OMEGA-3 FATTY ACIDS EFFECT ON WOUND HEALING

DISSERTATION

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By

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ABSTRACT

Prolonged wound healing is a monumental health problem in the United States (U.S.) In the year 2000, approximately 3-5 million Americans with non-healing wounds had a total annual treatment cost of approximately $25 billion, or 5 percent of the combined annual spending for Medicare and Medicaid. Estimations of individuals affected and health dollars spent are predicted to dramatically escalate as the U.S. population ages. Research studies are needed that consider the interdependent psycho-physiological relationships that affect wound healing processes.

Wound healing is known to be influenced by behavioral-related factors such as perceived stress, which have been found to alter immune function. In addition, cofactors such as aging, tobacco use and the presence of other co-morbidities such as diabetes may directly or indirectly affect neuro-endocrine and/or immune status and thus, diminish the wound healing process. Certain nutritional factors and behavioral-related nutritional choices such as the intake of omega-3 polyunsaturated fatty acids and the ratio of omega-6 polyunsaturated fatty acids to omega-3 fatty acids are also known to affect immune function. Increasing or decreasing polyunsaturated fatty acid intake is reflected in plasma fatty acid levels, which have demonstrated inverse correlations with levels of proinflammatory cytokines in peripheral blood. These cytokines assist in the expression and activation of several acute phase inflammatory cells. Therefore, a psychoneuroimmunological-based theoretical framework, which considers bidirectional...
pathways among behavioral, neuro-endocrine, and immune systems, provides a comprehensive foundation for research studies that investigate wound healing. The benefits of utilizing this theoretical framework to guide wound healing research and a discussion of how it was successfully applied to the present study are reported in the first manuscript.

Wound healing is an intricate, biological progression that begins with tissue injury and ends with scar formation. The three basic phases of wound healing are the inflammatory, proliferative, and maturation phases, which overlap and are dependent on one another for successful completion. Thus, physiological events in the initial, essential inflammatory stage of wound healing influence subsequent overlapping stages. Proinflammatory cytokines initiate and control molecular and cellular processes during the inflammatory stage. Polyunsaturated fatty acids alter proinflammatory cytokine production, but how this outcome specifically influences wound healing is still not clearly understood.

In the present study we examined effects of marine-derived omega-3 eicosapentaenoic and docosahexaenoic polyunsaturated fatty acids on proinflammatory cytokines in wound serum and time to complete healing in healthy, human skin. This randomized, experimental study of two groups, (N=30), compared plasma fatty acid levels at baseline and after four weeks of omega-3 supplements or placebo. Eight small blisters were created on all participants. Proinflammatory cytokines IL-1β, IL-6, and TNF-α were measured in blister fluid at 5 and 24 hours after creation. Wound area was calculated daily until 100% closure. We found that eicosapentaenoic and docosahexaenoic plasma fatty acid levels were significantly greater in participants who received the supplement for 4 weeks than the control group. Additionally, and contrary to our original hypothesis,
all three proinflammatory cytokine levels in blister fluid were higher in the active group than the placebo group at 24 hours post blistering, but the levels of IL-1β were significantly higher. Time to complete wound closure was not significantly different between the groups. These results suggest that omega-3 eicosapentaenoic and docosahexaenoic polyunsaturated fatty acids increase proinflammatory cytokine production at wound sites and thus, depending on the clinical context, have noninvasive, therapeutic potential to affect skin wound healing. The second manuscript contains a complete, detailed description of the study’s design, methods, results and implications for clinical application and future research endeavors.

The third manuscript describes the suction blister wound model employed in the present study to explore epidermal skin healing. It appraises the current opinions and data concerning existing measurement instruments appropriate to precisely calculate surface area of healing wounds. The stereophotogrammetry, single-camera computerized photogrammetry, and structured light techniques continue to demonstrate production of valid and reliable data and are particularly suitable for area measurements of small wounds such as those produced by a suction blister device. The pros and cons of employing a specific single-camera computerized photogrammetry system, Verge Videometer Measurement Documentation, to measure suction blister wounds, are also discussed. Although there are a few disadvantages to the single-camera computerized photogrammetry method for blister wound measurement, such as the initial cost investment, it provided precise, reliable blister wound measurements with ease in the current study and thus, is highly recommended for future research projects that utilize the blister wound model.
This dissertation is dedicated to the loving memory of my late husband, Dr. Richard Pelham, who prompted me to begin this intellectual journey. His belief in my ability to attain my highest aspirations sustained my momentum. I will be forever grateful for his insight, love and unwavering support. This is for you, Dick.
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ABBREVIATIONS

AA=arachidonic acid
ω=omega

BMI=body mass index
g=gram

DHA=docosahexaenoic acid
mL=milliliter

EPA=eicosapentaenoic acid
mg=milligram

FFQ=food frequency questionnaire
L=liter

GC/MS=gas chromatography/mass spectrometry
µL=microliter

GCRC=General Clinical Research Center
kcal=kilocalorie

IL-1β=Interleukin-1 beta
kg=kilogram

IL-6=Interleukin-6
µM/L=micromoles/liter

NDSR=Nutritional Data System for Research

PG=prostaglandins

PNI=psychoneuroimmunology

PSS=perceived stress scale

PUFA=polyunsaturated fatty acids

SAD=sagittal abdominal diameter

SCP=single camera digital photogrammetry

TNF-α=Tumor necrosis factor alpha

VeV MD=VergeVideometer Measurement Documentation
CHAPTER 1

UTILIZATION OF A PSYCHONEUROIMMUNOLOGICAL FRAMEWORK TO
GUIDE WOUND HEALING RESEARCH

Chronic wounds generate tremendous physical, psychological and financial burdens for the client, family and health care community (Park, 2000). Experts in the international Wound Healing Society recently estimated that approximately 3-5 million Americans with non-healing wounds generate treatment costs up to $25 billion dollars annually (Park, 2000). This figure represents approximately 5 percent of the combined annual spending for Medicare and Medicaid. According to the National Institute of Health (2000), non-healing wounds develop in over four million of our elderly each year. Associated costs are expected to dramatically escalate as the population of individuals 65 and older is predicted to double over the next 30 years (Park, 2000).

Psychological influences such as stress have been found to diminish neuroimmunological function, which subsequently slows wound healing (Hubner et al., 1996; Glaser et al., 1999; Kiecolt-Glaser, Marucha, Malarkey, Mercado & Glaser, 1995; Marucha, Kiecolt-Glaser, & Favagehi, 1998). Effectively designed research studies that measure the interrelated psychoneuroimmunological variables that influence the healing process are therefore essential. The purpose of this article is to clarify the advantages of utilizing a psychoneuroimmunological (PNI) theoretical framework in wound healing
research. The components of a generic PNI framework are described followed by the application of that model to a specific study design that examines omega-3 (ω-3) fatty acids effect on wound healing via their influence on proinflammatory cytokines.

Psychoneuroimmunology: Brief History and Tenets

Psychoneuroimmunology is the interdisciplinary study of the complex, bidirectional relationships among behavioral, neural and endocrine, and immune functions; the mind-body connections (Ader, 2000). The field of psychoneuroimmunology originally developed under the broader discipline of psychosomatic medicine. The term “psychoneuroimmunology” was first introduced by psychologist Robert Ader in 1980 (Ader, 1980).

The initial focus of psychoneuroimmunological research was the brain’s influence on the immunological system. The evidence for a bidirectional signaling network between the immune and the neuroendocrine systems eventually emerged and was strongly supported by the work of Hugo Besedovsky et al. (1975) Besedovsky’s identification of a proinflammatory cytokine, interleukin-1 (IL-1) as a potent stimulator of the hypothalamic-pituitary-adrenal axis (HPA) was ground-breaking (1977). The suggestion that the immune system could influence neuroendocrine function in a reciprocal relationship dramatically altered the course of PNI research. The opinions that brain peptides and their receptors reside within the immune system and that products of an activated immune system operate as neurotransmitters are now widely accepted.

Numerous studies over the past 25 years by psychologists, neuroendocrinologists and immunologists have continually supported the interdependence among psychological factors, behavior, neural and endocrine functions and the immune system (Blalock, 2005;
Elenkov, Wilder, Chrousos & Vizi, 2000). Investigating the mechanisms by which a host’s immunity is influenced by psychosocial and physiological interactions remains a primary goal in PNI. The belief that this complex network of defenses manages an organism’s homeostasis remains the basic tenet of the PNI discipline. A model based on a PNI framework is a dynamic, extremely valuable tool to utilize during the research design phase because it considers the multiple factors that may influence certain health outcomes such as wound healing.

PNI-based Framework for the Study of Health Outcomes

The science of PNI posits that a dynamic, highly evolved network of communication among the psychoneuroimmunological systems is responsible for health, which is a constantly changing phenomenon unique to an individual (Ader, 2000). Thus, a PNI-based framework provides a comprehensive template for studies that investigate associations among personal co-factors, psychosocial influences, neuroendocrine function, immune status and health outcomes such as wound healing (Kiecolt-Glaser, Page, Marucha, MacCallum & Glaser, 1998; Robinson, Mathews & Witek-Janusek, 2002; McCain, Gray, Walter, & Robins, 2005). A PNI-based framework therefore supports the use of quantitative and/or qualitative methodology to obtain comprehensive data about the variable(s) of interest.

An example of a generic PNI-based model is provided to illustrate how study designs can be constructed to investigate the relationships among psychoneuroimmunological variables and health outcomes (Figure 1). The primary elements in this model are co-factors, psychological influences, neurological influences, immunology, health and the lived experience (McCain et al., 2005). The Lazarus and Folkman cognitive-transactional
model of stress (perceived) is utilized within this PNI model (Lazarus & Folkman, 1984). Lazarus and Folkman define stress as a dynamic transaction among person factors, social-environmental factors, and illness-related stress factors that affect cognitive appraisal and coping strategies (Lazarus & Folkman, 1984). Coping strategies are thought to alter the stress response and hence neuro-endocrine-immune function (Kiecolt-Glaser et al., 1985; Fawzy et al., 1990, Glaser et al., 1992). Therefore, behavioral characteristics, such as individual interpretations of stress and coping patterns to relieve stress, are regarded as psychosocial moderators because their influence on the immune system is moderated through the neuroendocrine system. Neuroendocrine factors can have direct effects on the immune system and are considered mediators between psychological variables and immune function (McCain et al., 2005). Importantly, bidirectional pathways exist among all the major variables (Kiecolt-Glaser et al., 1998; Tsigos & Chrousos, 2002).

Co-factors include personal characteristics such as age, gender, exercise regimens, sleep patterns and nutritional status, which can have an effect on the type and degree of stress, coping skills and health status of an individual (McCain et al., 2005). Pre-treatment critical co-factors are conditions or circumstances specifically related to a particular health issue that can impact an individual’s stress perception and thus, their ability to adjust psychologically and physiologically. These factors may include disease acuity, ability to comprehend illness, and complexity of treatment regimens (McCain et al., 2005).

The psychological piece of this model speaks to the sociobehavioral characteristics or psychological status of an individual, which can impact the stress response. Emotional
factors such as depression, grief and distress may be included in this section (McCain et al., 2005). Studies have consistently shown that one way chronic stress can induce immunosuppression is via activation of the hypothalamic-pituitary-adrenal axis (HPA) and the sympathetic-adrenomedullary pathway (Marucha et al., 1998; Elenkov et al., 2000; Tsigos & Chrousos, 2002).

