Effects of Fish Oil Supplementation on Protease Levels in the Microenvironment of Chronic Venous Leg Ulcers: A Randomized Clinical Trial

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By

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Abstract

In the U.S. alone, the average annual incidence of chronic venous leg ulcers (CVLUs) in individuals aged 65+ is 2.2% with payer burden reaching ~$35 billion. Moreover, the incidence is expected to rise dramatically because CVLUs are associated with aging and obesity. CVLUs are difficult to treat, frequently recur, and lead to a reduced quality of life. Therefore, adjuvant therapies to standard care are needed to help improve healing outcomes. High levels of neutrophil-derived proteases in CVLU microenvironments are associated with slow healing. The bioactive components of fish oil, eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA], have been shown to reduce neutrophil activity in animal models of wounds, but have not been tested in humans with CVLUs. The purpose of this secondary analysis of data derived from a randomized, controlled study conducted at The Ohio State University Clinical Research Center was to determine the effects of EPA+DHA therapy on 1) levels of neutrophil-derived proteases in the fluid of venous leg ulcers, and 2) wound healing over an 8-week interval in patients with CVLUs. For 8 weeks, EPA+DHA Group participants (n=16) consumed an EPA+DHA supplement and Control Group participants (n=19) consumed a placebo. Plasma samples were evaluated at 0, 4 and 8 weeks to quantify EPA+DHA levels. Wound fluid was collected at the same time points to quantify neutrophil-derived proteases: matrix metalloproteinase-8 (MMP-8) and human neutrophil elastase (HNE). Sociodemographic and body mass index (BMI) data were also collected. Descriptive statistics, *t*-tests and Spearman's Rho were used to analyze data. On average, the age of the total group was 60.6 years (SD = 12.0) and BMI was 41.7 (SD = 11.5). The majority were male (60%), Caucasian (74%), and lived alone (51%). No significant differences in age or BMI emerged between groups. Plasma levels of EPA+DHA increased significantly in the EPA+DHA group at 4 weeks and at 8 weeks compared to baseline (p=0.05).

There were no significant differences in protease levels within or between groups at any time point, however there was a downward trend in levels of MMP-8 over time in the EPA+DHA Group and a significant negative relationship emerged between MMP-8 and healing at Week 8 ($p = 0.04$). In summary, the data indicate that higher levels of some neutrophil-derived proteases are associated with slower CVLU healing and suggest that EPA+DHA therapy may be helpful in reducing levels of some neutrophil-derived proteases in the microenvironment associated with slower healing. High BMIs suggest that interventions to help CVLU patients reduce weight may decrease risk for chronic diseases such as diabetes and cardiovascular disease that are also associated with healing complications. Additional studies are needed to test higher EPA+DHA doses for longer intervals in larger, more diverse samples of CVLU patients.

Chapter I: Statement of the Problem

Introduction

Chronic venous leg ulcers (CVLU) affect approximately 600,000 people annually in the United States, primarily those 65+ years of age (McDaniel, Roy, & Wilgus, 2013). Treatment-related costs of these frequently recurring wounds are now estimated to be ~$35 billion annually, a value expected to rise in tandem with increasing prevalence rates (Nherera, Woodmansey, Trueman, & Gibbons, 2016). Risk factors for CVLUs include obesity, diabetes mellitus, poor nutrition, and congestive heart failure, which are more prevalent in the aging population (Lazarus et al., 2014). Moreover, the chronic pain, reduced mobility, and increased healthcare costs associated with CVLUs collectively reduce quality of life. Currently, compression therapy, the gold standard of CVLU treatment, has only a 50-60% success rate. Therefore, new adjuvant therapies are greatly needed to improve healing outcomes and help prevent the recurrence of these problematic wounds.

To better understand, manage, and treat CVLUs, some studies have assessed the wound fluid from CVLUs in order to characterize factors in the microenvironment that might explain healing delays. Several studies have reported that higher levels of neutrophil-derives proteases such as matrix metalloproteinase-8 (MMP-8) and human neutrophil elastase (HNE) are associated with slower healing (Ferreira et al., 2016; Guo, Zheng; Wang, & Li, 2015; Serena et al., 2016). In normal wound healing, MMP-8 and HNE facilitate the healing process by degrading irregular portions of the extracellular matrix (ECM) (Latifa et al., 2016). However, sustained high levels of MMP-8 and HNE eventually destroy growth factors and contribute to the chronic inflammation that destroys the ECM and stops the progression of wound healing (Serra

et al., 2013). Therefore, it is essential to explore treatment options that have the potential to reduce the chronically high levels of MMP-8 and HNE often found in chronic wounds.

While many CVLU treatment options are available such as specialized dressings and bioengineered tissue products, they do not significantly improve healing outcomes (Lazarus et al., 2014). Recent studies suggest that the bioactive components of fish oil, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can promote healing. For example, Theilla et al., (2012) report that a diet enriched with fish oil (EPA+DHA) improves healing of pressure ulcers, but the mechanisms of action of EPA+DHA leading to these improvements are unclear. Some studies using cell and animal models report that EPA+DHA slow the infiltration of neutrophils into wound sites and thus may reduce levels of MMP-8 and HNE, however, to our knowledge, no human studies have quantified MMP-8 and HNE levels in CVLUs before and after EPA+DHA supplementation.

