NITRIC OXIDE METABOLITES IN WOUND FLUIDS
FROM PRESSURE ULCERS ON V.A.C.® THERAPY

By

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2004
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by

Beverly Bibera Childress
I dedicate this dissertation to my mother, Generosa Childress, for all her hard work to give me a better chance in life. To my stepfather, Andrew Childress, who accepted and loved me as his biological daughter.
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<td>Full Form</td>
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<tr>
<td>--------------</td>
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<td></td>
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<tr>
<td>AGU</td>
<td>aminoguanidine hemisulphate</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
<td></td>
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<tr>
<td>BH₄</td>
<td>tetrahydrobiopterin</td>
<td></td>
</tr>
<tr>
<td>DFU</td>
<td>diabetic foot ulcers</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent enzyme</td>
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</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
<td></td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
<td></td>
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<tr>
<td>GSNO</td>
<td>S-nitroso-glutathione</td>
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<td>HB-EGF</td>
<td>heparin binding epidermal growth factor</td>
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<td>IFN-γ</td>
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<td>IGF-I</td>
<td>insulin-like growth factor I</td>
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<td>IL-1β/1/2/6/12</td>
<td>interleukins 1 beta, 1, 2, 6, and 12</td>
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<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
<td></td>
</tr>
<tr>
<td>KGF</td>
<td>keratinocyte growth factor</td>
<td></td>
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<tr>
<td>KO</td>
<td>knockout</td>
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<tr>
<td>L-N̋-IL</td>
<td>L-N⁶-(1-iminoethyl)-lysine</td>
<td></td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MITU</td>
<td>S-methyl isothiouronium</td>
<td></td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
<td></td>
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<tr>
<td>NO₂⁻</td>
<td>nitrite</td>
<td></td>
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<tr>
<td>NO₃⁻</td>
<td>nitrate</td>
<td></td>
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<tr>
<td>NO⁺</td>
<td>nitric oxide</td>
<td></td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
<td></td>
</tr>
<tr>
<td>NOₓ</td>
<td>nitrate/nitrite</td>
<td></td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
<td></td>
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<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>SNAP</td>
<td>s-nitroso-N-acetylpenicillamine</td>
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<tr>
<td>TGF-α/β</td>
<td>transforming growth factor- alpha and -beta</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitors of metalloproteinase</td>
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<tr>
<td>TNF-α/β</td>
<td>tumor necrosis factor- alpha and beta</td>
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<tr>
<td>V.A.C.™</td>
<td>vacuum assisted closure™</td>
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<td>VEGF</td>
<td>vascular endothelin-derived factor</td>
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Compelling evidence suggests that nitric oxide (NO\textsuperscript{•}), a metabolite of arginine, plays an important role in wound healing. Arginine is a semi-essential amino acid that is metabolized by nitric oxide synthase and arginase. A model for regulation of wound healing proposes the importance of a strict reciprocal control of these enzymes in wounds. Thus, the purpose of this study was to investigate arginine metabolism in wound fluids of patients with pressure ulcers on Vacuum Assisted Closure (V.A.C.\textsuperscript{®}) therapy. This device, which has been shown to accelerate wound healing, also served as a tool to collect wound fluid.

Wound fluid extracts from the larger V.A.C.\textsuperscript{®} Study were used to determine nitrate and nitrite by the Griess reaction method. Quantitative measurement of tumor necrosis factor-alpha (TNF-\textalpha) and interleukin-1 beta (IL-1\textbeta) was performed by the enzyme-linked immunosorbent assay. Wound fluid analyses of arginine, citrulline, ornithine, and proline were performed by high-performance liquid chromatography. Eleven patients
between 31-92 years of age with Stage III or IV pressure ulcer on V.A.C.® therapy were enrolled. The subjects were recruited within a 50-mile radius of Gainesville. After informed consent was obtained, wound fluid was collected prior to V.A.C.® application and post-V.A.C.® within 24 hours, three days, and seven days. Subjects served as their own control in this prospective quasi-experimental repeated measures design.

There was no significant difference between pre- and post-V.A.C.® NO\(^\bullet\), citrulline, ornithine, and proline levels. However, there was a statistically significant decrease in arginine levels measured at baseline and day three of V.A.C.® therapy. Also, NO\(^\bullet\) measured at 24 hours of V.A.C.® placement decreased significantly at day seven of treatment. Furthermore, post-V.A.C.® levels of TNF-\(\alpha\) decreased significantly from baseline. Thus, a less cytotoxic environment is found indicating a healing wound.

Arginine and its metabolites are detectable in wound fluids from patients with pressure ulcers. To date, the metabolism of arginine has not been described in humans with pressure ulcers on V.A.C.® therapy. The determination of NO\(^\bullet\) in these wound environments provides baseline information on the mechanisms involved in aberrant wound healing, which has implications for nursing care.
CHAPTER 1
INTRODUCTION

The focus of this chapter is to introduce the problem, study variables, and purpose of the study. Specific aims with their respective research hypotheses are delineated. The significance of this study to wound research and the discipline of nursing is provided.

Background of the Problem

Despite advances in wound care treatment, the United States spends billions of dollars a year to care for almost one million Americans who develop chronic wounds (Mendez-Eastman, 1998). Chronic venous insufficiency, diabetes mellitus, and pressure ulcers account for 70% of all chronic wounds (Nwomeh, Yager, & Cohen, 1998). Management of pressure ulcers alone in 1994 was estimated at $1.335 billion for inpatient and outpatient facilities (Agency for Health Care Policy and Research). It costs approximately $2,731 to heal one pressure ulcer in hospital and long-term care settings. Furthermore, patients with a single pressure ulcer are 3.5 to 5 times more likely to stay in the hospital than those without ulcers (Maklebust & Sieggreen, 2001). In a recent article by Arnold (2003), the incidence of pressure ulcers in acute care settings was reported at 2%-29%. Also, the author reported that the cost of healing one pressure ulcer wound ranged from $2,000-$70,000. Clearly, the scope of the problem is not well documented in the literature. However, the problem remains and may worsen as the aging baby-boomers retire. As patient acuity increases in combination with technological advances to prolong life, the total expenditure in chronic wounds is expected to rise.
A plethora of commercial products are widely available in the market to treat chronic wounds. One such novel technology is the Vacuum Assisted Closure (V.A.C.®) device, which utilizes subatmospheric pressure upon topical application to acute, subacute, or chronic wounds (Argenta & Morykwas, 1997). The negative pressure created by this device is postulated to decrease wound exudates and bacterial colonization, increase tissue perfusion, and stimulate granulation tissue formation. The V.A.C.® creates an interstitial fluid environment that promotes healing, and as a result, the wound heals faster. However, little is known of the mechanism by which the V.A.C.® accelerates wound healing.

It is well established that normal wound healing occurs sequentially and is strictly regulated by pro-inflammatory cytokines and growth factors. Utilizing biological mediators to treat chronic wounds have been under intense investigation for several years. Clinically, growth factors have yet to be proven beneficial in the treatment of chronic wounds in human subjects (Goldman, 2004). It is imperative, therefore, to continue our search for novel mediators to improve healing outcomes. Recently, the importance of nitric oxide (NO•) in wound repair has been elucidated. Not only does it possess cytostatic and cytotoxic properties, but also regulatory functions to mediate epithelialization, angiogenesis, and collagen deposition crucial to the proliferative phase. Nitric oxide is synthesized from arginine by the constitutive and inducible nitric oxide synthases (cNOS and iNOS). In wounds, iNOS predominates and competes for its substrate with arginase. The by-products of arginase, ornithine and proline, are also essential in wound repair. Hence, a strict reciprocal regulation of these enzymes has been proposed to modulate wound healing (Shearer, Richards, Mills, & Caldwell, 1997).
gene technology advances, the possibility of treating chronic wounds with NO• releasing products or iNOS gene transfer exists. However, further study is needed to determine the role of nitric oxide in healing human wounds.

**Problem Statement**

Chronic, non-healing wounds of various etiologies are a major burden to society. Clinically regarded as the technology of the century, the V.A.C.® device accelerates wound healing by applying negative pressure to the edges of the wound (Mendez-Eastman, 1998). The efficacy of this technique, however, is not well understood at the cellular and molecular level. Compelling evidence suggests that NO• is vital to the wound healing process. As a free radical, it has cytotoxic properties as well as regulatory functions on various cell types involved in inflammation and proliferation (Schwentker & Billiar, 2002). Nitric oxide (NO•) is synthesized from L-arginine, a substrate for both nitric oxide synthase (NOS) and arginase (see Figure 1-1). In wounds, inducible NOS

![Figure 1-1. Arginine metabolism and phases of wound healing.](image-url)
Note: IL-1β = interleukins-1 beta; IFN-γ = interferon-gamma; TNF-α = tumor necrosis factor-alpha; iNOS = inducible nitric oxide synthase; NO• = nitric oxide; ROS = reactive oxygen species; RNS = reactive nitrogen species. Text in bold were measured.

(iNOS) catalyzes arginine to citrulline and NO•, whereas arginase converts arginine to ornithine and urea. Ornithine is a precursor for proline and polyamines, which are essential in normal wound healing (Wu & Morris, 1999).

**Study Purpose**

Human studies using impaired wound healing models are lacking in this area. Thus, the purpose of this study was to investigate the metabolic activity of arginine in wound fluids from pressure ulcer patients on V.A.C.® therapy. Wound fluid extracts from the ongoing larger V.A.C.® Study were used to analyze the metabolites of NO•, tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β). The main V.A.C.® Study is a repeated measures experimental design assessing the characteristics of pressure ulcer environments for pro-inflammatory cytokines, matrix metalloproteinases, tissue inhibitors of matrix proteinases, and amino acids. Additional information taken directly from the larger study included demographics and values of the amino acid profile.

**Research Aims and Hypotheses**

**Aim 1**

Evaluate the Effects of the V.A.C.® on Nitrate/Nitrite (NOx) levels in Wound Fluids from Non-healing and Healing Pressure Ulcers.