Thoughts, emotions and sensations are a result of sensory stimulation via cranial nerves, peripheral nerves or signals from inside the body that are processed in the cerebral cortex and limbic structures of the brain (Tsigos & Chrousos, 2002; Blalock, 2005). These thoughts and emotions signal a cascade of activity by the hypothalamus that affects immune function (Elenkov et al., 2000; Tsigos & Chrousos, 2002). It is therefore important to incorporate psychosocial measures in the PNI-based study design that are appropriate for the research question(s) and population of interest. Instruments that evaluate environmental stressors, coping behaviors and perceived emotional distress provide comprehensive data about the effects of psychological stress on a particular health outcome and add to the science of PNI (Kiecolt-Glaser et al., 1998; Robinson et al., 2002; McCain et al., 2005).

The neurological facet of the model characterizes the neuroendocrine changes that occur as a result of psychological and/or immune influences. When the cerebral cortex and limbic system of the brain processes perceive stressful stimuli, signals are transmitted to the hypothalamus (Tsigos & Chrousos, 2002). The HPA axis and sympathetic nervous system (SNS) pathways are then activated and stress hormones such as glucocorticoids and catecholamines are secreted, respectively, into the bloodstream. Both neuroendocrine and immune cells have receptors for these substances and thus are affected by their
release, supporting the belief that the systems are connected and communicating (Tsigos & Chrousos, 2002; Elenkov et al., 2000). Measurements of neuroendocrine activation may include cortisol or catecholamine levels in plasma, urine or saliva (Robinson et al., 2002). The decision about the substance to measure and in which body fluid is dependent on the chosen hypotheses, variable(s) of interest, sampling time points, population and cost factors.

The immunology section of the PNI model refers to immunological function. It considers the mechanisms by which the immune system is directly and indirectly affected by the stress response. Both immune and neuroendocrine cells contain receptors for stress mediators such as cortisol and catecholamines (Besedosky, Sorkin, Keller, & Mueller, 1975; Besedovsky, Sorkin, Felix & Haas, 1977; Elenkov et al., 2000; Tsigos & Chrousos, 2002). In addition, lymphoid tissue present in organs such as the spleen and lymph nodes is directly innervated by sympathetic nerve fibers (Elenkov et al., 2000). Activation of the immune system results in the stimulation of various cytokines, neurohormones and neuropeptides produced by immune cells that are responsible for regulating immune function (Elenkov et al., 2000; Glaser & Kiecolt-Glaser, 2005).

The complexity of the psycho-neuroendocrine-immune system and the bidirectional pathways that exist in the PNI-based framework for the study of health outcomes do contribute to methodological issues. Therefore, the PNI-based study design requires that immunological measurements also be as specific and appropriate as theoretically possible to the research question(s), the population and the health outcome(s) of interest. Centrally and peripherally-produced pro-inflammatory cytokines (i.e. IL-1, IL-6 and TNF-α) are of particular interest in the science of PNI as they have been found to be the principal
regulators of the innate inflammatory responses (Maes et al., 1998). In addition, investigators have found that they also affect psychobehavioral symptoms such as fatigue and depression related to illness (Appels, Bar, Bar, Bruggeman & Baets, 2000; Atanackovis, Kroger, Serke & Deter, 2004). Therefore, measuring pro-inflammatory cytokine production is one example of an appropriate measure of immune function to include in a PNI-based research design.

The health segment of this PNI-based model incorporates disease-specific health outcomes which may include psychosocial functioning, quality of life and physical health measurements. These health outcomes are congruent with the Lazarus and Folkman (1984) cognitive-transactional model of stress, which supports the belief that health outcomes are affected by numerous psychobehavioral and pathophysiological changes that are influenced by the stress response. Health outcomes may in turn impact the other major components of the model as well as the personal factors. These relationships are depicted in the model by “feedback” arrows.

The final component of this particular PNI-based theoretical framework is the “lived experience.” This particular concept represents a basic premise of PNI, which is that subjective experiences of living with an illness can alter the psychological, neuroendocrine and immunological well-being of an individual, thereby impacting health outcomes (McCain et al., 2005). In addition, sociocultural issues, economic influences and spirituality are considered to be aspects of the “lived experience” and thus, may play a role in altering the psychoneuroimmunological system. Psychoneuroimmunology
values the utilization of qualitative measures, as appropriate, to capture the phenomenon of the “lived experience” and enrich quantitative measures of the various components of the PNI framework.

Psychoneuroimmunology provides the scientific foundation for a theoretical framework to conceptualize how certain health outcomes may be influenced by cofactors, psychological, neuroendocrine and immunological variables and the lived experience. It is an ideal framework to utilize for studies designed to investigate the health outcome of wound healing because the PNI network is intricately involved in that process.

Wound Healing and PNI: The Connection

Wound healing is influenced by numerous co-factors such as age, nutritional status, alcohol and tobacco use, and exercise patterns (Kiecolt-Glaser et al., 1998; Clark, 1996; Clark, 2002). Co-factors can affect the basic health status of an individual and therefore influence the stress response and alter the neuroendocrine/immunological network that orchestrates the healing process. Co-factors may directly compromise the wound healing process or contribute to immune suppression, which can lead to a chronic wound. A PNI theoretical framework considers the interactions among co-factors, psychological variables such as stress, neuroendocrine activity and immune system function. It therefore provides a comprehensive foundation for investigating the wound healing phenomenon.

Wound healing is an intricate, biological process that begins with tissue injury and ends with scar formation (Clark, 1996; Clark, 2002). The three basic phases of wound healing are the inflammatory, proliferative, and maturation phases, which overlap and are dependent on one another for successful healing (Clark, 1996). The acute inflammatory
phase occurs during the first 24 hours after cellular injury when activation of the clotting system results in production of growth factors, cytokines and low-molecular-weight compounds (Werner & Grose, 2003). The later stages of wound repair are strongly dependent on the biological processes that occur during the initial inflammatory stage.

The immune system in particular is responsible for initiating and controlling essential functions in the wound healing process, especially in the preliminary inflammatory stage. Previous research has shown that proinflammatory cytokines, which are polypeptides, are crucial in mediating inflammatory and immune responses that affect wound healing. They assist in controlling infection and preparing tissue for further repair via their enhancement of phagocytic activity, synthesis of matrix proteins, stimulation of fibroblast and keratinocyte growth, and stimulation of various growth factors (Villarreal, Zagorski & Wahl, 2001; Rennekampff et al., 2000; Werner & Grose, 2003; Hubner et al., 1996).

An increased expression of proinflammatory cytokines within a few hours after tissue injury has been shown to correspond to the inflammatory stage of wound healing and normal repair (Grellner, Georg, & Wilske, 2000). Conversely, a diminished production of proinflammatory cytokines in the initial stage of wound healing was found in glucocorticoid-treated mice and was associated with impaired wound healing (Hubner et al., 1996). Cytokine production during the acute inflammatory stage is also affected by the presence of other inhibitors such as chronic stress, aging, sporadic sleep patterns and poor nutritional status as well as by the rate of cytokine degradation (Caughey, Mantzioris, Gibson, Cleland & James, 1995; Glaser et al., 1999; Appels et al., 2000; Villarreal et al., 2001).
Efficient wound healing generally occurs successfully, but if there is a problem with the initiating stimulus or if regulatory responses malfunction then a chronic, nonhealing wound may result. A PNI framework for wound healing considers the variables that can potentially alter psycho-neuro-immune activity and influence the healing process such as proinflammatory cytokine production.

Adapting a PNI-Based Theoretical Framework to Wound Healing Research

An illustration of a PNI-based model for wound healing research is provided to highlight a study that examines the specific effects of the co-factor ω-3 polyunsaturated fatty acid (PUFA) intake on “person factors” and thus, neuro-endocrine-immune function (Figure 2). The framework components will be described in detail and hopefully clarify the relationships among the co-factors, ω-3 fatty acid intake in particular, and the psycho-neuro-immune variables that affect the health outcome of an efficiently healed wound.

Background

Co-factors such as age (Kiecolt-Glaser et al., 2003; Solomon & Morley, 2001), gender (Goto & Nishioka, 1989; Marchetti et al., 2001; Kiecolt-Glaser & Glaser, 1988), alcohol use (Hosseini, Sepulveda, Lee & Watson, 2001), smoking (Silverstein, 1992; Clark, 2002) and nutritional behaviors (Conn, 1996; Kiecolt-Glaser & Glaser, 1988) such as the intake of ω-3 PUFAs (Caughey, Mantzioris, Gibson, Cleland & James, 1995; Simoupoulos, 2002; Grimble at al, 2002; Calder, 2002) have been found to ultimately affect immune function and thus, potentially wound healing.

The primary means through which omega-3 PUFAs contained in fish oils, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), influence immune function is by inducing anti-inflammatory effects on the body (Calder, 2003;
Simopoulos, 2002). It has been demonstrated that when EPA and DHA intake is increased, a therapeutic threshold is quickly reached, which causes a diminished inflammatory response by significantly reducing pro-inflammatory cytokine production in peripheral blood (Caughey et al., 1996; Alexander, 1998; Grimble et al., 2002; Calder, 2003; James, Gibson & Cleland, 2000). Ingestion of EPA and DHA in the form of encapsulated fish oil to a concentration of 1% of the total phospholipid fatty acids was found to decrease the production of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 by as much as 90%, 70% and 60% respectively (Meydani et al., 1991; Endres et al., 1989; Caughey et al., 1996). A total daily intake of EPA and DHA as low as 1.6 g and 1.1 g respectively has resulted in a decrease of proinflammatory cytokine levels in peripheral blood after only 4 weeks of consumption (Caughey et al., 1996).

The ability of marine-derived ω-3 PUFAs to affect positive health changes due to their systemic anti-inflammatory actions has been well established in the research literature however, their potential negative influence on wound healing by potentially decreasing necessary pro-inflammatory cytokine production at wounds sites, if local responses are similar to systemic responses, requires further investigation. A concern is that ω-3 PUFA supplement sales in the United States (U.S.) increased 50.3% in 2004 over 2002 and represented 11.5% of supplements’ total dollar sales in the U.S. (Uhland, Lewis, & Spehar, 2004). The American Heart Association has recommended specific doses of ω-3 PUFAs for cardiovascular benefits (Kris-Etherton, Harris, & Appel, 2002) and the FDA has approved the ω-3 PUFA DHA as a supplement to infant formula, therefore it is not surprising that consumer awareness and willingness to use these supplements are rising dramatically (Stone, 2002). It is important to consider that many individuals are
consuming varying doses of EPA and DHA through supplements, which could affect wound healing. This PNI-based study investigates if these specific ω-3 PUFAs could potentially diminish the wound healing process.