Background of the Problem

When the skin is injured, the body activates the healing cascade that involves blood cells, growth factors, cytokines, proteases, and ECM proteins for skin repair (Krejner, Litwiniuk, & Grzela, 2016). The normal wound healing process can be divided into three phases: hemostasis/inflammation, proliferation, and remodeling. In the beginning inflammatory stage, cells and molecules at the injured tissue site activate platelets and signal other inflammatory cells such as neutrophils to come to the wounded site. Neutrophils arrive within hours and generate free radicals that kill bacteria. During this inflammatory stage, neutrophils also produce the proteases MMP-8 and HNE to debride the wound by removing any non-functional host cells, bacterial-filled neutrophils, damaged matrix, foreign debris, and remaining bacteria (Zhao, Liang, Clarke, Jackson, & Xue, 2016). This allows the transition into the proliferative phase

where re-epithelialization, angiogenesis, and fibroplasia occur. At this stage, macrophages release growth factors and cytokines that stimulate the growth of fibrous tissue as well as blood vessels. During the last phase of skin repair, the remodeling phase, there is reorganization of the ECM and wound contraction (Krejner, Litwiniuk, & Grzela, 2016).

Generally, the wound healing process occurs in a timely manner, but studies suggest that chronic wounds are persistently inflamed with elevated protease activity, which, in turn, impedes healing (Zhao, Liang, Clarke, Jackson, & Xue, 2016). The unregulated activation of immune cells such as neutrophils leads to the persistent, unregulated release of proteases, including MMP-8 and HNE (Rohl, Murray, 2011). Total MMP-8 activity in chronic wound fluid has been found to be 30 times higher than in acute wound fluid (Serena et al., 2016). Consequently, the measurement and careful analysis of these neutrophil-derived proteases may help determine if a targeted intervention to reduce protease activity can facilitate healing.

According to previous studies using cell and animal models, EPA+DHA inhibit neutrophil recruitment and accumulation during the inflammatory stage of healing (Ji, Xu, Strichartz, & Serhan, 2011). The ability of EPA+DHA to reduce neutrophil activation may occur via several mechanisms. For example, as EPA+DHA levels rise in plasma and are incorporated into cell membranes they 1) alter cell membrane fluidity and receptor site function, 2) increase the production of eicosanoids with anti-inflammatory actions, and 3) inhibit the gene expression of pro-inflammatory molecules such as cytokines (Gould et al., 2015), all of which may contribute to reduced neutrophil activation. However, EPA+DHA therapy has not been tested specifically in CVLU patients to determine if high levels of neutrophil-derived proteases in wound fluid can be reduced and healing rates improved.

Purpose of the Study

The purpose of this randomized, double-blind study was to determine the effects of oral EPA+DHA therapy on levels of MMP-8 and HNE in the microenvironment of CVLUs and wound healing in a sample of older adults at two time points over an 8-week interval.

Significance of the Study

Patient outcomes as well as clinical practice will be improved if the safe, inexpensive treatment strategy of EPA+DHA oral supplementation, in addition to standard care, promotes healing in patients with CVLUs. Since CVLU recurrence and prevalence rates are rising, it is critical that novel adjunct therapies be tested. EPA+DHA systemic therapy has significant potential to enhance wound healing in patients with CVLUs and thus reduce the significant economic and personal burdens associated with these problematic wounds.

Conceptual Frame of Reference (Theory)

The study design was guided by Dr. Charles N. Serhan's theory about the anti-inflammatory actions of EPA+DHA. It is proposed that EPA+DHA reduce neutrophil infiltration and activation by down-regulating signaling molecules, like proinflammatory cytokines, that may, in turn, reduce neutrophil-derived protease levels (Serhan, 2014) (Figure 1).

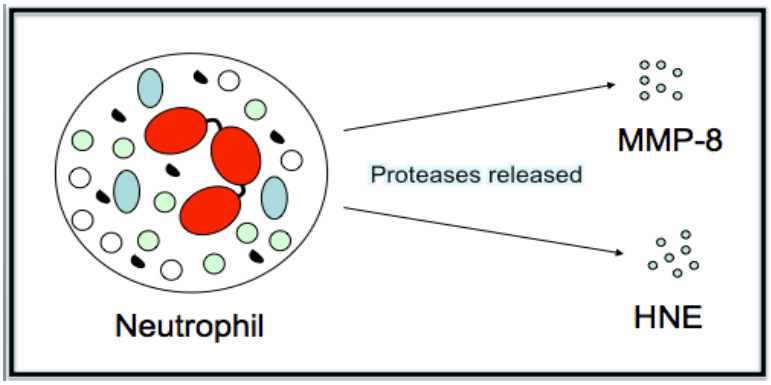

Figure 1. Neutrophils release proteases after infiltration into wound microenvironments. MMP-8 = matrix metalloproteinase; HNE = human neutrophil elastase

Research Question

In patients with CVLUs, does supplementing diets with EPA+DHA compared to placebo reduce levels of MMP-8 and HNE in the wound fluid and increase healing after 4 or 8 weeks of therapy?