**Hypothesis**

Pre- V.A.C.® concentrations of NOx will be high due to increased levels of TNF-α and IL-1β in chronic wounds. Post-V.A.C.® NOx will return to optimal levels that are supportive of healing within 24 hours followed by decreasing levels on days three and seven (see Figure 1-1).
Aim 2

Evaluate the Effects of the V.A.C.® on Arginine, Citrulline, Ornithine, and Proline in Wound Fluids from Non-healing and Healing Pressure Ulcers, which Reflects iNOS and Arginase Activities.

Hypothesis

There will be an increase in iNOS and arginase activities in chronic wounds as evidenced by high levels of citrulline, ornithine, and proline. After V.A.C.® placement, the NO•/citrulline pathway will predominate during days one and three followed by the ornithine/urea cycle (see Figure 1-1). Accordingly, arginine levels will decrease as it is utilized by iNOS and arginase. Data for these analyses were taken directly from the database of the larger V.A.C.® Study.

Study Assumptions

The following assumptions are used in this study.

1. Wound fluid reflects the biological wound environment.
2. The V.A.C.® changes the wound environment.
3. Variables of interest are detectable in the wound environment.

Study Limitations

Internal validity is an inherent threat to this proposed design. Without a control group, it is impossible to determine whether healing would have occurred over time without treatment of the V.A.C.®. It should be noted, however, that wounds are heterogeneous. The complexity of the wound healing process and the great variability that exist between patients further complicate comparison analyses (Stacey & Trengove, 1995).

A major limitation of convenience sampling is the potential bias of self-selection (Portney & Watkins, 2000). Such limitation cannot be avoided unless probability
sampling method is utilized. It should be noted, however, that the participating hospitals and clinics attracted all types of patients from the state of Florida. Unfortunately, the study sample was composed mainly of subjects of Caucasian descent. No medical complications were encountered as a result of the V.A.C.® intervention.

Another limitation of the proposed project is generalizability due to its small sample size. Only 11 subjects with pressure ulcers on the V.A.C.® were studied. In addition, the findings from this study cannot be generalized to all types of chronic wounds. The etiology of decubitus ulcers, for example, greatly differs from diabetic foot ulcers or venous ulcers. Hence, the results will only reflect the wound environment of pressure ulcers.

**Significance to Nursing**

This study examined the by-products of arginine metabolism in wound fluids extracted from patients with stage III or IV pressure ulcers. Specifically, the determination of NO• in these wound environments will provide baseline information on the mechanisms involved in aberrant wound healing. Gaining an insight to wound repair at the cellular and molecular level is vital to nursing care. At the bedside, nurses are integral in the assessment of skin integrity, risk factors, and nutritional needs of individuals. In most instances, nurses are first to identify skin breakdown and institute preventative measures such as positional and/or diaper (if incontinent) changes. At the same time, physicians and skin nurse specialists are alerted to further manage the patient especially those with complicated wounds. Knowledge of wound repair and measures that promote healing of chronic wounds is of the utmost importance. Nurses and other wound health care professionals can facilitate or impair wound repair. Thus, basic
wound research is paramount to the management of wound healing by providing evidence-based interventions for practice.
CHAPTER 2
LITERATURE REVIEW

The purpose of this chapter is to provide readers with background information. This includes an in-depth discussion of wound healing physiology and pathophysiology. Current models of acute and chronic wound healing are provided. A brief overview of nitric oxide (NO•) is presented. The role of NO• in wound healing is further elucidated with linking research to the inflammatory and proliferative phases of healing. Lastly, the Vacuum Assisted Closure (V.A.C.®) system is explained in detail with supporting evidence regarding its use and success in treating chronic wounds.

Acute Wound Healing Model

Current knowledge of normal wound healing physiology is based on the cutaneous wound healing model. Regardless of the cause and extent of tissue injury, the healing process includes three overlapping phases: (1) inflammation, (2) proliferation, and (3) maturation or remodeling (see Figure 2-1). Each stage is strictly regulated by cytokines, growth factors, and other cellular components of inflammation. These biochemical mediators stimulate or inhibit cellular actions that are critical for host defense, eradication of noxious agents, and facilitation of healing (Karukonda et al., 2000; Mast & Schultz, 1996).

Inflammatory Phase

Inflammation, the first phase of wound healing, is the body’s natural response to injury and lasts 1-5 days. Hemostasis occurs as clots form and blood vessels constrict. The clot consisting primarily of fibrin, trapped red blood cells, and aggregated platelets

8
not only halts bleeding, but also forms the provisional wound matrix. Platelets release cytokines such as basic fibroblast growth factor (bFGF), platelet-derived growth (PDGF), tumor growth factor-beta (TGF-β), tumor growth factor-alpha (TGF-α), platelet-derived epidermal growth factor (PDEGF), platelet-derived endothelial cell growth factor, 

![Cytokines, growth factors, and nitric oxide central to the wound healing process.](image)

Figure 2-1. Cytokines, growth factors, and nitric oxide central to the wound healing process. Modified from B.B. Childress and J.K. Stechmiller (2002) the “Role of Nitric Oxide in Wound Healing”. Biological Research of Nursing, 4(1), 5-15. Note: EGF = epidermal growth factor; FGF = fibroblast growth factor; IFN-γ = interferon-gamma; IGF-I = insulin-like growth factor I; IL 1/2 = interleukins 1 and 2; KGF = keratinocyte growth factor; NO* = nitric oxide; PDGF = platelet-derived growth factor; TGF α/β = transforming growth factor α and β; TNF-α = tumor necrosis factor α;
These proteins serve as mediators of the healing response by altering cellular functions. This is accomplished by the binding of cytokines to their receptors on cell membranes (Lawrence, 1998).

Vasoconstriction is immediately followed by vasodilation of local blood vessels in response to histamine, kinins, and prostaglandins. As vascular permeability and levels of chemoattractants increase, the number of leukocytes migrating to the injured site increases. This is in response to stimuli such as bacterial endotoxin, PDGF, tumor necrosis factor-alpha (TNF-α), and other chemotactic factors. As a result, neutrophils and monocytes infiltrate the area to remove damaged tissues and/or pathogens (Rote, 1998). Neutrophils, the first to arrive at the wounded tissue, clean up the wound environment via phagocytosis and breakdown extracellular matrix by releasing proteases. The proteolytic function of these enzymes differs greatly from the matrix metalloproteinases (MMPs) produced by fibroblasts in the subsequent stages. Protease activity is vital to wound debridement and the progression of the healing process to the next phase (Schultz & Mast, 1998).

The inflammatory response declines by the third day and is marked by the absence of neutrophils from the wound. Monocytes are transformed into activated macrophages through stimulation by T lymphocyte-derived interleukin-2 (IL-2) and interferon-sigma, and bacteria or viruses (Lawrence, 1998). Macrophages, which are also phagocytes, further decontaminate and prepare the wound for tissue repair. The breakdown of damaged matrix is mediated by collagenases and elastases that are secreted by macrophages, which are regulated by macrophage-derived tissue inhibitor of metalloproteinases (TIMPs) (Lawrence). Furthermore, macrophages stimulate fibroblasts
proliferation, collagen production, and other key healing processes by releasing cytokines and growth factors such as TNF-\(\alpha\), PDGF, TGF-\(\beta\), IL-1, insulin-like growth factor (IGF-1), fibroblast growth factor (FGF), and TGF-\(\alpha\) (Karukonda et al., 2000; Lawrence). In an autocrine manner, TGF-\(\beta\) stimulates macrophages to secrete additional TGF-\(\beta\) as well as other cytokines such as FGF, PDGF, TNF, and IL-1. Other products secreted by macrophage that are crucial to the wound healing process include oxygen metabolites, prostaglandins, and arginine (Lawrence, 1998).

**Proliferative Phase**

Central to the proliferative phase, which occurs from 3-16 days, is angiogenesis, reepithelialization, fibroplasia, and wound contraction (Maklebust & Sieggreen, 2001). These events are orchestrated in an orderly and timely manner by a multitude of cells including endothelial cells, epithelial cells, fibroblasts, myofibroblasts, and their biochemical mediators.

Angiogenesis, the formation of new blood vessels, provides all the metabolic needs of the healing tissue. Hypoxia, high lactic acid concentrations, and low pH stimulate endothelial cells to proliferate on capillary sprouts. In addition, macrophage-derived cytokines are directly and indirectly responsible for migration and proliferation of these cells. Vascular endothelin-derived growth factor (VEGF) and basic FGF are two of the most potent promoter angiogenesis. Other angiogenic stimulants include TGF-\(\alpha\), epidermal growth factor (EGF), TGF-\(\beta\), PD-ECGF, and TNF-\(\alpha\) (Karukonda et al., 2000; Lawrence, 1998).

Overlapping with inflammation, reepithelialization begins hours after injury with epithelial cells migrating to the wounded area in response to TGF-\(\alpha\), TGF-\(\beta\), and EGF.
By 24 hours, epithelial cells proliferate until a complete seal of the wound is formed to confine and protect the healing tissue (Karukonda et al., 2000; Rote, 1998). Proliferation of epithelial cells is mediated by TGF-α, EGF, heparin binding epidermal growth factor (HB-EGF), IGF, KGF, and bFGF. Key cytokines produced by epithelial cells include PDGF, TGF-β, and TGF-α (Lawrence, 1998).

Collagen deposition by fibroblasts is vital to tissue granulation formation and scar maturation. The synthesis of collagen is mainly stimulated by TGF-β, which is produced by pro-inflammatory cells and fibroblasts. External factors such as age, pressure, stress, and tension may directly affect the rate of collagen synthesis. PDGF, a stimulus for granulation of tissue, has been shown to indirectly limit cellular activity due to its influence on TGF-β expression (Lawrence, 1998). Furthermore, TNF-α and IL-1β stimulate fibroblasts to synthesize collagen, up regulate MMPs, and down regulate tissue inhibitors of metalloproteinases (Mast & Schultz, 1996). Thus, newly synthesized collagen is deposited in an extracellular matrix conducive to healing.