**Design**

The two-group, randomized, prospective, repeated measures, experimental design utilized for this PNI-based research study controls for extraneous variables. In addition to the strength of a randomized, two-group, experimental design, a benefit of a repeated-measures design is that each subject can serve as their own control, which increases power. A diagram to illustrate the design is provided.

\[
\begin{array}{ccccccccc}
X & 4 \text{ weeks of } \omega-3 & EPA \text{ and DHA supplement} & & & & & & \\
\sim X & 4 \text{ weeks of placebo} & & & & & & & \\
\text{PSS} = \text{Perceived Stress Scale} & & & & & & & & \\
\text{Sal. cortisol} = \text{salivary cortisol} & & & & & & & & \\
\text{O}_1 = \text{cytokines in blisters} & & & & & & & & \\
\text{O}_2 = \text{photos of healing} & & & & & & & & \\
\end{array}
\]

Evaluating perceived stress and coping patterns is important to the psychological grid of the PNI-based framework for wound healing research. In this study all subjects complete a Perceived Stress Scale (PSS) on admission to the General Clinical Research Center (GCRC). The PSS instrument measures the theoretical definition of stress utilized in the study and represents the subject’s cognitive appraisal of stress over the past month.
(Cohen, Karmarck & Mermelstein, 1983). In addition, salivary cortisol is measured on admission and at 5 and 24 hours post blistering to evaluate the potential stressful effects of the blistering procedure. The PSS explores the psychological stress response and the salivary cortisol reflects the physiological stress response. Salivary cortisol has excellent sensitivity and specificity with intra-assay and interassay coefficients of variation of less than 8% (Glaser et al., 1999).

Because immune function plays a primary role early in the wound healing cascade of events and specific pro-inflammatory cytokines have been found essential in orchestrating this occurrence, the cytokines IL-1β, TNF-α and IL-6 are measured in wound serum in this study at two time points in order to investigate the local inflammatory response (Glaser et al., 1999; Hubner et al., 1996; Grellner et al., 2000; Marucha et al, 1998). The assay is accomplished by filling the template wells over the blisters with an autologous serum-buffer solution. The solution is then aspirated from the wells to quantify the pro-inflammatory cytokines present by the enzyme-linked immunosorbent assay (ELISA) technique. Additional immune function measurements in a PNI-based wound healing study could include pro-inflammatory cytokines in blood and leukocyte counts in peripheral blood and in wound serum (Glaser et al., 1999). Immune function measurements are complex and sampling dilemmas do exist, hence it is important to carefully choose measures that are appropriate for each PNI-based study (Rabin, 1999).

The objective health outcome in the illustrated PNI-based model for wound research is wound healing. It is crucial that the biological measure of wound healing coincide with
the operational definition. In addition, it is imperative that the precision and validity of the measuring instrument be considered when choosing from the available methods.

For example, measuring wound area over time (days) until complete closure may be one operational definition of healing. Wound area measurement is usually calculated in square centimeters (cm²) and is either achieved by multiplying two perpendicular linear dimensions or tracing the wound edges either manually or via a computerized digital image. Three methods to achieve a wound tracing and calculate wound area in square centimeters are by digital planimetry, stereophotogrammetry (SPG) or single-camera computerized photogrammetry (SCP) system, which have been found to have similar intrarater and interrater reliability and validity (Thawer, Houghton, Woodbury, Keast & Campbell, 2002). These methods of measuring wound area take into consideration variation in wound shape.

The health outcome of wound healing is evaluated in this study for all subjects for eight consecutive days, then every 4 days until healing is complete. The chosen method of wound measurement is single digital camera computerized photogrammetry (SCP). Measuring the specific health outcome of wound healing will assist in demonstrating whether there is a valid connection among co-factors, psycho-neuro-immune-mediated activity and tissue repair.

Co-factors that may influence the stress response, neuro-endrocrine function and immune status are important to evaluate in a PNI-based research study. Since inadequate nutritional intake is associated with numerous immunological disorders (Kelley, 2001; Conn, 1996; Kiecolt-Glaser & Glaser, 1988) a nutritional assessment is essential to obtain in PNI-based research studies investigating wound healing. In this particular 2-group
investigation, subjects complete a 3-day food diary at baseline and again at 4 weeks. The nutritional data is evaluated using the Nutrition Data System for Research (NDSR) software program. In addition, a Food Frequency Questionnaire (FFQ) is completed to provide additional information about nutritional status. Body composition is also assessed in this study by recording weight and sagittal abdominal diameter (SAD) at baseline and again after 4 weeks of treatment. Sagittal abdominal diameter is an indirect measure of visceral fat and has a stronger correlation to total risk for cardiovascular disease and to metabolic risk than other anthropometric measures (Ohrvall, Berglund & Vessby, 2000). The baseline questionnaire collects additional co-factor information such as age, gender, ethnicity, smoking history, alcohol/drug use, exercise regimen, and sleep patterns, which may also influence person factors and hence, the stress response, neuro-endocrine function and immune status (Robinson et al., 2002).

Plasma fatty acids for both Group 1 (ω-3 supplement) and Group 2 (placebo) are evaluated at baseline and following the four weeks of ω-3 supplements or placebo to ensure that the two groups are comparable at onset and to evaluate effectiveness of supplementation.

A measurement of the “lived experience” component that explores the effects of living with an illness on other aspects of the model is not included in this particular study, however it may be considered in future research endeavors. The subjects in this study are young participants, the project is over a relatively short time span and there are no known diseases or illnesses present. A qualitative measure of the lived experience in future studies that investigate chronic wounds could provide additional insights.
Conclusion

Psychoneuroimmunology is a science that investigates the mind-body connection. Its tenets have been consistently supported by an abundance of research studies over the past 25 years. Psychoneuroimmunology views health as a dynamic state unique to the individual that is affected by psychosocial and physiological variables. A research study that utilizes a PNI-based framework considers the comprehensive psychosocial, neuroendocrine and immune network that influences specific health outcomes such as wound healing. A PNI-based framework also allows the use of qualitative as well as quantitative methodology, which contributes to improved health outcomes through research-based interventions that consider individual differences as well as similarities. It is an ideal theoretical framework to utilize to address the monumental health problem of nonhealing wounds, which are affected by the interdependent psychoneuroimmunological processes.
Figure 1.1
Generic model of the PNI-based theoretical framework

Figure 1.2
PNI-based model in wound healing.
PSS - Perceived Stress Scale; IL-1β – Interleukin Beta, IL-6 – Interleukin 6; TNF-α – Tumor Necrosis Factor Alpha.

LIST OF REFERENCES


CHAPTER 2
THE EFFECT OF OMEGA-3 FATTY ACIDS ON WOUND HEALING

Introduction

Wound healing is a complex, sequential, biological process that occurs in overlapping stages (Clark, 2001; Singer & Clark, 1999; Tonnesen, Fen & Clark, 2000). The inflammatory stage of healing is synchronized to a large degree by proinflammatory cytokine activity (Villarreal, Zagorski & Wahl, 2001; Rennekampff et al., 2000; Werner & Grose, 2003). The omega-3 (ω-3) polyunsaturated fatty acids (PUFAs) eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), present in high concentrations in fish oils, have been found to affect production of eicosanoids, which contribute to the regulation of proinflammatory cytokine production (Jia & Turek, 2004; Hankenson, Watkins, Schoenlein, Allen & Turek, 2000; Rola-Pleszczynski & Stankova, 1992). It has also been demonstrated that EPA and DHA modify the actual gene expression of proinflammatory cytokines at the level of transcription (Robinson et al., 1996; Simopoulos, 1996). The effect of ω-3 PUFAs on proinflammatory cytokine production during the inflammatory stage of wound healing has been minimally studied in human in vivo skin wounds and thus, is not yet clearly understood. Manipulating proinflammatory cytokine production, either positively or negatively, during the inflammatory stage of healing could noninvasively assist wound healing. The purpose of this study was to
examine the effects of EPA and DHA dietary supplementation on proinflammatory cytokine production at blister wound sites during the inflammatory stage of healing and subsequent time to complete skin healing in a healthy, human population (Figure 1).

The intricate, cascading progression of wound healing occurs in three basic stages known as the inflammatory, proliferative and maturation stages. The process begins with tissue injury and ends with scar formation (Clark, 2001; Singer & Clark, 1999; Tonnesen et al., 2000; Werner & Grose, 2003). The acute inflammatory stage occurs during the first 24 hours after cellular injury when neutrophils and monocytes, which mature into macrophages, arrive at the wound site in response to growth factors and cytokines produced by degranulating platelets and serum of injured blood vessels (Werner & Grose, 2003). These inflammatory cells produce proteinases, reactive oxygen species and additional growth factors and are the primary producers of proinflammatory cytokines, which facilitate activities during the inflammatory response that influence subsequent events (Werner & Grose, 2003; Hankenson et al., 2000; Hubner et al., 1996).

The proinflammatory cytokines Interleukin-1Beta (IL-1β), Interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) play essential roles in the signaling of biological processes during the inflammatory stage of wound healing by binding to receptors on target cells (Werner & Grose, 2003; Hubner et al., 1996; Diegelmann & Evans, 2004; Grellner, Georg & Wilske, 2000; Grellner, 2002). Collectively, this network of proinflammatory cytokines assists in controlling infection and preparing tissue for further repair by enhancing phagocytic activity, stimulating keratinocyte migration at wound edges, fibroblast chemotaxis and proliferation, breakdown of extracellular matrix proteins and by regulating the release of additional cytokines and growth factors (Villarreal et al.,
An increased expression of proinflammatory cytokines within a few hours after tissue injury has been shown to correspond to the inflammatory stage of wound healing and normal repair (Grellner et al., 2000). Conversely, a diminished production of proinflammatory cytokines in the initial stage of wound healing was found in glucocorticoid-treated mice and was associated with impaired wound healing (Hubner et al., 1996).

Studies have shown that increasing dosages of the ω-3 PUFAs EPA and DHA in the form of encapsulated fish oil to a concentration of 1% of the total phospholipid fatty acids will decrease lymphocyte proliferation and proinflammatory cytokines IL-1β, TNF-α and IL-6 by as much as 90%, 70% and 60% respectively (Meydani et al., 1991). A total daily intake of 1.6 g of EPA and 1.1 g of DHA resulted in a significant reduction in proinflammatory cytokine production after 4 weeks of consumption (Caughey et al., 1996). Based on dietary estimates by Institute of Medicine (IOM), the mean adult intake for EPA and DHA is 0.004 to 0.007 g per day and 0.052 to 0.093 g per day, respectively (2001), but a therapeutic threshold is quickly reached with dietary supplementation (Arterburn, Hall & Oken, 2006).

The exact mode of inhibition of TNF-α, IL-6 and IL-1β synthesis by ω-3 PUFAs is not completely known, but their direct effect may be due to their ability to alter eicosanoids, which help to regulate proinflammatory cytokine levels (Caughey et al., 1996). Omega-6 derived eicosanoids have been found to increase cytokine synthesis in blood and plasma. Omega-3 PUFAs compete with ω-6 PUFAs as substrates for cyclooxygenase and lipoxygenase, which results in the production of the less active prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT) than those produced by the omega-6 PUFAs.
Thus, proinflammatory cytokine production is influenced by the intake of both \( \omega-6 \) and \( \omega-3 \) fatty acids and the \( \omega-6/\omega-3 \) ratio (Grimble & Tappia, 1998; James, Gibson & Cleland, 2000; Simopoulos, 2002; Calder, 2006). Additionally, when integrated into cellular plasma membranes, these PUFAs may influence cytokine gene expression by altering membrane fluidity, cell to cell signaling, mobility of cells, interaction of receptors with their agonist, membrane function such as capping, and formation of secondary signals (Calder, 2003; Grimm, Mayer, Mayser & Eigenbrodt, 2002).

