomed Pharma As, Oslo, Norway) and chilled in an ice bath for 45 minutes. Approximately 15ml of heparinized whole blood was slowly overlaid on the gradient in each tube, and centrifuged for 45 minutes at 340xg and 15^{o}C. After centrifugation, the mononuclear cell layer between the plasma and gradient was removed with negative pressure through a pipet. The viable cell concentration was determined by trypan blue exclusion and the mononuclear cell percentage was determined by microscopic evaluation of a modified Wright's stained cytospin-prep slide. Viability was >90% in all samples and the cell population was typically 80-90% mononuclear cells. However, up to 30% neutrophils were present in a small number of isolates.

Evaluating Lymphocyte Proliferation using Tritiated Thymidine

Lymphocytes were suspended in triplicate in a 96-well U-shaped bottom plate at 2x10^{5} cells per well in 200µl of complete medium (RMPI with $NaHCO_3$, 25mM HEPES, 2-mercato ethanol, and pen strep glutamine) with 10% heat-inactivated autologous serum. Phytohemagglutinin-P or Concanavalin A was added to wells at a final concentration of 2µg/ml. No mitogens were added to control wells. After incubation at 37^{o}C in humidified air containing 5% CO_2 for 72 hours, 1 µCi of tritiated thymidine in 50µl of complete medium, without autologous serum was added to each well and the plates were allowed to incubate for an additional 4 hours. All plates were then frozen at -20^{o}C until time of harvest. Following defrost, the plates were harvested onto glass fiber filters (Tomtec Harvestor, Orange, CT) and incorporation of tritiated thymidine was determined by liquid scintillation spectrophotometry (Betaplate liquid scintillation counter 1205, LKB Wallac, Turku, Finland). Results were reported as the average counts per minute (CPM) of the triplicate wells. The stimulation index (SI) was determined by dividing the average CPM of the stimulated population by the average CPM of the unstimulated control population.

Lymphocyte Staining with Intracytoplasmic Fluorochrome

Five microliters of 5mM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) diluted 1:10 with dimethyl sulphoxide (DMSO) was added to 9x10^{6} lymphocytes in 1ml of complete medium and incubated in a 37^{o}C water bath for 10 minutes.

A separate aliquot of lymphocytes was also incubated without the addition of CFSE (negative control). Both aliquots of cells were washed twice, diluted to$1x10^6$ lymphocytes/ml with complete media plus 10% autologous serum, and transferred to 24-well flat bottom plates at 1ml/well. Phytohemagglutinin-P or Concanavalin A was added to wells at a final concentration of 2µg/ml. Each population of cells, determined by mitogen and presence or absence of CFSE, was cultured in triplicate wells. Plates were incubated at 37°C in humidified air containing 5% CO_2 for approximately 72 hours.

Immunophenotyping

Following 72 hours of incubation, cells from triplicate wells were combined and transferred to large falcon tubes. The cells were then washed with sterile PBS and resuspended with 150µl of PBS. Twenty-five microliters from each sample population was transferred into a small falcon tube for surface staining. Immunophenotyping was carried out by adding 10µl of mouse anti-canine CD4 (IgG1), anti-canine CD8α (IgG2a), or anti-canine CD21 (IgG1) monoclonal antibody (Leukocyte Antigen Biology Laboratory, University of California, Davis). Following 60 minutes of incubation at 4°C, cells were washed with PBS and 50µl of goat anti-mouse IgG (H+L) conjugated to Tricolor (TC) fluorochrome (Caltag Laboratories, Burlingame, CA) was added to each tube at a 1:100 dilution. In separate tubes also containing 25µl of sample, identification of activated lymphocytes was performed by adding 10µl of biotinylated recombinant human IL-2 or a negative control, biotinylated soybean trypsin inhibitor (R&D Systems, Inc. Minneapolis, MN). Following 60 minutes of incubation at 4°C, 10µl of avidin-phycoerythrin (PE) was added to the biotinylated samples. At this time, all samples were allowed to incubate an additional 30 minutes at 4°C and were then washed with PBS and resuspended with 200µl of 1% paraformaldehyde in PBS.

Evaluating Lymphocyte Proliferation by Flow Cytometric Analysis

All fluorochrome-stained lymphocyte culture preparations were analyzed within 24 hours by two-color analysis using a flow cytometer (FACScan, Becton Dickinson, San Jose, CA). The lymphocyte gate was based on light scatter parameters, cell size and internal granularity. Mitogen-stimulated cell divisions were determined by assessing the CFSE content (green fluorescence, FL1). Division parameters were set based on well defined peaks within a CFSE fluorescence versus events histogram to quantitate the number of cells in each generation. Cell counts above 200 were considered a division. Mitogen-stimulated samples were compared to medium controls to determine a reference point for non-dividing cells. Comparison was also made to the

autofluorescence control (unstimulated, unstained cells) to determine the end point of cell division. Division parameters were set on dual positive cells for CFSE and Tricolor (deep red fluorescence, FL3) to determine the divisional history of lymphocyte subsets. The percentage of activated cells was determined by measuring IL-2 receptor expression by enumerating IL-2-phycoerythrin-labelled cells (red fluorescence, FL2).

The number of events (cells) within each division was divided by 2 raised to the power of the division to calculate the percentage of original, undivided cells from which they arose. The sums of these gave the total undivided cohort for each culture. The sum of cohorts from each division was divided by the total number of undivided events to yield the proportion of the original population induced into cell division, designated the percentage proliferation (Lyons, 2000) (Figure and Table 3.1).

Enzyme Immunoassay for PGE_2

Peripheral blood mononuclear cells from 3 or 4 dogs of each group were isolated as described above. Mononuclear cells were isolated from 3 randomly chosen dogs of each group; isolation of too few cells was suspected from one dog of Group Fish oil + E, therefore an additional dog was randomly chosen from that group. The cells were incubated for 24 hours at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 2.0mM L-glutamine, 2.2g/L $NaHCO_3$, 25mM HEPES, 2.7 x 10^{-4} mg/L 2-mercaptoethanol, 100 U/ml penicillin, and 100µg/ml streptomycin sulfate with 10% heat-inactivated autologous serum in 24-well plates at a concentration of 1x10^6 cells/well. Macrophages were stimulated by adding lipopolysacchharide (LPS) from *Escherichia coli* (serotype 055:B5, Sigma Chemical, St. Louis, MO) at a concentration of 1µg/well. After 24 hours, the plates were frozen at -20°C. Twelve months later, all plates were defrosted and contents of each well plus 1ml of sterile saline flush/well were removed and placed in separate 15ml conical tubes. The tubes were centrifuged at 340xg for 10 minutes and supernatants were frozen at 20°C until time of analysis.

The supernatant PGE_2 concentration was determined using a competitive enzyme-linked immunoassay (EIA, Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions (Kay-Mugford et al., 2000). All reagents were provided. Briefly, 50µl of supernatant diluted 1:10 and 1:20 were added in duplicate to wells of a goat anti-mouse IgG coated 96-well plate provided by the manufacturer. Eight 1:2 serial dilutions of a provided standard were added to the wells. Fifty microliters of tracer compound consisting of PGE_2 conjugated to acetylcholinesterase (AChE), followed by the addition of 50µl of mouse anti-PGE_2 were added to

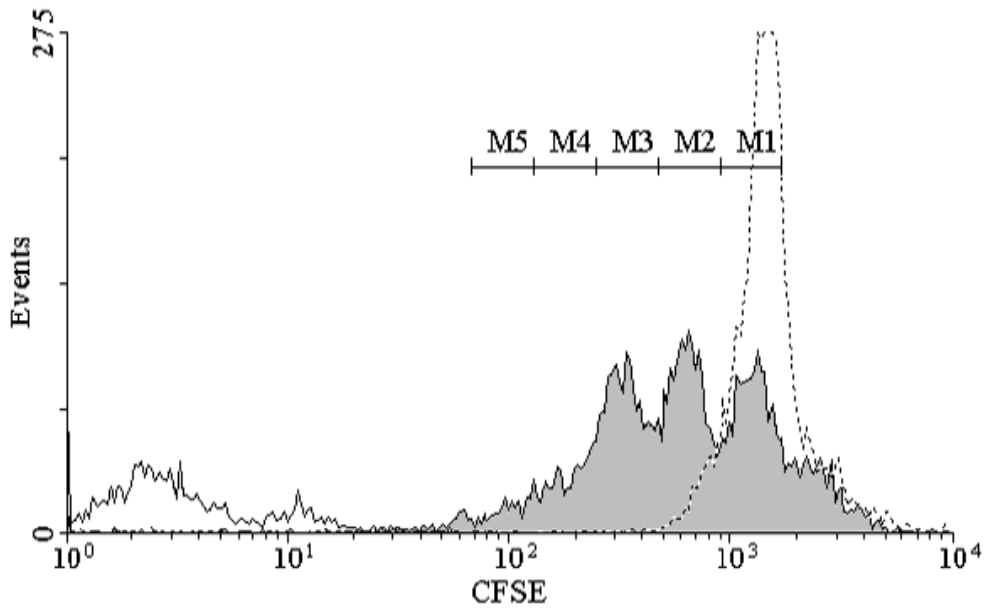

Figure 3.1 Histogram of CFSE fluorescence of stimulated lymphocytes (gray peaks) from Dog 9038 of Group Fish oil +E at week 12. Lymphocytes were incubated for 72 hours in complete medium with 10% autologous serum, 2ug/ml phytohemagglutinin (PHA) and 5µl of 5mM CFSE. Lymphocyte division was characterized by sequential halving of CFSE fluorescent dye, generating equally spaced peaks on the logarithmic scale. Markers above the gray peaks were used to enumerate the events within each division. Large dotted peak under the M1 marker denotes an unstimulated control cell population that displays maximal fluorescent intensity and no subsequent divisions. Small white peak denotes an unstimulated, unstained cell population serving as an autofluorescent control.

Table 3.1 Data generated from the histogram found in Figure 3.1.

Marker	Division (Dn)	Events	Divisor (2^{Dn})	Undivided Cohorts
M1	0	1756	1 (2^0)	1756
M2	1	1590	2 (2^1)	795
M3	2	1639	4 (2^2)	410
M4	3	493	8 (2^3)	62
M5	4	261	16 (2^4)	16
Cohort (Divisions 1-4)				**1283**
Total (Divisions 0-4)				**3039**
Percent Proliferation				**42.2%**
Number of Peaks				**4**

The number of events (cells) within each division was divided by 2 raised to the power of the division to calculate the proportion of original cells from which they arose (undivided cohorts). The sum of cohorts from each division (1-4) divided by the total undivided cohort number (0-4) represents the proportion of the original population induced into cell division, called the percentage proliferation.

each well. Following an18-hour incubation at 4°C, all wells were emptied and washed 5 times with buffer. After the addition of 200µl of fresh Ellman's Reagent (acetylthiocholine and 2-nitrobenzoic acid), the plate was allowed to develop on an orbital shaker for 90 minutes in the dark at room temperature. The intensity of the enzymatic reaction was determined spectrophotometrically at 415nm (EL808 Ultra Microplate Reader, Biotech Instruments, Winooski, VT). Sample PGE_2 concentrations were calculated using a log-logit curve generated by the eight standard dilutions.

Statistical Analysis

The number of lymphocyte peaks, determined by flow cytometry, was considered ordinal data and was evaluated for each of the 3 groups for a difference between 0 and 12 weeks using the Wilcoxon Rank-Sum test. An overall type I error of 0.05 was maintained for all 3 comparisons. Groups that behaved differently over time were considered different. PROC NPAR1WAY (SAS v8.0, SAS Institute, Cary, NC) was used for the analysis.

All other data was considered continuous and found to follow a normal distribution with rejection of the null hypothesis of normality at $p \leq 0.05$ using the Shapiro-Wilk test. The effect of diet and time was evaluated using a mixed effect linear model that accounted for the random variance of each dog, and the repeated measurements of in each dog. Where there was significant interaction of diet and time, least squares means comparisons were made between and within diet groups to determine where the significance effects occurred. If the groups behaved differently then they were considered to be different. If the groups showed similar significant changes over time, then between group comparisons were made at the significant time points. Type I error was maintained at 0.05 for all between and within group comparisons. All data were summarized as mean +/- SEM.

Results

There were no significant differences in lymphocyte proliferation between the three dietary groups measured by the tritiated thymidine or the flow cytometric method at week 0. Lymphocyte proliferation, as measured by incorporation of tritiated thymidine, decreased from week 0 to week 12 in all three groups when the results were expressed as both counts per minute and stimulation index. However, this decrease was only significant in the group supplemented with sunflower oil (Table 3.2), (Appendix IX).

Evaluation of lymphocyte proliferation by measuring the halving of CFSE through flow cytometry revealed a reduction in proliferation at week 12 in Groups Sunflower oil and Fish oil,

Table 3.2 Lymphocyte proliferation by incorporation of tritiated thymidine.

	Counts per Minute		Stimulation Index	
	Week 0	**Week 12**	**Week 0**	**Week 12**
Sunflower oil	21190.3 ± 8281.9*	2940.1 ± 1057.8†	55.2 ± 19.4*	8.9 ± 2.7†
Fish oil	12138.6 ± 2329.5	4175.5 ± 1928.5	24.3 ± 5.2	12.9 ± 7.4
Fish oil + E	14910.5 ± 5030.8	6103.9 ± 1692.7	46.8 ± 11.5	17.1 ± 4.7

Stimulation index determined by dividing the average CPM of the stimulated population by the average CPM of the unstimulated control population. Data are expressed as means +/- SEM, n = 5. Significant differences within a dietary group across time are denoted by different symbols (*, †).

when results were expressed as number of divisions or peaks. This decreased proliferation was once again significant in Group Sunflower oil only. However, when the flow cytometric results were expressed as percent proliferation, all three groups had reduced lymphocyte proliferation at week 12. In contrast to the tritiated thymidine method and the flow cytometric results expressed as number of peaks, only Group Fish oil had a significant decrease in proliferation at week 12 by this method (Table 3.3), (Appendix X).

Table 3.3 Lymphocyte proliferation and activation measured by flow cytometry.

	Number of Peaks		Percent Proliferation		Percent IL-2R+	
	Week 0	**Week 12**	**Week 0**	**Week 12**	**Week 0**	**Week 12**
Sunflower oil	3.6 + 0.2*	3.0 + 0†	27.9 ± 5.0	17.5 ± 2.8	60.8 ± 7.2	52.3 ± 8
Fish oil	3.6 + 0.2	3.2 + 0.4	44.8 ± 2.5*	26.0 ± 5.3†	78.1 ± 2.7	65.8 ± 5.9
Fish oil + E	3.2 + 0.4	3.2 + 0.2	34.9 ± 7.0	27.5 ± 5.9	74.4 ± 6.3	56.2 ± 8.1

Number of peaks (divisions) and percent proliferation were determined by evaluation of CFSE fluorochrome distribution as cultured lymphocytes divided. Proportion of lymphocytes that bound to phycoerythrin-labeled IL-2 determined the percent of IL-2 receptor expression. The data are expressed as mean +/- SEM, n = 5. Significant differences within a dietary group across time are denoted by different symbols (*, †). IL-2R = IL-2 receptor.

Flow cytometric evaluation of IL-2 receptor expression on activated lymphocytes revealed decreased binding of the phycoerythrin-labeled cytokine IL-2 in all three groups from week 0 to 12. However, none of the reductions within groups were significant. Furthermore, significant differences between groups were not detected at either time point (Table 3.3), (Appendix XI). The lymphocyte cultures could not be immunophenotyped (CD4, CD8, or CD21) due to nonspecific staining or expression of receptors on non-lymphoid cells: as determined by tricolor-staining of cells outside the lymphocyte gate.

The production of PGE_2 by stimulated mononuclear cells was not significantly different among the three groups of dogs at week 0. After 12 weeks of dietary treatment, production of PGE_2 by all three groups was diminished. However, the change from baseline was only statistically different for the two groups supplemented with fish oil. The PGE_2 concentrations of mononuclear cell cultures isolated from dogs receiving 12 weeks of fish oil supplementation were significantly lower when compared to the concentration of isolates from dogs supplemented with sunflower oil, but there was no difference between Groups Fish oil and Fish oil + E (Figure 3.2), (Appendix XII).

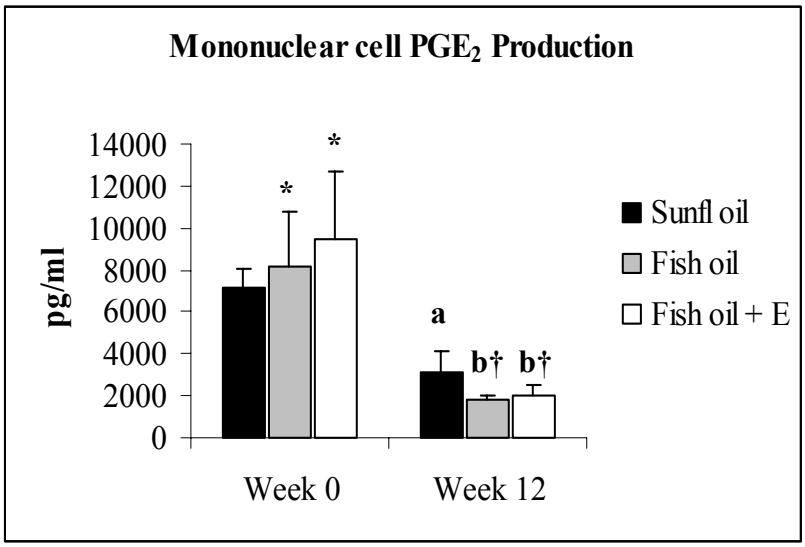

Figure 3.2 Effect of dietary supplementation for 12 weeks on peripheral mononuclear cell production of PGE_2 of three canine dietary groups. Data are expressed as means +/- SEM, n = 3 or 4. Significant differences between groups within a time point are denoted by different lower case letters (a, b); significant differences within a group across time are denoted by different symbols (*, †).

Discussion

Animal and human studies have shown that diets supplemented with or containing a high concentration of polyunsaturated fatty acids diminish the capacity of lymphocytes to proliferate when stimulated in culture (Calder et al., 1989; Soyland et al., 1993; Yaqoob et al., 1994; Jeffery et al., 1997a; Jolly et al., 1997; Kuratko, 2000). Several studies have suggested that n-3 fatty acids and eicosapentanoic acid (EPA) in particular are the most potent inhibitors of lymphoproliferation (Calder and Newsholme, 1992; Jeffery et al., 1996; Jolly et al., 1997; Kuratko, 2000). One study in dogs documented that n-3 fatty acid dietary supplementation significantly suppressed T-lymphocyte responsiveness based on a delayed-type hypersensitivity skin test (Wander et al., 1997). However, in contrast to that report and to studies evaluating *in vitro* lymphoproliferation in other species, the only study to actually measure canine lymphocyte proliferation after dietary supplementation with n-3 fatty acids found increased mitogen-induced proliferation (Kearns et al., 1999). Although PUFA and vitamin E intake, as well as the n-6:n-3 fatty acid ratios fed in these two canine studies differed (1.4:1 and 5:1, respectively), the contrasting assessments of lymphocyte function may simply be due to the differences in experimental methodology.