The last event of this phase is wound contraction, which lasts through the remodeling phase. Myofibroblasts responding to TGF-β and other substances mediate this process to promote wound closure (Karukonda et al., 2000; Rote, 1998).

**Remodeling or Maturation Phase**

The remodeling phase, which can last for several months, begins as cell proliferation and neovascularization ends. At this stage, the synthesis of new scar matrix and degradation of extracellular matrix components reach equilibrium. Fibroblasts produce stimulatory and inhibitory substances, which regulate this process. These cells are responsible for remodeling the new extracellular matrix by synthesizing collagen,
gelatin, and proteoglycans. Replacement of the old matrix requires the proteolytic activities of MMP-1, MMP-2, MMP-9, and MMP-3. The destructive nature of these enzymes to the healing tissues is inhibited by TIMP-1 and TIMP-2, which are also secreted by fibroblasts (Schultz, 2000; Schultz & Mast, 1998; Tarnuzzer & Schultz, 1996). The complex interaction between MMPs and TIMPs is key to tissue remodeling.

**Acute versus Chronic Wounds**

Acute and chronic wounds greatly differ in their etiology, healing time, and wound environment. In acute wounds, such as a clean cut, a sudden and quick insult to the skin occurs. Immediately thereafter, injured cells and platelets release cytokines and growth factors to elicit the inflammatory response. In chronic states, cellular injury results from a persistent stimulus such as repeated tissue trauma or ischemia. Over time, the affected area becomes an open wound, providing a good medium for bacterial growth. An inflammatory response is initiated by the influx of neutrophils and macrophages into the wound site. The process, therefore, bypasses the release of growth factors that signal the healing cascade to begin (as seen in acute injury). A vicious cycle occurs as inflammatory cells secrete cytokines, TNF-\(\alpha\) and IL-1\(\beta\), which in turn attract more inflammatory mediators (Schultz, 2000; Mast & Schultz, 1996). Further tissue damage ensues as the wound fails to move quickly and appropriately through the subsequent phases of healing.

Schultz and Mast (1998) best summarize wound environments at the molecular level. Based on fluid analysis from healing wounds and chronic ulcers, they discovered that healing wounds show high levels of mitogenic activity, growth factors, and functional fibroblasts, but low concentrations of cytokines and proteases. In contrast,
chronic wounds exhibit low mitotic activity, elevated levels of cytokines and proteases, low levels of growth factors, and senescent cells.

**Nitric Oxide and Wound Healing**

It is becoming evident from a decade of research that NO\(^*\) is essential to wound healing. Compelling data from animal and human studies clearly suggest that NO\(^*\) is an integral part of the inflammatory phase. Not only does it possess cytotoxic properties, but also regulatory functions to mediate epithelialization, angiogenesis, and collagen deposition crucial to the proliferative phase (see Figure 2-1). It is important to note, however, that the exact bioregulatory mechanism by which NO\(^*\) promotes wound repair has yet to be fully elucidated. Much research in this area is needed to understand how NO\(^*\) modulates wound healing in humans (P.C. Lee et al., 1999; Stallmeyer, Kämpfer, Kolb, Pfeilschifter, & Frank, 1999; Thornton et al., 1998).

Nitric oxide is a ubiquitous molecule that serves various biological functions in the body (see Figure 2-2). Existing for only seconds, NO\(^*\) readily reacts with molecular oxygen and water to form its stable end products, nitrate and nitrite (Snyder & Bredt, 1992). NO\(^*\) is generated by a family of enzymes called NOS from a semi-essential amino acid, L-arginine (Wu & Morris, 1999). The two constitutive NOS are (1) neuronal (nNOS or type I) that is expressed in the peripheral and central nervous system, and (2) endothelial NOS (eNOS or Type III) that is found in endothelial cells of the vascular system. Type II (iNOS) is only expressed in the presence of endotoxins and/or pro-inflammatory cytokines. Unique to iNOS is its ability to synthesize NO\(^*\) in high concentration for a period of time to sustain its toxic effects irrespective of intracellular Ca\(^{2+}\) levels (Lincoln, Hoyle, & Burnstock, 1997). A cytokine mixture of TNF-\(\alpha\), IL-\(\beta\),
and interferon-gamma (IFN-γ) can effectively induce human iNOS. Upon induction, the iNOS gene is transcribed and translated into a functional enzyme to generate NO• in the presence of its co-substrates, co-factors, and prosthetic groups (see Figure 2-3) (Taylor & Geller, 2001). Once released, NO• diffuses out of activated macrophages and destroys target cells through necrosis or apoptosis (Moncada & Higgs, 1995; Snyder & Bredt,
1992). Many of the iNOS inhibitors include TGF-β, PDGF, NO*, and IL-4, 6, 8, and 10 (Lincoln et al., 1997).

Interestingly, researchers have noted that NOS competes for its substrate with another enzyme called arginase. This enzyme converts arginine into ornithine and urea. Ornithine is an essential substrate for the synthesis of polyamines, which are important in
cellular proliferation and repair. Proline, also derived from ornithine, is key in collagen synthesis (Wu & Morris, 1999). Analyses of wound fluids (less than three days) showed high concentrations of NO• and citrulline in response to TNF-α, IL-β, IFN-γ and lipopolysaccharide (LPS). As the healing process progressed and the inflammatory response decreased (more than three days), arginase activity increased as indicated by high levels of ornithine (Shearer et al., 1997). In another study, R.H. Lee and others (2001) examined the biochemical activity of NOS over a 35-day period in rats. Gene expression of iNOS correlated highly with an elevated NO• concentration that peaked at 24 hours and declined steadily for the next 5-7 days with sustained levels up to the 10th day.

Research Linking iNOS and NO• during Inflammation

It is well established that NO• mediates the cytotoxic effects of macrophages during the inflammatory phase. Wound fluid studies, for example, consistently demonstrated high levels of NOx early and transiently from wounds of various etiologies. To determine the precise time at which concentrations of arginine metabolites predominated, Albina, Mills, Henry, and Caldwell (1990) implanted subcutaneous sponges in rats for 15 days. High levels of nitrite and citrulline were found within 3 days, whereas increasing levels of urea and ornithine were detected after five days post sponge implantation. Studies of iNOS knockout (KO) mice established the critical role of this enzyme in wound repair. In a study of iNOS-deficient mice, healing of excisional wounds was delayed by four days. Wound closure was prolonged by 31% in iNOS KO mice compared with wild types. After a topical application of an adenoviral-mediated iNOS gene transfer, however, the wound closed (Yamasaki et al., 1998). Thus, it is evident that iNOS is the...
key NO\(^\bullet\)-producing enzyme in wound healing, and gene therapy may prove beneficial for treating of chronic wounds.

Current evidence suggests that abnormalities in arginine metabolism may contribute to the pathogenesis of impaired healing in human extremity ulcers. In a group of 22 diabetic patients with diabetic foot ulcers (DFU), 22 diabetics, and 14 controls; iNOS and arginase activities were significantly increased in DFU patients when compared to the other two groups. Furthermore, the higher concentration of NO\(^\bullet\) found in the diabetic groups was attributed to low levels of TGF-\(\beta_1\) (Jude, Boulton, Ferguson, & Appleton, 1999). It is suggested by Abd-El-Aleem et al. (2000) that the destructive effects of peroxynitrite on tissues may contribute to the pathogenesis of chronic venous ulcers. In this study, the investigators enrolled 16 normal subjects and 18 patients with chronic venous ulcers. Biochemical and immunohistological analysis of biopsied samples revealed high levels of NOS and arginase in subjects with ulcers compared with normal skin. Recall that in normal repair, arginase enhances extracellular matrix deposition; however, when in excess it may lead to callus formation.

**Research Linking iNOS and NO\(^\bullet\) during Proliferation**

Central to the proliferative phase is collagen deposition by fibroblasts. One group of researchers examined the effects of an iNOS inhibitor, S-methyl isothiouronium (MITU), in mice with implanted polyvinyl alcohol sponges. After 10 days, the group that received the highest dose of MITU (100mg/kg/day) exhibited low levels of NO\(^\bullet\) in wound fluids and cell culture supernatants. This was highly correlated with decreased collagen accumulation and wound breaking strength (Schäffer, Tantry, Gross, Wasserkrug, & Barbul, 1996). Similar results were found on a subsequent study using
aminoguanidine hemisulphate (AGU) (Schäffer, Tantry, Thornton, & Barbul, 1999). To further elucidate the role of iNOS in wound healing, iNOS-KO fibroblasts synthesized less collagen than the wild-type fibroblast. Restoration of collagen production was observed after low concentrations of an NO• donor, s-nitroso-N-acetylpenicillamine (SNAP), was administered to the iNOS-KO cells (Shi, Most, Efron, Tantry, Fischel, & Barbul, 2001). On the other hand, collagen accumulation was shown to increase in rats following iNOS gene transfection (Thornton et al., 1998). NO-deficiency associated with diabetes demonstrates poor healing. Diabetes-induced rats given exogenous molsidomine, a nitric oxide donor, showed increased hydroxyproline content and wound breaking strength (Witte, Kiyama, & Barbul, 2002). Such findings suggest that NO• is vital to tissue repair as evidenced by impaired healing in a wound environment with low levels of NO•. Potential treatments for impaired healing may involve administration of nitric oxide donors and/or gene manipulation.

In a cutaneous wound repair study, Frank and colleagues (1998) revealed that iNOS was significantly expressed during inflammation, reepithelialization, and granulation of tissue. Within minutes of injury, epithelial cells will normally migrate from wound edges immediately post-injury and proliferates within the first 24 hours. Under NO• deficient states, reepithelialization was severely delayed when a specific iNOS inhibitor, L-N6- (1-iminoethyl)-lysine (L-NIL) was introduced to wounded mice (Stallmeyer et al., 1999).