Though many studies exist regarding the correlation between increased levels of \( \omega-3 \) PUFAs and diminished proinflammatory cytokine production in peripheral blood monocytes and plasma, a few studies have shown enhancement in the production of certain proinflammatory cytokines in cells such as fibroblasts and peritoneal macrophages treated with \( \omega-3 \) PUFAs (Jia & Turek, 2004; Hardardottir & Kinsella, 1991; Petursdottir, Olafsdottir & Hardardottir, 2002). Furthermore, a positive correlation was found between collagen production in EPA-treated porcine medial collateral ligament fibroblasts and proinflammatory IL-6 production in \textit{in vitro} wounds (Hankenson et al, 2000). The mixed study findings concerning the effects of \( \omega-3 \) PUFAs on proinflammatory cytokine levels may be related to variations in study designs and cell types. Glaser et al. (1999) found no correlation between elevated proinflammatory IL-8 levels in blister fluid and levels from peripheral blood, which showed no change over time, suggesting that proinflammatory activity at wound sites either precedes or is independent of peripheral blood activity.
We attempted to clarify the effects of ω-3 EPA and DHA PUFAs on the healing of epidermal skin tissue in vivo in healthy individuals because the potential relationships among increased EPA/DHA consumption, decreased proinflammatory production and diminished wound healing had not been explored in previous studies in this population. Our hypotheses were that individuals consuming ω-3 EPA/DHA dietary supplements for 4 weeks prior to blistering would have significantly lower levels of proinflammatory cytokines at blister wound sites and a slower time to complete wound healing than those in the control group consuming a placebo for the same interval of time.

Materials and Methods

This randomized, double-blind, repeated measures, experimental study was conducted at the General Clinical Research Center (GCRC) at The Ohio State University with an independent variable (IV) of ω-3 EPA/DHA supplement and dependent variables (DV) of wound healing time and proinflammatory cytokines in blister wound fluid.

The majority of the sample was recruited from the surrounding academic area and medical center. Based on a power analysis with an estimated large effect size of .40, power at .80, and .05 significance, 14 per group were necessary to conduct a 2-group repeated measures analysis of variance (ANOVA) (Stevens, 2002). Effect size was based on documented reductions in proinflammatory cytokine levels due to ingestion of EPA and DHA (Meydani et al., 1991; Blok, Katan & van der Meer, 1996; Endres et al., 1989). One additional subject (11%) per group was added to account for possible attrition based on previous studies using a wound initiation procedure (Glaser et al., 1999) for a total number of 30 subjects. The sample included 13 men and 17 women who were randomly assigned to either the ω-3 EPA/DHA supplement or placebo group.
Participants were healthy individuals between 18-45 years of age with the ability to read and write English. Individuals were excluded if they were taking non-steroidal anti-inflammatory drugs, aspirin, lipid-lowering medications, nutritional supplements or corticosteroids and those experiencing chronic inflammatory skin diseases or were pregnant or lactating. Individuals who had immunologic related health problems such as cancer, autoimmune diseases, diabetes mellitus or peripheral vascular disease, difficulties with wound healing, surgery in the past year, self-reported current smokers or those reporting drinking 10 or more alcoholic beverages per week were also excluded.

After Institutional Review Board (IRB) approval, participants were recruited through advertisements placed in the university newspaper and on university bulletin boards. Interested individuals were evaluated for inclusion and exclusion criteria. Following a complete explanation of the study including potential risks and benefits, informed written consent was obtained. In addition to IRB approval, all research methods conducted were in compliance with the ethical rules for human experimentation stated in the 1975 Declaration of Helsinki.

Participants were assigned by computerized random sort to either experimental or control group and blinded as to treatment. During visit one at the GCRC demographic data were collected including age, gender, ethnicity and education level. Body measurements consisted of height, weight, sagittal abdominal diameter (SAD) and body mass index (BMI). All participants were given verbal and written instructions by the primary investigator (PI), who was also blinded to group assignment, to take five softgels (Group 1- ω-3 softgels, Group 2 - placebo softgels) at bedtime until study completion. A specific date to begin the softgels was assigned to each participant. A total daily intake of
1.6 g of EPA and 1.1 g DHA has been determined to decrease pro-inflammatory cytokine production in peripheral blood after 4 weeks of consumption and, thus was the chosen dosage and time frame for this study (Caughey et al., 1996). All softgels were the same in appearance and packaged in like containers by J.R. Carlson Laboratories, Inc. (Arlington Heights, IL). Verbal and written instructions were given to participants to maintain their usual diets, but to exclude fish, seafood, kelp and flaxseeds until study completion. Blood was collected for plasma fatty acid analysis after an 8-hour fast. The food frequency questionnaire (FFQ) was completed, which reflected micro and macro nutrient contents in the diet for the three months prior to study enrollment.

Four weeks after beginning the softgels each participant was admitted in the morning for a 26-hour stay at the GCRC and was discharged the following morning. Parking (or bus) expenses and controlled meals were provided. Participants received $150 after completing the 5-week study. A diagram to illustrate the design and variable measurement points during the GCRC stay and post-blistering period is provided.

\[ X = 4 \text{ weeks of } \omega-3 \text{ EPA and DHA supplement} \]
\[ \sim X = 4 \text{ weeks of placebo} \]
\[ \text{PSS = Perceived Stress Scale} \]
\[ \text{Sal. cortisol = salivary cortisol} \]

\[ \text{Blistering=creation of eight } 8 \text{ mm. blisters on nondominant forearm} \]
\[ \text{O}_1 = \text{cytokines in blisters} \]
\[ \text{O}_2 = \text{photos of healing} \]

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**Blister Initiation**

A suction blister protocol was modeled after one utilized in studies at the National Institute of Allergy and Infectious Diseases and at The Ohio State University (Glaser et al., 1999; Kuhn, DeCarlo, Hawk & Gallin, 1992; Kuhns & Gallin, 1995; Zimmerli & Gallin, 1987). A similar suction blister device was also used (Electronic Diversities, Finksburg, MD). A plastic template was taped to the volar surface of the nondominant forearm and a vacuum of 350 mm Hg was applied through a pump attached to a regulator until blisters formed (1-1½ hours) (Electronic Diversities, Finksburg, MD). The dermoepidermal junction was separated by the gentle suction and 8 sterile 8-mm blisters were formed. Fluid was aspirated from each blister (0.05-0.1 mL) with a 27 gauge needle and syringe; the blister roof (epidermis) was removed with sterile scissors. A plastic template was placed over the blister wounds. Using a syringe with an attached angiocath the template wells were filled with 0.8 to 1.0 mL of 70% autologous serum, consisting of serum from the subjects’ own blood combined with Hanks’ balanced salt solution, and the top sealed with sterile tape. The autologous serum-buffer solution was aspirated via an angiocath attached to a syringe from half the wells 5 hours after the blisters formed. In 24 hours the remaining solution was aspirated from the other 4 wells and the template removed. A standard wound care protocol was initiated prior to discharge. Participants were informed that small scabs would form over the site of the blisters and would fall off in one to two weeks.
After Discharge

Daily assessments of the healing of the superficial blister sites occurred for eight consecutive days after discharge and then every 4 days until complete healing. The PI, blinded to group assignment, utilized the single digital camera photogrammetry method of wound assessment to quantify daily wound area yet to be healed (VergeVideometer Measurement Documentation (VeV MD), Winnipeg, Manitoba, Canada). The 10 minute follow-up appointments took place in an office at the College of Nursing on The Ohio State University campus.

Measures

Omega-3 Fatty Acid Supplement

Both groups maintained their normal diet except for the exclusion of any fish, seafood, kelp or flaxseeds for the duration of the study. Group 1 subjects, the active group, received ω-3 fatty acid supplements supplied as a total daily intake of 1.6 g EPA and 1.1 g DHA in five opaque softgels (J.R. Carlson Laboratories, Inc., Arlington Heights, IL). Group 2 subjects, the placebo group, received softgels containing a total daily intake of 2.4 ml of mineral oil. Plasma fatty acid analysis was performed for both groups at baseline and again at 4 weeks to evaluate effectiveness.

Plasma Fatty Acids

The fatty acids evaluated in plasma included total ω-3 PUFAs, ω-3 EPA and DHA, total ω-6 PUFAs, ω-6 arachidonic acid (AA), and the ω-6/ω-3 ratio and were quantified by gas chromatography/mass spectrometry (GC/MS) at Metametrix Clinical Laboratory
(Norcross, GA). Fasting blood samples (1.0mL) were collected in EDTA vacutainers and centrifuged at 720 g for 30 minutes at room temperature to isolate the plasma fraction. Plasma obtained was stored at -80°C prior to analysis.

Sample preparation consisted of a methyl esterification reaction followed by liquid/liquid extraction prior to analysis. To a 16 x 100 mm glass screw top tube, 2 mL of internal standard solution was added to 200 µL of plasma. Samples were vortex mixed followed by a 1.5 mL addition of reaction solution (1:3 v/v, acetyl chloride:iso-octane). Sample tubes were capped, vortex mixed, and placed in a heat block at 100 ºC for 1 hour. Samples were cooled and neutralized with 4 mL of potassium carbonate (100 mg/L in H2O). The samples were vortex mixed and centrifuged at 3500 RPMs for 10 minutes. The top layer of the biphasic sample solution was extracted into amber auto-sampler vials and loaded on instrument. The samples were analyzed using an Agilent 6890N GC with autosampler and an Agilent 5973N mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA). The analytical separation was performed on a HP-23 (Cis/Trans FAME capillary column) 60 m x 0.25 mm x 0.25 mm film thickness. The instrumental and data analysis were performed using MSD Chem Station (CV < 10%).

**Stress**

Increased stress has been associated with diminished proinflammatory production and delayed wound healing and thus, is essential to consider in an *in vivo* wound healing study (Kiecolt-Glaser, 2005; Head, Farrow, Sheridan & Padgett, 2006). In this study all subjects completed a Perceived Stress Scale (PSS) on admission to the GCRC (after four weeks of treatment or placebo). The PSS instrument corresponded to the theoretical definition of stress utilized in the study and captured the subject’s cognitive appraisal of
stress (Cohen, Kamarck & Mermelstein, 1983). It is a 10-item questionnaire with a scale of 0 (never) to 4 (very often) that measures how unpredictable, uncontrollable and overloaded one perceives their daily life in the previous month. Possible scores range from 0-40 with a higher score suggesting greater perceived stress. The PSS has demonstrated a high internal consistency with a Cronbach $\alpha = .91$ (Glaser et al., 1999).

In addition, salivary cortisol was measured on admission to the GCRC and at 5 and 24 hours post blistering to appraise the potential stressful effects of the blistering procedure. Saliva was obtained from a dental cotton roll and assayed using the solid-phase radioimmunoassay procedure, wherein I-125 labeled cortisol competes for a fixed time with cortisol in the patient sample for antibody sites (Diagnostic Products Corporation, Los Angeles, CA). Mean %CV was 4.66 in the current study. Salivary cortisol is a valid and reliable measure of unbound hormone in the blood and reflects the physiological stress response (Kirschbaum & Hellhammer, 1994). Only unbound cortisol reaches target tissue and elicits glucocorticoid effects.

**Proinflammatory Cytokines**

Proinflammatory cytokines (IL-1$\beta$, TNF$\alpha$ and IL-6) were measured in the blister fluid at 5 and 24 hours post blister formation. Determinations were made using electrochemiluminescence Multiplex System Sector 2400 imager (Meso Scale Discovery (MSD), Gaithersburg, MD). Samples were assayed in duplicate using the Human ProInflammatory II 4-Plex Ultra-Sensitive Kit measuring IL-1$\beta$, IL-6, and TNF-$\alpha$, which demonstrates a sensitivity range from 0.7 pg/ml to 2.4 pg/ml. Coefficient variations of standard curve data provided by MSD were largely below 5%. Within the first 6 plates,
the CVs of the spiked controls (24 measurements for each control) were less than 10% for all cytokines. The interplate CVs were slightly higher than the intraplate CVs.