Chapter II: Review of the Literature

Introduction

CVLUs negatively impact patients' lives physically, emotionally, and financially. For example, treatment-related costs are nearly $35 billion dollars per year in the U.S. alone (Nherera, Woodmansey, Trueman, & Gibbons, 2016). CVLUs comprise the largest single group of leg ulcers treated in wound care clinics in the U.S. (Lazarus et al., 2014). Moreover, 15% of venous ulcers never heal, and recurrence occurs one or more times in up to 71% of cases. Additionally, there is an increased risk of developing CVLUs in individuals aged $\geq$ 65 years, which is a population that is expected to grow to approximately 71 million by 2030 in the U.S. (U.S. Census Bureau, 2010). CVLUs are associated with pain, infections, and loss of functionality, and can lead to amputations or sepsis (Zhao, Liang, Clarke, Jackson, & Xue, 2016). Despite the current gold standard treatment of compression therapy and debridement, only 50-60% of venous leg ulcers heal (Lazarus et al., 2014). Therefore new treatment options are critically needed to help improve healing rates and help prevent recurrence.

Chronic Venous Leg Ulcers and Inflammation

Inflammation is an essential, nonspecific, innate immune response that involves the breakdown of tissue and removal of cellular, extracellular, and pathologic debris. The normal skin repair process is divided into three overlapping phases: hemostasis/inflammation, proliferation, and remodeling (Guo, Zheng, Wang, & Li, 2015). Although the inflammatory phase is essential, chronic inflammation impedes the progression to the subsequent healing phases and may lead to increased scarring (Moro, Nagahashi, Ramanathan, Takabe, & Wakai, 2016). The sustained venous hypertension associated with CVLUs and the persistent high numbers of activated neutrophils in the microenvironment releasing proteases such as MMP-8

and HNE foster chronic inflammation (Gould et al., 2015). The profuse neutrophil infiltration and its associated reactive oxygen species (ROS) and destructive enzymes propagate the inflammatory cycle and keep healing from progressing (Zhao, Liang, Clarke, Jackson, & Xue, 2016).

Since CVLUs fail to progress through the normal phases of healing and are fixed in a self-perpetuating inflammatory stage, topical treatments that do not target chronic inflammation are often ineffective. Thus topical or systemic therapies that can target the specific factors contributing to the chronic inflammation associated with CVLUs are greatly needed. The active metabolites of EPA+DHA, resolvins and protectins, have been shown to facilitate the resolving phase of acute inflammation and may prevent chronic inflammation (Gould et al., 2015). Studies show that resolvins and protectins reduce neutrophil activation and signal non-inflammatory monocytes to help moderate inflammation and reduce excessive tissue injury (Moro, Nagahashi, Ramanathan, Takabe, & Wakai, 2016). Thus, there is the clinical potential for EPA+DHA therapy to increase levels of resolvins and protectins that may help chronically inflamed wounds such as CVLUs heal more efficiently.

MMP-8 and HNE

Neutrophils release proteases such as MMP-8 and HNE. Thus prolonged neutrophil infiltration into wound sites causes the persistent release of high numbers of MMP-8 and HNE, which can degrade the ECM and growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-beta, and keep the wound inflamed (Guo, Zheng, Wang, & Li, 2015). Fluids from chronic wounds show elevated levels of HNE (Ferreira et al., 2016). In addition to HNE, total MMP-8 activity in chronic wound fluid has been reported to be 30 times higher than that in acute wound fluid (Zhao, Liang, Clarke, Jackson, & Xue, 2016), and a study

by Serena et al. (2016), concluded that high levels of HNE and MMP-8 activity are correlated with non-healing wounds.

EPA+DHA and Inflammation

The omega-3 PUFAs EPA and DHA are naturally found in fish oil and their anti-inflammatory effects have been noted since the early studies in Greenland Eskimos who demonstrated a lower incidence of cardiovascular disease than the general population (Paunescu et al., 2013). Since then, numerous studies have generated additional support for the inflammation-resolving actions of EPA+DHA. The study by Paunescu et al. (2013) examined Greenland Eskimos and determined that the resolvins and protectins generated from EPA+DHA have anti-inflammatory, inflammation-resolving, and immunomodulatory effects. This study concluded that EPA+DHA induce anti-inflammatory effects on bones by decreasing the production of pro-inflammatory cytokines, and thus improved the bone health of Inuit women. Further, according to Moro et al., "EPA+DHA from fish oil have anti-inflammatory effects due to their metabolites, resolvins and protectins," (Moro, Nagahashi, Ramanathan, Takabe, & Wakai, 2016). A study of acute wounds done by McDaniel, Belury, Ahijevych, & Blakely (2008), reported that proinflammatory cytokine synthesis and activity are also affected by EPA+DHA therapy. Additionally, Duvall and Levy (2016), reported that the anti-inflammatory actions of EPA+DHA include their ability to inhibit neutrophil migration, enhance macrophage phagocytosis of apoptotic neutrophils, and suppress of pro-inflammatory cytokines and chemokines in diseases with chronic airway inflammation, such as asthma and COPD.