In angiogenesis, both iNOS and eNOS are postulated to be equally important in synthesizing NO•. Newly formed blood vessels are governed by one of the most potent angiogenic factors, VEGF. NO• is believed to effectively enhance the expression of VEGF by keratinocytes during tissue repair. To illustrate the effects of exogenous NO•,
in vitro cultures of human keratinocyte cell line HaCaT were exposed to purified growth factors, cytokines and/or S-nitroso-glutathione (GSNO). A potent keratinocyte inducer of VEGF mRNA expression, GSNO-treated cultures with TGF-β1, keratinocyte growth factor, IL-1β, or IFN-γ exhibited high levels of VEGF and proteins. Similar results were also observed in vivo with L-NIL-treated rats showing decreased levels of VEGF mRNA during inflammation (Frank et al., 1999). One can infer from these studies that the underlying function of NO• in repair is to induce keratinocytes to express VEGF. In a recent study, eNOS KO mice and wild types were wounded to determine the requirement of this enzyme in wound closure and strength. By day 10, wound strength was reduced by 38% in eNOS KO mice. Furthermore, a delay of 9.2 days in wound closure was observed in the eNOS KO group compared with the wild type controls (P.C. Lee et al., 1999). Undoubtedly, angiogenesis is essential in wound healing and NO• may regulate this process with iNOS and eNOS as major contributors.

In summary, the cytotoxic properties of NO• are vital to the inflammatory phase of wound healing. NO• continues to play a significant role in acute wound healing as a signaling molecule. As a messenger, NO• upregulates and downregulates wound cellular functions. Additionally, the vasodilatory effects of NO• in old and newly formed blood vessels are vital for wounded sites. Evidence to support the above assertions is summarized in Table 2-1.

**Vacuum Assisted Closure™ (V.A.C.®)**

FDA-approved since 1995, the V.A.C.® (KCI, San Antonio, TX) device promotes rapid healing of chronic wounds refractory to conventional treatment (Mendez-Eastman, 1998). Clinically, the V.A.C.® has been shown to enhance granulation tissue formation
and increase healing rates. This may be in part due to increased vascularity, decreased bacterial burden, and increased growth factor to MMP ratios. It is indicated for acute/traumatic wounds, flaps and grafts, chronic wounds open wounds (diabetic and subacute wounds (dehisced incisions). The V.A.C.® system consists of V.A.C.® unit or pump, foam dressings, canister, drapes, and extension tubing.

Simultaneously treatment of several wound sites is possible through the use of Y-connector (Kinetic Concepts, Incorporation, The Clinical Advantage, 2000).

A special porous dressing is positioned in the wound cavity to distribute localized negative pressure to the edges of the wound. This acts to mechanically draw the tissue inward thereby stimulating epithelial migration and cellular proliferation (Argenta & Morykwas, 1997). Furthermore, removal of interstitial fluid from the surrounding tissues improves blood supply and eliminates bacterial contaminants (Mendez-Eastman, 1998).

Table 2-1: Animal Studies of NO* and Wound Healing

<table>
<thead>
<tr>
<th>Intervention</th>
<th>NOx</th>
<th>Epithelialization</th>
<th>Angiogenesis</th>
<th>OHP</th>
<th>WBS</th>
<th>Collagen Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molsidomine (diabetic rats)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MITU</td>
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<tr>
<td>AGU</td>
<td></td>
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<tr>
<td>iNOS gene transfection</td>
<td></td>
<td></td>
<td>↑</td>
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<tr>
<td>iNOS-KO fibroblasts</td>
<td></td>
<td></td>
<td>↓</td>
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<td></td>
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<tr>
<td>iNOS-KO cells with SNAP</td>
<td></td>
<td></td>
<td>↑</td>
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</tr>
<tr>
<td>eNOS-KO</td>
<td></td>
<td></td>
<td>↓</td>
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<td></td>
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<tr>
<td>L-NIL</td>
<td></td>
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</tbody>
</table>

Note: NOx = nitrate/nitrite; OHP = hydroxyproline; WBS = wound breaking strength; MITU = S-methyl isothiourea; AGU = aminoguanidine hemisulphate; iNOS = inducible nitric oxide; KO = knockout; SNAP = s-nitroso-N-acetylpenicillamine; eNOS = endothelial NOS; L-NIL = L-N6- (1-iminoethyl)-lysine.
Within three to four days of V.A.C.® treatment, the number of bacteria in the wound was shown to decrease significantly (Argenta & Morykwas). In a six week randomized trial of V.A.C.® versus standard therapy of chronic wounds, 64% of granulation tissue formation occurred in the V.A.C.® group (Joseph et al., 2000) in comparison to the saline-wet-to-moist dressing group. Deva and colleagues (2000) also reported positive healing outcomes in 26 out 30 pressure ulcer patients on the V.A.C.®. Thus, the environment created from the V.A.C.® therapy is conducive to the healing process, which ultimately leads to wound closure.

Clinical outcomes of V.A.C.® therapy for acute and chronic wound treatment have shown promising results. The exact mechanism by which this device accelerates healing, however, is not well understood at the cellular and molecular level. Furthermore, the role of nitric oxide in wound repair has yet to be fully elucidated in animal and human studies. Undoubtedly, there is a need for further research in this area to better understand the phenomena.
CHAPTER 3
METHOD

This chapter is divided into four sections. The first section presents subject characteristics and sampling method. Second, the materials section provides information on the instruments used and variables tested in the study. The procedures are presented in the third section with specifics on study design, protocol, and data collection. A description of data management and statistical analyses of the two aims used examine the model shown in Figure 1.1.

Subjects

Sample and Sampling Method

Eleven adults 21 years of age and over were selected as subjects by convenience sampling. Stage III or IV pressure ulcer patients who were scheduled for V.A.C.® therapy were recruited. V.A.C.® treatment was indicated for patients without fistulas, necrotic tissue, untreated cellulitis or osteomyelitis, connective tissue disorder, or malignancy in the wound. The inclusion and exclusion criteria specific to the study were as follows:

- 21 years of age or older with stage III or IV pressure ulcers.
- Patient required V.A.C.® therapy on an outpatient or in-patient basis
- Pressure ulcer(s):
  - present for more than one month
  - no previous treatment with dermal skin substitutes
  - received little to no wound treatment for one week prior to V.A.C.® (enzymatic debridement agent)
no hyperbaric oxygen or warm-up therapy
no fistulas, necrotic tissue with eschar, untreated cellulitis or osteomyelitis, connective tissue disorder, and no malignancy in the wound
debridement recently performed

- no active systemic infection (normal white blood count), anemia (hematocrit less than 26) or immune deficiency diseases.
- no smoking within the past six months.
- not receiving steroids, immuno-suppressive or cytotoxic medications.

Setting
The study sites were within a 50-mile radius of Gainesville, Florida. The University of Florida and Veterans Administration Institutional Review Board approval of the study were obtained from each facility. The Plastic and Reconstructive Surgeons, Advanced Registered Nurse Practitioner, and Clinical Nurse Specialists contacted the principal investigator (PI) and/or sponsor when subjects or their family members agreed to talk about participation in the study. The PI or faculty sponsor recruited potential subjects from the inpatient and outpatient settings of the hospitals and nursing homes. Written informed consent was obtained prior to review of medical records and all procedures.

Materials
Vacuum Assisted Closure™ (V.A.C.®)
In this study, the V.A.C.® System was applied and maintained according to the manufacturer’s protocol and followed by the subject’s wound care team. These included the Clinical Nurse Specialist, Wound, Continence, and Ostomy Nurse Specialists, or Advanced Registered Nurse Practitioner who were all employed by the participating institution. Ten of the patients were on the classic V.A.C.®, while one subject was on the
mini-V.A.C.®. In all patients, the physician and/or nurse practitioner ordered V.A.C.® therapy to sacral wound on continuous therapy at 125 mmHg. Using the black polyurethane foam, the appropriate health care staff changed the dressing three times a week or as needed. Therapy was disrupted when patients were out of their rooms for medical tests, clinic visits, or physical therapy.

**Measurement of serum nitrate/nitrite (NO₃⁻) concentrations**

Due to its volatile nature, NO⁺ has a short half-life (t½ = seconds) and is oxidized to its stable end products, nitrate (NO₃⁻) and nitrite (NO₂⁻) (Taylor & Geller, 2001). Since NO₂⁻ is converted to NO₃⁻ in most bodily fluids, the primary metabolite present is NO₃⁻. Numerous studies in wound healing have estimate NO₃⁻ and NO₂⁻ (NOₓ) in wound fluids as an indirect measure of NO⁺ synthesis in the healing process. The simplest and most widely used technique is spectrophotometric quantification of NO₂⁻ by using the Griess diazotization reaction. Assays of total NO₂⁻ + NO₃⁻; therefore, are necessary to account for NO₃⁻ that is undetected by the Griess method (Moshage, 1997; Sun, Zhang, Broderick, & Fein, 2003).

The Cayman Chemical Nitrate/Nitrite Colorimetric Assay Kit (Ann Arbor, MI) was used to quantify total NOₓ in the wound fluid. The assay has a sensitivity of 2 µM and is outlined in the manufacturer’s instruction as a two-step process. First, it involves the conversion of nitrate to nitrite followed by the addition of Greiss reagents to determine nitrite concentrations. Pre-assay preparation included washing of laboratory ware to decrease NOₓ contamination (Ishibashi et al., 2000; Makela et al., 1997), spin rinsing of filters, ultrafiltration of wound fluid to reduce absorbance background, and preparation of
reagents that were provided with the kit. The following supplies were pre-washed as follows:

1. **Disposable (polyethylene) gloves (Fisher Scientific, Pittsburg, PA)** – exterior surfaces were washed five times with molecular grade water (Mediatech, Inc Cellgro) and air dried. This type of glove was shown to have the least amount of NO$_x$ contamination (Makela et al., 1997) in comparison to gloves made of vinyl, latex, or non-latex synthetic polymers. Our recent study on potential sources of NO$_x$ contamination showed that nitrile gloves (Kimberly-Clarke Safeskin Purple Nitrile) contained high amounts of NO$_x$ (Davis, Childress, & Stechmiller, 2004). Therefore, this type of glove was avoided in the analysis.