**Wound Healing**

This was defined as the advance of the wound margins toward the wound center. Daily area yet to be healed was measured by single camera digital photogrammetry (SCP) and the wound measurement software (VeV MD, Vista Medical, Winnipeg, Manitoba, Canada) on 8 consecutive days after blister initiation and every 4 days thereafter until complete (100%) healing occurred. An orientation card of known dimensions was placed next to the blister sites in view of the camera. The blister images were then downloaded to the VeV MD computer software program. Blister wounds were compared to the known size of the orientation card per the computer software. The wound perimeters were outlined on the computer with a cursor by the PI and the sum of the areas yet to be healed for all 8 blisters was then calculated. The VeV measurement system is a noncontact method to compute wound length, width and surface area immediately, print color reports to document the healing process, store additional patient data and is easily transported.

Total surface area yet to be healed for all eight blisters at each time point for 15 randomly selected subjects was recalculated by the same PI who was blinded to group assignment (active or placebo) and compared to initial calculations to evaluate intrarater reliability of the VeV measurement system. Significant Pearson’s correlation coefficients ranged from .59 to .99 with an average of .88 signifying that the VeV measurement system demonstrated high test-retest intrarater reliability in the current study.
Statistical Analyses

Statistical analyses were conducted using the SPSS statistical package. Descriptive statistics were used to characterize the subjects. To examine the two groups for differences in sociodemographic information and body measurements, chi-square (nominal and ordinal data) and t-test (interval or ratio data) were used. Macro and micronutrient data regarding potential confounding nutritional influences were generated from the FFQs completed at baseline and compared between the intervention and control groups with t-tests, as were PSS scores. A 2-group (with and without \( \omega-3 \) fatty acid EPA/DHA supplement) across time (at GCRC admission, 5 and 24 hours after blistering) within and between group ANOVA was used to compare salivary cortisol levels. A t-test was used to examine any differences between groups on plasma fatty acid levels (0 and 4 weeks) and a paired t-test was used to evaluate within group differences at the two time points. Plasma fatty acid analyses were completed to evaluate compliance with the protocol and effectiveness of supplementation. Significance levels were set a priori at \( \alpha = 0.05 \).

To evaluate whether the quantities of cytokines at the blister wound sites differed by group at 5 and 24 hours after blister wound initiation, we applied a t-test. Significance levels were set a priori at \( \alpha = 0.05 \).

The differences between the active and placebo groups in the total area yet to be healed (cm\(^2\)/day) for all 8 blisters at each time point were compared with a t-test. A wound size of 0 cm\(^2\) was considered complete wound closure by SCP. A total lapsed time (days) from initiation of blisters to first occurrence of 0 cm\(^2\) wound size was also compared between the two groups and evaluated with a t-test.
Results

Demographics, Nutritional and Stress Influences

Demographic characteristics describing participants in the active and placebo groups are displayed in Table 1 with similar data for both groups. Nutritional data obtained from the FFQs showed no statistically significant differences between the active and placebo groups in regard to nutrients that could potentially influence wound healing (Table 2).

Statistical analyses of PSS scores (at GCRC admission) and salivary cortisol levels (at GCRC admission and at 5 and 24 hours post blistering) demonstrated that participants in both groups were similar in their psychological and physiological reactions to stress. Salivary cortisol levels at times 1 and 3 were significantly higher than at time 2 for all participants (F=17.558, df=1, p<.001), however the interaction between treatment groups and salivary cortisol levels was not significant (Table 3). The correlation between PSS scores and salivary cortisol levels was not significant perhaps because PSS scores reflect perceived psychological stress in the preceding month while salivary cortisol levels represent the physiological stress response at the time the salivary sample was collected.

Plasma Fatty Acid Measures

As expected, at 4 weeks post enrollment, those who received the EPA/DHA supplement for four weeks, had significantly higher plasma EPA (F=58.56, df=1,28, p<.001), DHA (F=61.87, df=1,28, p<.001), total ω-6 fatty acids (F=7.15, df=1,28, p<.001), total ω-3 fatty acids (F=64.68, df=1,28, p<.001), ratio of AA to EPA (F=41.60, df=1,28, p<.001) and ratio of total ω-3 to total ω-6 fatty acids (F=120.60, df=1,28, p<.001) than the placebo group (Table 4).
Fatty acid plasma levels were also examined for each individual to evaluate change from baseline to four weeks. Not surprisingly, the change in plasma fatty acid measures for participants who received the EPA/DHA supplement was significant for EPA ($t=-6.70$, $df=14$, $p<.001$), DHA ($t=-5.93$, $df=14$, $p<.001$), AA ($t=3.64$, $df=14$, $p<.05$), total ω-6 fatty acids ($t=3.58$, $df=14$, $p<.05$), total ω-3 fatty acids ($t=-6.62$, $df=14$, $p<.001$), ratio of AA to EPA ($t=5.75$, $df=14$, $p<.001$) and ratio of total ω-3 to total ω-6 fatty acids ($t=8.3$, $df=14$, $p<.001$). There were no significant individual differences found in the placebo group for changes in plasma fatty acid content from baseline to four weeks. Thus, it appears that EPA/DHA supplements were taken appropriately to yield these increases.

**Proinflammatory Cytokine Responses**

As anticipated, IL-1β, IL-6 and TNF-α proinflammatory cytokines levels in blister wound serum increased across the two time points, 5 to 24 hours post blistering, for both groups. Unexpectedly however, and contrary to our original hypothesis, participants who received EPA/DHA supplementation to their diet for 4 weeks, had higher rather than lower levels of the three proinflammatory cytokines in the blister fluid at 24 hours post blistering than the placebo group, although not all statistically significant (Figure 2).

A t-test revealed that participants in the active group who consumed the ω-3 fatty acid EPA/DHA supplement for 4 weeks had significant increases in IL-1β cytokine levels at blister wound sites at 24 hours post blistering when compared to the placebo group ($t=2.52$, $df=25$, $p<.05$) (Figure 2a). To examine the relationships among predictor variables and IL-1 values at 24 hours post blistering fifteen possible variables were chosen for a forward stepwise multiple regression procedure. Interestingly, using that procedure the model chose gender and the ratio of AA/EPA at week 4 as the two primary
predictors of IL-1 levels at 24 hours post blistering. Together those two variables explained 47.4% of the variance in the IL-1 values at that time point. Males and participants with lower AA:EPA ratios had higher IL-1 levels. In addition, lower AA:EPA ratios were significantly correlated (p<.001) with the active treatment group.

Similarly, there were higher quantities of IL-6 cytokines (Figure 2b) in wound serum for both groups across time, but again, participants who consumed the EPA/DHA supplement for 4 weeks produced higher levels of IL-6 cytokine levels in blister wound serum at 24 hours post blistering than the placebo group, though not statistically significant. And finally, following the same pattern, the proinflammatory cytokine TNF-\(\alpha\) levels were higher across time for both groups, but once again the active group produced nonsignificant higher levels of TNF-\(\alpha\) than the placebo group (Figure 2c).

**Wound Healing Measures**

The overall wound size in cm\(^2\) for participants who received the \(\omega-3\) fatty acid EPA/DHA (fish oil) supplement for 4 weeks compared to those who consumed the placebo for the same interval, was not significantly different at any time point.

Time to complete wound healing was then evaluated (Figure 3). The number of days to complete wound healing (100% closure) was not significantly different between the two groups, although the mean number of days to complete healing for those who consumed the EPA/DHA supplement for 4 weeks prior to the blistering procedure, was slightly longer (10.7) than the mean (9.8 days) for those in the placebo group.
Discussion

Past research studies related to effects of dietary ω-3 fatty acids on wound healing have been limited and have produced conflicting results, most likely due to variations in study designs, dosages and durations of supplement intake or in vitro cell exposure. In the present study, we observed that with an EPA/DHA dose of 1.6 g/d and 1.2 g/day respectively, there were significant increases in plasma fatty acids levels for both EPA and DHA from baseline to four weeks for individuals in the active group, which were consistent with several past studies examining various physiological changes resulting from EPA/DHA supplementation (see review Arterburn et al., 2006). In addition, there were significantly diminished quantities of AA and ratio of total ω-6 to ω-3 fatty acids in individuals consuming the EPA/DHA supplement after four weeks, which were outcomes also aligned with a number of previous studies (Caughey et al., 1996; Mayer et al., 2003). Collectively, the plasma fatty acid evaluations from the present study validated the usage of the supplements by the participants in the active group during the study interval and the ability of the supplied dose to alter plasma fatty acid levels. No significant changes were evident in any plasma fatty acid levels for participants in the placebo group who consumed the placebo, mineral oil, for four weeks.

Interestingly, one recent study revealed that when EPA and DHA were provided as supplements to the diet in a total dose as low as 1 g/day in the form of ethyl esters, which are taken up much more slowly than the more rapidly absorbed triacylglycerols from fish, EPA and DHA plasma levels rose from 0.6% to 1.4% and 2.9% to 4.3 % respectively within only 10 days (Rupp, Wagner, Rupp & Schulte, 2004). Additionally, both PUFAs returned to near baseline values within 10 days of discontinuing the supplements. In the
current study, the decision to use the particular dosage of EPA/DHA, obtained from fish oil, was based on work by Caughey et al. (1996) that showed plasma proinflammatory cytokine inhibition after 4 weeks with that same quantity. It may be beneficial in future studies to use dosages of EPA/DHA in smaller quantities, in the form of both ethyl esters and triacylglycerols, for shorter time periods in a stratified design to more clearly delineate their influence on proinflammatory cytokine responses at wound sites and in peripheral blood to compare values and to determine minimal effective quantities.

In this study, IL-β, IL-6 and TNF-α proinflammatory cytokine production in blister fluid was significantly increased across time from 5 to 24 hours after blister wound initiation in both groups in response to tissue trauma, which is consistent with earlier studies using suction blister models (Hubner et al., 1996; Glaser et al., 1999; Kuhns et al., 1992). Interestingly, and contrary to our original hypothesis, participants who took the EPA/DHA supplement for the 4 week period, had statistically significant higher IL-1β proinflammatory cytokine levels at blister sites at 24 hours post blistering, when compared to the placebo group. Nevertheless, these findings are consistent with a study that observed higher IL-1 and TNF-α expression with EPA treatment in both nonirradiated and UVB-irradiated keratinocytes (Pupe et al., 2002). Additionally, a recent study found that EPA-induced collagen in fibroblasts could be regulated with different fatty acid ratios, which was thought to be via PGE2 and PGE receptor subtype responses (Jia & Turek, 2004). Because IL-1 assists in regulating fibroblast chemotaxis and the production of collagen it can be posited that its upregulation at the wound site, as a result of EPA/DHA dietary supplementation, could be another pathway to influence collagen formation and thus, would be important to investigate in future studies.
In the present study, again divergent to our original hypothesis, higher quantities of IL-6 proinflammatory cytokines were also evident in blister fluid at 24 hours post blistering in the active group, though not statistically significant, when compared to the placebo group. This finding is however, rather consistent with two studies that observed a positive correlation with ω-3 PUFA-exposed medial collateral ligament fibroblasts, IL-6 production, and collagen formation in *in vitro* wounds with increased proliferation and migration by fibroblasts (Jia & Turek, 2004; Hankenson et al., 2000). These findings were valuable because healthy collagen is vitally important to skin healing and scar formation that is strong, but not excessive or fibrotic. The amount of collagen formation necessary for efficient healing varies with the type of injury (Jia & Turek, 2004). An increased production of healthy collagen is necessary for connective tissue healing while excessive amounts may cause fibrosis in internal organs or keloids on the skin surface. Although our study findings do not support our initial hypothesis they do bear up the correlation between EPA/DHA supplementation and increased IL-6 levels in blister fluid at 24 hours post blistering, but slightly slower wound healing. Although we did not measure fibroblast collagen formation in this study, it would be important to assess in future studies to substantiate the promising link among dietary supplementation of EPA/DHA, IL-6 upregulation at wound sites, increased healthy collagen formation and scar tissue integrity.