Although both methods used in the current study (tritiated thymidine incorportation and flow cytometry) indicated diminished lymphocyte proliferation in all three dietary groups, statistical significance varied depending on the method used for evaluation. Measurement of tritiated thymidine uptake, when reported as counts per minute and stimulation index, revealed a significant reduction in lymphocyte proliferation in dogs supplemented with sunflower oil. Quantification of the number of peaks derived by sequential halving of CFSE agreed that only the sunflower oil group had a significant reduction in proliferation. However, when the percent proliferation was calculated from the CFSE histograms, only the fish oil group was found to have a significant reduction. The reasons for the discrepancies between the methodologies are likely to be multivariate. First, while tritiated thymidine is incorporated into the cell's DNA as it progresses through S-phase, DNA synthesis does not always correlate with complete cell division (Maurer, 1981). Given this potential circumstance, measurement utilizing tritiated thymidine could overestimate the proliferation of a stimulated lymphocyte culture and explain the discrepancy in the assessment of proliferation in Group Fish oil. Secondly, using the tritiated thymidine method proliferation is only measured during the time the culture is pulsed with tritium, usually the last 4-6 hours of culture. Unlike the CFSE method, the proliferation that may have occurred previously during the 72 hour culture period is not assessed. Finally, 'cold' thymidine released from dead

cells can dilute the tritiated thymidine added to a lymphocyte culture (Kristensen et al., 1982). In this scenario, the CPM would underestimate the number of cell divisions of the lymphocyte culture, again explaining the differing results between the methodologies used here. For example, stimulated lymphocyte cultures of Dog 9062 at week 0 showed significant proliferation when stained with CFSE and evaluated using flow cytometry (Figure 3.3). The percent proliferation of this culture was 49.9 % (Mean +/- SEM for Group Fish oil, 44.8% +/- 2.5). This same lymphocyte culture also exhibited a high level of activation (82.9%) by increased IL-2 receptor expression (Mean +/- SEM for Group Fish oil, 78.1 +/- 2.7) (Figure 3.4). However, after pulsing with tritiated thymidine, evaluation of this same stimulated lymphocyte culture revealed a modest lymphoproliferative response at best: CPM were13535.6 (Mean +/- SEM for Group Fish oil, 12138.6 +/- 2329.5), and the SI was 17.3 (Mean +/- SEM for Group Fish oil, 24.3 +/- 5.2). Based

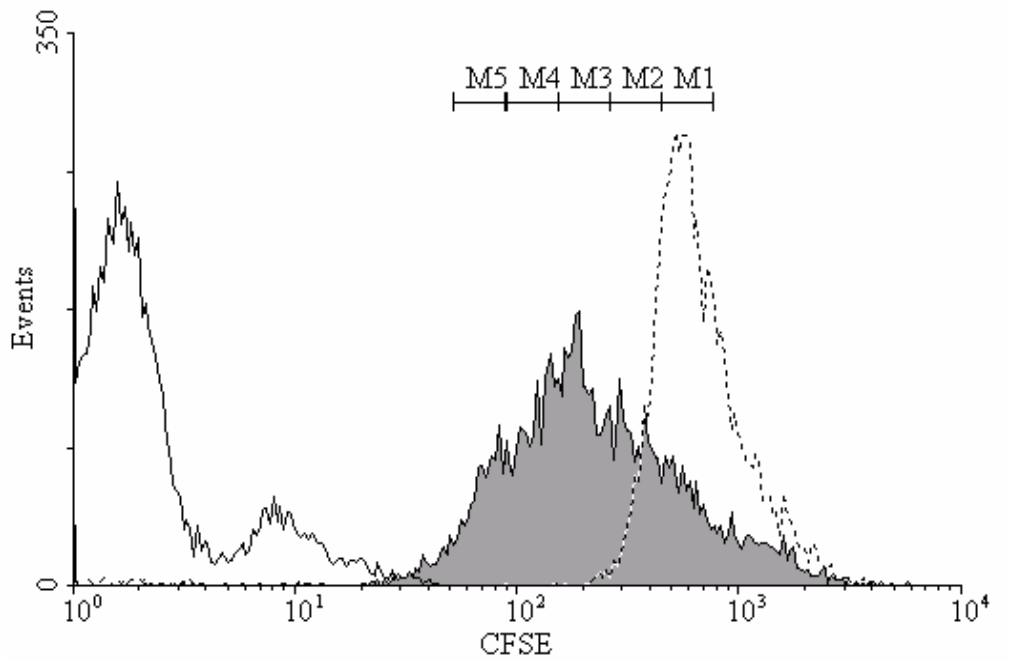

Figure 3.3 Histogram of CFSE fluorescence of stimulated lymphocytes (gray peaks) from Dog 9062 of Group Fish oil at week 0. Marked cell proliferation is denoted by the large volume of the gray peaks to the left of the unstimulated dotted peak. The minimal volume of gray peaks within the region of expected unstimulated cells may represent senescence of unstimulated cells; cell senescence could lead to a release of cold thymidine and explain the low CPM of these cells when pulsed with tritiated thymidine.

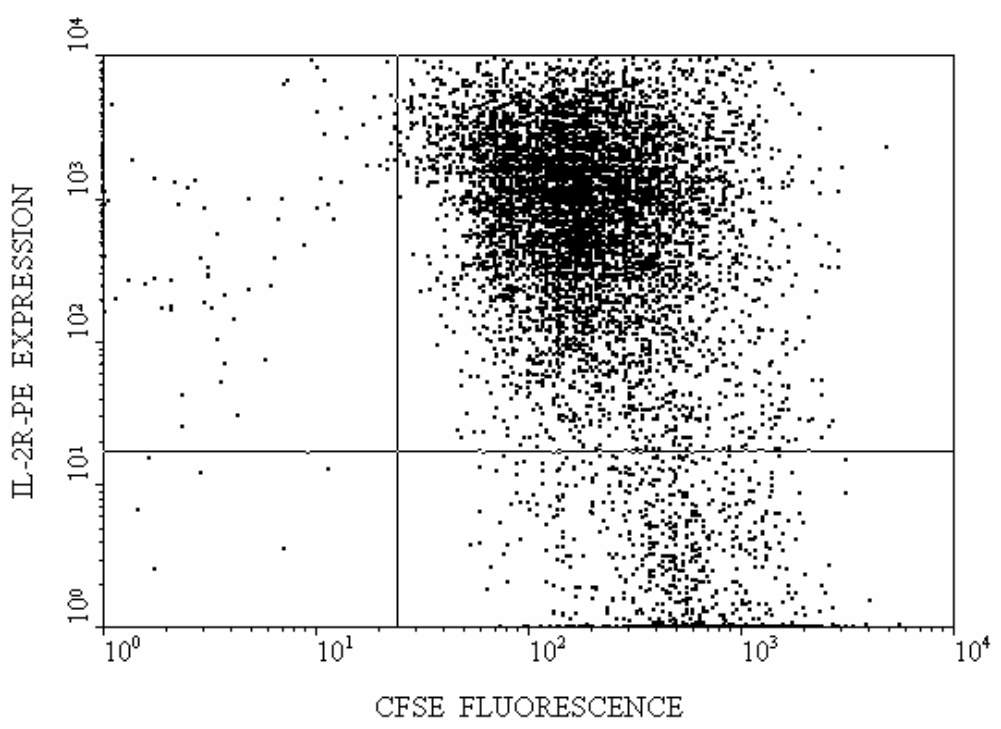

Figure 3.4 Dot plot of CFSE vs. phycoerythrin (PE)-labeled IL-2R of stimulated lymphocytes from Dog 9062 of Group Fish oil at week 0 (same cell culture as Figure 3.4). Dots in upper right quadrant represent dual positive cells that are both activated and proliferating. IL-2R = IL-2 receptor.

on the CFSE histogram this stimulated lymphocyte population (Figure 3.3), senescence of the unstimulated cells is apparent. Thus, incorporation of cold thymidine released from these cells may account for the discordantly lower values obtained when evaluating CPM and SI.

Given these potential pitfalls of the tritiated thymidine method, the percent proliferation as derived from the flow cytometric-CFSE method appears to be more accurate at assessing the true lymphoproliferative response. This method has proven very useful and accurate in the measurement of lymphoproliferative responses in other species (Lyons, 2000; Sathiyaseelan and Baldwin, 2000). Counting the number of generation peaks within the CFSE histogram appeared to agree with the tritiated thymidine-derived results in the current study. However, this rather crude measurement does not take into account the number of cells present within each division.

The finding here that a low n-6:n-3 fatty acid ratio diet consisting of 0.65% n-3 fatty acids (DMB) acted as a more potent suppressor of lymphocyte proliferation, based on evaluation of the percent lymphocyte proliferation as measured by the flow cytometric-CFSE method, agrees with reports in other species but contrasts with the findings of the only other similar canine study (Kearns et al., 1999). These authors speculated that their unique findings of enhanced lymphoproliferative response after supplementation with n-3 fatty acids were due in part to maintained plasma levels of vitamin E levels during their study. The results reported in the current study would seem to support a positive effect of vitamin E on lymphocyte proliferation, since the decrease in lymphocyte proliferation was not significant in dogs receiving the low n-6:n-3 fatty acid ratio diet plus vitamin E supplementation (Group Fish oil + E) but was significant in dogs receiving n-3 fatty acids alone, based on the flow cytometric technique. Other studies have shown vitamin E to have a protective effect against n-3 fatty acid-induced inhibition of lymphocyte proliferation (Kramer et al., 1991), or to promote lymphocyte proliferation outright (Corwin and Shloss, 1980; Meydani et al., 1990; De la Fuente et al., 2000). An n-3 fatty acid-induced decrease in the lymphocyte-mediated immune response of canine subjects, based on delayed-type hypersensitivity skin testing, has been attributed to reduced plasma vitamin E concentrations and increased lipid peroxidation (Wander et al., 1997). However, despite maintained plasma vitamin E concentrations compared to baseline (Figure 2.1) dogs in the current study supplemented with sunflower oil and fish oil had diminished lymphoproliferative responses. Furthermore, an increase in plasma lipid peroxides was not detected in any of the three dietary groups (Figure 2.4). Therefore, based on results of this study, the reduction of lymphocyte proliferation induced by a low n-6:n-3 fatty acid diet does not appear to involve reduced vitamin E levels or increased lipid peroxidation. Although oxidative damage would potentially be detrimental to the immune response, a lack of correlation between lipid peroxidation and n-3 fatty acid-induced diminished lymphoproliferation has also been observed by other researchers (Soyland et al., 1993).

Other mechanisms have been suggested to account for the effect of polyunsaturated fatty acids on lymphocyte proliferation. It has been speculated that inhibition of lymphocyte proliferation revolves around the production of PGE_2; PGE_2 is an inhibitor of lymphocyte proliferation and other functions. However, several studies suggest that prostaglandin-independent mechanisms are probably more important (Santoli and Zurier, 1989; Calder et al., 1992; Soyland et al., 1993). Despite a significant n-3 fatty acid induced-reduction in mononuclear cell production of PGE_2 (Figure 3.2), lymphocyte proliferation was also reduced in the present study, again

suggesting PGE_2 independent suppression. Decreased synthesis of the lymphocyte proliferation-promoting cytokine IL-2 (Santoli and Zurier, 1989; Calder and Newsholme, 1992; Kumar et al., 1992; Endres et al., 1993; Terada et al., 2001) or decreased expression of its receptor, IL-2R (Soyland et al., 1994; Terada et al., 2001), has also been repeatedly implicated as a potential cause for the reduction in lymphocyte proliferation induced by polyunsaturated fatty acids, and specifically n-3 fatty acids. Furthermore, n-3 fatty acid-induced reduction of IL-2 secretion has been related to decreased production of intracellular lipid second messengers (Jolly et al., 1997). Although IL-2 production was not measured here, all three diets resulted in a decreased percentage of lymphocytes expressing IL-2 receptor. However, these changes were not found to be statistically significant and a role for IL-2 or its receptor could neither be confirmed nor denied.

Attempts to immunophenotype lymphocyte cultures in this study were unsuccessful. Analysis of forward versus side scatter dot plots of cells staining positively for CFSE and tri-color fluorochromes revealed the presence of dual positive non-lymphoid cells from cultures of several dogs. This occurred with isolates labeled for all three CD markers, so the finding was attributed to the non-specific binding of our secondary antibody labeled with the tri-color fluorochrome. Non-specific binding of labeled antibodies to cultured cells via Fc (IgG) receptors has been previously described (Imamura et al., 1991). Furthermore, canine neutrophils express CD4 receptors (Moore et al., 1992). Therefore, expression of this receptor with subsequent specific binding of anti-CD4 antibodies in the specific case of neutrophils cannot be ruled out.

Based on the findings in this study, a diet consisting of 0.65% n-3 fatty acids (DMB) with an n-6:n-3 fatty acid ratio of 3.4:1 appears to suppress, not promote, lymphocyte proliferation in healthy,young adult dogs. Although high plasma levels of vitamin E appear to inhibit this suppression of the lymphoproliferative response, this n-3 fatty acid-induced effect is not solely the result of diminished vitamin E levels or increased production of lipid peroxides. Regardless, this study supports the use of low n-6:n-3 fatty acid ratio diets as part of an anti-inflammatory arsenal against chronic inflammatory diseases of the canine.

References

Adolfsson O, Huber B, Meydani S. 2001. Vitamin E-enhanced IL-2 production in old mice: Naive but not memory T cells show increased cell division cycling and IL-2-producing capacity. J Immunol 167:3809-3817.

Beharka A, Wu D, Han S, Meydani S. 1997. Macrophage prostaglandin production contributes to the age-associated decrease in T cell function which is reversed by the dietary antioxidant vitamin E. Mech Ageing Dev 93:59-77.

Calder P. 1998. Dietary fatty acids and the immune system. Nutr Rev 56:S70-S83.

Calder P, Bevan S, Newsholme E. 1992. The inhibition of T-lymphocyte proliferation by fatty acids is via an eicosanoid-independent mechanism. Immunology 75:108-115.

Calder P, Bond J, Newsholme E. 1989. Fatty acid inhibition of concanavalin A-stimulated lymphocyte proliferation. In: 631st Meeting of Biochemical Society. Guildford. p 1042-1043.

Calder P, Newsholme E. 1992. Polyunsaturated fatty acids suppress human peripheral lymphocyte proliferation and interleukin-2 production. Clin Sci 82:695-700.

Corwin L, Shloss J. 1980. Influence of vitamin E on the mitogenic response of murine lymphoid cells. J Nutr 110:916-923.

De la Fuente M, Carazo M, Correa R, Del Rio M. 2000. Changes in macrophage and lymphocyte functions in guinea-pigs after different amounts of vitamin E ingestion. Brit J Nutr 84:25-29.

Endres S, Meydani S, Ghorbani R, Schindler R, Dinarello C. 1993. Dietary supplementation with n-3 fatty acids suppresses interleukin-2 production and mononuclear cell proliferation. J Leukocyte Biol 54:599-603.

Imamura N, Ota H, Kuramoto A. 1991. CD7 false-positive acute myelogenous leukemia and promyelocytic leukemia cell line HL-60: characterization of CD7 epitopes by four monoclonal antibodies. Am J Hematol 38:72-74.

Jeffery N, Cortina M, Newsholme E, Calder P. 1997a. Effects of variations in the proportions of saturated, monounsaturated and polyunsaturated fatty acids in the rat diet on spleen lymphocyte functions. Brit J Nutr 77:805-823.

Jeffery N, Newsholme E, Calder P. 1997b. Level of polyunsaturated fatty acids and the n-6 to n-3 polyunsaturated fatty acid ratio in the rat diet alter serum lipid levels and lymphocyte functions. Prostaglandins, Leukotrienes, and Ess. Fatty Acids 57:149-160.

Jeffery N, Sanderson P, Sherrington E, Newsholme E, Calder P. 1996. The ratio of n-6 to n-3 polyunsaturated fatty acids in the rat diet alters serum lipid levels and lymphocyte functions. Lipids 31:737-745.

Jolly C, Jiang Y-H, Chapkin R, McMurray D. 1997. Dietary (n-3) polyunsaturated fatty acids suppress murine lymphoproliferation, interleukin-2 secretion, and the formation of diacylglycerol and ceramide. J Nutr 127:37-43.

Kay-Mugford P, Benn SJ, LaMarre J, Conlon P. 2000. In vitro effects of nonsteroidal anti-inflammatory drugs on cyclooxygenase activity in dogs. Am J Vet Res 61:802-810.

Kearns R, Hayek M, Turek J, Meydani M, Burr J, Greene R, Marshall C, Adams S, Borgert R, Reinhart G. 1999. Effect of age, breed, and dietary omega-6 (n-6) : omega-3 (n-3) fatty acid ratio on immune function, eicosanoid production, and lipid peroxidation in young and aged dogs. Vet Immuno Immunopathol 69:165-183.

Kramer T, Schoene N, Douglass L, Judd J, Ballard-Barbash R, Taylor P, Bhagavan H, Nair P. 1991. Increased vitamin E intake restores fish oil-induced suppressed blastogenesis of mitogen-stimulated T lymphocytes. Am J Clin Nutr 54:896-902.

Kristensen F, Walker C, Kristensen B, Vandevelde M, De Weck A. 1982. Technical aspects of low ^{3}H-thymidine incorportation by mitogen stimulated canine peripheral blood lymphocytes *in vitro*. Vet Immuno Immunopathol 3:557-566.

Kumar G, Das U, Kumar K, Madhavi N, Das N, Tan B. 1992. Effect of n-6 and n-3 fatty acids on the proliferation of human lymphocytes and their secretion of TNF-alpha and IL-2 in vitro. Nutr Research 12:815-823.

Kuratko C. 2000. Proliferation of colonic lymphocytes in response to inflammatory cytokines is lower in mice fed fish oil than in mice fed corn oil. Cancer Letters 148:27-32.

Lyons A. 2000. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. J Immunol Methods 243:147-154.

Maurer H. 1981. Potential pitfalls of [^{3}H] thymidine techniques to measure cell proliferation. Cell Tissue Kinet 14:111-120.

Meydani S, Barklund M, Liu S, Meydani M, Miller R, Cannon J, Morrow F, Rocklin R, Blumberg J. 1990. Vitamin E supplementation enhances cell-mediated immunity in healthy elderly subjects. Am J Clin Nutr 52:557-563.

Meydani S, Endres S, Woods M, Goldin B, Soo C, Morrill-Labrode A, Dinarello C, Gorbach S. 1991. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. J Nutr 121:547-555.

Moore PF, Rossitto PV, Danilenko DM, Wielenga JJ, Raff RF, Severns E. 1992. Monoclonal antibodies specific for canine CD4 and CD8 define functional T-lymphocyte subsets and high-density expression of CD4 by canine neutrophils. Tissue Antigens 40:75-85.

Muggli R. 1989. Dietary fish oils increase the requirement for vitamin E in humans. In: Chandra R, editor. Health effects of fish and fish oils. St. John's, Newfoundland: ARTS Biomedical Publishers and Distributors. p 201-210.

Reinhart G. 1996. Review of omega-3 fatty acids and dietary influences on tissue concentrations. In: Carey D, Norton S, Bolser S, editors. Recent advances in canine and feline nutritional research: Proceedings of the 1996 Iams international nutrition symposium. Wilington: Orange Frazer Press. p 235-242.

Santoli D, Zurier R. 1989. Prostaglandin E procursor fatty acids inhibit human IL-2 production by a prostaglandin E-independent mechanism. J Immunol 143:1303-1309.

Sathiyaseelan T, Baldwin C. 2000. Evaluation of cell proliferation by bovine T cells in polyclonally activated cultures using carboxyfluorescein succinimidyl ester (CFSE) loading and flow cytometric analysis. Res Vet Sci 69:275-281.

Sharon J. 1998. Basic Immunology. Baltimore: Williams and Wilkins.

Soyland E, Lea T, Sandstad B, Drevon A. 1994. Dietary supplementation with very long-chain n-3 fatty acids in man decreases expression of the interleukin-2 receptor (CD25) on mitogen-stimulated lymphocytes from patients with inflammatory skin diseases. European J Clin Investigation 24:236-242.

Soyland E, Nenseter M, Braathen L, Drevon C. 1993. Very long chain n-3 and n-6 polyunsaturated fatty acids inhibit proliferation of human T-lymphocytes in vitro. Euro J Clin Invest 23:112-121.

Terada S, Takizawa M, Yamamoto S, Ezaki O, Itakura H, Akagawa K. 2001. Suppressive mechanisms of EPA on human T cell proliferation. Microbiol Immunol 45:473-481.

Wander R, Hall J, Gradin J, Du S, Jewell D. 1997. The ratio of dietary (n-6) to (n-3) fatty acids influences immune system function, eicosanoid metabolism, lipid peroxidation and vitamin E status in aged dogs. J Nutr 127.