While there is a significant body of evidence from randomized, controlled trials supporting the effectiveness of EPA+DHA therapy for inflammation reduction in diseases that have an inflammatory component, there have been no studies focusing on people with chronic

wounds such as CVLUs. EPA+DHA therapy has significant potential for reducing the chronic inflammation associated with CVLUs and facilitating healing.

Chapter III: Methodology

Research Design

This project was a secondary analysis of data generated from an experimental, randomized, double-blind, study of CVLU patients that took place between 2012 and 2015. The parent project studied two groups of individuals with CVLUs at 0, 28 and 56 days after eligibility screening, informed consent, and enrollment to the study. Participants were randomly assigned to one of two groups on day-0: EPA+DHA Group participants (n=16) consumed EPA+DHA containing softgels and 81mg aspirin (ASA) tablets daily until the final study visit. ASA enhances the action of the resolvin species. Control Group participants (n=19) consumed identical-looking placebo softgels and 81mg ASA tablets for the same schedule. The time points were chosen to allow adequate time for the supplements to affect plasma PUFA levels and for some CVLU healing to occur. Neutrophil-derived proteases (MMP-8 and HNE) in wound fluid were quantified at 0, 28 and 56 days because of their known influence on inflammation and to determine their associations to the independent variable: EPA+DHA supplementation. The variables' associations to wound healing were determined by quantifying re-epithelialization at the same time points. Body mass index (BMI) was calculated at the three time points to evaluate potential changes associated with EPA+DHA supplementation.

Population and Sample Design

Participants were recruited by a research nurse at the Comprehensive Wound Center (CWC) from a pool of patients who were scheduled to begin receiving CVLU treatment with a Silver-coated dressing beneath a 4-layer compression dressing.

Inclusion criteria: men and women, ages 18-85 years, identified from CWC medical records as having at least one existing CVLU between the ankle and knee for ≥ 3 months; medical

clearance to take 81mg/d of ASA; prescribed compression therapy; ambulatory; ankle brachial pressure index (ABPI) of ≥ 0.8; positive venous duplex with reflux; target wound of 10 cm^2 to 40 cm^2; understood English and could sign own consent.

Rationale for inclusion criteria: The age range expanded on the peak prevalence age range for CVLUs (60-80), but reduced the risk of adding confounding variables that might be present with a broader age range. Aspirin facilitates production of EPA- and DHA-derived resolvins. Compression therapy is standard care for CVLU. The wound fluid collection protocol required that the CVLU be located on the lower extremity and that participants be ambulatory versus bed-bound. An ABPI ≥ 0.8 and positive venous duplex with reflux confirm diagnosis of venous ulcer. It is difficult to obtain adequate fluid from smaller wounds (< 10 cm^2) for the planned analyses. It is more likely that participants who understand English comprehend study requirements than those who do not. A participant's ability to sign his/her own consent provides assurance they want to participate.

Exclusion criteria: allergy to fish or seafood; exposed bone, tendon or fascia around target wound; receiving warfarin or Plavix therapy; immunologic related condition (e.g. Crohn's disease, systemic lupus erythematosus); chronic inflammatory skin diseases (e.g., psoriasis); requiring non-steroidal anti-inflammatory drugs > 2x a week, nutritional supplements or corticosteroids; chronic renal insufficiency; and already in a study related to CVLU.

Rationale for exclusion criteria: Supplements contained fish oil. There are additional healing complications when bone, tendon or fascia are involved. Though some studies report that fish oil supplementation does not increase the risk of abnormal bleeding when administered concomitantly with warfarin and that aspirin does not increase bleeding time after high-dose n-3 supplementation, other studies report decreases in coagulation when fish oil supplementation is

added to warfarin therapy (n=1). Immunological related conditions, inflammatory skin diseases, anti-inflammatory drugs, nutritional supplements, corticosteroids, and chronic renal insufficiency may affect one or all outcome measures. Multiple study protocols may conflict. Other factors (lipid lowering medications, oral antibiotics, diabetes) that have the potential to affect the outcome measures were noted and controlled for statistically during data analysis.

EPA+DHA softgels: Five opaque EPA+DHA softgels provided a total daily intake of 2.2 g EPA and 0.6 g DHA. The U.S. Federal Drug Administration (FDA) evaluated the safety of EPA and DHA and concluded that a daily intake of EPA+DHA of up to 3.0 g/d is acceptable for the general public.