TNF-α levels were also found to be nonsignificantly higher in blister serum in the active group at 24 hours post blistering when compared to the placebo group in this study. These findings were similar to those from a study by Pupe et al. (2002), which found that EPA treatment of human keratinocytes in skin resulted in higher TNF-α levels.
both in nonirradiated and UVB irradiated keratinocytes. An interesting finding in the present study was that increased levels of TNF-α in blister fluid at 24 hours post blistering were associated with slightly slower healing in the active group, which differs from a prior study that demonstrated diminished levels of TNF-α, from neutrophils and macrophages, in healing-impaired glucocorticoid-treated mice (Hubner et al., 1996).

The present study design was the first to explore the influence of dietary ω-3 PUFA’s influence on skin expression of proinflammatory cytokines in healthy human skin in response to wounding. Just as there have been mixed results in earlier studies, which examined the outcome of EPA/DHA supplementation on proinflammatory cytokine production in peripheral blood monocytes or plasma (see review Blok et al., 1996; Caughey et al., 1996; Petursdottir et al., 2002; Wu, Han, Meydani & Meydani, 2004), there have also been variable results in a limited number of studies that have investigated dietary EPA/DHA’s effect on skin expression of proinflammatory cytokines in in vitro or animal models (Pupe et al., 2002; Shahbakhti et al., 2004) and none in healthy human skin. Several of these studies were observing the effects in UVB or UVC irradiated skin. Further research is needed to clarify the effects of dietary EPA/DHA supplementation on various cell types at the wound site and their subsequent influence on proinflammatory cytokine production. In addition, it would be important to always evaluate the time to complete skin healing, levels of collagen production, and scar appearance and integrity to more clearly establish how to manipulate the skin healing process via administration of dietary PUFAs.

The variation in salivary cortisol levels, with significantly higher measures for all participants at times 1 and 3 when compared with time 2, was likely due to the fact that
salivary samples for times 1 and 3 were collected in the early mornings when cortisol levels are known to be higher than in the early afternoon when the sample for time 2 was collected (Kirschbaum & Hellhammer, 1994).

In this study, the single digital camera photogrammetry (SCP) method of assessing wound healing demonstrated that although the time to complete wound healing (100% closure) for all eight blisters did not differ significantly between the two groups, those in the group who consumed the EPA/DHA supplement for 4 weeks, took approximately one day longer to heal than those in the placebo group, which could translate to clinical significance. This important finding illustrated that after controlling for other known influencing variables such as stress, EPA/DHA PUFA dietary supplementation, in a dose that affected plasma fatty acid content after 4 weeks of consumption, did influence time to complete healing, though not significantly. The higher levels of proinflammatory cytokines at 24 hours post blistering in the active group suggest a more intense early inflammatory response. Therefore, it could have taken longer for the exudative phase of the inflammatory response to resolve, which resulted in a slightly longer time to complete wound closure. This particular finding was consistent with a previous study of wound healing in dog models, which observed a longer time for re-epithelialization in surgical wounds after ω-3 PUFA supplementation when compared with other dietary alterations (Mooney et al., 1998). In addition, topical administration of ω-3 PUFAs applied to surgical wounds of mice was associated with slower wound closure in the first 10 days after surgery (Cardoso, Souza, Ferro, Favoreto & Pena, 2004). On the other hand, a study that investigated PUFAs influence on wound healing in rat intestinal epithelium, found improved reconstitution of epithelial integrity following mucosal injury with both
ω-3 and ω-6 PUFA-treated cells (Ruthig & Meckling-Gill, 1999). Once again, these variable findings support the need for additional well-designed research studies focusing on the influence PUFAs have on wound healing, so that data can be accurately compared in similarly designed studies.

Delayed wound healing is a serious, costly quandary that affects the individual, family and healthcare community. Successful wound healing is a complex process that occurs in sequential, but overlapping stages. Factors such as malnutrition, excessive alcohol and tobacco consumption, and prolonged stress have been found to diminish the healing process through various psychoneuroimmunological pathways (Kiecolt-Glaser et al., 1998). For example, high levels of cortisol, present in individuals who experience chronic stress, have been associated with delayed wound healing as a result of diminished proinflammatory cytokine production in wound serum (Glaser et al., 1999). Omega-3 PUFAs EPA and DHA have also been associated with diminished systemic proinflammatory cytokine production and thus, have been found to be beneficial for diseases associated with increased inflammation such as rheumatoid arthritis and coronary heart disease (Meydani et al., 1991; Caughey et al., 1996; Wu et al., 2004).

In summary, this study is the first to examine the effects of dietary ω-3 (EPA/DHA) fatty acid supplementation on proinflammatory cytokine production in blister wound fluid and subsequent influences on skin healing in a healthy human population. The results presented from this study linked the EPA/DHA dietary supplementation in the active group, which resulted in lowering of the AA:EPA ratio from baseline, with a significantly higher production of the proinflammatory cytokine IL-1β at blister wound sites at 24 hours post blistering when compared with the placebo group, and
nonsignificantly slower wound healing. Although not consistent with the original hypothesis, the study findings still support previous evidence that dietary ω-3 fatty acids may be promising nonpharmaceutical adjuncts when designing noninvasive, therapeutic interventions to improve skin healing. They could provide a mechanism to manipulate particular aspects of the healing process, such as collagen production, which is associated with the upregulation of specific proinflammatory cytokines. Adequate production of healthy collagen is important for connective tissue repair while an overproduction is associated with problems such as keloid formation and fibrosis of vital organs after injury. Manipulating PUFA dosages may be beneficial in preventing the high incidence of keloid configurations after skin trauma in African American and Hispanic populations, therefore the continual study of their effects on collagen production in humans, and subsequent scar tissue development, would contribute to improved wound care for vulnerable populations.
### Demographic Characteristics of Participants (N=30)

<table>
<thead>
<tr>
<th>Demographic Category</th>
<th>Active (n = 16)</th>
<th>Placebo (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean years (SD)</td>
<td>23 (5.4)</td>
<td>28 (8.4)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>44</td>
<td>43</td>
</tr>
<tr>
<td>White (%)</td>
<td>76</td>
<td>79</td>
</tr>
<tr>
<td>African American (%)</td>
<td>12.5</td>
<td>7</td>
</tr>
<tr>
<td>Asian (%)</td>
<td>12.5</td>
<td>14</td>
</tr>
<tr>
<td>Educational level (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>College graduates</td>
<td>39</td>
<td>57</td>
</tr>
<tr>
<td>Undergrad. Students</td>
<td>56</td>
<td>43</td>
</tr>
<tr>
<td>High school graduates</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>
## Table 2.2
Dietary Characteristics of Participants @ Baseline
(N=30) Mean (SD)

<table>
<thead>
<tr>
<th></th>
<th>Active (n = 16)</th>
<th>Placebo (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFQ data (visit 1) estimates daily intake for preceding 3 mos.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean vitamin C mg/day (SD)</td>
<td>125.3 (69.0)</td>
<td>136.9 (84.5)</td>
</tr>
<tr>
<td>Mean protein g/day (SD) * (RDAs 46-56 g/d)</td>
<td>91.3 (32.1)</td>
<td>91.4 (53.5)</td>
</tr>
<tr>
<td>Mean kilocalories/day (SD) ** (RDAs 1,848 – 3,141 kcal/day)</td>
<td>2046.6 (562.6)</td>
<td>2188.0 (1084.4)</td>
</tr>
<tr>
<td>Mean EPA g/day (SD)</td>
<td>.09 (.17)</td>
<td>.04 (.04)</td>
</tr>
<tr>
<td>Mean DHA g/day (SD) ** (AI .50 g/day EPA + DHA)</td>
<td>.24 (.40)</td>
<td>.09 (.07)</td>
</tr>
<tr>
<td>Data below from demographic questionnaire (visit 1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cups of caffeine/day (SD)</td>
<td>1.4 (1.8)</td>
<td>1.6 (1.5)</td>
</tr>
<tr>
<td>Mean alcoholic drinks/wk. (SD)</td>
<td>1.2 (2.2)</td>
<td>2.2 (3.7)</td>
</tr>
</tbody>
</table>

Dietary Characteristics of Participants
RDA = Recommended Dietary Allowances Al= Acceptable Intake
* Based on 0.8 g/kg body weight for reference body weight for adults 18->70 yrs. of age.
** Estimated Energy Requirements (EER) for men and women 30 years of age; For each year below 30, add 7 kcal/day for women and 20 kcal/day for men. For each year above 30, subtract 7 kcal/day for women and 20 kcal/day for men. Requirements vary with BMI and physical activity level.
*** SOURCE: International Society for Study of Fatty Acids and Lipids; Recommendations for Dietary Intake of Polyunsaturated Fatty Acids in Healthy Adults (2004)
### Stress Characteristics of Participants

<table>
<thead>
<tr>
<th></th>
<th>Active (n = 16)</th>
<th>Placebo (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean PSS Scores (SD)</strong></td>
<td>13 (3.4)</td>
<td>12 (6.5)</td>
</tr>
<tr>
<td>Higher score (0-40) = greater stress over preceding month</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean Salivary Cortisol Levels</strong> (µg/dl; SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time 1 (GCRC entry-a.m.)</td>
<td>0.40 (0.2)</td>
<td>0.39 (0.2)</td>
</tr>
<tr>
<td>Time 2 (5 hrs. post blistering-p.m.)</td>
<td>0.16 (0.2)*</td>
<td>0.14 (0.1)*</td>
</tr>
<tr>
<td>Time 3 (24 hrs. post blistering-a.m.)</td>
<td>0.34 (0.2)</td>
<td>0.39 (0.3)</td>
</tr>
</tbody>
</table>

Table 2.3
Stress Characteristics of Participants
* = significantly different from both Times 1 and 3 within groups (p < .001)
Table 2.4
Plasma Fatty Acid Measurements

AC = Active; PL = Placebo; EPA = eicosapentanoic acid; DHA = docosahexaenoic acid; AA = arachidonic acid
a = significantly different from baseline – within group (p < .05)
b = significantly different between groups @ week 4 (p < .05)