Yaqoob P, Newsholme E, Calder P. 1994. The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation. Immunology 82:603-610.

CHAPTER IV: EFFECTS OF DIETARY FISH OIL SUPPLEMENTATION ON INFLAMMATORY MEDIATOR PRODUCTION IN THE DOG

Introduction

Cytokines are multifunctional peptides that are produced predominantly by activated lymphocytes and macrophages during immune and inflammatory reactions (Collins, 1999). Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 are among the most important cytokines produced by macrophages (Calder, 2001). TNF-α and IL-1 are the first cytokines to be released in response to many stimuli. Their release from macrophages peaks 1-2 hours after lipopolysaccharide (LPS) challenge (Glauser, 1996). Increased production of IL-6 quickly follows in response to high levels of TNF-α and IL-1 (Calder, 1997). Stimuli that promote macrophage production of these cytokines, aside from bacterial endotoxin or LPS, include immune complexes such as complement and C-reactive protein, fibrin degradation products, and thermal or radiation injury (Dinarello, 1996; Collins, 1999). Enhanced release of TNF-α, IL-1 and IL-6 is considered one of the hallmarks of sepsis and hemorrhagic shock (Jarrar et al., 1999). These cytokines promote a multitude of changes: fever; increased production of acute phase proteins; hypotension; activation and infiltration of neutrophils; increased cytokine synthesis by lymphocytes, increased synthesis of lipid mediators such as prostaglandins, leukotrienes, and platelet-activating factor (PAF); and, many others (Dinarello, 1996; Calder, 1997; Collins, 1999). These effects are crucial in the acute response against pathogens and other injuries; however, over production of these cytokines can be severely detrimental to the host. Inappropriate or prolonged production has been associated with cartilage breakdown, organ dysfunction, anorexia and cachexia, and non-regenerative anemia (Gelin et al., 1991; Strassmann and Kambayashi, 1995; Dinarello, 1996; Jarrar et al., 1999).

Cytokines are not the only important mediators of immunity and inflammation. Many of the same stimuli also activate cellular phospholipases that lead to the synthesis of prostaglandins, leukotrienes, and platelet activating factor (PAF). Prostaglandin E_2 (PGE_2) is an arachidonic acid metabolite produced predominantly by macrophages that is very important in local acute inflammatory responses. Localized swelling and erythema is largely due to PGE_2-induced vasodilation and increased permeability (Collins, 1999; Tilley et al., 2001). Fever is also an important component of acute inflammation in which PGE_2 plays a critical role. One study indicates that the induction of fever in response to PGE_2, IL-1, and LPS is in fact mediated through a single PGE_2 receptor (Ushikubi et al., 1998). There is evidence that PGE_2 also plays a

role in some chronic inflammatory diseases such as cystic fibrosis (Greally et al., 1994), ulcerative colitis, and Crohn's disease (Hommes et al., 1996). Interestingly, PGE_2 has anti-inflammatory and immunosuppressive effects as well. PGE_2-mediated suppression of *in vitro* and *in vivo* TNF-α synthesis has been reported (Renz et al., 1988; Spinas et al., 1991). PGE_2 has also been found to suppress lymphocyte proliferation (Goodwin et al., 1977; Chouaib et al., 1985; Calder et al., 1992) and the production of the pro-lymphocyte growth and activation cytokine IL-2 (Rappaport and Dodge, 1982; Chouaib et al., 1985; Calder, 2002a).

"Platelet-activating factor" is actually a family of structurally similar bioactive phospholipid-derived mediators (Collins, 1999; McManus and Pinckard, 2000). PAF synthesis is dependent on phospholipase A_2-mediated hydrolysis of membrane phospholipids. Similar to PGE_2 and other arachidonic acid metabolites, it is produced by platelets, neutrophils, macrophages, endothelial cells and epithelial cells, all of which appear to possess a specific serpentine type membrane receptor for PAF. Therefore, it functions as an autocrine and paracrine mediator (Bulger and Maier, 2000; McManus and Pinckard, 2000). The name, platelet-activating factor, is derived from its earliest discovered function of stimulating rabbit platelet aggregation (Benveniste et al., 1972). PAF is now known to have many pro-inflammatory effects on blood vessels, small airways and leukocytes, including increased vascular permeability, increased bronchoconstriction, and increased adhesion and chemotaxis of neutrophils and eosinophils (Kroegel et al., 1994; Christie and Henderson, 2002). PAF has also been shown to enhance the synthesis of TNF-α and IL-1 by stimulated macrophages (Floch et al., 1989; Poubelle et al., 1991). Intravenous injection of PAF leads to symptoms of shock similar to those seen in clincal cases of sepsis (Mathiak et al., 1997), so it may play a relatively direct role in the response to endotoxemia. Increased concentrations of serum PAF has been associated with development of thrombocytopenia and neutropenia in a canine endotoxemia model as well (Tsuchiya et al., 1999).

Finding ways to inhibit the synthesis or block the biological effects of these inflammatory mediators is an active area of research. The ability to control these mediators offers the potential to minimize the negative impact of many chronic inflammatory diseases. However, commercially available anti-inflammatory medications can be expensive and many have significant toxicities in humans and animals, especially with long-term use. Dietary supplementation with n-3 fatty acids obtained from fish or fish oils has been suggested as a way to modulate the immune response while avoiding some these toxicities, and appears to have some beneficial anti-inflammatory

effects. The principal n-3 fatty acids within fish or fish oils are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

Endres and coworkers were among the earliest to show an effect of dietary n-3 fatty acid supplementation on cytokine production. They supplemented healthy humans with 18g fish oil a day and found significantly decreased TNF-α and IL-1 production from their LPS-stimulated peripheral blood mononuclear cells in culture (Endres et al., 1989). Cultured peripheral blood mononuclear cells from young and older women supplemented with 2.4g of n-3 fatty acids per day produced significantly less IL-1, IL-6, TNF-α and PGE_2 compared to baseline levels (Meydani et al., 1991). Men and women over the age of 40 receiving a low fat diet supplemented with 1.23g of EPA and DHA (n-6:n-3 fatty acid ratio of 3.6:1) also had significant reductions in stimulated mononuclear cell production of IL-1, IL-6, TNF-α, and PGE_2, but not granulocyte-macrophage-colony stimulating factor (GM-CSF) (Meydani et al., 1993). Rodent studies have yielded similar results. Activated peritoneal macrophages from mice fed a high fat diet supplemented with fish oil (10.6g EPA and 10.1g DHA/100g of total fat) synthesized significantly less IL-1, TNF-α, and PGE_2 compared to mice supplemented with other oils (Wallace et al., 2000). Mice fed this same diet also had reduced plasma concentrations of IL-1, IL-6 and TNF-α when injected intraperitoneally with LPS compared to mice supplemented with safflower oil (Sadeghi et al., 1999). Concentrations of PGE_2 released from stimulated alveolar macrophages were significantly lower in rats consuming a diet of 20% fish oil (Mancuso et al., 1997).

Dietary supplementation with n-3 fatty acids reduces PAF synthesis by many cells. Rats supplemented with high levels of α-linolenic acid, the fatty acid precursor for EPA and DHA, had reduced PAF synthesis by stimulated neutrophils compared to that of controls (Horii et al., 1991). Hypoxic brain tissue of young mice previously fed a standard diet supplemented with 10% by weight fish oil endogenously synthesized significantly less PAF compared to controls (Akisu et al., 2002). Finally, preincubation of cultured human endothelial cells with EPA and DHA markedly suppressed TNF-α-stimulated synthesis of PAF (Mayer et al., 2002).

Few reports describe clinical trials in which patients were supplemented with n-3 fatty acids alone. Oral EPA supplementation of human patients with unresectable pancreatic adenocarcinoma resulted in reduced serum concentrations of the acute phase protein C-reactive protein, and decreased production of IL-6 by peripheral blood mononuclear cells stimulated in culture (Wigmore et al., 1997). Systemic lupus erythrematous (SLE) is a chronic inflammatory disease characterized by vasculitis, glomerulonephritis, arthritis, and cutaneous rash; SLE patients

supplemented with high doses of EPA and DHA (162mg and 144mg, respectively) had significantly prolonged remission times (Das, 1994). Clinical improvement has also been reported in human patients with rheumatoid arthritis that receive n-3 fatty acids (Kremer et al., 1987). With exception to the previously mentioned studies, the majority of clinical trials have used the commerical product IMPACT® which contains arginine, yeast RNA and n-3 fatty acids (Calder, 2002b). It is difficult to make conclusions about the benefits of n-3 fatty acid supplementation in these trials without knowing the effects of the other nutrients.

Dietary n-3 fatty acids reduce the production of PGE_2, an n-6 fatty acid metabolite, by competitive inhibition. Once dietary n-6 and n-3 fatty acids are incorporated into membrane phospholipids, they compete directly as substrates for the same enzymes (Bauer, 1992; Reinhart, 1996). Changes in cytokine production related to n-3 fatty acid supplementation are not the result of a reduction in PGE_2, since this prostaglandin decreases TNF-α and IL-1 production. Several animal studies have demonstrated an effect of dietary fish oil on cytokine gene expression (Calder, 2002a). Evidence suggests a reduction in gene expression may be the result of down regulation of the nuclear transcription factor NFκB (nuclear factor kappa B) (Xi et al., 2001). A study evaluating the mechanism of n-3 fatty acid-induced reductions in PAF synthesis also found low levels of a transacylase enzyme thought to be crucial to one of the earliest steps of PAF production (Oh-hashi et al., 1997).

Studies evaluating inflammatory mediator production in normal dogs supplemented with n-3 fatty acids have yielded mixed results. Maintaining young and older dogs on a diet consisting of 1.65% fish oil (n-6:n-3 ratio of 5:1) for 60 days had no effect on IL-1, IL-6, TNF-α, or PGE2 production by peritoneal macrophages or peripheral blood mononuclear cells (Kearns et al., 1999). In contrast, fish oil supplementation reduced PGE_2 production by peripheral blood mononuclear cells of dogs receiving a diet consisting of 4.4% fish oil (n-6:n-3 ratio of 1.4:1) for 12 weeks (Wander et al., 1997). Production of leukotriene B_4 (LTB_4), another pro-inflammatory arachidonic acid metabolite, was decreased in neutrophils and skin of dogs fed diets with n-6:n-3 fatty acid ratios of 5.3:1 and 10.4:1 where n-3 fatty acids comprised 3.4% and 1.8% of the total dietary fatty acids, respectively (Vaughn et al., 1994). A few clinical trials assessing the response to n-3 fatty acid supplementation in dogs with naturally occurring disease have also been reported. Pruritus associated with atopy, a common allergic dermatosis, was controlled in 44.4% of dogs fed a commercial diet with an n-6:n-3 fatty acid ratio of 5.5:1 (Scott et al., 1997). Other clinical trials in atopic dogs treated with n-3 fatty acid supplements suggest beneficial effects in 11.1 to 18.3% of

dogs (Scott and Buerger, 1988; Lloyd, 1989). Diminished pain associated with hip dysplasia has been reported in dogs receiving n-3 fatty acid supplements (Miller, 1989). Finally, dogs diagnosed with heart failure were supplemtented with 27mg/kg EPA and 18mg/kg DHA each day for 8 weeks (Freeman et al., 1998). Circulating plasma and stimulated peripheral blood mononuclear cell cultures from these dogs revealed only a significant decrease in mononuclear cell production of IL-1. TNF-α and PGE_2 concentrations in these dogs were not significantly different from controls.

There is overwhelming evidence that cytokines such as IL-1, IL-6, and TNF-α, as well as lipid mediators such as PGE_2 and PAF, play an important role in many inflammatory diseases. Data suggest that dietary n-3 fatty acid supplementation can influence the production of these inflammatory mediators, and potentially reduce the severity of some diseases. However, studies evaluating the effects of dietary n-3 fatty acid supplementation on these mediators in the dog are limited and contradictory with respect to many reports in other species. The purpose of this study was to evaluate the *in vitro* and *in vivo* production of IL-1, IL-6, TNF-α, PGE_2, and PAF in dogs fed a maintenance diet supplemented with 7g of fish oil per day, resulting in a dietary n-6:n-3 fatty acid ratio of 3.4:1 and an n-3 fatty acid percentage of 0.65 (DMB).

Material and Methods

The *in vitro* and *in vivo* experiments reported in this chapter were conducted using the same experimental animals and diet formulations reported in Chapter II.

Experimental Design

Peripheral blood mononuclear cells (PBMCs) for cytokine and PAF synthesis analysis were isolated as described in Chapter III. PBMCs were collected at weeks 0 and 12 of the dietary trials.

After the dogs consumed the experimental diets for 12 weeks, a sterile 16-gauge catheter (Venocath-16, Abbott Ireland, Sligo, Ireland), was aseptically placed in one of the jugular veins of each dog for the 6 hour experiment. Approximately 20ml of whole blood was obtained through the catheter for baseline analysis of circulating IL-1, IL-6, TNF-α, PAF, PGE_2 and collection of PBMCs. Each dog was treated with an intravenous injection of lipopolysaccharide (LPS) from *Escherichia coli* (serotype 055:B5, Sigma Chemical, St. Louis, MO) in sterile saline at 0.1 μg/kg to stimulate the production of inflammatory mediators (LeMay et al., 1990). Following the LPS treatment, approximately 20ml of whole blood was drawn at 0.5, 1, 1.5, 2, 3, 4, and 6 hours to determine the circulating blood concentrations of cytokines and lipid mediators of inflammation.

During this time, the dogs were monitored for signs of illness, and rectal temperatures were taken hourly. Following the 6 hour experiment, 500ml of lactated Ringer's solution was administered intravenously to each dog for replacement of fluid loss, and the catheters were removed.

Serum Cytokine Bioassays (IL-1, IL-6, TNF-α)

Ten milliliters of whole blood (from the 20ml drawn at each time point) was placed into plain vacutainer tubes, allowed to clot, and centrifuged for the isolation of serum. The serum was separated into 0.5ml aliquots and frozen at -80^{o}C until analysis for cytokine activity. These bioassays do not utilize specific antibodies and the cytokines were not sequenced. IL-1-, IL-6-, and TNF-α-like activities were measured based on the proliferation or death of the target cell lines.

Cell Culture and Preparation of Cytokine Standards

All three target cell lines used for the cytokine bioassays were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2.0mM L-glutamine, 2.2g/L $NaHCO_3$, 25mM HEPES, 2.7 x 10^{-4} mg/L 2-mercaptoethanol, 100 U/ml penicillin, and 100µg/ml streptomycin sulfate. The B13.29 clone B9 cells used for the IL-6 bioassay were additionally supplemented with 75 U/ml of recombinant murine IL-6 (R&D Systems, Minneapolis, MN). Standard curves for each assay were generated by 1:3 serial dilutions of recombinant human IL-1, IL-6, or TNF-α (Sigma Chemical, St. Louis, MO). Each recombinant cytokine promoted a dilution-dependent proliferation/death of the target cell line.

Mononuclear Cell Culture and Stimulation

Peripheral blood mononuclear cells were incubated for 24 hours at 37^{o}C and 5% CO_2 in RPMI 1640 medium supplemented with 2.0mM L-glutamine, 2.2g/L $NaHCO_3$, 25mM HEPES, 2.7 x 10^{-4} mg/L 2-mercaptoethanol, 100 U/ml penicillin,100µg/ml streptomycin sulfate and 10% heat-inactivated autologous serum in 24-well plates. Autologous serum was inactivated by incubation in a 56^{o}C water bath for 45 minutes. Mononuclear cells were cultured at a concentration of 1x10^{6} cells/well for the determination of PAF concentration and 5x10^{6} cells/well for the determination of IL-1, IL-6, and TNF-α activity. Macrophages were stimulated by adding lipopolysacchharide (LPS) from *Escherichia coli* (serotype 055:B5, Sigma Chemical, St. Louis, MO) at a concentration of 1µg/well. After 24 hours, the plates were frozen at -20^{o}C. Approximately 12 months later, all plates were defrosted and contents of each well plus 1ml of sterile saline flush per well were removed and placed in 15ml conical tubes. The tubes were centrifuged at 340xg for 10 minutes and supernatants were frozen at -20^{o}C until time of analysis.

Bioassay for IL-1

The cytotoxicity assay for interleukin-1 was performed using the human melanoma cell line A375.S2 (Bueno et al., 1999). Triplicate serum samples from each time point were diluted 1:10 in 100ul of culture medium and placed in 96-well flat-bottom plates. An additional 100ul of culture medium containing 2 x 10^3 A375.S2 cells was placed in each well (the final dilution of the serum sample was 1:20). LPS-stimulated macrophage culture supernatant was assayed for IL-1 using the same method with a final sample dilution of 1:7. Plates were sealed to prevent evaporation and incubated at 37°C in 5% CO_2 for 96 hours. Following removal of medium, the plates were washed once with CMF-PBS (pH 7.2-7.4). The remaining cells were fixed by adding 50ul of 100% methanol to each well and allowed to incubate at room temperature for 30 minutes. Once the methanol was removed, the cells were stained with 50ul of 0.5% crystal violet in 20% ethanol for 30 minutes at room temperature. Following a thorough wash with tap water, 100ul of 50% methanol was added to each well to elute the incorporated violet stain. Cell survival was determined by reading optical density of the plates at 595 nm in a multiwell scanning spectrophotometer (Dynatech Laboratories, Chantilly, VA). The sensitivity of the assay for IL-1 was 7.4 U/ml.

Bioassay for IL-6

The cytoproliferative activity of serum and macrophage supernatant samples was assayed using the murine hybridoma IL-6 dependent cell line B13.29 clone B9 (LeMay et al., 1990; Bueno et al., 1999). Serum samples were diluted 1:100 with complete medium and placed in a 96-well flat-bottom plate at 100ul/well. An additional 100ul of medium containing 2 x 10^3 B9 cells was added to each well (the final dilution of the serum sample was 1:200). Macrophage supernatants were assayed for IL-6 using the same method with a final sample dilution of 1:100. Plates were sealed and incubated at 37°C in 5% CO_2 for 72 hours. For the last 4 hours of incubation, the cells were pulsed with 1.0 μCi of tritiated thymidine/well. The plates were harvested onto glass fiber filters (Tomtec Harvestor, Orange, CT) and incorporation of tritiated thymidine was determined by liquid scintillation spectrophotometry (Betaplate liquid scintillation counter 1205, LKB Wallac, Turku, Finland). The sensitivity of the assay for IL-6 was 0.001 U/ml.

To determine if the cytoproliferative activity observed was specific to IL-6, the bioactivity was neutralized by incubating cells with a mouse anti-human CD126 (IL-6 Receptor) IgG1 monoclonal antibody (Serotec, Raleigh, NC). Prior to the assay, B9 cells were incubated for 1 hour at 37°C at a concentration of 7.25μg antibody/2 x 10^3 cells.

Bioassay for TNF-α

Tumor Necrosis Factor-α (TNF-α) cytotoxicity activity against the mouse fibrosarcoma cell line WEHI 164 clone 13 was determined for serum and macrophage supernatant samples (LeMay et al., 1990; Bueno et al., 1999). First, 96-well flat bottom plates were seeded with 2 x 10^3 WEHI 164 clone 13 cells in 100µl of complete medium per well and allowed to incubate overnight at 37°C in 5% CO_2. Approximately 18 hours later, 55µl of media was removed from each well and discarded. This volume was replaced by 5µl of actinomycin D at 20µg/ml and 50µl of serum to be assayed at a 1:50 dilution (the final dilution of the serum sample was 1:100). Macrophage supernatant samples were assayed with a final dilution of 1:50. The plates were sealed and allowed to incubate at 37°C in 5% CO_2. Approximately 20 hours later, 10µl of tetrazolium dye 3-(4,5 dimethylthiazole-2yl)-2,5 triphenyl tetrazolium bromide (MTT; 5mg/ml in calcium and magnesium free (CMF)-phosphate buffered saline solution (PBS)) was added to each well and incubated for an additional 4 hours at 37°C in 5% CO_2. After incubation, 100µl of 0.04N HCl in isopropanol was vigorously pipetted into each well to dissolve the purple formazan crystals. After 30 minutes at room temperature, the optical density of the plates was read at 595 nm in a multiwell scanning spectrophotometer (Dynatech Laboratories, Chantilly, VA). The sensitivity of the assay was 0.06 U/ml.