Placebo softgels: Five opaque placebo softgels provided a total daily intake of 2.5 mL of mineral oil, which is well below the therapeutic dose of 10 mL for constipation. Mineral oil is chemically inert and on ingestion the majority (98%) remains unabsorbed in the feces. All softgels were identical in appearance and lemon-flavored to reduce the risk of "fish burps." The softgels were compounded and packaged in like containers by J.R. Carlson Laboratories, Inc.

Data Collection Procedures

Visit 1: Baseline: Following a complete explanation of the study, informed consent was obtained via a form approved by the IRB. Sociodemographic data were collected. Body measurements were recorded (height, weight) for BMI calculations. Blood and wound fluid were collected so that study variables could be quantified. The area of the CVLU was measured using single digital camera photogrammetry (SCP). Verbal and written instructions were given to the participants to maintain their usual diets, but to exclude any fish, seafood, algae, kelp and nutritional supplements until study completion. Participants were also instructed to refrain from using NSAIDS (Tylenol permitted), other than the 81 mg/d of ASA, because of NSAID's

inhibitory effect on inflammation. All participants received a pill bottle containing the subsequent month's supply of softgels and one containing the subsequent month's supply of ASA. Participants were given verbal and written instructions to store the softgels in the refrigerator and to take 5 softgels and 1 aspirin tablet every day with their evening meal. They began taking the softgels at the end of the enrollment day, Visit 1. Participants brought empty bottles to Visit 2. Weekly phone calls were made to participants during the study to answer questions and to remind them of upcoming appointments, and, if possible, to refrain from NSAID use.

Visit 2: 28 days later: Blood and wound fluid samples were collected. BMI was calculated. SCP of CVLU occurred. Pill bottles were collected to evaluate compliance and new bottles containing the month's supply of softgels and ASA were provided.

Visit 3: 56 days later: Samples of blood and wound fluid were collected. BMI was calculated and a final assessment of the CVLU was completed with SCP. Pill bottles were collected.

Data Collection Instruments

Wound fluid was collected using a standard wound fluid collection technique that has been used successfully in previous studies. Briefly, the CVLU was washed with sterile water prior to the collection of wound fluid, followed by the application of a transparent occlusive film over the wound. The subject's leg was placed in a dependent position and the fluid was aspirated from beneath the dressing after approximately 1 hour with a 26G x 0.5" needle and syringe and transferred into plain collection tubes. The wound fluid samples were centrifuged at 14,000g for 10 minutes to remove any particulate matter and to collect PMN. The samples were frozen and stored at -80 ^{0}C until further analysis.

Differential counts and fluorescence-activated cell sorting (FACS) of polymorphonuclear leukocytes (PMNs – neutrophils) were accomplished by assessing aliquots of cells obtained from blood and CVLU fluid for total and differential leukocyte counts via light microscopy to identify individual cell types (i.e., neutrophil, monocyte, etc.). Samples were transported in plain collection tubes in a Styrofoam cooler within 30 minutes of collection to Dr. Roy's laboratory. Cells and fluid were separated via centrifugation. Cells were fixed and analyzed using flow cytometry. Fluid was aliquoted in 200 ul portions then frozen and stored at -80^0 C until further analysis. For flow cytometry analysis, cells were stained with CD45-FITC to gate for all leukocytes. In the total gated (CD45+) leukocyte population, PMN was easily distinguished in a forward scatter (FSC)/side scatter (SSC) plot. A dual staining approach using PE conjugated antibodies was also used to identify specific cell types: CD15 and CD18 for human PMN, CD14 for human monocytes. Activated PMN was detected using CD11b, CD177, CD66b.

Matrix metalloproteinase-8 (MMP-8) was measured as a biomarker of PMN activation in the CVLU fluid using the MMP-8, neutrophil collagenase, Biotrak enzyme-linked immunosorbent assay (ELISA) kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Standards, blanks, and samples (1.0 μg of wound fluid extracts or biopsy lysates in triplicate) were incubated in the microplate wells, precoated with anti-MMP-8 antibody, for 2 hours at 25^oC. Wells were washed and a peroxidase-labeled FAB' antibody to MMP-8 was added. After further incubation and washing, the TMB substrate was added to the wells and the plate incubated for 30 minutes at 25^oC. The reaction was stopped and plotting the optical density against a purified MMP-8 standard generated a standard curve.

Human neutrophil elastase (HNE) is another neutrophil-specific protease that was measured as a biomarker of neutrophil activation in the CVLU fluid using the Innuozyme™ Human Neutrophil Elastase Immunocapture Activity Assay Kit (Calbiochem, EMD Biosciences Inc., San Diego, CA). The assay used a monoclonal antibody on a 96-well plate to specifically bind HNE.

Re-epithelialization of CVLUs was quantified over time using single digital camera photogrammetry (SCP). This method provided precise objective information on wound size, shape, outline, area, color, and surrounding tissue changes using the photogrammetry technique, which is based on color- and light-balanced computerized photographic image capture. The SCP method utilized an orientation card of known dimensions that was placed next to the wound in view of the camera. Wound images were downloaded to the Verge Videometer (VeV) Wound Measurement computer software program. Wounds were oriented and compared to the known size of the orientation card per the computer software. CVLU epithelial margins were outlined with the cursor. The area yet to be re-epithelialized was calculated by the software program. The rate of wound closure was expressed as percent reduction in wound area at 28 days and 56 days (compared with the initial wound area at 0 days).