<table>
<thead>
<tr>
<th>Fatty Acids (µM/L)</th>
<th>Baseline AC (n=16)</th>
<th>Baseline PL (n=14)</th>
<th>Week 4 AC (n=16)</th>
<th>Week 4 PL (n=14)</th>
<th>Change AC (n=16)</th>
<th>Change PL (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPA</td>
<td>20.9 ± 14.9</td>
<td>22.0 ± 29.7</td>
<td>125.2 ± 53.1ab</td>
<td>15.1 ± 8.6b</td>
<td>99.9 ± 57.8ab</td>
<td>-6.9 ± 30.0b</td>
</tr>
<tr>
<td>DHA</td>
<td>110.1 ± 38.4</td>
<td>102.2 ± 41.2</td>
<td>179.4 ± 42.2ab</td>
<td>83.8 ± 17.9b</td>
<td>67.3 ± 43.9ab</td>
<td>-18.4 ± 35.5b</td>
</tr>
<tr>
<td>AA</td>
<td>668.7 ± 144.1</td>
<td>644.9 ± 84.4</td>
<td>576.8 ± 121.1ab</td>
<td>651.0 ± 102.2b</td>
<td>-77.8 ± 105.5ab</td>
<td>6.1 ± 70.4b</td>
</tr>
<tr>
<td>Total ω-6</td>
<td>2490.6 ± 482.1</td>
<td>2599.6 ± 357.0</td>
<td>2218.7 ± 403.1ab</td>
<td>2589.8 ± 349.9b</td>
<td>-259.1 ± 290.5ab</td>
<td>-9.8 ± 255.2b</td>
</tr>
<tr>
<td>Total ω-3</td>
<td>182.2 ± 57.3</td>
<td>187.7 ± 85.0</td>
<td>371.8 ± 94.7ab</td>
<td>158.3 ± 31.2b</td>
<td>181.3 ± 107.7ab</td>
<td>-29.3 ± 83.9b</td>
</tr>
<tr>
<td>Ratios AA/EPA</td>
<td>43.8 ± 24.3</td>
<td>49.0 ± 21.8</td>
<td>5.5 ± 2.7ab</td>
<td>57.2 ± 32.0b</td>
<td>-38.1 ± 25.7ab</td>
<td>8.3 ± 22.9b</td>
</tr>
<tr>
<td>Ratios ω-6/ω-3</td>
<td>14.4 ± 3.2</td>
<td>15.2 ± 4.0</td>
<td>6.3 ± 1.6ab</td>
<td>16.8 ± 3.5b</td>
<td>-8.0 ± 3.7ab</td>
<td>1.6 ± 2.6b</td>
</tr>
</tbody>
</table>
Figure 2.1
Conceptual model of biophysiological components of study
Figure 2.2
Proinflammatory Cytokine Production from Wound Serum. Mean Values
Figure 2.3
Days to Complete Healing

p > .05


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CHAPTER 3
METHODS FOR SUCTION BLISTER WOUND MEASUREMENT IN RESEARCH

Introduction

Several important decisions are made during the design phase of an in vivo research study investigating the wound healing process. One of those decisions involves choosing a suitable representative wound model so that the specific physiological process of interest can be accurately studied. For example, a suction blister model may be used to investigate epidermal re-epithelization and/or the inflammatory stage of wound healing (Koivukangas & Oikarinen, 2003). A second decision entails defining the concept of wound healing so that its meaning is clearly communicated to the reader. As an illustration, the concept of wound healing in a study utilizing a blister model could be defined as time in days to complete (100%) wound closure and measured daily as surface area (cm²) yet to be healed. A third critical decision that occurs during the study design phase requires selecting the instrument to accurately measure the concept of wound healing as defined in the study. Precise measurement instrumentation for blister wound calculations is the primary focus of this article.

An accurate measurement of wound size is important to the management of acute and chronic wounds in the clinical setting, but it is a crucial principle to consider in the research setting. Precise wound dimensions are necessary for the production of accurate, reliable data and thus, to correctly depict the healing trajectory. There are several
measurement techniques and tools available to compute the size of a wound. The purpose of this article is to describe the suction blister wound model and appraise the current opinions and data concerning existing measurement instruments to utilize in research studies exploring the wound healing process. Recommendations will then be made for the most appropriate devices to use in studies utilizing a blister wound model. In addition, the pros and cons of employing one of the recommended instruments to measure blister wounds, the Verge Videometer Measurement Documentation (VEV MD) system, in a recent study “Omega 3 Fatty Acids Effect on Wound Healing” will be discussed.

The Suction Blister Wound Model

In the design phase of a wound healing research study it is vitally important to choose an *in vitro* or *in vivo* wound healing model that accurately depicts the physiological process of interest. The resulting data will then assist in suitably answering the specific research questions. For example, an *in vitro* model that utilizes a single cell system model type may be suitable when studying biological processes such as cell migration, proliferation, protein synthesis or wound contraction (Gottrup, Agren, & Karlsmark, 2000). *In vivo* animal or human models designed to study the acute or impaired wound healing process may examine excisional wounds, incisional wounds, burn wounds or superficial wounds. In a superficial tissue wound model a researcher may create the environment with suction blister wounds, dermatome wounds, abrasions or tape stripping (Gottrup et al., 2000).

If the focus of a research question is on the physiological processes occurring during or influenced by the initial inflammatory stage of wound healing such as proinflammatory cytokine production or epidermal regeneration then the *in vivo* blister
wound model is a particularly suitable choice (Alexis, Wilson, Todhunter, & Stiller, 1999; Gottrup et al., 2000; Koivukangas & Oikarinen, 2003). Epidermal suction blistering has been utilized for many years in the research setting (Falabella, 2000; Kiistala & Mustakallio, 1964) to separate the dermoepidermal junction and has evolved over time to facilitate the study of collagen synthesis, quantify cytokines in skin, evaluate the influence of various pharmacological agents on skin and measure wound healing (Glaser et al., 1999; Ihlberg et al., 1993; Rommain, Brossard, Piron, & Smets, 1991; Svedman, Svedman, & Njalsson, 1991).

Blister creation can be achieved with instrumentation devices that produce 150 to greater than 500 mm Hg negative pressure over a template that contains uniform sized openings from 3-10 mm. in diameter (Alexis et al., 1999; Falabella, 2000). The vacuum separates the epidermis from the dermis at the lamina lucida in the basement membrane and creates small, standardized epidermal blisters filled with tissue fluid (Koivukangas & Oikarinen, 2003). The heated, higher pressure device producing 508 mmHg of negative pressure created uniform blisters in 24-40 minutes in subjects who reported no pain or discomfort during the procedure (Alexis et al., 1999).

The advantages of employing the blister wound model in research studies are that the wounds are of equal size, produced at the same time under identical conditions making comparisons more reliable. The disadvantages are that the blisters represent only superficial wounds and therefore study results and therapeutic implications can only be applied to similar types of wounds in the clinical setting (Gottrup et al., 2000).
Wound Measurement Tools Appropriate for the Suction Blister Model

Multiple measurement techniques are available to calculate the size of a wound, but may not be appropriate to precisely measure the surface area of small superficial blister wounds specifically created to study a certain physiological process during wound healing. A review of the literature supports the appropriateness of four primary measurement tools for wound healing research because of the excellent reliability and validity they possess in measuring superficial surface area (Goldman & Salcido, 2002; Langemo et al., 1998; Plassmann & Jones, 1998; Thawer, Houghton, Woodbury, Keast, & Campbell, 2002). They are the manual wound tracing technique with digital planimetry area calculation, stereophotogrammetry (SPG), single digital camera photogrammetry (SCP) and the structured light technique (Table 1).

In addition to precision, a study’s definition of wound healing, cost parameters, and ease of use are criteria to consider when deciding on the measurement tool of choice. Although all four methods could be utilized to calculate the surface area of blister wounds in wound healing research it is important to consider the pros and cons of each before making a final decision (Table 2).

The SCP method, in the form of the Verge Videometer (VeV) measurement system, was chosen for the study ‘Omega-3 Fatty Acid Effect on Wound Healing’ primarily because it demonstrated the highest interrater reliability and the most precise calculations when measuring smaller wounds when compared to the manual wound tracing technique with digital planimetry area calculation (Thawer et al., 2002). In addition, the required equipment was lightweight and simple to transport.
Single Digital Camera Photogrammetry-The VeV Measurement Tool

The VeV measurement documentation system was developed in 1999 (Verg Inc./Vista Medical Ltd., Winnipeg, Manitoba, Canada) and was designed as a noncontact method for precise wound measurement, assessment, documentation and tracking of healing progression over time (Keast et al., 2004; Williams, 2000). The initial start-up package requires the purchase of the VeV computer software package. In addition, a digital or video camera and a computer, all with recommended minimum requirements, are necessary purchases (Thawer et al., 2002; Williams, 2000).

An orientation card of known dimensions that approximates the size of the wound is placed in the same plane as the wound. The digital images are captured by the camera and transferred to the computer (Thawer et al., 2002). The computer software analyzes the orientation card dimensions by photogrammetry and calibrates the system (Goldman & Salcido, 2002). The wound perimeter is outlined with a computer-pointing device on the computer screen by the operator and the calculations of the wound area and perimeter are then displayed (Goldman and Salcido, 2002). The VeV measurement system also has the ability to evaluate wound length, width, depth, volume, hue, and condition of surrounding tissue.

In a 2002 study (Thawer, et al.) a comparison was made between the SCP (VeV Measurement Documentation, Vista Medical, Winnipeg, Manitoba, Canada) method for wound size measurement and the known reliable and valid digital planimetry method (Planix 7 Tamaya Digital Planimeter accuracy of ± 0.2% and resolution of 0.1 cm³). Concurrent validity of the VeV method compared to the digital planimetry technique using a single assessor and the average of three repeated measurements were excellent
when evaluating surface area of human wounds using the intraclass correlation coefficient (ICC). The ICC is a reliability coefficient that is calculated using variance estimates from analysis of variance (ANOVA). In the concurrent validity study, ICC was 0.94 for single measurement and 0.98 for an average of 3 measurements in human wounds.

Intrarater reliability for single surface area measurements of both human and animal wounds using either the manual or computerized technique was excellent (ICC (3,1) > 0.75) (Thawer, 2002). When measuring the surface area of animal wounds, which were smaller, the computerized SCP method was slightly more precise than the digital planimetry method. SCP demonstrated an ICC of 0.99 for both human and animal wounds with standard error of measurement (SEM) of 0.18 and 0.0096 respectively (Thawer et al., 2002). In addition, the SCP does not require direct contact with the wound bed, as does the planimetry method, thus there is less risk of contamination and damage to the wound bed and less pain for the research subject (Thawer, et al., 2002).

Although more costly than the digital planimetry method (approximately $5000 for the software package in addition to the digital camera and laptop computer), the SCP VeV measurement system has been shown to provide a higher degree of reliability and precision compared with other wound measurement methods, which is essential for the research setting (Williams, 2000; Thawer, et al., 2002; Langemo et al., 1998). It is a noncontact wound measurement technique, computes wound length, width and surface area immediately, can print color reports to document the healing process, is flexible in its use and enables the researcher to store a variety and abundance of data. The multiple
wound assessment abilities of the VeV measurement made it an ideal choice for the measurement of suction blister wounds in the study ‘Omege-3 Fatty Acids Effect on Wound Healing’.

Wound Measurement Methodology-Omega-3 Fatty Acids Effect on Wound Healing

Design

This 2-group (omega-3 supplement or placebo), randomized, double-blind, repeated measures, experimental study was conducted at the General Clinical Research Center (GCRC) at The Ohio State University with an independent variable of ω-3 eicosapentaenoic and docosahexaenoic acids (EPA/DHA) as a dietary supplement and dependent variables of wound healing time and inflammatory stage of wound healing measured by proinflammatory cytokines. The primary aim of the study was to evaluate effects of 4 weeks of ω-3 dietary supplementation on wound healing and proinflammatory cytokines in blister wound serum.