To determine if the cytotoxicity observed was due to TNF-α, the WEHI cells were incubated with rat anti-mouse CD120a (TNF-Receptor I) IgG2a monoclonal antibody (HyCult Biotechnology b.v.). Prior to the assay, WEHI cells were incubated for 1 hour at 37°C at a concentration of 5µg antibody/2 x 10^3 cells.

Scintillation Proximity Assay for PAF

Due to active acetylhydrolases in peripheral blood, PAF is quickly converted to an inactive metabolite, lyso-PAF (McManus and Pinckard, 2000). After collection, whole blood (1.5ml) was immediately extracted with 4.5ml of ice cold methanol to inactivate acetylhydrolase activity. Plasma was isolated by centrifugation and frozen at -80°C until time of analysis. Semipurification of the plasma and macrophage supernatant samples was performed using a modified Bligh-Dyer lipid phase extraction (Yamada et al., 1988). Briefly, 3ml samples were mixed with 1ml of distilled water and 4ml of chloroform. The lower phase was collected and evaporated to dryness under a stream of N_2 gas. The residue was dissolved in 200µl of chloroform and applied to a C-18 Amprep minicolumn (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England). The column was washed with 3ml of chloroform, 2ml of chloroform-methanol (3:2) and 3ml of

chloroform-methanol-28% aqueous ammonia (10:5:1), and then eluted with 2ml of chloroform-methanol-28% aqueous ammonia (50:50:7). The eluate was evaporated under a stream of N_2 gas and the residue was dissolved in 200µl of assay buffer.

The PAF concentration of the purified samples was determined for each time point using a scintillation proximity assay (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England) according to manufacturer's instructions. All reagents were provided. Briefly, 100µl of sample were combined with 100µl of the tracer compound ([^{3}H]-PAF), 100µl of anti-PAF antibody and 100µl of SPA protein A (bound to fluomicrospheres). Each sample was prepared in duplicate and incubated on an orbital shaker for approximately 20hrs at room temperature. After incubation, the amount of [3H]-PAF bound to the fluomicrospheres was determined by an automated β-scintillation counter (Beckman LS 5000TD Liquid Scintillation System, Beckman Instruments, Fullerton, CA) and reported as counts per minute. Average counts per minute were calculated for each replicate. The concentration of PAF within each sample was determined using a log-logit curve generated by seven standard dilutions. The sensitivity of the assay was approximately 20pg/sample. The specificity of this assay for PAF (1-hexadecyl-2-acetyl GPC-PAF (C16:0)) is 100% with 40% cross reactivity for PAF (1-octadecyl-2-acetyl GPC-PAF (C18:0)) but <0.01% with Lyso-PAF and other choline-containing phospholipids.

Enzyme Immunoassay for PGE_2

Four milliliters of whole blood were added to an EDTA vacutainer tube with 10µM indomethacin to prevent conversion of PGE_2 to more stable metabolites. Plasma was isolated by centrifugation and frozen at -80^{o}C until time of analysis. Plasma PGE_2 concentrations were determined using a competitive enzyme-linked immunoassay for a stable PGE_2 derivative (Bicyclo Prostaglandin E2 EIA Kit, Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions (Kay-Mugford et al., 2000). Briefly, 500µl sample aliquots were derivatized by adding 150µl of 1 M carbonate buffer and incubated overnight at 37^{o}C. Buffered samples were further diluted to 1:10 and 1:20 and added in duplicate to wells of a goat anti-mouse IgG coated 96-well plate provided by the manufacturer. Eight 1:2 serial dilutions of a provided standard were made and added to the appropriate wells. Next, 50 µl of tracer compound consisting of PGE_2 conjugated to acetylcholinesterase (AChE) was added to each well, followed by the addition of 50µl of mouse anti-PGE_2. Following an18-hour incubation at 4^{o}C, all wells were emptied and washed 5 times with buffer. After the addition of 200µl per well of fresh Ellman's Reagent (acetylthiocholine and 2-nitrobenzoic acid), the plate was allowed to develop on an

orbital shaker for 90 minutes in the dark at room temperature. The intensity of the enzymatic reaction was determined spectrophotometrically at 415nm (EL808 Ultra Microplate Reader, Biotech Instruments, Winooski, VT). Sample PGE_2 concentrations were calculated using a log-logit curve generated by the eight standard dilutions. The sensitivity of the assay was approximately 2pg/ml. The specificity of the assay was 100% with <1% cross-reactivity with other eicosanoids.

Statistical Analysis

All data were considered continuous and found to follow a normal distribution with rejection of the null hypothesis of normality at $p \leq 0.05$ using the Shapiro-Wilk test. For the 6 hour *in vivo* curves, the area under the curve (concentration-time) was calculated using the trapezoid method to make a global comparison between the diet types (Stewart, 1995). The concentration-time was evaluated using a mixed effect linear model that accounted for the random variance of dog. For macrophage production of mediators, the effect of diet and time was also evaluated using a mixed effect linear model that accounted for the random variance of dog, and the repeated measurements on each dog. Where there was significant interaction of concentration-time (6 hour *in vivo*) or diet and time (macrophage) at $p \leq 0.05$, least squares means comparisons were made between and within diet groups to determine where the significant effects were occurring. Type I error was maintained at 0.05 for all between and within group comparisons. The data were summarized as mean +/- SEM. PROC NPAR1WAY (SAS v8.0, SAS Institute, Cary, NC) was used for the analysis.

Results

During the 6 hour *in vivo* experiment, 3 of 5 Group Sunflower oil dogs, 1 of 5 Group Fish oil dogs, and 2 of 5 Group Fish oil +E dogs vomited 1-2 hours after receiving their intravenous injection of LPS. Rectal temperatures for all dogs remained within normal limits during the experiment (Appendix XIII). All dogs appeared bright, alert, and had a good appetite after receiving their post-experiment intravenous fluids.

Immediately prior to LPS injection, there was no significant difference in serum cytokine activity or concentrations of PAF and PGE_2 between the three dietary groups. Total serum IL-1 activity (area under the curve) of the two groups supplemented with fish oil was significantly lower than the activity levels of Group Sunflower oil after intravenous LPS injections (Figure 4.1), (Appendix XIV). However, significant differences between groups at individual time points were only found at hours 4 and 6. The IL-1 activity of Group Sunflower was significantly higher than

Group Fish oil at 4 hours and higher than Groups Fish oil and Fish oil + E at 6 hours. Groups Fish oil and Fish oil +E were not significantly different at any time point or in total IL-1 activity.

Stimulated mononuclear cell culture IL-1 activity was not significantly different between groups at weeks 0 or 12. Culture levels of IL-1 activity in all three groups significantly declined after 12 weeks of dietary supplementation (Figure 4.2), (Appendix XV).

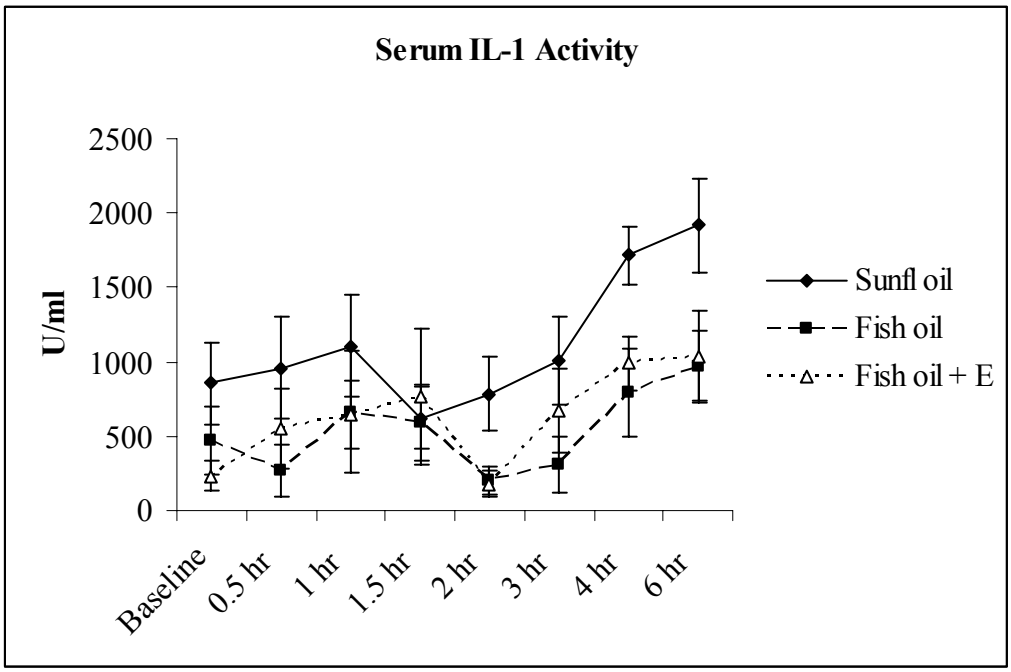

Figure 4.1 Serum IL-1 activities of three canine dietary groups following intravenous LPS injection. Total IL-1 activity (area under the curve) of Group Sunflower oil was significantly higher compared to the total activity of the two fish oil groups. The data are expressed as means +/- SEM, n = 5.

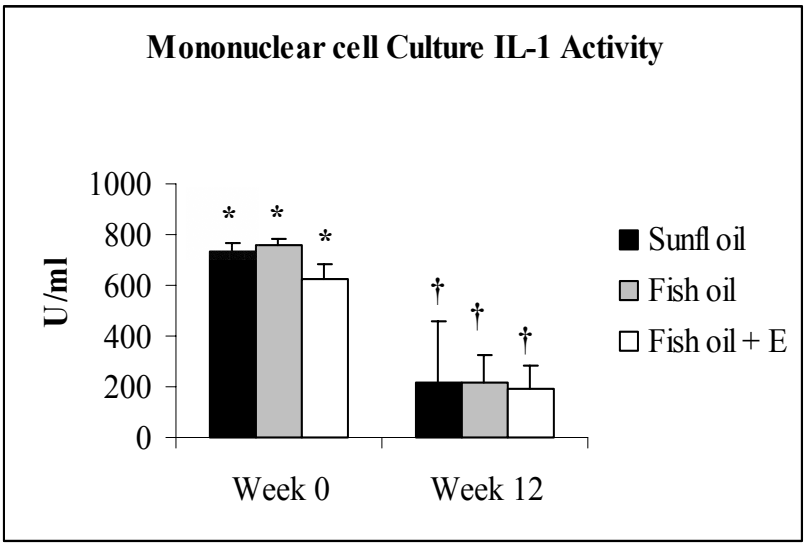

Figure 4.2 Effect of dietary supplementation for 12 weeks on mononuclear cell culture IL-1 activity of three canine dietary groups. The data are expressed as means +/- SEM, n = 3-4. Significant differences within a dietary group across time are denoted by different symbols (*,†).

Intravenous LPS stimulated a significant increase in the serum IL-6 activity among dogs of all three dietary groups. IL-6 activity levels were significantly increased above baseline at 1.5, 2, and 3 hours for all three groups and at 4 hours for Groups Sunflower oil and Fish oil + E. IL-6 activity levels of Group Fish oil were significantly lower than levels of Group Sunflower oil at hours 1.5 and 4. Evaluation of total serum IL-6 activity showed Groups Fish oil and Fish oil +E levels to be significantly lower than levels of Group Sunflower oil. Groups Fish oil and Fish oil +E were not significantly different at any time point or in total IL-6 activity (Figure 4.3), (Appendix XVI).

Stimulated mononuclear cell culture IL-6 activity was not significantly different between dietary groups at week 0. Culture levels of IL-6 activity in all three groups increased significantly after 12 weeks of dietary treatment. Increases in IL-6 activity were not significantly different between the dietary groups (Figure 4.4), (Appendix XV). The effect of mononuclear cell supernatant on the target cell line was completely neutralized by the addition of the anti-human IL-6 receptor antibody, supporting the specificity of this assay for canine IL-6 (data not shown).

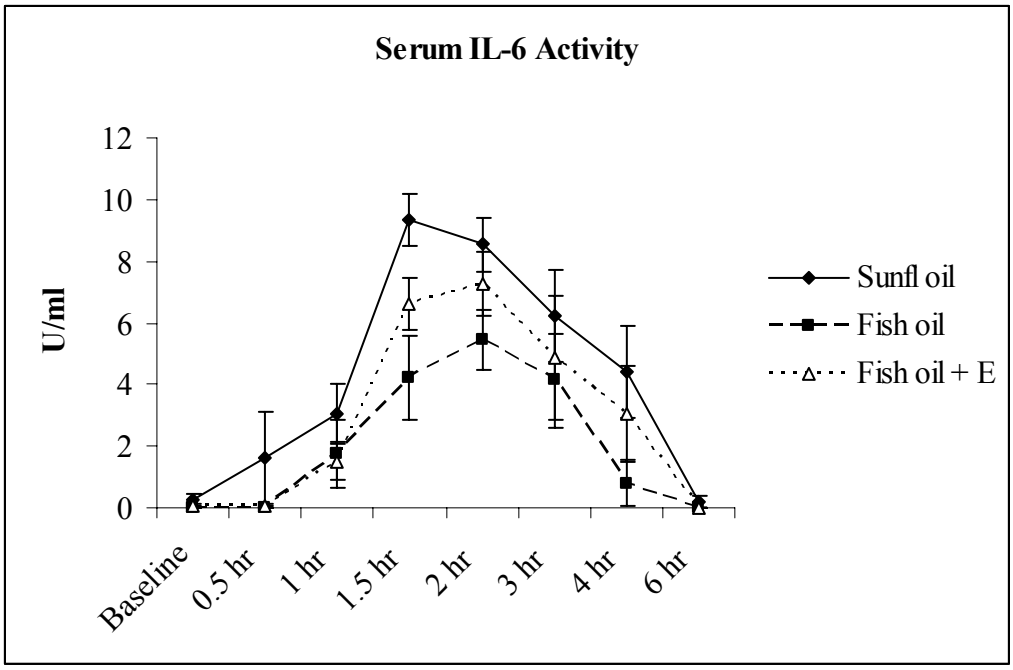

Figure 4.3 Serum IL-6 activities of three canine dietary groups following intravenous LPS injection. Total IL-6 activity (area under the curve) of Group Sunflower oil was significantly higher compared to the total activity of Groups Fish oil and Fish oil + E. The data are expressed as means +/- SEM, n = 5.

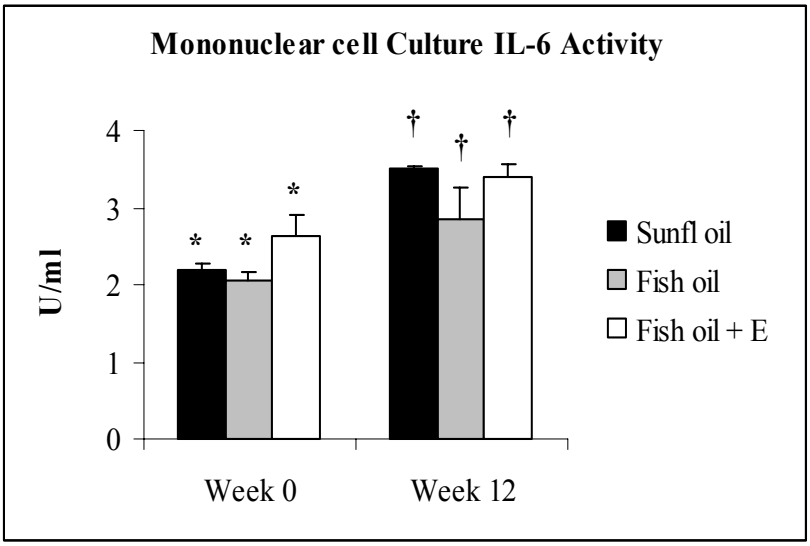

Figure 4.4 Effect of dietary supplementation for 12 weeks on mononuclear cell culture IL-6 activity of three canine dietary groups. The data are expressed as means +/- SEM, n = 3-4. Significant differences within a dietary group across time are denoted by different symbols (*,†).

Serum TNF-α activities among dogs of all three dietary groups were significantly increased 1 hour after receiving an intravenous LPS injection. Activity levels remained significantly above baseline at 1.5 hours for Group Fish oil +E. Significant differences between groups were not found at any time point, including 1 and 1.5 hours post LPS injection. Total serum TNF-α activity was not significantly different between the three groups (Figure 4.5), (Appendix XVII).

Detectable TNF-α activity was not consistently found in the mononuclear cell culture supernatants of any dietary group at either week 0 or week 12. Thus, addition of the anti-mouse TNF-α-receptor had no effect on recombinant human TNF-α-induced death of the target cells.

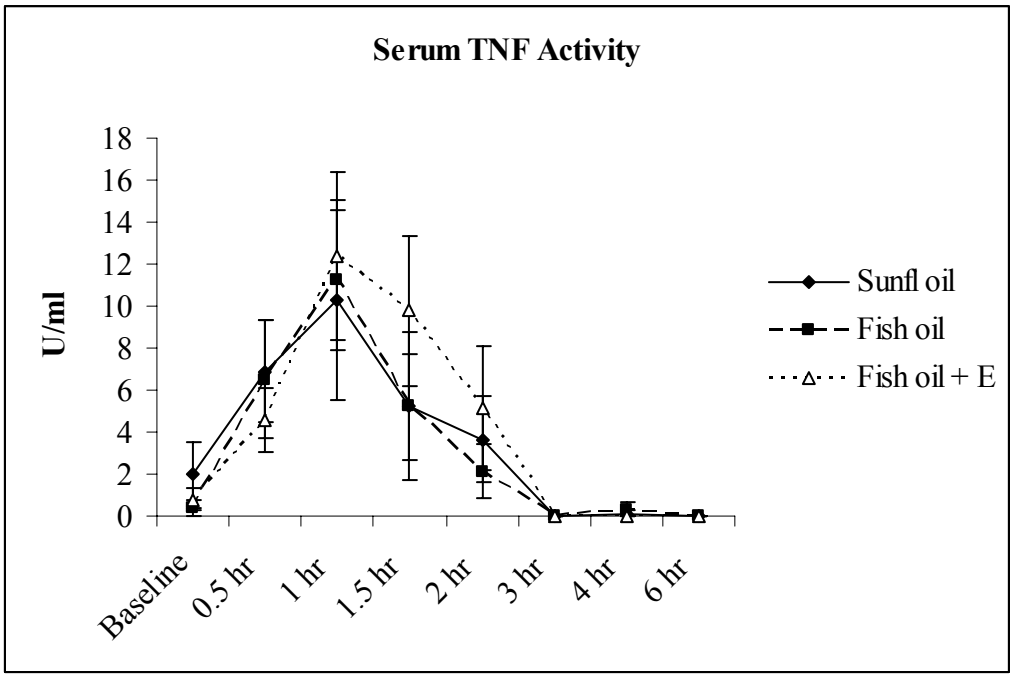

Figure 4.5 Serum TNF-α activities of three canine dietary groups following intravenous LPS injection. No significant differences in total TNF-α activity (area under the curve) were found. The data are expressed as means +/- SEM, n = 5.

Intravenous LPS injections produced significant changes in serum PAF concentration compared to baseline in all three dietary groups. However, the significant elevations at 0.5, 1.5, and 2 hours post LPS injection did not differ between groups. Significant differences in total PAF concentrations between dietary groups were not detected (Figure 4.6), (Appendix XVIII).