Chapter IV: Results

Participant Characteristics

Data were collected from 35 adults from Central Ohio, ages 28 to 81 years, who had one or more CVLUs for at least 3 months (Table 1). On average, the EPA+DHA Group was 60.3 (±12.6) years of age and the Control Group was 60.9 (±11.8) years of age. The majority of participants for both the EPA+DHA Group (62.5%) and Control group (58%) were male. The majority of the EPA+DHA group were widowed/divorced/single (56%), but the majority of the Control Group were married or living with someone (53%). The majority of the EPA+DHA group were either high school graduates (37.5%) or college graduates (37.5%), and the majority of the Control Group reported having "some college" education (42.1%). At baseline, the EPA+DHA Group had an average BMI of 40.4 (±8.2) and the Control Group had a mean BMI of 42.7 (±13.8), both calculations indicating morbid obesity. There were no significant differences between groups in terms of sociodemographic or wound data at baseline.

Table 1. Sociodemographic and Wound Characteristics of Participants

Sociodemographic and Wound Characteristics of Participants (N=35)		
	EPA+DHA [a,] (n = 16)	Control [a,] (n = 19)
Age, mean years (SD)	60.3 (12.6)	60.9 (11.8)
Male (%)	10 (62.5)	11 (58)
Female (%)	6 (37.5)	8 (42)
White (%)	12 (75)	14 (75)
African American (%)	4 (25)	5 (26)
Marital status		
Married/living with someone (%)	7 (44)	10 (53)
Widowed/divorced/single (%)	9 (56)	9 (47)
Education		
Some high school (%)	0	1 (5.3)
High school graduate (%)	6 (37.5)	5 (26.3)
Some college (%)	4 (25)	8 (42.1)
College graduate (%)	6 (37.5)	5 (26.3)
Annual income		
< $10,000 (%)	5 (33.3)	3 (15.8)
$10,000 - $24,999 (%)	5 (33.3)	7 (36.8)
$25,000 - $44,999 (%)	2 (12.5)	2 (10.5)
> $45,000 (%)	4 (25)	7 (36.8)
BMI, kilograms/meter^2 - mean (SD)		
Baseline	40.4 (8.2)	42.7 (13.8)
28 days	40.9 (8.5)	42.7 (8.5)
56 days	40.6 (8.9)	42.1 (13.7)
Wound Characteristics		
Size, baseline (cm^2) - mean (SD)	15.6 (34.4)	19.7 (23.2)
Estimated wound age		
< 6 months (%)	8 (50)	7 (36.8)
> 6 months (%)	8 (50)	12 (63.2)

[a] No significant differences between groups
SD=standard deviation
BMI = body mass index

EPA+DHA Data: At Days 28 and 56, there were significantly higher levels of EPA in the plasma as compared to Day 0 in the EPA+DHA Group and significantly higher levels at Days 28 and 56 compared to the Control Group (Figure 2A). Similarly, at Days 28 and 56, there were significantly higher levels of DHA compared to Day 0 in the EPA+DHA Group, and significantly higher levels at Days 28 and 56 compared to the Control Group (Figure 2B).

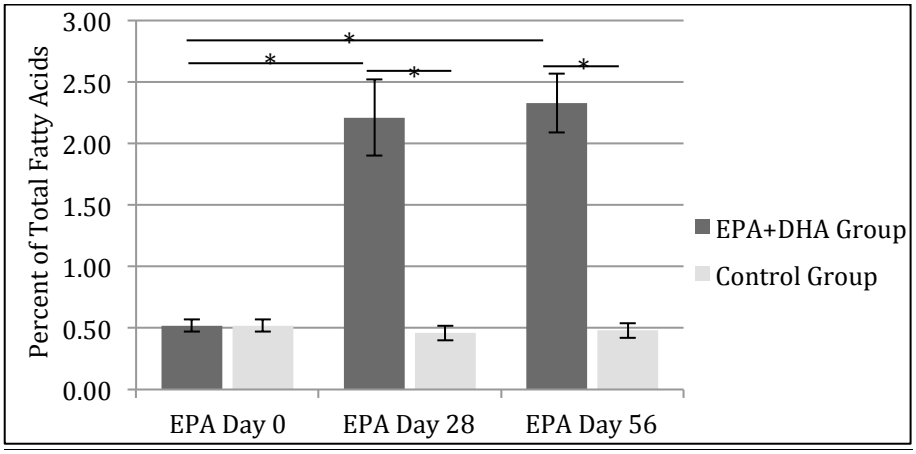

Figure 2A. Comparison of EPA levels (% of total fatty acids in plasma) at Days 0, 28 and 56 within and between the two groups (*p < 0.05)