2. **Plastic graduated cylinders (Fisherbrand), beakers (Fisherbrand), and troughs (Corning, NY)** – the inner surfaces of these supplies were washed five times by rinsing them with molecular grade water. Glass laboratory wares were not used in the analysis since they contain considerable amounts of NO$_x$ (Makela et al.).

3. **1.5 ml graduated microcentrifuge tubes (Fisherbrand)** – filled with molecular grade water, these tubes were capped and inverted several times. Water was removed after vigorously shaking them.

4. **Pre-sterilized pipette tips 20 µl (Fisherbrand), 100 µl and 1000 µl (Molecular Bioproducts, San Diego, CA)** - the outer and inner surfaces of these tips were washed with molecular grade water as described by Ishibashi et al. (2000). Briefly, the pipette tips were attached to the appropriate mechanical pipette (Rainin Instruments, Oakland, CA) and dipped into water to a depth of two-thirds of the tip’s length. This procedure washed the outside of the tips and was repeated five times. For the interior surfaces, water was aspirated in a larger volume than the set volume. Then, water was expelled out of the tip until no water droplet was visible. This was repeated five times.

5. **Microcon YM-10 centrifugal filter device (Millipore, Bedford, MA)** – the inner and outer surfaces of the sample reservoir were washed three times (per manufacturer guideline) as well as the filtrate vial as described above. Then, 200 µl of molecular grade water was placed in the sample reservoir and spun for 15 minutes at 14,000 g at room temperature (Eppendorf Centrifuge 5804, Brinkman Instruments, Westbury, NY).

After washing all the necessary laboratory supplies, 100-128 µl of wound fluid was ultrafiltered through a 10 kDa molecular weight cut-off filter (Microcon YM-10) for 30 minutes at 14,000 g (Eppendorf Centrifuge) at room temperature. In addition to
decreasing hemoglobin’s interference in the analysis, ultrafiltration of samples increased color formation in the presence of the Greiss reagents.

Wound fluid samples were diluted three-fold with the assay buffer provided. This dilution factor was determined previously from two study patients. The manufacturer’s instructions for the preparation of nitrate standard and reagents were adhered to closely. To avoid mistakes during the assay, a template of the 96-well plate configuration was made. The first two columns of the plate contained the nitrate standard, which was done in duplicate. The standard stock, 200 µM, was prepared by the addition of 0.1 ml of the reconstituted nitrate standard into a clean test tube containing 0.9 ml of assay buffer. As configured in the template, the standard curve were placed in wells with the appropriate volume of assay buffer resulting in final nitrate concentration of 0, 5, 10, 15, 20, 25, 30, and 35 µM. To create the blanks wells, 200 µl of assay buffer was pipetted into two columns of the plate. No other reagents were added to these wells. Immediately thereafter, 40-80 µl of samples per well were placed in the plate in quantiplicates as outlined by the template. Assay buffer was added to wells containing 40 µl of samples to obtain a final volume of 80 µl. After adding 10 µl of the enzyme cofactor and nitrate reductase to each of the wells (standards and unknowns), the plate was incubated for three hours at room temperature. Following incubation, 50 µl of Greiss reagents 1 and 2 were added to each of the standards and unknowns. After 10 minutes of incubation period, absorbance was read spectrophotometrically at 540 nm (Elx 800 Microplate Reader, Winooski, VT).

Sample nitrate and nitrite concentrations were calculated from the nitrate standard curve generated by least squares regression analysis. The average absorbance values of
the standards and blank wells were determined. The standard curve was plotted once the average of the blanks was subtracted from the average of the standards (Fig 3.1). In Figure 3-1, the optical density for the standards is on the vertical (y) axis and the concentration of the standard is on the horizontal (x) axis expressed in µM. Depending on the volume and dilution factor used, the sample NO\textsubscript{x} was quantified by using the following formula given by the manufacturer:

\[
\text{[Nitrate + Nitrite]} = \frac{(A_{540} - \text{y-intercept})/\text{slope} * (200 \mu\text{l} / \text{volume of sample used} \mu\text{l}) * \text{dilution}}{}
\]

The coefficient of variation for sample replicates was less than 10%.

![Image of nitrate standard curve for nitrate/nitrite assay.](image)

Figure 3-1. Nitrate standard curve for nitrate/nitrite assay. Note: y = 0.0332x + 0.0275, \(R^2 = 0.9888\); blue = standards, black = suppressed standards.

**Analysis of TNF-\alpha and IL-1\beta in Wound Fluid**

Quantitative measurement of TNF-\alpha and IL-1\beta was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit from
Amersham Biosciences (Buckinghamshire, England). The assay sensitivity is less than one pg/ml for IL-1β and less than five pg/ml for TNF-α. The “sandwich” enzyme immunoassay technique is employed in both of these kits. The ELISAs were performed separately; however, both assay procedures are similar and will be discussed simultaneously in subsequent paragraphs.

All reagents and working standards were prepared according to the manufacturer’s instruction manual. Prior to running both assays, a plate template identifying the locations of the samples, standards, and blanks was created in Microsoft Excel program. Based on previous analyses, the wound fluid was diluted with the provided diluent to 100-fold and five-fold for IL-1β and TNF-α, respectively. A 1:2.5 serial dilution was prepared for both standard curves. Five standards, one zero and unknown samples, were run in duplicates. All empty wells were filled with sample diluent. A three hour and two hour incubation time at room temperature were observed after 50 µl of the appropriate biotinylated antibody reagent was added to all wells for IL-1β and TNF-α, respectively. At the end of each period, the plates were manually washed three times using a squirt bottle and blotted on paper towel. A 100 µl of pre-diluted streptavidin-HRP (horseradish peroxidases) conjugate was pipetted immediately into each wells using a multi-channel pipette (Rainin Instruments) and incubated for 30 minutes at room temperature. Using the same procedure described above, the plates were washed three times. Then, 100 µl of pre-mixed TMB substrate was added to each well and incubated at room temperature for 30 minutes in the dark. Using a plate reader set at 450 nm, the optical density of each well was determined within 30 minutes of the addition of the stop solution. KC junior (Bio-Tek Instruments), a curve- fitting statistical software package, generated a standard
curve for each ELISA (see Fig 3.2). Based on this curve, levels of IL-1β and TNF-α were quantified in the wound fluid. Note that the best-fit curve for TNF-α is not a line. Instead, a four-parameter logistic curve fit was plotted as suggested by the manufacturer with $R = .9963$. The coefficient of variation for sample duplicates was less than 10%.

Figure 3-2. TNF-α standard curve for enzyme-linked immunosorbent assay. Note: $y = [(1.8273-2.3367)/(1 + (x/36.8707)^{0.5182} ) + 2.3367], R^2 = 0.9963$, h = human, TNF-α = tumor necrosis factor-alpha
**Quantification of Total Protein**

To correct for the dilutional effect in the assays, total protein content was analyzed by using the BCA™ Protein Assay Kit (Pierce, Rockford, IL). In this particular assay, the microplate procedure was used due to a smaller volume requirement. The preparation of diluted bovine serum albumin and working reagent was done per manufacturer’s instructions. In duplicates, 25 µl of each standard and unknown sample were pipetted into a microplate well (Nunc Brand Products, Denmark). Then, 200 µl of the prepared working reagent (25 ml of Reagent A with 1 ml of Reagent B) was added onto each well and mixed thoroughly on a plate shaker for 30 seconds (MaxQ 2000, Barnstead Lab-line, Melrose Park, IL). After incubation for 30 minutes at 37 oC, the plate was read at 540 nm on a plate reader. Using KCjunior software (Bio-Tek Instruments), a four-parameter curve was used (see Fig 3.3) as recommended by the manufacturer to determine the concentration of protein in the wound fluid. The coefficient of variation for sample
duplicates was less than 10%. The inflammatory cytokines, TNF-α and IL-1β, were then normalized to total protein content and expressed as pg/ug of protein.

**Amino Acid Analysis**

Based on the larger V.A.C. Study, levels of arginine, citrulline, ornithine, and proline of wound fluids were analyzed by high performance liquid chromatography (HPLC) method (Waters, Millford, MA).

![Figure 3-4. Protein assay standard curve. Note: 4 parameter: y = (4.14633-0.0331)/(1 + (x/2070)^1.1775 + 0.0331), R^2 = 0.9994; blue = standards, black = suppressed standard](image)

**Study Design**

A prospective quasi-experimental repeated measures design was utilized to investigate the metabolic activity of arginine in wound fluids of patients with pressure ulcers on V.A.C. therapy. Every subject was on V.A.C. therapy and evaluated at each time interval. Therefore, each subject served as his/her own control (Portney & Watkins,
Wound fluid was collected at baseline prior to V.A.C.® application and within 24 hours, three days, and seven days of V.A.C.® placement (see Figure 3-4).

Consent

In accordance to the Health Insurance Portability and Accountability (HIPAA) guidelines, clinicians informed V.A.C.® candidates of the study and notified investigators of potential subjects. Subjects or health surrogates with power-of-attorney were approached in person or via phone. The purpose, risks, and benefits of the study were discussed in detail (Appendix A). Furthermore, the subject’s right to withdraw from the project at anytime without consequences was explained. For consents obtained over the phone, a witness, such as the patient’s nurse, was involved in the consent process. After the appropriate parties consented, a brief review of the medical record for subject eligibility was conducted. Clinicians were notified if the subject met all the study criteria. Coordination of time for V.A.C.® placement was vital to baseline wound fluid collection.

Study Protocol

The diagram (see Figure 3-5) illustrates the study protocol used in this study.