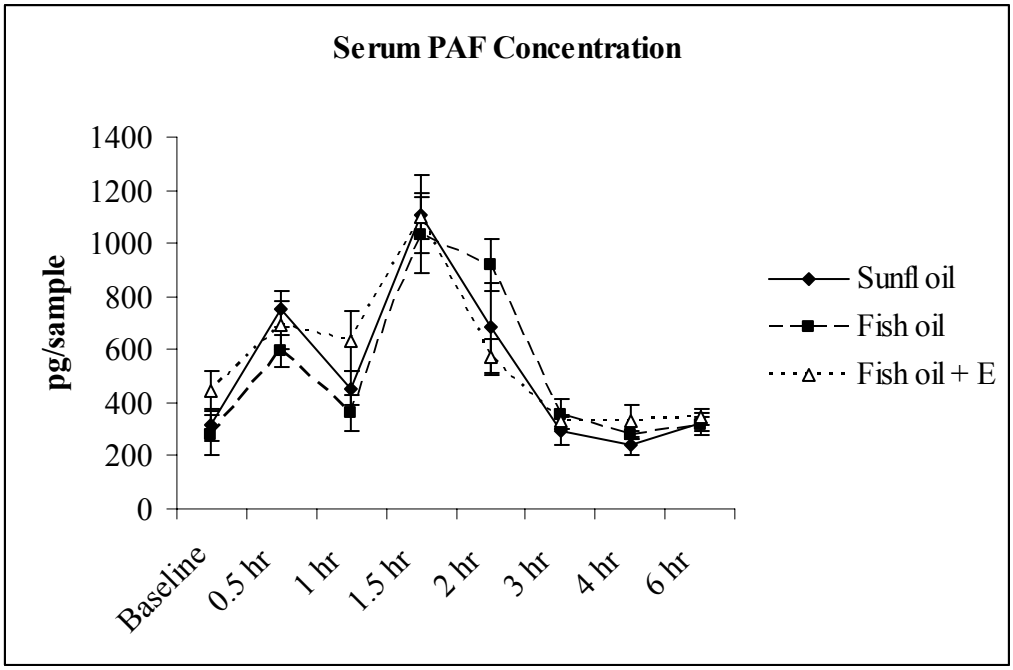

Figure 4.6 Serum PAF concentrations of three canine dietary groups following intravenous LPS injection. No significant differences in total PAF concentrations (area under the curve) were found. The data are expressed as means +/- SEM, n = 5.

Mononuclear cell culture concentrations of PAF were not significantly different between dietary groups at week 0. After 12 weeks of dietary supplementation, both groups supplemented with fish oil had a decreased supernatant concentration of PAF, while PAF concentration of mononuclear cell culture supernatants of Group Sunflower oil increased. However, none of these changes were statistically significant, and there were no significant differences between the dietary groups. (Figure 4.7), (Appendix XIX).

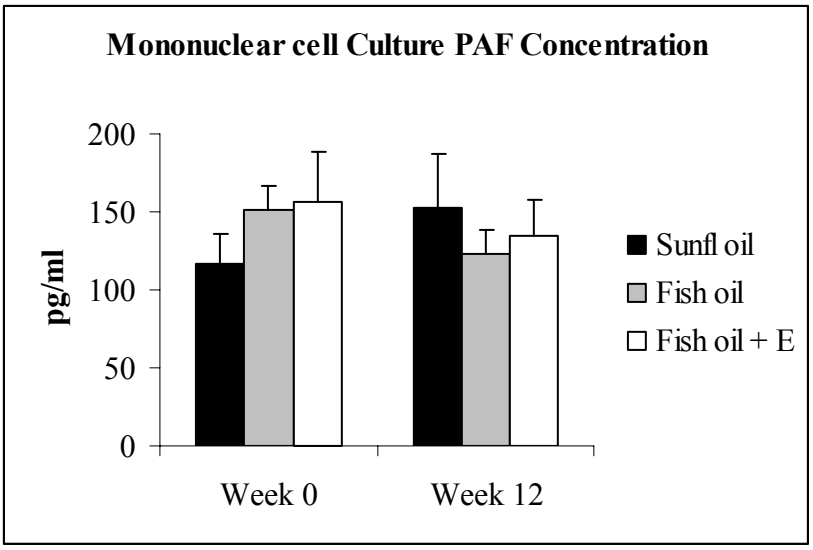

Figure 4.7 Effect of dietary supplementation for 12 weeks on mononuclear cell production of PAF of three canine groups. The data are expressed as means +/- SEM, n = 3-4.

Elevations significantly above the baseline serum PGE_2 concentration were found at 0.5, 1, 1.5, and 2 hours after LPS injection among dogs in Group Sunflower oil. Dogs in Group Fish oil + E had significantly increased serum PGE_2 concentration 1.5 hours post LPS injection. However, there were no significant changes in serum PGE_2 among dogs in Group Fish oil. Total serum PGE_2 concentration as well as the 0.5, 1, 1.5, and 2 hr concentrations of Groups Fish oil and Fish oil + E were significantly decreased compared to Group Sunflower oil. Significant differences in serum PGE_2 concentrations between Groups Fish oil and Fish oil + E were not found (Figure 4.8), (Appendix XX).

Discussion

Human and animal studies suggest that dietary supplementation with n-3 fatty acids reduces the synthesis of many inflammatory mediators and improves the clinical progression of many chronic inflammatory diseases. However, canine studies evaluating the effects of dietary n-3 fatty acids on cytokine and lipid mediator production have often yielded results contrary to those obtained in other species (Kearns et al., 1999) and have been limited in the number of mediators evaluated (Vaughn et al., 1994; Wander et al., 1997). Previous work has also varied greatly in both the dietary ratio of n-6:n-3 fatty acids and the quantity of n-3 fatty acids used in the experimental diets. The ideal n-3 fatty acid enriched diet suited for diminishing the inflammatory response in dogs while minimizing potential side effects has not been established.

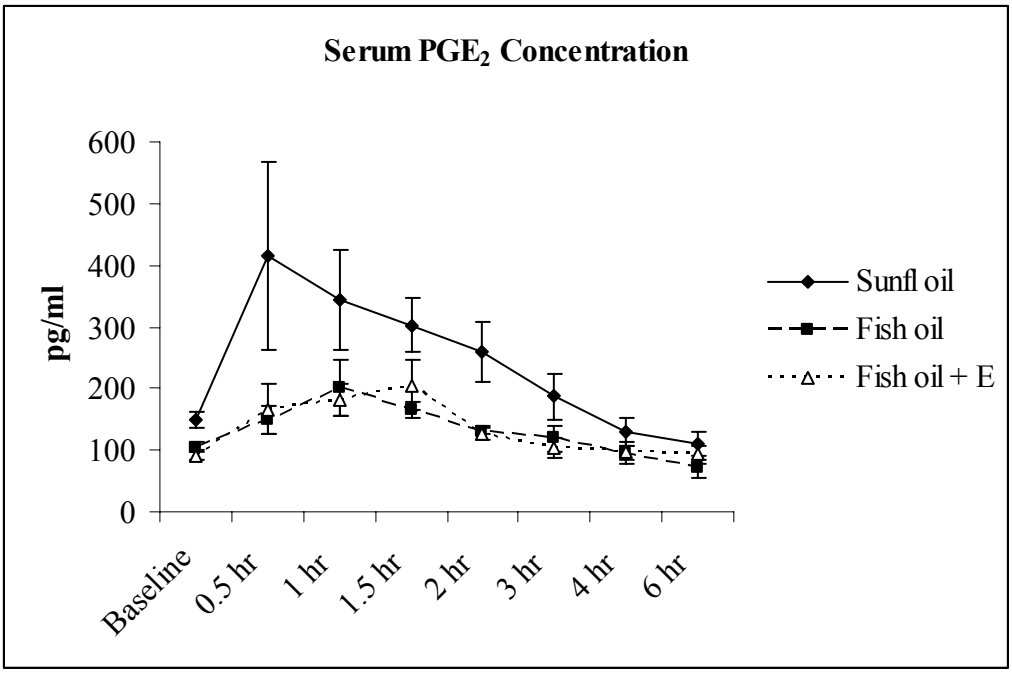

Figure 4.8 Serum PGE_2 concentrations of three canine dietary groups following intravenous LPS injection. Total serum PGE_2 concentrations (area under the curve) of Group Sunflower oil was significantly higher compared to the total concentrations of the two fish oil groups. The data are expressed as means +/- SEM, n = 5.

The main objective of this study was to evaluate *in vivo* and *in vitro* inflammatory mediator production in healthy, young adult dogs fed a n-6:n-3 fatty acid ratio diet of 3.4:1 (0.65% n-3 fatty acids, DMB). The effects of this diet on the production of interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α), prostagladin E_2 (PGE_2), and platelet-activating factor (PAF) were evaluated because of their importance in the inflammatory response. Our results demonstrate that feeding a diet with this quantity and ratio of n-6:n-3 fatty acids has significant effects on the synthesis of several of these mediators in young adult dogs.

The total serum activity (area under the curve) for each cytokine in each dietary group peaked following LPS administration at time points in agreement with previous reports (LeMay et al., 1990; Glauser, 1996). Total serum IL-1 activity of the two groups supplemented with fish oil was significantly lower than the control group supplemented with sunflower oil. However, significant differences between the 3 dietary groups at individual time points were not apparent until hours 4 and 6. The total serum IL-1 activity of Group Sunflower did not significantly

increase above baseline until 4 and 6 hours after the LPS injection. This delayed increase in activity is unexpected based on previous reports (Glauser, 1996). It is possible that the cytotoxicity of the A375.S2 cell line at these time points was not related to an increase in IL-1 activity. Therefore, the actual total serum IL-1 activity may not be significantly different between groups.

The IL-1 activity of LPS-stimulated peripheral blood mononuclear cell cultures was significantly decreased by dietary supplementation in all three groups after 12 weeks. Suppression of IL-1 production by stimulated mononuclear cells of dogs receiving n-3 fatty acid supplementation was expected based on human reports (Endres et al., 1989; Meydani et al., 1991; Meydani et al., 1993). The lack of significant difference between groups is not surprising given the unexpected increase in n-3 fatty acid concentration within the plamsa phospholipids of the control group (Group Sunflower oil). The n-3 fatty acid-induced reductions in IL-1 activity within mononuclear cell supernatants, but not in serum IL-1 activity, are consistent with previously reported data in the canine (Freeman et al., 1998).

IL-6, unlike TNF-α, is synthesized by many cell lines including macrophages, endothelial cells, fibroblasts, and activated T-lymphocytes in response to endotoxin and other cytokines, like TNF and IL-1 (Abbas et al., 2000). As previously reported (Sadeghi et al., 1999), total serum IL-6 activity after the intravenous LPS injection was significantly lower in the dietary groups supplemented with fish oil as compared to Group Sunflower oil, even though there was no difference between the groups in TNF-α and IL-1 activities. This implies that dietary n-3 fatty acid supplementaion may have attenuated the direct induction of IL-6 synthesis by LPS.

Evaluation of IL-6 activity from LPS-stimulated peripheral blood mononuclear cells in all 3 dietary groups revealed surprising results. The observation that increased LPS-stimulated mononuclear cell production of IL-6 was increased in all groups does not correlate with the serum IL-6 levels in these same dogs. It was anticipated that the mononuclear cell culture activity of IL-6 would either remain similar to baseline or decrease, with the most pronounced reduction in cultures from dogs supplemented with fish oil. This discrepancy in IL-6 activity between serum and LPS-stimulated peripheral blood mononuclear cells could be the result of variable effects of n-3 fatty acids on different cell lines. Serum IL-6 activity may reflect IL-6 synthesis by endothelial cells, as opposed to macrophage production of IL-6 within mononuclear cell cultures. It is also possible optimal conditions for the growth of the target cell line were different from weeks 0 to 12, despite similar levels of IL-6. Regardless, the n-3 fatty acid-induced increase in macrophage

synthesis of IL-6 is in contrast to the findings of previous studies (Meydani et al., 1993; Kearns et al., 1999).

There was no significant difference in total serum TNF-α activity between the dietary groups in this study. This is consistent with previous work in dogs that failed to show a significant effect of dietary n-3 fatty acids on mononuclear cell production of TNF-α (Freeman et al., 1998; Kearns et al., 1999). Addition of an anti-mouse TNF-α receptor antibody failed to inhibit target cell death induced by human recombinant TNF-α. The affinity of the antibody may not have been strong enough to inhibit the cytokine from binding to the receptor and preventing cell killing. Secondly, binding of the antibody to the receptor may not have inhibited binding of TNF-α to the receptor. Nevertheless, since the human recombinant TNF-α used in the study consistently produced dilution-dependent cell death of the WEHI cell line, the assay should have been appropriate for detecting TNF-α activity in canine samples.

Based on the detection of TNF-α activity in serum samples, detectable levels of TNF-α activity in the stimulated mononuclear cell supernatants were expected. While the TNF-α activities within mononuclear cell supernatants as reported here may be accurate, excessive proliferation of the target cell line is also possible. Mononuclear cells in this experiment were frozen in culture media for approximately 1 year at -20^{o}C. Prolonged contact of these cells with supernatant may have allowed liberation of growth promoting substances, and impacted the death of the target cell line. Mononuclear cell components may also have affected the integrity of TNF-α. Freezing of stimulated mononuclear cells for unspecified periods of time has been reported (Meydani et al., 1991; Freeman et al., 1998). However, separation of the supernatants from the mononuclear cells 18-24 hours after stimulation (Kurzman et al., 1993; Tappia and Grimble, 1994; Kearns et al., 1999) may have allowed for detectable levels of TNF-α activity.

Dietary n-3 fatty acid supplementation has previously been reported to suppress PAF synthesis in other species (Horii et al., 1991; Oh-hashi et al., 1999; Akisu et al., 2002; Mayer et al., 2002). Serum PAF concentrations did not differ significantly between groups in this study. Decreased PAF concentrations were observed in the stimulated mononuclear cell cultures of dogs receiving n-3 fatty acid supplementation, but this change did not attain statistical significance.

Several human and animal studies have documented significant reductions in PGE_2 synthesis by mononuclear cells following n-3 fatty acid supplementation (Meydani et al., 1991; Meydani et al., 1993; Wander et al., 1997; Wallace et al., 2000). The current study also documented significant reductions in PGE_2 concentrations within mononuclear cell supernatants

(results discussed in Chapter III) and serum following LPS stimulation of dogs supplemented with n-3 fatty acids when compared to baseline values. This effect of dietary n-3 fatty acid supplementation on both cellular and systemic levels of PGE_2 was expected based on the well characterized relationship between fatty acids and prostaglandin production (Bauer, 1992; Reinhart, 1996).

Direct influence of vitamin E or lipid peroxidation on cytokine production is rarely addressed (Meydani et al., 1991). Low levels of vitamin E may allow increased lipid peroxidation, which could inhibit lymphocyte or macrophage function, including cytokine production. Significant differences in cytokine production were not observed between the two fish oil groups consuming different concentrations of dietary vitamin E. However, this is not surprising considering that neither group had elevations in plasma lipid peroxides or decreased concentrations of vitamin E.

In conclusion, a decreased dietary n-6:n-3 fatty acid ratio, a simple increase in intake of n-3 fatty acids, or both was associated with significant reduction in peripheral blood mononuclear cell culture IL-1 activity and PGE_2 concentration in this study. This dietary manipulation also resulted in decreased serum PGE_2 concentrations and IL-6 activity. Lower dietary n-6:n-3 fatty acid ratios or increased absolute intake of n-3 fatty acids holds promise for clinical suppression of pro-inflammatory mediator synthesis.

References

Abbas A, Lichtman A, Pober J. 2000. Cellular and Molecular Immunology, 4th ed. Phiadelphia: W. B. Saunders Company.

Akisu M, Huseyinov A, Baka M, Yalaz M, Kultursay N. 2002. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the generation of platelet-activating factor and leukotriene B(4) in hypoxic-ischemic brain in young mice. Prostaglandins Leukot Essent Fatty Acids 67:429-433.

Bauer J. 1992. The potential for dietary polyunsaturated fatty acids in domestic animals. Aust Vet J 71:342-345.

Benveniste J, Henson PM, Cochrane CG. 1972. Leukocyte-dependent histamine release from rabbit platelets. The role of IgE, basophils, and a platelet-activating factor. J Exp Med 136:1356-1377.

Bueno A, Seahorn T, Cornick-Seahorn J, Horohov D, Moore R. 1999. Plasma and urine nitric oxide concentrations in horses given a low dose of endotoxin. Am J Vet Res 60:969-976.

Bulger EM, Maier RV. 2000. Lipid mediators in the pathophysiology of critical illness. Crit Care Med 28:N27-36.

Calder P. 2001. Polyunsaturated fatty acids, inflammation, and immunity. Lipids 36:1007-1024.

Calder P. 2002a. Dietary modification of inflammation with lipids. Proc Nutr Soc 61:345-358.

Calder P, Bevan S, Newsholme E. 1992. The inhibition of T-lymphocyte proliferation by fatty acids is via an eicosanoid-independent mechanism. Immunology 75:108-115.

Calder PC. 1997. n-3 polyunsaturated fatty acids and cytokine production in health and disease. Ann Nutr Metab 41:203-234.

Calder PC. 2002b. Fatty acids and gene expression related to inflammation. Nestle Nutr Workshop Ser Clin Perform Programme 7:19-36; discussion 36-40.

Chouaib S, Welte K, Mertelsmann R, Dupont B. 1985. Prostaglandin E2 acts at two distinct pathways of T lymphocyte activation: inhibition of interleukin 2 production and down-regulation of transferrin receptor expression. J Immunol 135:1172-1179.

Christie PE, Henderson WR, Jr. 2002. Lipid inflammatory mediators: leukotrienes, prostaglandins, platelet-activating factor. Clin Allergy Immunol 16:233-254.

Collins T. 1999. Acute and chronic inflammation. In: Cotran R, Kumar V, Collins T, editors. Robbins Pathologic basis of disease, 6th ed. Philadelphia: WB Saunders. p 70-74.

Das U. 1994. Beneficial effect of eicosapentaenoic and docoahexaenoic acids in the management of systemic lupus erythematous and its relationship to the cytokine network. Prostaglandins, Leukotrienes, and Ess. Fatty Acids 51:207-213.

Dinarello CA. 1996. Biologic basis for interleukin-1 in disease. Blood 87:2095-2147.

Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, van der Meer JW, Cannon JG, Rogers TS, Klempner MS, Weber PC, et al. 1989. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med 320:265-271.

Floch A, Bousseau A, Hetier E, Floc'h F, Bost PE, Cavero I. 1989. RP 55778, a PAF receptor antagonist, prevents and reverses LPS-induced hemoconcentration and TNF release. J Lipid Mediat 1:349-360.

Freeman LM, Rush JE, Kehayias JJ, Ross JN, Jr., Meydani SN, Brown DJ, Dolnikowski GG, Marmor BN, White ME, Dinarello CA, Roubenoff R. 1998. Nutritional alterations and the effect of fish oil supplementation in dogs with heart failure. J Vet Intern Med 12:440-448.

Gelin J, Moldawer L, Lonnroth C, Sherry B, Chizzonite R, Lundholm K. 1991. Role of endogenous tumor necrosis factor α and interleukin 1 for experimental tumor growth and the development of cancer cachexia. Cancer Res 51:415-421.

Glauser MP. 1996. The inflammatory cytokines. New developments in the pathophysiology and treatment of septic shock. Drugs 52 Suppl 2:9-17.

Goodwin JS, Bankhurst AD, Messner RP. 1977. Suppression of human T-cell mitogenesis by prostaglandin. Existence of a prostaglandin-producing suppressor cell. J Exp Med 146:1719-1734.

Greally P, Cook AJ, Sampson AP, Coleman R, Chambers S, Piper PJ, Price JF. 1994. Atopic children with cystic fibrosis have increased urinary leukotriene E4 concentrations and more severe pulmonary disease. J Allergy Clin Immunol 93:100-107.