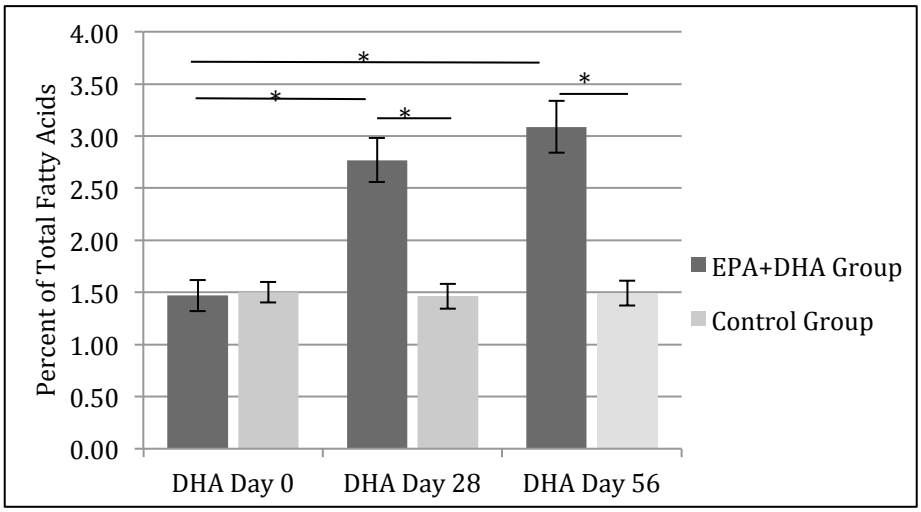

Figure 2B. Comparison of DHA levels at Days 0, 28 and 56 within and between groups. (*p < 0.05) (*T*-test, EPA+DHA Group: n=16; Control Group: n=19)

MMP-8 Data: Even though there were no significant differences in MMP-8 levels within or between groups at days 0, 28, and 56, MMP-8 levels steadily declined over time in the EPA+DHA group (Figure 3). Additionally, a significant negative relationship emerged between MMP-8 and healing at day 56 ($p = 0.04$).

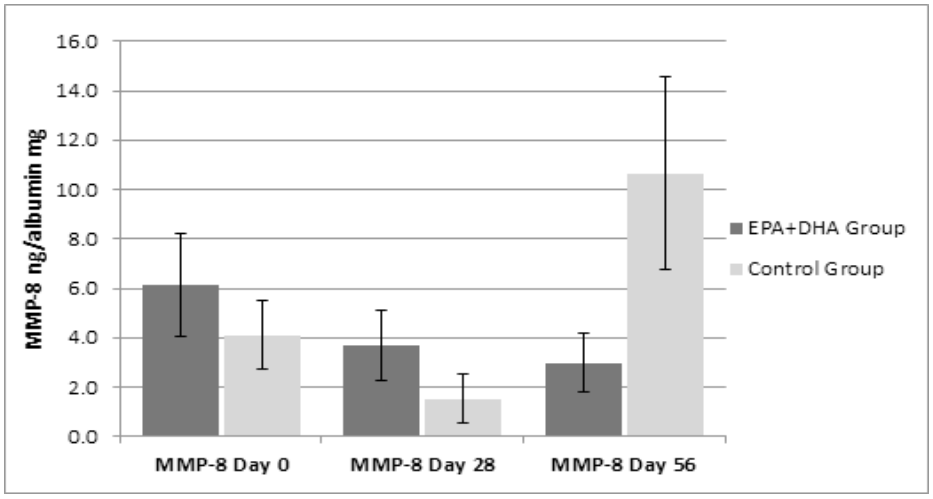

Figure 3. Comparison of levels of MMP-8 in CVLU fluid at Days 0, 28 and 56 between EPA+DHA Group and Control Group. Each bar shows mean levels of MMP-8 in ng per mg of albumin ± SEM. (Student's *t* test, EPA+DHA Group: n=14; Control Group: n=19)

Wound Area Data: At both days 28 and 56, there is a greater percent (%) reduction in the wound area compared to day 0 in the EPA+DHA group than in the Control group (Figure 4).

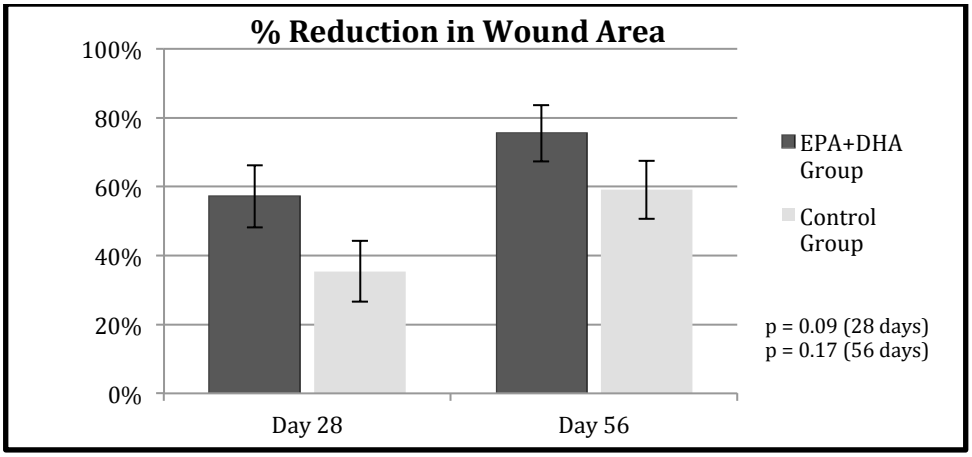

Figure 4. Comparison of % reduction in wound area at Days 28 and 56 between EPA+DHA Group and Control Group. Each bar shows mean % reduction ± SEM. (*T*-test, EPA+DHA Group: n=16; Control Group: n=19)