[Diagram of study protocol]

Figure 3-5. Diagram of study protocol
Wound Fluid Collection and Storage

Once consent was obtained, a transparent polyurethane occlusive dressing (Tegaderm, 3M, St. Paul, Minnesota) was placed over the pressure ulcer prior to the initiation of V.A.C.® therapy. Hydration status of the patients was standardized through intake of 500 ml of fluids by mouth. Three of the eleven subjects were placed on maintenance intravenous fluids and/or other intravenous medications. Subjects were placed on their side for at least an hour, which facilitated fluid collection. After this period, the fluid was aspirated from beneath the dressing using a sterile needless tuberculin syringe being careful to avoid injury to the underlying tissue (Stacey & Trengove, 1995). This procedure was repeated two or three more times if not enough fluid was present. If no fluid was found on the third attempt, 1 ml of normal saline (NS) was injected into the wound. Three of the eleven subjects had 1 ml of NS added into the wound.

Approximately 0.5-2 ml of fluid was collected in a 15-ml Fisherbrand disposable sterile centrifuge tube (Fisher Scientific, Pittsburg, PA). This specimen was placed immediately on ice and transferred to the laboratory in a biohazard container. The sample was pipetted into 1.5-ml microtubes (Fisher Scientific, Pittsburg, PA) and centrifuged (Eppendorf Centrifuge, Westbury, NY) at 8000 rpm for 15 minutes. Small but visible pellets were discarded. The supernatant was aliquoted into separate microvials and stored at –80 °C until analyzed. For the amino acid profile, one unspun vial was stored in the same manner. These specimens served as baseline data.

Within 24 hours following application of the V.A.C.®, additional wound fluid was collected from the tubing of the V.A.C.® System. This was accomplished by clamping off both ends of the tubing and stopping the therapy. First, the clamp proximal to the
patient was clamped off followed by the second one, which was distal to the patient. Then, therapy was stopped for approximately 10-15 seconds. The tube was disconnected and fluid was allowed to drain into the 15-ml tubes. Immediately thereafter, the tubes were reconnected and unclamped. The patient was informed that a quick gentle-like massage or suction would to be felt once the V.A.C.® is placed back on therapy. Tubing connection and V.A.C.® pressure settings were verified prior to leaving the patient’s bedside. This procedure was repeated for the 3rd and 7th day collection. Using the same steps to transfer, handle, and store the specimens as previously described.

Wound fluid was collected from the V.A.C.® system at least two hours after dressing and/or tubing changes. It was reported by Childress and colleagues (2003) that certain biochemical markers were altered upon exposure to the V.A.C.® components. In this study, the impact of time (0, 1, and 6 hour) and wound exposure to V.A.C.® foam and tubing was investigated. Preliminary findings indicated that there was an immediate decrease in IL-1β levels upon wound fluid exposure to foam and tubing. These levels, however, remained constant over the six-hour time period. No conclusions could be made regarding TNF-α. In a separate analysis, NOx levels were noted to slightly increase within an hour of exposure to V.A.C.® foam, but remained constant over time. Whereas, the V.A.C.® tubing did not appear to alter NOx levels at all time period. Furthermore, the V.A.C.® components, foam and tubing, were found to contain very low to undetectable amounts of NOx.

**Data Management and Analysis**

The subject’s baseline profile and other pertinent data were compiled in a folder with his/her initials and identification number. These folders were secured in a file cabinet in a locked office. The office was centrally located for easy accessibility. A
spreadsheet was created in Microsoft Excel program, which was exported to statistical software for analysis. Summary measures were generated from SPSS (SPSS Inc., Chicago, IL) on a Windows based computer. Sample size was calculated using two-sided one sample t-test with an overall type I error .05. Power was determined to be over 80% with sample size nine as long as delta ÷ sigma ≥ 0.31 (Splus Software). Delta represents the expected difference between NO\textsubscript{x} levels before and after V.A.C.® placement, and sigma denotes the standard deviation of the difference. Based on preliminary data available, the study sample size was deemed sufficient to address the study aim.

Since the assumptions of normality were violated, nonparametric statistical techniques were used to determine significance at .05 for the aims of this particular study. The specific aims, hypotheses testing, statistical tests, and outcome measures are summarized in Table 3-1.

<table>
<thead>
<tr>
<th>Specific Aims</th>
<th>Hypothesis Testing</th>
<th>Statistical Test</th>
<th>Outcome Measure</th>
</tr>
</thead>
</table>
| **Aim 1:** Evaluate the effects of the V.A.C.® on NO\textsubscript{x} levels | a) H\textsubscript{0}: \( \mu_1 = \mu_2 \)  
H\textsubscript{1}: \( \mu_1 > \mu_2 \)  
b) H\textsubscript{0}: \( \mu_2 = \mu_3 = \mu_4 \)  
H\textsubscript{1}: H\textsubscript{0} is not true under \( \mu_2 \geq \mu_3 \geq \mu_4 \) | a) Wilcoxon Signed-Ranks Test  
b) Friedman Two-way Analysis of Variance  
c) Spearman Correlations. | NO\textsubscript{x} concentrations |
| **Aim 2:** Evaluate the effects of the V.A.C.® on citrulline, ornithine, and proline | a) H\textsubscript{0}: \( \mu_1 = \mu_2 \)  
H\textsubscript{1}: \( \mu_1 > \mu_2 \)  
b) H\textsubscript{0}: \( \mu_2 = \mu_3 = \mu_4 \)  
H\textsubscript{1}: H\textsubscript{0} is not true under \( \mu_2 \geq \mu_3 \geq \mu_4 \) | a) Wilcoxon Signed-Ranks Test  
b) Friedman Two-way Analysis of Variance  
c) Spearman Correlations | Levels of citrulline, ornithine, and proline |
CHAPTER 4
RESULTS

The primary aim of this study was to evaluate the effects of the Vacuum Assisted Closure (V.A.C.®) on nitrate/nitrite (NO\textsubscript{x}) levels in wound fluids from non-healing and healing pressure ulcers. The secondary aim of the study was to evaluate the effects of the V.A.C.® on arginine citrulline, ornithine, and proline in wound fluids from non-healing and healing pressure ulcers. The presence of these metabolites reflects inducible nitric oxide synthase (iNOS) and arginase activities. This chapter first presents descriptive results including mean, median, range, standard deviation, and frequency data for each of the variables. The two hypotheses posed in Chapter 1 are addressed using the following nonparametric tests, Wilcoxon-Signed Ranks Test, Friedman two-way analysis of variance by ranks, and Spearman Rank Correlation.

**Statistical Procedure**

Values considered below the detection limit of the assays used in this study were corrected and included in the data analyses. This was accomplished by taking from the smallest value of the specific assay divided by two. All data files were prepared in the Microsoft Excel program and then imported into SPSS for analysis. Prior to performing any of the statistical analysis, the raw data were checked for accuracy. Then, the normality of the distribution of values for all continuous variables was assessed. This was accomplished by obtaining descriptive statistics, which included the mean, standard deviation, range, skewness, and kurtosis. The tests of normality were performed to obtain the Kolmogorov-Smirnov and Wilks-Shapiro statistics. Additional information
was gained through visual inspection of histograms, normal Q-Q plots, detrended normal Q-Q plots, and box-plots. Many of the variables were positively skewed, thus, violating the normality assumptions. Log transformations were performed in an attempt to normalize the variables. Unfortunately, the data remained abnormally distributed. As a result, the non-parametric statistics were used to analyze the data.

**Descriptive Results**

**Subject Demographics**

Twenty-eight subjects were invited to participate in this study. Four patients were contacted by phone, and the remainder was invited to participate in person. Thirteen subjects did not meet the study criteria. Two subjects were not interested in participating in the study for various reasons. One subject was on nitric oxide (NO\(^*\)) inhalation therapy for pulmonary hypertension. Only one subject was dropped from the study because the V.A.C.\(^\circledR\) was found to hinder rehabilitative activities.

Subject demographics expressed in numbers and percentages included age, gender, race, diabetes, and stage of pressure ulcer. Table 4-1 identifies the subject demographics, which are expressed in numbers and percentages.

**Clinical Measurements**

Table 4-2 lists the main clinical measurements for the entire study. The mean age was 61 years with a range of 31 to 92.
Table 4-1: Subject Demographic Summary

<table>
<thead>
<tr>
<th>Variables</th>
<th>N (total = 11)</th>
<th>Percent</th>
</tr>
</thead>
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<tr>
<td><strong>Age</strong></td>
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<td>30-39</td>
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<td>40-49</td>
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<td>70-79</td>
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<td>9.1</td>
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<td>No</td>
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<td>45</td>
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<tr>
<td>Yes</td>
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<td>55</td>
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<td><strong>Stage of Pressure</strong></td>
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</tr>
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</tr>
<tr>
<td>Stage IV</td>
<td>6</td>
<td>55</td>
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</table>

Table 4-2: Summary Statistics of NO\textsubscript{x}, Cytokines, and Amino Acid

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<thead>
<tr>
<th>Variables</th>
<th>N</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Median</th>
<th>Mean</th>
<th>Std. Deviation</th>
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<tr>
<td>Age</td>
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<td>92</td>
<td>67.1</td>
<td>61</td>
<td>16.56</td>
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<tr>
<td><strong>NO\textsubscript{x} (µM)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>baseline</td>
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<td>6.30</td>
<td>86.78</td>
<td>29.48</td>
<td>33.04</td>
<td>22.41</td>
</tr>
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<td>24-hr</td>
<td>11</td>
<td>7.17</td>
<td>83.79</td>
<td>23.96</td>
<td>28.58</td>
<td>24.58</td>
</tr>
<tr>
<td>3d</td>
<td>11</td>
<td>1.50</td>
<td>64.87</td>
<td>14.04</td>
<td>19.72</td>
<td>19.42</td>
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<tr>
<td>7d</td>
<td>10</td>
<td>0.07</td>
<td>26.97</td>
<td>16.50</td>
<td>14.70</td>
<td>8.27</td>
</tr>
<tr>
<td><strong>IL-1β (pg/mg protein)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>baseline</td>
<td>11</td>
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<td>0.5746</td>
<td>0.0592</td>
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<td>0.2209</td>
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<td>0.4763</td>
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</tr>
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<td>0.0070</td>
<td>0.6320</td>
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<td>7d</td>
<td>10</td>
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<td>0.1552</td>
<td>0.2220</td>
<td>0.2481</td>
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<tr>
<td><strong>TNF-α (pg/mg protein)</strong></td>
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<td></td>
</tr>
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<td>11</td>
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<td>0.1293</td>
<td>0.0192</td>
<td>0.0274</td>
<td>0.0355</td>
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<td>0.0078</td>
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<td>0.0119</td>
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<td>0.0030</td>
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<td>7d</td>
<td>10</td>
<td>0.0036</td>
<td>0.0201</td>
<td>0.0054</td>
<td>0.0072</td>
<td>0.0050</td>
</tr>
<tr>
<td><strong>Proline (µM/L)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>baseline</td>
<td>10</td>
<td>28</td>
<td>779</td>
<td>335</td>
<td>384.95</td>
<td>268.63</td>
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Table 4-2. Continued