Hommes DW, Meenan J, de Haas M, ten Kate FJ, von dem Borne AE, Tytgat GN, van Deventer SJ. 1996. Soluble Fc gamma receptor III (CD 16) and eicosanoid concentrations in gut lavage fluid from patients with inflammatory bowel disease: reflection of mucosal inflammation. Gut 38:564-567.

Horii T, Satouchi K, Kobayashi Y, Saito K, Watanabe S, Yoshida Y, Okuyama H. 1991. Effect of dietary alpha-linolenate on platelet-activating factor production in rat peritoneal polymorphonuclear leukocytes. J Immunol 147:1607-1613.

Jarrar D, Chaudry IH, Wang P. 1999. Organ dysfunction following hemorrhage and sepsis: mechanisms and therapeutic approaches (Review). Int J Mol Med 4:575-583.

Kay-Mugford P, Benn SJ, LaMarre J, Conlon P. 2000. In vitro effects of nonsteroidal anti-inflammatory drugs on cyclooxygenase activity in dogs. Am J Vet Res 61:802-810.

Kearns R, Hayek M, Turek J, Meydani M, Burr J, Greene R, Marshall C, Adams S, Borgert R, Reinhart G. 1999. Effect of age, breed, and dietary omega-6 (n-6) : omega-3 (n-3) fatty acid ratio on immune function, eicosanoid production, and lipid peroxidation in young and aged dogs. Vet Immuno Immunopathol 69:165-183.

Kremer JM, Jubiz W, Michalek A, Rynes RI, Bartholomew LE, Bigaouette J, Timchalk M, Beeler D, Lininger L. 1987. Fish-oil fatty acid supplementation in active rheumatoid arthritis. A double-blinded, controlled, crossover study. Ann Intern Med 106:497-503.

Kroegel C, Virchow JC, Jr., Luttmann W, Walker C, Warner JA. 1994. Pulmonary immune cells in health and disease: the eosinophil leucocyte (Part I). Eur Respir J 7:519-543.

Kurzman ID, Shi F, MacEwen EG. 1993. In vitro and in vivo canine mononuclear cell production of tumor necrosis factor induced by muramyl peptides and lipopolysaccharide. Vet Immunol Immunopathol 38:45-56.

LeMay DR, LeMay LG, Kluger MJ, D'Alecy LG. 1990. Plasma profiles of IL-6 and TNF with fever-inducing doses of lipopolysaccharide in dogs. Am J Physiol 259:R126-132.

Lloyd D. 1989. Essential fatty acids and skin disease. J Small Anim Pract 30:207-212.

Mancuso P, Whelan J, DeMichele SJ, Snider CC, Guszcza JA, Claycombe KJ, Smith GT, Gregory TJ, Karlstad MD. 1997. Effects of eicosapentaenoic and gamma-linolenic acid on lung permeability and alveolar macrophage eicosanoid synthesis in endotoxic rats. Crit Care Med 25:523-532.

Mathiak G, Szewczyk D, Abdullah F, Ovadia P, Rabinovici R. 1997. Platelet-activating factor (PAF) in experimental and clinical sepsis. Shock 7:391-404.

Mayer K, Merfels M, Muhly-Reinholz M, al e. 2002. ω-3 fatty acids suppress monocyte adhesion to human endothelial cells: role of endothelial PAF generation. Am J Physiol Heart Circ Physiol 283:H811-H818.

McManus LM, Pinckard RN. 2000. PAF, a putative mediator of oral inflammation. Crit Rev Oral Biol Med 11:240-258.

Meydani S, Endres S, Woods M, Goldin B, Soo C, Morrill-Labrode A, Dinarello C, Gorbach S. 1991. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. J Nutr 121:547-555.

Meydani S, Lichtenstein A, Cornwall S, Meydani M, Goldin B, Rasmussen H, Dinarello C, Schaefer E. 1993. Immunologic effects of national cholesterol education panel step-2 diets with and without fish-derived acid enrichment. J Clin Invest 92:105-113.

Miller W. 1989. Fatty acid supplements as anti-inflammatory agents. In: Kirk R, editor. Current Veterinary Therapy X. Philadelphia: WB Saunders Co. p 563-565.

Oh-hashi K, Takahashi T, Tanabe A, Watanabe S, Okuyama H. 1999. Dietary alpha-linolenate suppresses endotoxin-induced platelet-activating factor production in rat kidney. Lipids 34:31-37.

Oh-hashi K, Takahashi T, Watanabe S, Kobayashi T, Okuyama H. 1997. Possible mechanisms for the differential effects of high linoleate safflower oil and high alpha-linolenate perilla oil diets on platelet-activating factor production by rat polymorphonuclear leukocytes. J Lipid Mediat Cell Signal 17:207-220.

Poubelle PE, Gingras D, Demers C, Dubois C, Harbour D, Grassi J, Rola-Pleszczynski M. 1991. Platelet-activating factor (PAF-acether) enhances the concomitant production of tumour necrosis factor-alpha and interleukin-1 by subsets of human monocytes. Immunology 72:181-187.

Rappaport RS, Dodge GR. 1982. Prostaglandin E inhibits the production of human interleukin 2. J Exp Med 155:943-948.

Reinhart G. 1996. Review of omega-3 fatty acids and dietary influences on tissue concentrations. In: Carey D, Norton S, Bolser S, editors. Recent advances in canine and feline nutritional research: Proceedings of the 1996 Iams international nutrition symposium. Wilingtion: Orange Frazer Press. p 235-242.

Renz H, Gong JH, Schmidt A, Nain M, Gemsa D. 1988. Release of tumor necrosis factor-alpha from macrophages. Enhancement and suppression are dose-dependently regulated by prostaglandin E2 and cyclic nucleotides. J Immunol 141:2388-2393.

Sadeghi S, Wallace F, Calder P. 1999. Dietary lipids modify the cytokine response to bacterial lipopolysaccharide in mice. Immunology 96:404-410.

Scott D, Buerger R. 1988. Nonsteroidal anti-inflammatory agents in the management of canine puritus. J Am Anim Hosp Assoc 24:425-428.

Scott D, Miller W, Reinhart G, Mohammed H, Bagladi M. 1997. Effect of an omega-3/omega-6 fatty acid-containing commerical lamb and rice diet on pruritus in atopic dogs: results of a single-blinded study. Can J Vet Res 61:145-153.

Spinas GA, Bloesch D, Keller U, Zimmerli W, Cammisuli S. 1991. Pretreatment with ibuprofen augments circulating tumor necrosis factor-alpha, interleukin-6, and elastase during acute endotoxinemia. J Infect Dis 163:89-95.

Stewart J. 1995. Approximate Integration. In: Calculus. Early Transcendentals. Pacific Cove: Brooks/Cole Publishing Co. p 459.

Strassmann G, Kambayashi T. 1995. Inhibition of experimental cancer cachexia by anti-cytokine and anti-cytokine-receptor therapy. Cytokines Mol Ther 1:107-113.

Tappia PS, Grimble RF. 1994. Complex modulation of cytokine induction by endotoxin and tumour necrosis factor from peritoneal macrophages of rats by diets containing fats of different saturated, monounsaturated and polyunsaturated fatty acid composition. Clin Sci (Lond) 87:173-178.

Tilley S, Coffman T, Koller B. 2001. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. J Clin Invest 108:15-23.

Tsuchiya R, Kyotani K, Scott MA, Nishizono K, Ashida Y, Mochizuki T, Kitao S, Yamada T, Kobayashi K. 1999. Role of platelet activating factor in development of thrombocytopenia and neutropenia in dogs with endotoxemia. Am J Vet Res 60:216-221.

Ushikubi F, Segi E, Sugimoto Y, Murata T, Matsuoka T, Kobayashi T, Hizaki H, Tuboi K, Katsuyama M, Ichikawa A, Tanaka T, Yoshida N, Narumiya S. 1998. Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. Nature 395:281-284.

Vaughn D, Reinhart G, Swaim S, Lauten S, Garner C, Boudreaux M, Spano J, Hoffman C, Conner B. 1994. Evaluation of effects of dietary n-6 to n-3 fatty acid ratios on leukotriene B synthesis in dog skin and neutrophils. Vet Dermatol 5:163-173.

Wallace F, Miles E, Calder P. 2000. Activation state alters the effect of dietary fatty acids on pro-inflammatory mediator production by murine macrophages. Cytokine 12:1374-1379.

Wander R, Hall J, Gradin J, Du S, Jewell D. 1997. The ratio of dietary (n-6) to (n-3) fatty acids influences immune system function, eicosanoid metabolism, lipid peroxidation and vitamin E status in aged dogs. J Nutr 127.

Wigmore SJ, Fearon KC, Maingay JP, Ross JA. 1997. Down-regulation of the acute-phase response in patients with pancreatic cancer cachexia receiving oral eicosapentaenoic acid is mediated via suppression of interleukin-6. Clin Sci (Lond) 92:215-221.

Xi S, Cohen D, Brave S, Chen L. 2001. Fish oil suppressed cytokines and nuclear factor kappaB induced by murine AIDS virus infection. Nutrional Research 21:865-878.

Yamada K, Asano O, Yoshimura T, Katayama K. 1988. Highly sensitive gas chromatographic--mass spectrometric method for the determination of platelet-activating factor in human blood. J Chromatogr 433:243-247.

SUMMARY

This study examined the impact of dietary n-3 fatty acid supplementation on hematologic, serum biochemical, and immunological parameters in healthy, young adult dogs. The long chain n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) are principally obtained from fish or fish oil. These fatty acids act as anti-inflammatory agents in part by directly inhibiting the metabolism of arachidonic acid (AA). The pathways leading to decreased production of AA metabolites, namely 2-series prostaglandins and 4-series leukotrienes, are well characterized (Bauer, 1992; Reinhart, 1996).

The benefits of dietary n-3 fatty acid supplementation do not come without potential side effects. The long, unsaturated carbon chains of EPA and DHA are easy targets for oxygen free radicals (Mylonas and Kouretas, 1999). The oxidation of these fatty acids contained within cell membrane phospholipids initiates an autocatalytic reaction called lipid peroxidation. Increased levels of lipid peroxidation can lead to cell dysfunction and death. Dietary vitamin E supplementation is suggested when high levels of n-3 fatty acids are consumed to help prevent this process (National-Research-Council, 1985; Muggli, 1989). Evidence of increased lipid peroxidation and significantly reduced plasma vitamin E levels has been reported in dogs supplemented with n-3 fatty acids (Wander et al., 1997). However, plasma lipid peroxide and serum vitamin E concentrations among the dogs in the current study did not change significantly from baseline, despite a diet consisting of 1.65% oil (combined sunflower and fish oil) and an n-6:n-3 fatty acid ratio of 3.4:1. The vitamin E content of these diets was well above the recommended intake for this level of fatty acid supplementation.

Dietary n-3 fatty acids have been shown to inhibit platelet aggregation in both animals and man, perhaps most notably in Greenlandic Eskimos consuming a diet high in fish products (Dyerberg and Bang, 1979; Adan et al., 1999). Similar abnormalities have not previously been documented in healthy or tumor-bearing dogs (Boudreaux et al., 1997; McNiel et al., 1999). These findings were confirmed here, since no significant inhibition of platelet aggregation could be found in dogs consuming the n-3 fatty acid enriched diets.

Dietary n-3 fatty acid supplementation has been shown to reduce serum triglyceride concentrations in healthy humans, humans suffering from primary hyperlipidemia, rats and pigs (Bang et al., 1971; Harris, 1996). In contrast to previous canine reports, dietary n-3 fatty acid supplementation resulted in significant reduction of serum triglyceride concentrations in the dogs

studied here. This finding has potential clinical application in the treatment of canine idiopathic hyperlipidemia.

Dietary supplementation of fish oil resulted in increased levels of n-3 fatty acids within plasma phospholipids. However, a similar change was observed in the control group supplemented with sunflower oil, a source rich in n-6 fatty acids. This result may be attributed to several factors, including alterations in desaturase enzyme activity and preferential n-3 fatty acid esterification within phospholipids.

The main object of this study was to evaluate dietary n-3 fatty acid supplementation-induced effects on lymphocyte proliferation and inflammatory mediator production. Most work examining n-3 fatty acid supplementation in humans and animals shows significant suppression of lymphocyte proliferation and interleukin (IL)-2 production, an important autocrine and paracrine growth promoter in lymphocytes (Meydani et al., 1991; Calder and Newsholme, 1992; Jeffery et al., 1997). The few canine studies that have assessed lymphocyte proliferation and function after dietary n-3 fatty acid supplementation report contrasting results (Wander et al., 1997; Kearns et al., 1999). Lipid peroxide and plasma vitamin E concentrations have been related to reduced lymphocyte proliferation and function, and have also been investigated in people (Meydani et al., 1993). Finally, although prostaglandin-independent mechanisms are also suggested (Calder et al., 1992), prostaglandin E_2 (PGE_2) is known to be a potent suppressor of lymphocyte proliferation (Goodwin et al., 1977; Chouaib et al., 1985). Significant suppression of lymphocyte proliferation was observed in the current study among dogs supplemented with fish oil despite unaltered plasma lipid peroxide and serum vitamin E concentrations, and decreased serum levels and mononuclear cell production of PGE_2. These data suggest that n-3 fatty acid-induced suppression of lymphocyte proliferation is independent of lipid peroxidation, vitamin E status, and PGE_2 production. Some studies indicate that the effects of n-3 fatty acids on lymphocyte proliferation are mediated, at least in part, through decreased IL-2 production or suppressed expression of the IL-2 receptor (IL-2R) (Calder and Newsholme, 1992; Terada et al., 2001). A decreased percentage of lymphocytes expressing IL-2 receptor was demonstrated here; however, these changes were not statistically significant and a role for IL-2 or its receptor could neither be confirmed nor denied.

The importance of IL-1, IL-6, tumor necrosis factor (TNF)-α, and platelet-activating factor (PAF) in the inflammatory response are well described. Dietary n-3 fatty acid supplementation reduces the synthesis of these inflammatory mediators in human and numerous animal species (Endres et al., 1989; Horii et al., 1991; Meydani et al., 1991; Wallace et al., 2000). However,

diminished synthesis in dogs supplemented with dietary n-3 fatty acids appears inconsistent based on published reports (Wander et al., 1997; Freeman et al., 1998; Kearns et al., 1999). Dietary n-3 fatty acid supplementation of the current study population was associated with significant reductions in peripheral blood mononuclear cell culture IL-1 activity and PGE_2 concentration, *in vitro*. More importantly, this dietary manipulation resulted in decreased *in vivo* production PGE_2 and IL-6.

Future research directed towards evaluation of varying n-6:n-3 fatty acid ratios and absolute quantities of dietary n-3 fatty acids is clearly needed. Significant changes in serum triglycerides, lymphocyte proliferation and production of certain inflammatory mediators were observed here. Low dietary n-6:n-3 fatty acid ratios or an increased absolute dietary level of n-3 fatty acids may permit beneficial manipulation of the inflammatory response. Future investigation may lead to clinically beneficial therapy for chronic inflammatory diseases and hyperlipidemia.

References

Adan Y, Shibata K, Sato M, Ikeda I, Imaizumi K. 1999. Effects of docosahexaenoic and eicosapentanoic acid on lipid metabolism, eicosanoid production, platelet aggregation, and atherosclerosis in hypercholesterolemic rats. Biosci Biotechnol Biochem 63:111-119.

Bang HO, Dyerberg J, Nielsen AB. 1971. Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos. Lancet 1:1143-1145.

Bauer J. 1992. The potential for dietary polyunsaturated fatty acids in domestic animals. Aust Vet J 71:342-345.

Boudreaux M, Reinhart G, Vaughn D, Spano J, Mooney M. 1997. The effects of varying dietary n-6 to n-3 fatty acid ratios on platelet reactivity, coagulation screening assays, and antithrombin III activity in dogs. J Am Anim Hosp Assoc 33:235-243.

Calder P, Bevan S, Newsholme E. 1992. The inhibition of T-lymphocyte proliferation by fatty acids is via an eicosanoid-independent mechanism. Immunology 75:108-115.

Calder P, Newsholme E. 1992. Polyunsaturated fatty acids suppress human peripheral lymphocyte proliferation and interleukin-2 production. Clin Sci 82:695-700.

Chouaib S, Welte K, Mertelsmann R, Dupont B. 1985. Prostaglandin E2 acts at two distinct pathways of T lymphocyte activation: inhibition of interleukin 2 production and down-regulation of transferring receptor expression. J Immunol 135:1172-1179.

Dyerberg J, Bang HO. 1979. Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. Lancet 2:433-435.

Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, van der Meer JW, Cannon JG, Rogers TS, Klempner MS, Weber PC, et al. 1989. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med 320:265-271.

Freeman LM, Rush JE, Kehayias JJ, Ross JN, Jr., Meydani SN, Brown DJ, Dolnikowski GG, Marmor BN, White ME, Dinarello CA, Roubenoff R. 1998. Nutritional alterations and the effect of fish oil supplementation in dogs with heart failure. J Vet Intern Med 12:440-448.

Goodwin JS, Bankhurst AD, Messner RP. 1977. Suppression of human T-cell mitogenesis by prostaglandin. Existence of a prostaglandin-producing suppressor cell. J Exp Med 146:1719-1734.

Harris WS. 1996. n-3 fatty acids and lipoproteins: comparison of results from human and animal studies. Lipids 31:243-252.

Horii T, Satouchi K, Kobayashi Y, Saito K, Watanabe S, Yoshida Y, Okuyama H. 1991. Effect of dietary alpha-linolenate on platelet-activating factor production in rat peritoneal polymorphonuclear leukocytes. J Immunol 147:1607-1613.

Jeffery N, Newsholme E, Calder P. 1997. Level of polyunsaturated fatty acids and the n-6 to n-3 polyunsaturated fatty acid ratio in the rat diet alter serum lipid levels and lymphocyte functions. Prostaglandins, Leukotrienes, and Ess. Fatty Acids 57:149-160.

Kearns R, Hayek M, Turek J, Meydani M, Burr J, Greene R, Marshall C, Adams S, Borgert R, Reinhart G. 1999. Effect of age, breed, and dietary omega-6 (n-6) : omega-3 (n-3) fatty acid ratio on immune function, eicosanoid production, and lipid peroxidation in young and aged dogs. Vet Immuno Immunopathol 69:165-183.

McNiel E, Ogilvie G, Mallinckrodt C, Richardson K, Fettman M. 1999. Platelet function in dogs treated for lymphoma and hemangiosarcoma and supplemented with dietary n-3 fatty acids. J Vet Intern Med 13:574-580.

Meydani S, Endres S, Woods M, Goldin B, Soo C, Morrill-Labrode A, Dinarello C, Gorbach S. 1991. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. J Nutr 121:547-555.

Meydani S, Lichtenstein A, Cornwall S, Meydani M, Goldin B, Rasmussen H, Dinarello C, Schaefer E. 1993. Immunologic effects of national cholesterol education panel step-2 diets with and without fish-derived acid enrichment. J Clin Invest 92:105-113.

Muggli R. 1989. Dietary fish oils increase the requirement for vitamin E in humans. In: Chandra R, editor. Health effects of fish and fish oils. St. John's, Newfoundland: ARTS Biomedical Publishers and Distributors. P 201-210.

Mylonas C, Kouretas D. 1999. Lipid peroxidation and tissue damage. In vivo 13:295-310.

National Research Council. 1985. Nutrient Requirements of Dogs. Wasington, DC: National Academy Press.

Reinhart G. 1996. Review of omega-3 fatty acids and dietary influences on tissue concentrations. In: Bolser S, editor. Recent advances in canine and feline nutritional research: Proceedings of the 1996 Iams international nutrition symposium. Wilingtion: Orange Frazer Press. P 235-242.

Terada S, Takizawa M, Yamamoto S, Ezaki O, Itakura H, Akagawa K. 2001. Suppressive mechanisms of EPA on human T cell proliferation. Microbiol Immunol 45:473-481.

Wallace F, Miles E, Calder P. 2000. Activation state alters the effect of dietary fatty acids on pro-inflammatory mediator production by murine macrophages. Cytokine 12:1374-1379.