Sample

Participants were healthy individuals between 18-45 years of age with the ability to read and write English. Individuals were excluded if they were taking non-steroidal anti-inflammatory drugs, aspirin, lipid-lowering medications, nutritional supplements or corticosteroids and those experiencing chronic inflammatory skin diseases or were pregnant or lactating. Additional exclusion criteria were those who had immunologic related health problems such as cancer, autoimmune diseases, a history of diabetes mellitus or peripheral vascular disease, difficulties with wound healing, surgery in the past year, self-reported current smokers or those reporting drinking 10 or more alcoholic beverages per week.
Procedures- Suction Blister Creation

A suction blister protocol was modeled after one utilized in studies at the National Institute of Allergy and Infectious Diseases and at The Ohio State University (Glaser et al., 1999; Kuhns, DeCarlo, Hawk, & Gallin, 1992; Kuhns & Gallin, 1995; Zimmerli & Gallin, 1987). A similar suction blister device was used in the omega-3 study (Electronic Diversities, Finksburg, MD). A plastic template was taped to the volar surface of the nondominant forearm and a vacuum of 350 mm Hg was applied through a pump attached to a regulator until blisters formed (1-1½ hours) (Electronic Diversities, Finksburg, MD). The dermoeipidermal junction was separated by the gentle suction and 8 sterile 8-mm blisters were formed. To evaluate and compare the initial inflammatory response in the wound healing process between the two groups, three proinflammatory cytokines (Interleukin 1-Beta, Interleukin 6 and Tumor Necrosis Factor-Alpha) were quantified in the blister fluid at 5 and 24 hours after blister initiation. Fluid was initially aspirated from each blister (0.05-0.1 mL) with a 27 gauge needle and syringe; the blister roof (epidermis) was then removed with sterile scissors. A plastic template was placed over the blister wounds. Using a syringe with an attached angiocath the template wells were filled with 0.8 to 1.0 mL of 70% autologous serum in a Hanks’ balanced salt solution and the top sealed with sterile tape. The autologous serum-buffer solution was aspirated via an angiocath attached to a syringe from half the wells 5 hours after the blisters formed. In 24 hours the remaining solution was aspirated from the other 4 wells and the template removed (Glaser et al., 1999). A standard wound care protocol was initiated prior to discharge. Participants were informed that small scabs would form over the site of the blisters and would fall off in one to two weeks.
Wound Measurement

‘Wound healing’ was defined as the advance of the wound margins toward the wound center. Daily area yet to be healed was measured by single camera digital photogrammetry (SCP) and the wound measurement software (VeV MD, Vista Medical, Winnipeg, Manitoba, Canada) on 8 consecutive days after blister initiation and every 4 days thereafter until complete (100%) healing had occurred. An orientation card of known dimensions was placed next to the blister sites in view of the camera. The blister images were then downloaded to the VeV MD computer software program. Blister wounds were oriented and compared to the known size of the orientation card per the computer software. The wound perimeters were outlined on the computer with a cursor by the primary investigator (PI) and the sum of the areas yet to be healed for all 8 blisters was then calculated. The wound area calculations for a single blister ranged from .01 to 5.28 cm² over the selected measurement time span.

Data Analysis

To compare the differences between treatment and placebo groups in the total area yet to be healed (cm²/day) for all 8 blisters at each time point, a t-test was utilized. A wound size of 0 cm² using the SCP technique was considered complete wound closure. A total lapsed time in days from initiation of blisters to first occurrence of 0 cm² wound size was also compared between the two groups and evaluated with a t-test.
Results

The overall wound size in cm² of the group who received the ω-3 fatty acid EPA/DHA (fish oil) supplement for 4 weeks and the control group, who consumed the placebo for the same interval, was not significantly different at any of the 9 time points (every day for 8 days and then every 4 days or until 100% wound closure).

Time to complete wound healing was then evaluated. The number of days to complete wound healing (100% closure) was not significantly different between the two groups, although the mean number of days to complete healing for those who consumed the EPA/DHA supplement for 4 weeks prior to the wound blistering procedure, was slightly longer (10.7) than the mean (9.8 days) for those who took the placebo for the same interval.

Intrarater reliability of the VeV measurement system in the current study was evaluated. Half the sample was randomly selected and blister wound measurements were repeated by the same individual who was blinded to group assignment (omega-3 supplement or placebo). Total surface area yet to be healed for all eight blisters at each time point was compared to the initial calculations. Significant Pearson’s correlation coefficients ranged from .59 to .99 with an average of .88. It was concluded that the VeV method of measuring blister wound area had high test-retest intrarater reliability in the study ‘Omega-3 Fatty Acids Effect on Wound Healing’.

Discussion

Accurate measurements of wound size over time are essential when determining wound healing progress in research studies. It is generally agreed that accuracy, precision, cost effectiveness and ease of use are criteria for choosing an appropriate
measurement instrument. In addition, the type of wound model chosen to suitably answer
the research questions and the elected definition of the concept of wound healing also
need to be considered before selecting the measurement device.

Several appropriate wound measurement tools are available that appear to be
appropriate for research studies examining suction blister wound healing defined as
surface area yet to be healed over time until 100% closure. In recent years the accuracy
and precision of the available methods for wound area calculations have been studied and
compared (Goldman & Salcido, 2002; Keast et al., 2004; Langemo et al., 1998; Thawer
et al., 2002), but not specifically discussed in reference to their ability to accurately and
precisely measure the surface area of multiple small blister wounds (3-10 mm in
diameter) in close proximity created by a negative pressure devise in the research setting.
The SPG or SCP computer-assisted techniques are highly recommended for research
purposes because of their precision and data storage capabilities. The SGP, SCP and
structured light technique were found to be particularly suitable although pros and cons
exist for each method.

The SCP technique in the form of the VeV measurement documentation system (Verg
Inc./Vista Medical Ltd., Winnipeg, Manitoba, Canada) was chosen for a recent study to
measure the small (8 mm.) suction blister wounds produced to evaluate the effects that
omega-3 fatty acid supplements had on epidermal skin healing. Precise wound
measurements were produced in the exemplified study with little difficulty using the VeV
measurement system. It is highly recommended for measuring multiple small suction
blister wounds in close proximity, storing patient wound photographs and related data,
and tracking wound healing over time. Several suggestions can be offered however, for
future research endeavors utilizing this technique, such as purchasing the manufacturer’s recommended camera and computer, to deter potential problems and contribute to the production of the most accurate and precise data possible (Table 3). Clinical decisions, related to superficial epidermal skin healing, that are based on valid data produced from well designed research studies that have incorporated suitable, precise wound measurement techniques will positively impact the monumental health problem of impaired wound healing.
<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Manual Wound Tracing with Digital Planimetry</td>
<td>Direct wound contact technique using freehand tracing of wound outline onto transparency and calculating surface area with mechanical planimetry. Image can be digitized to computer screen using stylus and wound calculated using computer software (Goldman &amp; Salcido, 2002)</td>
</tr>
<tr>
<td>Stereophotogrammetry (SPG)</td>
<td>Noncontact wound measurement where two photographs of wound are taken by specialized stereo camera /projector to create three-dimensional images from which surface area and volume can be calculated by specialized computer system (Thawer et al., 2002)</td>
</tr>
<tr>
<td>Single digital camera photogrammetry (SCP)</td>
<td>Noncontact wound measurement utilizing single digital camera and customized computer software to calculate wound size (Williams, 2000)</td>
</tr>
<tr>
<td>Structured light technique</td>
<td>Noncontact wound measurement using projector that illuminates wound area with parallel laser light stripes or dots. Image is recorded with electronic camera connected to image processing computer. Three-dimensional representation of wound area produced by triangulation (Plassmann &amp; Jones, 1998).</td>
</tr>
</tbody>
</table>

Table 3.1
Wound Healing Instruments to Measure Surface Area in Research
<table>
<thead>
<tr>
<th>METHOD</th>
<th>ADVANTAGES</th>
<th>DISADVANTAGES</th>
</tr>
</thead>
</table>
| Manual Wound Tracings with Digital Planimetry | • Inexpensive  
• No special skills or training required  
• Immediate results  
• Accurate measurements with computer assistance | • Requires direct wound contact (risk of contamination, pain and damage to wound bed)  
• May have difficulty identifying wound edge  
• Manual technique found to overestimate actual wound size (Thawer et al., 2002)  
• Reliability lessens with decreasing wound size (Thawer et al., 2002) |
| Stereophotogrammetry (SPG)         | • Noninvasive  
• Very accurate—especially with small wounds  
• Photographic or digitized record | • Expensive, cumbersome equipment  
• Lengthy meas. process  
• Requires specialized training  
• Wound boundaries manually delineated on computer (Thawer et al., 2002) |
| Single digital cameral photogrammetry (SCP) (i.e. VeV Measurement System) | • Noninvasive  
• Provides precise, reliable wound area calculations with coordinate measurements  
• Photographic or digitized record produced quickly  
• Flexible in use  
• Large data base capabilities for subjects’ histories, assessments, measurement documentations and trackings  
• Less training, expense and processing than SPG | • High initial cost investment for software, camera, laptop  
• Boundaries of wound are manually delineated on computer (Thawer et al., 2002) |
| Structured light technique         | • Noninvasive  
• Provides precise, reliable area measurements  
• Automated delineation of wound parameters and area calculation  
• Relatively fast photograph acquisition | • Requires some specialized training  
• Reliability with small wounds not known  
• Camera configuration somewhat cumbersome  
• Cost factor (Thawer et al., 2002) |

Table 3.2  
Performance of Area Measurement Methods
- Purchase recommended minimum camera requirements
  - Resolution of 760 x 480 pixels digital camera
  - Hi-8mm video camera
  - Macro focus ability (Vista Medical, 1999)

- Purchase recommended minimum computer requirements
  - Pentium or equivalent
  - 64 MB memory
  - 4.3+ GB hard drive
  - SVGA 64,000 colors, 800 x 600 resolution
  - 17 inch monitor (Vista Medical, 1999)

- Ensure that orientation card is carefully placed in same plane as wound(s) to be measured (Vista Medical, 1999)

- Ensure that orientation card is approximately same size as wound(s); for best results it should not be > than 4 times larger or < ¼ smaller than size of wound(s). Best accuracy achieved if large orientation card with a hole in the middle to surround the wound is used. (Vista Medical, 1999)

- If poor image quality: Increase lighting, improve camera steadiness, change camera focus area (Vista Medical, 1999)

- If orientation rating low: ensure angle between camera and target plate is < 45º from vertical, ensure target plate is in focus and in same plane as wound, recapture image (Vista Medical, 1999)

- If colors appear incorrect: ensure capture settings (brightness contrast, hue, saturation and gamma) are same for images being compared; may need to recapture images, sample color of white area of target plates in images being compared and compare red-green-blue values of each (Vista Medical, 1999)

- Become familiar with camera prior to study use (read instruction manual and practice capturing images)

- Become familiar with software database program prior to study use by practicing image capturing with target plate, bringing images into computer and VeV software, adding information to database and measuring image parameters

- Ensure that images are labeled with subject identification number and date/time taken

- Although program considers variations in lighting and distance from wound(s), ideal to capture images in same locale using same lighting and similar distances

- Capture more than one image per time point to allow for potential photographic error

- Ensure efficiency by carrying extra camera batteries and misc. supplies to photographic site

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Table 3.3
VeV Measurement System – Operational Suggestions
LIST OF REFERENCES