<table>
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<tr>
<th>Variables</th>
<th>N</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Median</th>
<th>Mean</th>
<th>Std. Deviation</th>
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</thead>
<tbody>
<tr>
<td>24-hr</td>
<td>10</td>
<td>72</td>
<td>929</td>
<td>397</td>
<td>427.8</td>
<td>236.74</td>
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<td>3d</td>
<td>10</td>
<td>3.5</td>
<td>2841</td>
<td>345</td>
<td>551.65</td>
<td>849.19</td>
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<tr>
<td>7d</td>
<td>9</td>
<td>37.5</td>
<td>1234</td>
<td>360</td>
<td>508.94</td>
<td>434.83</td>
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<tr>
<td>Citrulline (µM/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>baseline</td>
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<td>302.5</td>
<td>102</td>
<td>116.95</td>
<td>89.33</td>
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<td>24-hr</td>
<td>10</td>
<td>74</td>
<td>695</td>
<td>131.5</td>
<td>191.9</td>
<td>185.38</td>
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<td>3d</td>
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<td>444</td>
<td>110</td>
<td>128</td>
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</tr>
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<td>9</td>
<td>198</td>
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<td>127.01</td>
<td>61.99</td>
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<td>Ornithine (µM/L)</td>
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<td>4</td>
<td>541.25</td>
<td>167.5</td>
<td>215.13</td>
<td>163.55</td>
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<td>24-hr</td>
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<td>133</td>
<td>743</td>
<td>251</td>
<td>301.1</td>
<td>174.19</td>
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<td>3d</td>
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<td>890</td>
<td>260</td>
<td>345.5</td>
<td>263.57</td>
</tr>
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<td>7d</td>
<td>9</td>
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<td>495</td>
<td>149</td>
<td>193.08</td>
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</tr>
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<td>Arginine (µM/L)</td>
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<td></td>
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<tr>
<td>baseline</td>
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<td>17</td>
<td>345</td>
<td>102.5</td>
<td>122.9</td>
<td>96.39</td>
</tr>
<tr>
<td>24-hr</td>
<td>10</td>
<td>35</td>
<td>168</td>
<td>87</td>
<td>95.9</td>
<td>46.09</td>
</tr>
<tr>
<td>3d</td>
<td>10</td>
<td>7</td>
<td>169</td>
<td>42</td>
<td>57.58</td>
<td>48.98</td>
</tr>
<tr>
<td>7d</td>
<td>9</td>
<td>12.5</td>
<td>159</td>
<td>95</td>
<td>77.72</td>
<td>52.33</td>
</tr>
</tbody>
</table>

Analytic Results

Statistical Analysis of Change for NO₅

Aim 1

To evaluate the effects of the V.A.C.® on nitrate/nitrite (NOₓ) levels in wound fluids from non-healing and healing pressure ulcers.

A visual examination of the data shows decreasing levels of NOₓ over time (see Table 4-2, Figure 4-1). Variability is low between time points as indicated by the close approximation of the error bars (Fig 4-1). The Friedman two-way analysis of variance by ranks, however, did not yield statistical significance with time as an independent variable. A Wilcoxon signed-ranks test was performed to determine the difference in NOₓ levels in wound fluids prior to and after V.A.C.® application. There was no significant difference in NOₓ levels at baseline with the post-V.A.C.® levels measured at 24 hours, three days,
and seven days (Table 4-3). However, there was a statistically significant difference in NO\textsubscript{x} levels from 24 hours to 7 days of V.A.C.\textsuperscript{®} therapy ($z = -2.395$, $p = 0.017$).

![Graph showing concentration of NO\textsubscript{x}](image)

**Figure 4-1.** Concentration of NO\textsubscript{x} at baseline and at 24 hours, 3 days, and 7 days of V.A.C.\textsuperscript{®} treatment. Values are expressed as means +/- SD.

**Table 4-3: Paired Differences for NO\textsubscript{x} by Ranks**

<table>
<thead>
<tr>
<th>Differences</th>
<th>Ranks</th>
<th>N</th>
<th>Mean Rank</th>
<th>Sum of Ranks</th>
<th>Z</th>
<th>p (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO7d &lt; NObase Negative</td>
<td>6</td>
<td>6</td>
<td>7.00</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO7d &gt; NObase Positive</td>
<td>4</td>
<td>4</td>
<td>3.25</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO7d - NObase Total</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>-1.478</td>
<td>.139</td>
</tr>
<tr>
<td>NO7d &lt; NO24hr Negative</td>
<td>8</td>
<td>8</td>
<td>6.38</td>
<td>51</td>
<td>-2.395</td>
<td>0.017</td>
</tr>
<tr>
<td>NO7d &gt; NO24hr Positive</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO7d - NO24hr Total</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The graph shown in Fig 4-2 depicts a drastic drop in mean levels of TNF-α from baseline to 24 hours of V.A.C.\textsuperscript{®} therapy. Then, the mean levels stabilized by days three and seven of treatment. To determine if significant differences existed, the Wilcoxon signed-ranks test was conducted (see Table 4-4). There was a statistically significant difference in TNF-α levels from baseline to 24 hours ($p = .016$, $z = -2.401$), baseline to three days ($p = .010$, $z = -2.578$), and baseline to seven days ($p = 0.028$, $z = -2.191$).
Furthermore, the Friedman test showed a significant difference in TNF-\(\alpha\) concentrations over time (\(\chi^2 = 6.84, \text{df} = 3, p = .039\), one-tailed).

Figure 4-2. Concentration of TNF-\(\alpha\) at baseline and at 24 hours, 3 days, and 7 days of V.A.C.\textsuperscript{®} treatment. Values are expressed as means +/- SD.

Table 4-4: Paired Differences for TNF-\(\alpha\) by Ranks

<table>
<thead>
<tr>
<th>Differences</th>
<th>Ranks</th>
<th>N</th>
<th>Mean Rank</th>
<th>Sum of Ranks</th>
<th>Z</th>
<th>p (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF24hr &lt; TNFbase</td>
<td>Negative</td>
<td>8</td>
<td>7.5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TNF24hr &gt; TNFbase</td>
<td>Positive</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF24hr - TNFbase</td>
<td>Total</td>
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<td>-2.40</td>
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<td>TNF3d &lt; TNFbase</td>
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</tr>
<tr>
<td>TNF3d &gt; TNFbase</td>
<td>Positive</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF3d - TNFbase</td>
<td>Total</td>
<td>11</td>
<td></td>
<td>-2.58</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>TNF7d &lt; TNFbase</td>
<td>Negative</td>
<td>7</td>
<td>7</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF7d &gt; TNFbase</td>
<td>Positive</td>
<td>3</td>
<td>2</td>
<td>6</td>
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<td></td>
</tr>
<tr>
<td>TNF7d - TNFbase</td>
<td>Total</td>
<td>10</td>
<td></td>
<td>-2.19</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

**Correlational Analysis for NO\textsubscript{x} and Cytokines**

As part of aim 1, the relationship between NO\textsuperscript{x} levels with TNF-\(\alpha\) and IL-1\(\beta\) was investigated using the Spearman rank order correlation. There was a positive correlation between TNF-\(\alpha\) and IL-1\(\beta\) (rho = .5811, n = 11, p < 0.030, one-tailed), with TNF-\(\alpha\) levels
associating moderately with IL-1β concentrations. A very weak positive correlation of NO* with IL-1β and TNF-α existed; however, it was not statistically significant.

**Statistical Analysis of Change for Arginine, Citrulline, Ornithine, and Proline**

**Aim 2**

Evaluate the effects of the V.A.C.® on arginine, citrulline, ornithine, and proline in wound fluids from non-healing and healing pressure ulcers.

Levels of arginine decreased from baseline to 24 and 72 hours, but increased marginally by day seven of V.A.C.® therapy (Fig 4.3). Citrulline levels increased in 24 hours, then decreased to baseline. Whereas, ornithine and proline levels increased from baseline to 24 and 72 hours of V.A.C.® treatment (see Figure 4-4.). Both levels decreased by day seven with ornithine levels falling below pre- V.A.C.® levels (see Fig 4-4 and Table 4-2). Variability is low between time points for all amino acids as indicated by the close approximation of the error bars (see Figure 4-4 and 4-5).

A Wilcoxon signed-Ranks test was conducted to determine the differences in pre- and post- V.A.C.® levels of arginine, citrulline, proline, and ornithine in wound fluids (Table 4-5). Arginine levels at baseline were different from the post- V.A.C.® levels at day three (z = -1.89, p = .03). There were no significant differences in citrulline, ornithine, and proline concentrations before and after V.A.C.® application. Furthermore, the Friedman two-way analysis of variance by ranks, did not yield significance for any of the variables tested over time.
Figure 4-3. Levels of arginine and citrulline at baseline and at 24 hours, 3 days, and 7 days of V.A.C.® treatment. Values are expressed as means +/- SD.