Wander R, Hall J, Gradin J, Du S, Jewell D. 1997. The ratio of dietary (n-6) to (n-3) fatty acids influences immune system function, eicosanoid metabolism, lipid peroxidation and vitamin E status in aged dogs. J Nutr 127.

APPENDIX I: DIET FORMULATIONS

	Kibble	Sunfl oil	Fish oil	Basal Diet	Group Sunfl oil	Group Fish oil	Group Fish oil+E
units	%wt/wt	%wt	%wt	grams	grams	grams	grams
Protein	25.83	/wt	/wt	/day	/day	/day	/day
Cr. Fiber	2.3				500g	500g	500g
Moisture	9.31			800g	kibble +	kibble +	kibble +
Cr. Fat	8.9			kibble	oil	oil	oil
T Sat. FA	2.76	11	26.3	22.08	15.164	15.707	15.707
T MonoUn	3.37	23.1	19.9	26.96	19.7144	18.3816	18.3816
T PolyUn	2.04	57.1	41.4	16.32	17.2804	13.4406	13.4406
T Unsat	5.41	80.2	61.3	43.28	36.9948	31.8222	31.8222
Total Fat	8.17	91.2	87.6	65.36	52.1588	47.5292	47.5292
C14:0	0.13	0.07	6.63	1.04	0.65868	1.11452	1.11452
C15:0	0.02	0	0.7	0.16	0.1	0.149	0.149
C16:0	1.7	6.16	15.1	13.6	9.26384	9.59396	9.59396
C16:1	0.24	0.11	10.1	1.92	1.21364	1.90766	1.90766
C16:2	0	0	1.91	0	0	0.1337	0.1337
C16:3	0	0	1.66	0	0	0.1162	0.1162
C17:0	0.05	0	0.55	0.4	0.25	0.2885	0.2885
C18:0	0.81	3.41	2.97	6.48	4.47284	4.27836	4.27836
C18:1	3.07	22.6	8.44	24.56	18.1524	16.0764	16.0764
C18:2 n6	1.88	56.8	1.39	15.04	16.4432	9.8381	9.8381
C18:3 n6	0	0	0.14	0	0	0.0098	0.0098
C18:3 n3	0.1	0.16	1.63	0.8	0.51984	0.61506	0.61506
C18:4 n3	0.02	0.18	3.28	0.16	0.12232	0.33068	0.33068
C20:0	0.02	0.24	0.19	0.16	0.12976	0.11474	0.11474
C20:1	0.05	0.38	1.2	0.4	0.29712	0.33628	0.33628
C20:2 n6	0.01	0	0.19	0.08	0.05	0.0633	0.0633
C20:3 n6	0	0	0.24	0	0	0.0168	0.0168
C20:3 n3	0	0	0.24	0	0	0.0168	0.0168
C20:4 n6	0.02	0	0.75	0.16	0.1	0.1525	0.1525
C20:4 n3	0	0	1.59	0	0	0.1113	0.1113
C20:5 n3	0	0	10.7	0	0	0.749	0.749
C21:5 n3	0	0	0.61	0	0	0.0427	0.0427
C22:0	0	0.77	0	0	0.09548	0.00462	0.00462
C22:4 n6	0	0	0.27	0	0	0.0189	0.0189
C22:5 n6	0	0	0.34	0	0	0.0238	0.0238
C22:5 n3	0	0	2.18	0	0	0.1526	0.1526
C22:6 n3	0.01	0	13	0.08	0.05	0.96	0.96
C24:0	0.02	0.3	0	0.16	0.1372	0.1018	0.1018
Vitamin E				39.2	32.97	25.05	196.59
IU/g	0.049	0.67	0	IU/day	IU/day	IU/day	IU/day

APPENDIX II: BODY WEIGHT

(lbs)	Week 3	Week 6	Week 9	Week 12
Group Sunflower Oil				
Dog 9002	50.4	47.7	46.3	48
Dog 9028	51.1	50.6	49.7	52
Dog 9040	57.8	58.1	61.4	61.9
Dog 9057	53.9	55.7	56.7	58.9
Dog 9060	57	55.6	55.9	55.9
Mean	54.04	53.54	54	55.34
SD	3.347088	4.255937	5.988322	5.501182
SEM	1.496863	1.903313	2.678059	2.460203
Group Fish Oil				
Dog 9003	55.6	58.3	61.4	60.8
Dog 9031	46.2	46.8	48.5	47.7
Dog 9039	48.7	47.7	46.7	46.2
Dog 9049	58.7	61.4	55.2	55.6
Dog 9062	58.1	55.4	59.1	60
Mean	53.46	53.92	54.18	54.06
SD	5.677411	6.455773	6.43405	6.806468
SEM	2.539016	2.887109	2.877395	3.043945
Group Fish Oil + E				
Dog 9005	53.7	48.7	46.8	50.7
Dog 9038	47.6	46	46.1	47.2
Dog 9050	53.5	54.7	52.7	52.7
Dog 9058	50	50.5	51.6	51.5
Dog 9061	59.4	57.7	60.3	60.8
Mean	52.84	51.52	51.5	52.58
SD	4.464639	4.684229	5.703946	5.030606
SEM	1.996647	2.094851	2.550882	2.249756

APPENDIX III: SERUM VITAMIN E

(ug/mL)	Week 0	Week 6	Week 12
Group			
Sunflower oil			
Dog 9002	9.35	8.43	8.32
Dog 9028	4.24	4.18	5.14
Dog 9040	5.98	10.78	8.74
Dog 9057	2.85	5.46	5.97
Dog 9060	4.33	6.9	6.1
Mean	5.35	7.15	6.854
SD	2.495967	2.576567	1.580658
SEM	1.11623	1.152276	0.706892
Group			
Fish oil			
Dog 9003	7.18	6.92	6.29
Dog 9031	4.95	5.39	5.69
Dog 9039	4.33	3.82	7.54
Dog 9049	4.19	4.9	3.8
Dog 9062	1.38	4.14	7.56
Mean	4.406	5.034	6.176
SD	2.073651	1.222162	1.554873
SEM	0.927365	0.546567	0.69536
Group			
Fish oil + E			
Dog 9005	7.66	10.76	13.37
Dog 9038	7.89	8.01	14.54
Dog 9050	6.13	13.8	13.43
Dog 9058	5.63	14.39	14.71
Dog 9061	4.92	10.38	11.28
Mean	6.446	11.468	13.466
SD	1.289702	2.627693	1.368404
SEM	0.576772	1.17514	0.611969

APPENDIX IV: PLATELET AGGREGATION

(%LT)	Week 0		Week 6		Week 12	
	ADP	Collagen	ADP	Collagen	ADP	Collagen
Group Sunflower oil						
Dog 9002	76	59	64	31	60	47
Dog 9028	70	45	70	39	79	52
Dog 9040	40	34	72	33	52	52
Dog 9057	78	50	77	54	68	52
Dog 9060	57	62	60	50	68	38
Mean	64.2	50	68.6	41.4	65.4	48.2
SD	15.8177116	11.2472219	6.69328021	10.2127371	10.0895986	6.09918027
SEM	7.07389567	5.02991053	2.99332591	4.5672749	4.51220567	2.72763634
Group Fish oil						
Dog 9003	80	62	85	47	69	45
Dog 9031	58	46	62	33	79	42
Dog 9039	55	47	39	14	86	51
Dog 9049	48	30	49	39	61	25
Dog 9062	58	58	85	78	53	67
Mean	59.8	48.6	64	42.2	69.6	46
SD	12.0083304	12.481987	20.8326667	23.4243463	13.2966161	15.1986842
SEM	5.37028863	5.58211429	9.31665176	10.4756861	5.9464275	6.79705819
Group Fish oil + E						
Dog 9005	68	60	48	15	70	50
Dog 9038	74	69	78	28	78	59
Dog 9050	52	46	68	8	60	30
Dog 9058	69	60	52	40	52	52
Dog 9061	61	52	80	58	62	44
Mean	64.8	57.4	65.2	29.8	64.4	47
SD	8.5264295	8.76356092	14.6696967	19.97999	9.93981891	10.9087121
SEM	3.81313519	3.91918359	6.56048779	8.93532316	4.44522215	4.87852437

APPENDIX V: HEMATOLOGY / CHEMISTRY WEEK 0

Analyte	**Glucose**	**ALT**	**ALP**	**BUN**	**Creatinine**	**HCT**	**PLT**	**WBC**
units	mg/dL	U/L	U/L	mg/dL	mg/dL	%	$x10^3$/uL	$x10^3$/uL
Group								
Sunflower Oil								
9002	88	22	43	18	0.8	40	331	16.1
9028	98	53	67	19	0.9	49.5	285	16.8
9040	89	38	43	28	1.1	55.7	198	13.6
9057	87	36	39	15	1.1	48.3	245	15.6
9060	76	72	22	20	0.8	52.3	261	15.1
Mean	87.6	44.2	42.8	20	0.94	49.16	264	15.44
SD	7.829432	19.03155	16.0686	4.84768	0.151658	5.860717	49.13247	1.205404
SEM	3.501428	8.511169	7.186098	2.167948	0.067823	2.620992	21.97271	0.539073
Group								
Fish Oil								
9003	84	23	56	23	0.8	49.5	282	13.7
9031	94	128	49	20	1	50	271	15.1
9039	96	33	31	34	1	52.6	290	18.9
9049	77	43	38	16	1.1	54.9	203	15.4
9062	94	40	34	26	0.8	56.2	317	14.9
Mean	89	53.4	41.6	23.8	0.94	52.64	272.6	15.6
SD	8.185353	42.40637	10.54988	6.797058	0.134164	2.941598	42.4535	1.954482
SEM	3.660601	18.9647	4.71805	3.039737	0.06	1.315523	18.98578	0.874071
Group								
Fish Oil + E								
9005	94	22	69	18	0.8	39.8	553	17.7
9038	97	25	42	22	0.9	45.8	293	12.6
9050	86	45	20	18	1.1	55.3	182	14.3
9058	98	45	50	21	1.1	50.2	223	11.6
9061	87	36	42	27	0.9	54.5	294	13.4
Mean	92.4	34.6	44.6	21.2	0.96	49.12	309	13.92
SD	5.59464	10.83051	17.62952	3.701351	0.134164	6.445696	144.5182	2.336022
SEM	2.501999	4.843552	7.884161	1.655295	0.06	2.882603	64.63049	1.044701

APPENDIX VI: HEMATOLOGY / CHEMISTRY WEEK 6

Analyte	Glucose	ALT	ALP	BUN	Creatinine	HCT	PLT	WBC
units	mg/dL	U/L	U/L	mg/dL	mg/dL	%	$x10^3$/uL	$x10^3$/uL
Group								
Sunflower Oil								
9002	86	42	38	15	1	44.5	284	13.4
9028	87	49	49	17	1.1	52.5	294	14.6
9040	96	39	45	20	0.9	48	255	10.7
9057	89	36	52	29	1	45.5	214	12.4
9060	86	62	33	21	0.8	50.2	309	13.3
Mean	88.8	45.6	43.4	20.4	0.96	48.14	271.2	12.88
SD	4.207137	10.35857	7.829432	5.366563	0.114018	3.297423	37.5726	1.448102
SEM	1.881489	4.632494	3.501428	2.4	0.05099	1.474653	16.80298	0.647611
Group								
Fish Oil								
9003	74	27	41	21	1	49.3	355	19.3
9031	84	37	38	25	1	46.2	339	12.2
9039	93	34	23	24	1	52.9	386	15
9049	98	49	42	23	1	45.9	225	13.4
9062	89	92	48	27	0.7	48.5	299	13.2
Mean	87.6	47.8	38.4	24	0.94	48.56	320.8	14.62
SD	9.181503	25.95573	9.343447	2.236068	0.134164	2.829841	62.05804	2.802142
SEM	4.106093	11.60776	4.178516	1	0.06	1.265543	27.7532	1.253156
Group								
Fish Oil + E								
9005	99	46	32	24	1.1	46.1	443	17.6
9038	84	26	41	17	0.9	50.5	306	11.9
9050	88	43	26	24	1	51.2	247	14.6
9058	98	44	35	22	1	53.1	268	14.3
9061	98	52	48	18	1	49.1	199	11.7
Mean	93.4	42.2	36.4	21	1	50	292.6	14.02
SD	6.913754	9.705668	8.443933	3.316625	0.070711	2.613427	92.52729	2.403539
SEM	3.091925	4.340507	3.776242	1.48324	0.031623	1.16876	41.37946	1.074895

APPENDIX VII: HEMATOLOGY / CHEMISTRY WEEK 12

Analyte	Glucose	ALT	ALP	BUN	Creatinine	HCT	PLT	WBC
units	mg/dL	U/L	U/L	mg/dL	mg/dL	%	$x10^3$/uL	$x10^3$/uL
Group								
Sunflower Oil								
9002	95	36	41	25	1	53	249	12.1
9028	90	42	58	17	1.2	54.9	276	12.4
9040	78	28	49	23	1	45.9	236	14.7
9057	74	37	41	27	0.9	48.3	217	19.7
9060	97	42	14	30	0.9	48.7	219	11.7
Mean	86.8	37	40.6	24.4	1	50.16	239.4	14.12
SD	10.28105	5.744563	16.4408	4.878524	0.122474	3.683477	24.29609	3.330465
SEM	4.597826	2.569047	7.352551	2.181742	0.054772	1.647301	10.86554	1.489429
Group								
Fish Oil								
9003	76	27	26	24	1.1	53.3	324	13
9031	91	42	30	22	1	44.9	311	14.4
9039	94	27	31	25	1.1	46.2	339	15.1
9049	75	46	36	22	1.1	46.5	217	16.6
9062	87	35	30	28	0.9	53.6	284	13.4
Mean	84.6	35.4	30.6	24.2	1.04	48.9	295	14.5
SD	8.677557	8.619745	3.577709	2.48998	0.089443	4.198214	48.05726	1.43527
SEM	3.880722	3.854867	1.6	1.113553	0.04	1.877498	21.49186	0.641872
Group								
Fish Oil + E								
9005	84	35	36	30	1.1	51.1	410	15.2
9038	87	25	97	15	0.9	47.9	357	16.2
9050	78	41	21	20	1	51.3	219	21.1
9058	80	33	34	25	1.1	49.3	276	16.9
9061	86	49	39	22	0.9	49.2	324	15.1
Mean	83	36.6	45.4	22.4	1	49.76	317.2	16.9
SD	3.872983	8.988882	29.65299	5.59464	0.1	1.427585	73.45543	2.462722
SEM	1.732051	4.01995	13.26122	2.501999	0.044721	0.638436	32.85027	1.101363

APPENDIX VIII: LIPID PEROXIDES

(uM)	Week 0	Week 6	Week 12
Group			
Sunflower oil			
Dog 9002	4.03	4.42	5.63
Dog 9028	6.5	5.33	5.67
Dog 9040	5.54	3.25	1.95
Dog 9057	1.17	5.03	4.33
Dog 9060	2.17	3.38	6.63
Mean	3.882	4.282	4.842
SD	2.231069	0.942799	1.811717
SEM	0.997765	0.421633	0.810225
Group			
Fish oil			
Dog 9003	4.68	8.06	4.94
Dog 9031	6.89	5.37	3.53
Dog 9039	6.24	1.17	3.55
Dog 9049	1.69	4.77	4.31
Dog 9062	5.46	5.92	5
Mean	4.992	5.058	4.266
SD	2.023579	2.502593	0.715772
SEM	0.904972	1.119193	0.320103
Group			
Fish oil + E			
Dog 9005	8.66	2.34	5.01
Dog 9038	7.54	3.32	6.67
Dog 9050	3.47	6.24	4.85
Dog 9058	4.29	6.08	3.81
Dog 9061	1.95	5.98	3.51
Mean	5.182	4.792	4.77
SD	2.820863	1.826614	1.243302
SEM	1.261528	0.816887	0.556022

APPENDIX IX: LYMPHOCYTE PROLIFERATION BY TRITIATIED THYMIDINE

(CPM)	Week 0	Week 12
Group		
Sunflower oil		
Dog 9002	6817.82	2149.85
Dog 9028	13166.4	3374.64
Dog 9040	11790.7	466.24
Dog 9057	21167	1963.38
Dog 9060	53009.8	6746.6
Mean	21190.34	2940.142
SD	18518.81	2365.202
SEM	8281.862	1057.751
Group		
Fish oil		
Dog 9003	6179.61	6189.97
Dog 9031	18083	10510.9
Dog 9039	7277.45	262.6
Dog 9049	15617.3	371.29
Dog 9062	13535.6	3542.54
Mean	12138.59	4175.46
SD	5208.867	4312.158
SEM	2329.476	1928.456
Group		
Fish oil + E		
Dog 9005	32717.8	6289.8
Dog 9038	18800.7	9971.1
Dog 9050	8833.16	4373.76
Dog 9058	4359.63	9221.78
Dog 9061	9841.47	663.33
Mean	14910.55	6103.954
SD	11249.26	3784.975
SEM	5030.823	1692.692

(SI)	Week 0	Week 12
Group		
Sunflower oil		
Dog 9002	15.3613	7.34991
Dog 9028	30.212	12.048
Dog 9040	55.4074	1.55367
Dog 9057	47.0054	6.26817
Dog 9060	127.815	17.1299
Mean	55.16022	8.86993
SD	43.45011	5.935792
SEM	19.43148	2.654567
Group		
Fish oil		
Dog 9003	13.1043	15.17
Dog 9031	30.9455	40.6816
Dog 9039	18.7529	1.08365
Dog 9049	41.3222	0.86194
Dog 9062	17.3451	6.94861
Mean	24.294	12.94916
SD	11.60175	16.55988
SEM	5.18846	7.405802
Group		
Fish oil + E		
Dog 9005	84.142	22.6301
Dog 9038	51.5737	24.3911
Dog 9050	51.671	10.0766
Dog 9058	16.1611	26.127
Dog 9061	30.4068	2.0608
Mean	46.79092	17.05712
SD	25.73139	10.49673
SEM	11.50743	4.69428

APPENDIX X: LYMPHOCYTE PROLIFERATION BY FLOW CYTOMETRY

(Peaks)	Week 0	Week 12
Group		
Sunflower oil		
Dog 9002	3	3
Dog 9028	4	3
Dog 9040	4	3
Dog 9057	3	3
Dog 9060	4	3
Mean	3.6	3
SD	0.547	0
SEM	0.244	0
Group		
Fish oil		
Dog 9003	4	3
Dog 9031	4	3
Dog 9039	3	2
Dog 9049	4	4
Dog 9062	3	4
Mean	3.6	3.2
SD	0.547	0.836
SEM	0.244	0.374
Group		
Fish oil + E		
Dog 9005	3	3
Dog 9038	4	4
Dog 9050	3	3
Dog 9058	2	3
Dog 9061	4	3
Mean	3.2	3.2
SD	0.836	0.447
SEM	0.374	0.2

(% Proliferation)	Week 0	Week 12
Group		
Sunflower oil		
Dog 9002	19.1	11.9
Dog 9028	26.7	20.6
Dog 9040	40.1	11.8
Dog 9057	15.3	16.8
Dog 9060	38.3	26.5
Mean	27.9	17.52
SD	11.12025	6.223906
SEM	4.973128	2.783415
Group		
Fish oil		
Dog 9003	37.7	32.1
Dog 9031	44.7	26
Dog 9039	50.7	7.2
Dog 9049	41.1	25.5
Dog 9062	49.9	39.2
Mean	44.82	26
SD	5.58856	11.88634
SEM	2.49928	5.315731
Group		
Fish oil + E		
Dog 9005	12.9	19.9
Dog 9038	35.1	42.2
Dog 9050	50.8	36.7
Dog 9058	27.2	29.2
Dog 9061	48.6	9.3
Mean	34.92	27.46
SD	15.68302	13.15876
SEM	7.013658	5.884777