Chapter V: Conclusions and Recommendations

Summary of Findings

The purpose of this secondary analysis of data generated from an experimental, randomized, double-blind study of CVLU patients was to evaluate the effects of EPA+DHA on levels of neutrophil-derived MMP-8 and HNE in CVLU fluid. An important finding from this study is that MMP-8 levels steadily declined over time in the EPA+DHA Group, a pattern not detected in the Control Group. Additionally, a significant negative relationship emerged between MMP-8 and healing at Day 56 ($p = 0.04$). Finally, there was a greater percent reduction in wound area at both Days 28 and 56 versus Day 0 in the EPA+DHA Group than in the Control Group. The findings of reduced MMP-8 levels in the EPA+DHA Group are similar to reports from other studies exploring the effects of EPA and DHA on neutrophil activation using cell and animal models (Paunescu et al., 2013 & Moro, Nagahashi, Ramanathan, Takabe, & Wakai, 2016). Also, our findings that higher levels of MMP-8 in CVLU fluid are associated with less wound healing, supports findings from previous studies (Zhao, Liang, Clarke, Jackson, & Xue, 2016). The effect of EPA+DHA therapy noted in this study may have been more remarkable in a larger sample and, thus, these data may be used to design larger intervention studies of patients with CVLUs or other types of chronic wounds. Greater reductions in MMP-8 and HNE levels may have been detected in the EPA+DHA group if 1) other sources of proteases (e.g., *Pseudomonas*), and 2) cytokines that can enhance or inhibit protease synthesis had been controlled for in the current study.

The current study data also indicate that the majority of participants were morbidly obese. Obesity increases the risk for other comorbidities, such as cardiovascular disease and diabetes, both chronic conditions that also contribute to healing delays (Tsukinoki et al., 2012).

Cardiovascular disease and diabetes compromise blood circulation, and therefore reduce oxygen supply to the extremities that may lead to local tissue inflammation, tissue breakdown, and an increased risk for wound complications (Cianfarani et al., 2013).

Conclusions

In summary, although there was a downward trend in MMP-8 levels noted in the EPA+DHA Group over the study interval that was not seen in the Control Group, and there were greater percent reductions in wound area at 28 and 56 days in the EPA+DHA Group when compared to the Control Group. The collective findings support the hypothesis that EPA+DHA therapy may curtail high levels of some neutrophil-derived proteases in the CVLU microenvironment and facilitate healing. Additional studies using a larger, more diverse sample of CVLU patients are needed to evaluate the effects of EPA+DHA on MMP-8 and HNE levels and wound healing before EPA+DHA therapy can be recommended as an adjuvant to standard care for CVLUs. The high group mean BMI—indicating morbid obesity—increases the risk for chronic diseases such as diabetes mellitus and cardiovascular disease, which also contribute to wound complications.

Implications for Nursing Practice

An interdisciplinary team of nurses, physicians, and registered dietitians can provide holistic care for CVLU patients. It may essential that all CVLU patients have a dietary assessment at his or her initial visit to a wound clinic. Based on the assessments, an effective plan can then be implemented to address nutritional needs, and consider financial barriers to a healthy diet. Also, in the future it may be important to assess plasma levels of EPA+DHA and to quantify levels of the proteases MMP-8 and HNE in the wound microenvironment to determine if EPA+DHA therapy is indicated for the targeting of high levels of these destructive proteases.

Interventions such as exercise and healthy food choices (e.g., fish containing high levels of EPA+DHA, fruits, and vegetables) may improve healing outcomes and contribute to the overall well-being of CVLU patients. Nurses work closely with CVLU patients and thus are critical to the planning and implementation of evidence-based strategies to improve healing outcomes of these serious wounds that have rising prevalence rates.

Recommendations

Additional studies using larger sample sizes of more diverse populations of CVLU patients are needed to more fully address the research question, "in patients with CVLUs, does supplementing diets with EPA+DHA compared to placebo reduce levels of MMP-8s and HNE in the wound fluid?" Since EPA+DHA supplementation could potentially reduce inflammation and facilitate CVLU healing, this therapy should be tested in patients who present with other types of chronic wounds too, such as diabetic foot ulcers and pressure ulcers since the pathogenesis of these wounds also involves chronic inflammation and high levels of neutrophil-derived proteases.

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