APPENDIX XI: PERCENT IL-2 RECEPTOR EXPRESSION BY FLOW CYTOMETRY

(%)	Week 0	Week 12
Group		
Sunflower oil		
Dog 9002	42.17	50.61
Dog 9028	64.56	52.47
Dog 9040	76.04	28.3
Dog 9057	45.79	51.54
Dog 9060	75.44	78.59
Mean	60.8	52.302
SD	16.07095	17.82293
SEM	7.187148	7.970655
Group		
Fish oil		
Dog 9003	76.4	74.07
Dog 9031	76.31	65.51
Dog 9039	84.96	44.28
Dog 9049	69.7	66.35
Dog 9062	82.91	78.61
Mean	78.056	65.764
SD	6.05888	13.19071
SEM	2.709614	5.899066
Group		
Fish oil + E		
Dog 9005	53.97	73.91
Dog 9038	84.45	70.54
Dog 9050	89.77	47.03
Dog 9058	68.87	59.56
Dog 9061	74.83	29.76
Mean	74.378	56.16
SD	14.01238	18.12266
SEM	6.266529	8.104702

APPENDIX XII: MONONUCLEAR CELL CULTURE PGE_2 CONCENTRATION

(pg/mL)	Week 0	Week 12
Group		
Sunflower oil		
Dog 9028	8371.3	1962.5
Dog 9040	7656.6	5136.25
Dog 9057	5362.7	2245.3
Mean	7130.2	3114.683
SD	1571.859	1756.429
SEM	907.5133	1014.075
Group		
Fish oil		
Dog 9031	5767.4	1697.4
Dog 9039	5158	2244.4
Dog 9049	13470.8	1357.6
Mean	8132.067	1766.467
SD	4633.508	447.4162
SEM	2675.157	258.3158
Group		
Fish oil + E		
Dog 9005	10396.8	1573
Dog 9038	17954.9	3325.2
Dog 9058	6866.9	1968.4
Dog 9061	2770.6	1220.6
Mean	9497.3	2021.8
SD	6442.246	921.0584
SEM	3221.123	460.5292

APPENDIX XIII: RECTAL TEMPERATURE AFTER INTRAVENOUS LPS INJECTIOIN

(^{o}F)	Baseline	0.5 Hr	1 Hr	1.5 Hr	2 Hr	3 Hr	4 Hr	5 Hr	6 Hr
Group									
Sunflower oil									
Dog 9002	101.6	101.2	100.8	100.5	101.2	101.3	100.9	101.3	100.8
Dog 9028	101.6	101.8	101.8	101.5	102.1	102.1	101.5	101.3	101.2
Dog 9040	101.8	101.7	100.2	100.9	101	101.4	100.8	100.7	100.7
Dog 9057	101.8	101.4	101.8	102	102	101.1	101.8	100.7	100.9
Dog 9060	100.8	102.1	102.3	101.7	101.7	101.6	101.5	101.4	100.8
Group									
Fish oil									
Dog 9003	102.2	101.6	101.6	101.8	101.5	102.7	102.2	102.2	101.9
Dog 9031	101.4	102.8	102.9	102.6	102.2	102.1	101.8	101.3	101
Dog 9039	101.6	101.3	101.7	101.6	101.4	100.5	100.3	100.1	100.4
Dog 9049	102	101.4	101.3	102	101.6	100.9	101.4	101.7	101
Dog 9062	102.4	101.7	102	102.2	101.9	101.7	101.6	100.9	100.3
Group									
Fish oil + E									
Dog 9005	102	102.1	102.1	101.8	101.9	101.7	101.8	101.7	101.1
Dog 9038	101.2	102.1	102.2	102.1	101.2	100.8	101.2	100.8	101
Dog 9050	102	101.7	101.9	101.7	102	102.1	101.7	101.1	100.8
Dog 9058	101.6	101.6	101.5	101.4	101.3	100.8	101.1	100.9	100.9
Dog 9061	101.6	102.5	102.2	101.7	101.1	101.5	101.5	101.3	100.9

APPENDIX XIV: SERUM IL-1 ACTIVITY AFTER INTRAVENOUS LPS INJECTION

Group Sunflower oil (U/mL)	9002	9028	9040	9057	9060	Mean	SD	SEM
Baseline	578.66	41.33	875.86	1014.29	1758.86	853.8	628.7263	281.1749
0.5 Hr	925.33	0	775.86	964.29	2124.57	958.01	760.4162	340.0685
1 Hr	2078.66	1796.88	500	550	610.29	1107.166	765.7474	342.4526
1.5 Hr	1392	503.55	337.93	657.14	216	621.324	461.978	206.6028
2 Hr	1185.33	228	151.72	971.43	1393.14	785.924	564.8325	252.6008
3 Hr	1165.33	539.11	382.76	921.43	2038.86	1009.498	653.0105	292.0352
4 Hr	1985.33	1081.33	1486.21	1885.71	2153.14	1718.344	432.4389	193.3925
6 Hr	1632	1076.88	1648.27	2321.43	2907.43	1917.202	707.7707	316.5247

Group Fish oil	9003	9031	9039	9049	9062	Mean	SD	SEM
Baseline	138.66	1220.75	0	265.5	741.71	473.324	502.4697	224.7113
0.5 Hr	538.66	0	0	0	827.43	273.218	387.7998	173.4293
1 Hr	1705.33	0	0	0	1616	664.266	910.1318	407.0233
1.5 Hr	1238.66	737.73	0	0	998.86	595.05	571.363	255.5213
2 Hr	452	164.15	0	0	370.29	197.288	208.4173	93.20706
3 Hr	485.33	0	93.1	0	958.85	307.456	415.6201	185.8709
4 Hr	1045.33	1333.96	131	31	1421.71	792.6	665.2954	297.5291
6 Hr	1038.66	1541.5	886.2	427.58		973.785	459.1731	229.5865

Group Fish oil + E	9005	9038	9050	9058	9061	Mean	SD	SEM
Baseline	605.33	152.44	226.66	0	187.43	234.372	224.4526	100.3783
0.5 Hr	865.33	165.77	1469.8	0	250.29	550.238	609.3898	272.5274
1 Hr	1378.66	596.88	803.14	0	438.86	643.508	505.9863	226.2839
1.5 Hr	525.33	165.77	2536.47	0	587.43	763	1021.195	456.6925
2 Hr	465.33	41.33	265.88	0	113.14	177.136	190.294	85.10206
3 Hr	358.66	468	1661.96	0	884.57	674.638	635.7189	284.3021
4 Hr	912	623.55	1383.53	591.37	1450.29	992.148	408.0396	182.4808
6 Hr		703.77	1658		741.71	1034.493	540.3057	311.9457

APPENDIX XV: MONONUCLEAR CELL CUTLURE
IL-1 AND IL-6 ACTIVITY

IL-1 (U/mL)	Week 0	Week 12
Group		
Sunflower oil		
Dog 9028	680.44	0
Dog 9040	740.15	541.46
Dog 9057	791.62	104.43
Mean	737.4033	215.2967
SD	55.64087	287.2513
SEM	32.12427	244.8432
Group		
Fish oil		
Dog 9031	773.09	276.59
Dog 9039	721.62	369.3
Dog 9049	787.5	0
Mean	760.7367	215.2967
SD	34.63376	192.1283
SEM	19.99581	110.9253
Group		
Fish oil + E		
Dog 9005	742.21	0
Dog 9038	513.68	284.16
Dog 9058	563.09	
Dog 9061	694.85	284.16
Mean	628.4575	189.44
SD	107.6946	164.0599
SEM	53.8473	94.72

IL-6 (U/mL)	Week 0	Week 12
Group		
Sunflower oil		
Dog 9028	2.35	3.53
Dog 9040	2.2	3.45
Dog 9057	2.03	3.52
Mean	2.193333	3.5
SD	0.160104	0.043589
SEM	0.092436	0.025166
Group		
Fish oil		
Dog 9031	1.82	2.99
Dog 9039	2.17	2.06
Dog 9049	2.14	3.48
Mean	2.043333	2.843333
SD	0.193993	0.721272
SEM	0.112002	0.416427
Group		
Fish oil + E		
Dog 9005	2.59	3.49
Dog 9038	2.15	2.94
Dog 9058	3.45	3.57
Dog 9061	2.31	3.59
Mean	2.625	3.3975
SD	0.579281	0.308045
SEM	0.289641	0.154022

APPENDIX XVI: SERUM IL-6 ACTIVITY AFTER INTRAVENOUS LPS INJECTION

Group Sunflower oil (U/mL)	9002	9028	9040	9057	9060	Mean	SD	SEM
Baseline	0	0.23	0	1.03	0.03	0.258	0.44212	0.197722
0.5 Hr	0	0.18	7.6	0.43	0	1.642	3.335284	1.491584
1 Hr	2.24	6	0.07	2.86	4.06	3.046	2.196311	0.98222
1.5 Hr	7.57	9.61	12.5	8.43	8.54	9.33	1.914354	0.856125
2 Hr	5.65	9.13	10.8	9.34	7.77	8.538	1.939116	0.867199
3 Hr	6.95	7.92	9.99	5.33	1.07	6.252	3.352062	1.499088
4 Hr	6.35	7.81	6.16	1.87	0	4.438	3.327232	1.487983
6 Hr	0	0	0	0.98	0	0.196	0.438269	0.196

Group Fish oil	9003	9031	9039	9049	9062	Mean	SD	SEM
Baseline	0	0	0	0	0	0	0	0
0.5 Hr	0	0	0	0	0	0	0	0
1 Hr	1.53	6	0.094	0	1.32	1.7888	2.454237	1.097568
1.5 Hr	5.13	5.16	0.745	1.67	8.4	4.221	3.072549	1.374086
2 Hr	5.68	6.19	5.09	2.13	8.22	5.462	2.203672	0.985512
3 Hr	6.2	5.49	1.125	0	7.94	4.151	3.418162	1.528648
4 Hr	3.79	0	0	0	0.21	0.8	1.673932	0.748605
6 Hr	0	0	0	0	0	0	0	0

Group Fish oil + E	9005	9038	9050	9058	9061	Mean	SD	SEM
Baseline	0	0	0.27	0	0	0.054	0.120748	0.054
0.5 Hr	0	0.04	0.2	0	0	0.048	0.086718	0.038781
1 Hr	1.36	2.5	3.28	0.08	0.38	1.52	1.365357	0.610606
1.5 Hr	6.45	6.73	9.63	5.55	4.62	6.596	1.887003	0.843893
2 Hr	7.8	8.61	10.1	5.36	4.39	7.252	2.346608	1.049435
3 Hr	6.76	7.03	10.2	0	0.42	4.882	4.476753	2.002065
4 Hr	2.28	4.81	8.23	0	0	3.064	3.50453	1.567273
6 Hr	0	0	0	0	0	0	0	0

APPENDIX XVII: SERUM TNF-α ACTIVITY AFTER INTRAVENOUS LPS INJECTION

Group Sunflower oil (U/mL)	9002	9028	9040	9057	9060	Mean	SD	SEM
Baseline	8.2	1.6	0	0	0	1.96	3.556403	1.590472
0.5 Hr	11.4	11.1	2.1	0	9.9	6.9	5.420793	2.424252
1 Hr	26.8	10.9	0	1.77	11.9	10.274	10.65384	4.764544
1.5 Hr	18.1	7.7	0	0	0.5	5.26	7.886888	3.527123
2 Hr	10.9	5.7	0	0	1.7	3.66	4.669368	2.088205
3 Hr	0	0	0	0	0	0	0	0
4 Hr	0.7	0	0	0	0	0.14	0.31305	0.14
6 Hr	0	0	0	0	0	0	0	0

Group Fish oil	9003	9031	9039	9049	9062	Mean	SD	SEM
Baseline	0	0	1.8	0	0	0.36	0.804984	0.36
0.5 Hr	0	9.4	8.8	14.2	0	6.48	6.274711	2.806136
1 Hr	24.4	7.9	6.3	9.6	7.9	11.22	7.459692	3.336076
1.5 Hr	14.5	0	3.2	6.2	2.2	5.22	5.646415	2.525153
2 Hr	6.8	0	2.7	1.1	0	2.12	2.840246	1.270197
3 Hr	0	0	0	0	0	0	0	0
4 Hr	0	0	0	1.6	0	0.32	0.715542	0.32
6 Hr	0	0	0	0	0	0	0	0

Group Fish oil + E	9005	9038	9050	9058	9061	Mean	SD	SEM
Baseline	0	0	2.2	1.8	0	0.8	1.104536	0.493964
0.5 Hr	6.9	0	7.9	5.9	2.2	4.58	3.346192	1.496462
1 Hr	21.7	10.3	20.5	9.5	0	12.4	8.925805	3.991741
1.5 Hr	16	2.8	17.9	12.2	0	9.78	7.98198	3.56965
2 Hr	16.2	4.4	5.1	0	0	5.14	6.62782	2.964051
3 Hr	0	0	0	0	0	0	0	0
4 Hr	0	0	0	0	0	0	0	0
6 Hr	0	0	0	0	0	0	0	0

APPENDIX XVIII: SERUM PAF CONCENTRATION AFTER INTRAVENOUS LPS INJECTION

Group Sunflower oil (pg/sample)	9002	9028	9040	9057	9060	Means	SD	SEM
Baseline	136.3	282.2	351.6	495.2	320.9	317.24	129.258	57.806
0.5 Hr	742.4	610.9	1008.4	637.9	760.9	752.1	157.194	70.299
1 Hr	425.4	277.8	422.5	471	679.4	455.22	144.864	64.785
1.5 Hr	806.1	734.9	1149.5	1354.8	1504.8	1110.02	335.562	150.068
2 Hr	459	287.4	1280	616.9	763.8	681.42	378.803	169.406
3 Hr	160.4	186.4	338.3	333.2	436.4	290.94	115.278	51.554
4 Hr	221.8	198.5	319.7	369.7	309.3	283.8	71.480	31.967
6 Hr	228.6	298.9	352.6	317.4	438.7	327.24	76.967	34.4211

Group Fish oil	9003	9031	9039	9049	9062	Means	SD	SEM
Baseline	142.6	248.1	238.2	206.3	562.5	279.54	163.462	73.102
0.5 Hr	494.2	462.3	804.1	659.4	557.8	595.56	138.764	62.057
1 Hr	204.9	287.7	406	295.1	602.8	359.3	153.753	68.760
1.5 Hr	661.6	928.3	889.2	1177.46	1493.4	1029.992	317.163	141.839
2 Hr		720.8	1004.6	1022.2		915.866	169.161	97.665
3 Hr	223	244.7	332.1	516.8	465.9	356.5	130.940	58.558
4 Hr	240.4	305.3	311.5	299.9	247.9	281	33.992	15.201
6 Hr	185.2	351.8	403.2	298.5	327.6	313.26	81.236	36.330

Group Fish oil + E	9005	9038	9050	9058	9061	Means	SD	SEM
Baseline	564.3	239.2	569.9	562	288.9	444.86	166.013	74.243
0.5 Hr	724.6	510.6	1023.3	615.9	589.1	692.7	200.065	89.472
1 Hr	363.9		543.2	865.8	757.1	632.5	223.662	111.831
1.5 Hr	948.6	1012.7	1426.8	1119.6	1005.7	1102.68	191.430	85.610
2 Hr		706.2	506.4		502.5	571.7	116.496	67.259
3 Hr	336.5	239.4	309.9	407.9	354.1	329.56	61.852	27.661
4 Hr	299.7	215.7	264.5	586.6	278.4	328.98	147.286	65.868
6 Hr	360.2	239.2	334.1	437.1	366.8	347.48	71.537	31.992

APENDIX XIX: MONONUCLEAR CELL CULTURE
PAF CONCENTRATION

(pg/mL)	Week 0	Week 12
Group		
Sunflower oil		
Dog 9028	94.8	117.1
Dog 9040	154.8	221.7
Dog 9057	101.5	117.2
Mean	117.0333	152
SD	32.87801	60.36199
SEM	18.98213	34.85001
Group		
Fish oil		
Dog 9031	125.3	92.8
Dog 9039	179.7	137.9
Dog 9049	148.1	140.1
Mean	151.0333	123.6
SD	27.31837	26.69625
SEM	15.77227	15.41309
Group		
Fish oil + E		
Dog 9005	174.7	106.4
Dog 9038	230.2	148.8
Dog 9058	81.9	194.4
Dog 9061	140.9	87.8
Mean	156.925	134.35
SD	62.10402	47.4795
SEM	31.05201	23.73975

APPENDIX XX: SERUM PGE2 CONCENTRATION AFTER INTRAVENOUS LPS INJECTION

Group Sunflower oil (pg/mL)	9002	9028	9040	9057	9060	Mean	SD	SEM
Baseline	165.5	135.7	183.2	143.8	116.6	148.96	25.959	11.609
0.5 Hr	278.9	318.4	1022.1	244.7	216.7	416.16	340.860	152.437
1 Hr	620.8	354.2	377	221.4	144.4	343.56	182.127	81.449
1.5 Hr	419.1	297.5	378.5	246.5	171.3	302.58	99.681	44.579
2 Hr	382.9	186.3	370	211.4	151.1	260.34	108.232	48.402
3 Hr	315.7	93.3	195	168.9	161.1	186.8	81.249	36.335
4 Hr	179.6	54.1	174.3	113.1	133.5	130.92	51.178	22.887
5 Hr	125.8	50.2	160.4	132.5	113.8	116.54	40.843	18.265
6 Hr	127.7	80.7	124.8	121.9	95.1	110.04	20.942	9.365

Group Fish oil	9003	9031	9039	9049	9062	Mean	SD	SEM
Baseline	118.2	86.2	129.4	97	92.1	104.58	18.388	8.223
0.5 Hr	171.9	218.7	157.2	97.8	104.4	150	50.1381	22.422
1 Hr	308	316.9	143.2	108.3	126.6	200.6	102.896	46.016
1.5 Hr	179.6	158.5	170.5	116.4	198.3	164.66	30.642	13.703
2 Hr	137.1	116.2	139.8	134.8	125.5	130.68	9.71941	4.346
3 Hr	76.1	84.4	191.3	120.1	122.9	118.96	45.500	20.348
4 Hr	55.6	65.5	143.7	118.7	94.8	95.66	36.585	16.361
5 Hr	65.2	41.5	124.1	105.9	103.5	88.04	33.711	15.076
6 Hr	42.9	33.5	113.5	102.5	59.2	70.32	35.816	16.017

Group Fish oil + E	9005	9038	9050	9058	9061	Mean	SD	SEM
Baseline	75.3	101.4	112.4	72.8	100.1	92.4	17.441	7.800
0.5 Hr	119.6	328.7	128.9	134.9	120.1	166.44	90.930	40.665
1 Hr	235.3	218	210.7	123	121.5	181.7	55.003	24.598
1.5 Hr	305.3	293.6	199	122.3	106.7	205.38	92.800	41.501
2 Hr	116.9	158.3	145.5	128	89	127.54	26.774	11.973
3 Hr	55.4	121.1	150.5	107.3	81	103.06	36.578	16.358
4 Hr	53.6	119.3	100	105.2	107.5	97.12	25.334	11.329
5 Hr	47.6	108	93.7	124	122.1	80.34	31.263	13.981
6 Hr	50.7	104	77.8	124.7	106.1	92.66	28.797	12.878

VITA

Casey John LeBlanc was born to James and Jacqueline LeBlanc on December 4, 1970, in Lake Charles, Louisiana. He completed pre-veterinary curriculum requirements at McNeese State University, Lake Charles, Louisiana, in 1992. He received the degree of Doctor of Veterinary Medicine (D.V.M.) in 1996 from the School of Veterinary Medicine at Louisiana State University. Following veterinary school, he completed a one-year internship in small animal medicine and surgery at the Animal Medical Center in New York, New York. In 1997, he was accepted into a combined residency/doctoral degree program in the Department of Veterinary Pathology at Louisiana State University. In 1999, he was appointed as an instructor in veterinary clinical pathology in the Department of Veterinary Pathology. He married Amy in 2003.