Figure 4-4. Levels of ornithine and proline at baseline and at 24 hours, 3 days, and 7 days of V.A.C.® treatment. Values are expressed as means +/- SD.
Table 4-5: Paired Differences for Arginine by Ranks

<table>
<thead>
<tr>
<th>Differences</th>
<th>Ranks</th>
<th>N</th>
<th>Mean Rank</th>
<th>Sum of Ranks</th>
<th>Z</th>
<th>p (1-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg3d &lt; Argb</td>
<td>Negative</td>
<td>8</td>
<td>8.25</td>
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<td>Positive</td>
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<td></td>
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<tr>
<td>Arg3d - Argb</td>
<td>Total</td>
<td>10</td>
<td></td>
<td>-1.89</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

Note: arg = arginine, b = baseline

Correlational Analysis for iNOS and Arginase

To determine the relationship between citrulline, proline, ornithine, and arginine levels at baseline, the Spearman rank order correlation was performed. All pre- V.A.C.® levels correlated highly and significantly. A strong positive correlation existed between proline and citrulline (rho = .782, p = .008), between proline and ornithine (rho = .879, p = .001), and between proline and arginine (rho = .830, p = .003). Baseline levels of citrulline correlated moderately with baseline levels of arginine (rho = .770, p = .009) and highly with baseline levels of ornithine (rho = .879, p = .001). Pre- V.A.C.® levels of ornithine moderately correlated with pre- V.A.C.® levels of arginine (rho = .697, p = .025).

The Spearman correlations were conducted to determine the relationships of citrulline with the by-products of arginase, ornithine and proline, on the 7th day of V.A.C.® placement. A negative and very weak inverse correlation existed between citrulline and proline (rho = -.075, p = .847) as well as between citrulline and ornithine (rho = -.0133, p = .732). Furthermore, citrulline weakly and negatively correlated with arginine (rho = -.017, p = .966). As one can see, all correlations were not statistically significant, however, the existing relationship has been established in the literature.

A very weak correlation existed between NOx, citrulline, and arginine at baseline. Interestingly, a statistically high and moderate relationship exits between citrulline and
arginine at baseline (rho = .770, p = .009). This relationship was not observed at 7th day of V.A.C.® therapy. Instead, a statistically significant correlation existed between NO• and arginine (rho = .833, p = .002). An inverse but very weak relationship was noted between NO• and citrulline levels (rho = -0.17, p = .966) seven day post- V.A.C.® placement.
CHAPTER 5
DISCUSSION AND CONCLUSIONS

In this chapter, the descriptive and analytic results addressed in chapter 4 will be discussed in detail. Conclusions regarding the research hypotheses are provided with rationales as supported in the literature. In addition, implications for clinical practice and recommendations for future research are provided.

Discussion of Results

Demographics

Fifty-five percent (N = 6) of the subjects in the study were men and 45% were women (N = 5). Ninety-one percent were Caucasian and 9% were African-American. Fifty-four percent were between 50-69 years of age with 18% accounting for below 50 and 27% above 70 years of age. Almost half of the sample was non-diabetic (45%), while 55% were diabetics. Approximately 45% had stage III pressure ulcer, and the remainder with stage IV pressure ulcer (55%).

Clinical Characteristics

Of the 11 subjects who were enrolled in the study, only nine completed the study protocol. The other two subjects were excluded in some but not all of the statistical analyses. An insufficient amount of wound fluid resulted in loss of data for the amino acid profile for one subject. A similar problem is attributed to the other subject with difficulty in obtaining a specimen for the 7th day of V.A.C.® therapy.
NO\textsubscript{x} Results

Aim 1

To evaluate the effects of the V.A.C.\textsuperscript{®} on nitrate/nitrite (NO\textsubscript{x}) levels in wound fluids from non-healing and healing pressure ulcers.

The null hypothesis for Aim 1 was that there is no difference between pre- V.A.C.\textsuperscript{®} and post- V.A.C.\textsuperscript{®} NO\textsubscript{x} concentrations. We accept the null hypothesis and conclude that there was no significant difference in NO\textsubscript{x} levels from baseline to day one, three, and seven of V.A.C.\textsuperscript{®} placement. Notably, NO\textsubscript{x} measured at 24 hours of V.A.C.\textsuperscript{®} therapy was significantly different from day seven (p = 0.017). Further evaluation of the data show decreasing levels at all four time points (see Table 4-2 and Figure 4-1). Although, the correlational analysis did not yield statistical significance, a fair degree of relationship existed between baseline NO\textsubscript{x} levels and at 24 hours (rho = .245, p = .467) of V.A.C.\textsuperscript{®} treatment. Pre-V.A.C.\textsuperscript{®} NO\textsubscript{x} levels became inversely correlated with post-V.A.C.\textsuperscript{®} levels on days three (rho = -.2, p = .555) and seven (rho = -.612, p = .03, one-tailed) of therapy.

Based on the above findings, one can conclude that a different wound environment exists after the V.A.C.\textsuperscript{®} is applied. Wound fluids from non-healing pressure ulcers contain high amounts of NO\textsubscript{x}. Within 24 hours of V.A.C.\textsuperscript{®} placement, NO\textsubscript{x} dropped consistently and persistently over the study period. This result is consistent with previous studies in experimental wounds. During the early phase of inflammation, macrophage and other wound cells express iNOS to synthesize NO\textsuperscript{•}. The activity of this enzyme peaks within 24-72 hours post-injury (Albina et al., 1990; Becker et al., 1993, Carter et al., 1994; R.H. Lee, et al., 2001, Reichner et al., 1999). According to Albina and colleagues, NO\textsuperscript{•} concentrations in wound fluids were highest before day three of sponge
implantation in rats. One can infer that a pseudo-acute wound environment is created by the V.A.C.®. Additionally, a physiological level of NO* that is conducive to healing is achieved by the 7th day of V.A.C.® therapy.

Secondary statistical analyses were performed to determine the role of diabetes mellitus in the study sample. In a recent study by Jude et al. (1999), subjects who had diabetic foot ulcers were shown to have increased iNOS and arginase activities. For this study, the Mann-U Whitney test did not yield statistical significant difference in NO levels between subjects with and without diabetes. This could be due to one of the limitations of the study, the sample size.

**Relationship between NOx and Pro-inflammatory Cytokines**

Little to no relationship exists between baseline NOx and IL-1β levels (rho = .009, p > .05), and between NOx and TNF-α (rho = .018, p > .05). Thus, NO* does not covary with IL-1β and TNF-α at baseline. In addition to IL-1β and TNF-α, other inducers of iNOS expression include IFN-γ and LPS (Taylor & Gaylor, 2001).

**Relationship between IL-1β and TNF-α**

A strong positive correlation between IL-1β and TNF-α levels (rho = .582, n = 11, p < 0.03, one-tailed) exists at baseline. Thus, a change in IL-1β levels is proportionally related to a change in TNF-α in chronic pressure ulcer wounds. Interestingly, baseline levels of TNF-α are significantly different from post- V.A.C.® therapy at 24 hours (p = .016), three days (p = .010), and seven days (p = .028). This was demonstrated in two statistical analyses using the Wilcoxon Signed-Ranks test and Friedman’s two-way analysis of variance by ranks. This clearly suggests a decrease in inflammation of pressure ulcer wounds as it heals over time (Mast & Schultz, 1996). Similarly, Trengove
and colleagues (2000) showed high levels of TNF-α in wound fluids from patients with chronic venous leg ulcers. Within two weeks, the levels significantly decreased in the healing wounds. Similar trends in TNF-α levels are observed in this project, but the TNF-α present in chronic wound of pressure ulcers are 10 times less than are reported by Trengove et al. (2000).

**Results of the Amino Acid profiles**

**Aim 2**

To evaluate the effects of the V.A.C.® on arginine, citrulline, ornithine, and proline in wound fluid from non-healing and healing pressure ulcers.

The null hypothesis for Aim 2 was that there are no significant differences in pre- and post- V.A.C.® levels of arginine, citrulline, ornithine, and proline in wound fluids. We reject the null hypothesis and conclude that there was a significant difference in arginine levels measured at baseline and day three of V.A.C.® therapy. We accept the null hypothesis and conclude that there were no significant differences in pre- and post-V.A.C.® levels of citrulline, ornithine, and proline in wound fluids.

Abnormalities in arginine metabolism have been cited as the pathogenesis of chronic venous ulcers and diabetic foot ulcers in humans (Jude et al., 1999; Abd-El-Aleem et al., 2000). In these types of wounds, increased iNOS and arginase activities were found resulting in high levels of NO•, citrulline, and ornithine. For this particular study sample, the pressure ulcer wounds contained high levels of arginine (Fig 5-1) and NOx (Fig 5-2). In contrast, citrulline and ornithine were present at lower concentrations (Fig 5-2). Since iNOS appears to be the predominantly active enzyme in the chronic
wound, its substrate is sustained at a higher level. It appears that NO$^*$ along with the pro-inflammatory cytokines are maintaining the pressure ulcer wound in its chronic state.

Figure 5-1. Bar graph representing pre-V.A.C.$^\text{®}$ and post-V.A.C.$^\text{®}$ levels of arginine, citrulline, ornithine, and proline. Values are expressed as means.

Figure 5-2. Bar graph representing pre-V.A.C.$^\text{®}$ and post-V.A.C.$^\text{®}$ NO$_x$ levels. Values are expressed as means.
In terms of substrate availability, arginine appears to be utilized by iNOS and arginase at 24 hours of V.A.C.® therapy. This increased in catabolic activity is evident by the high levels of NO_x, citrulline, ornithine, and proline at 24 hours of V.A.C.® therapy. Within 72 hours, arginine reached its lowest level as arginase activity peaked. This is reflected by a concurrent increased in levels of ornithine and proline. Simultaneously, iNOS activity decreased as seen by the lower levels of NO_x and citrulline from 24 hours. Arginine supply is slowly replenished as both activities of iNOS and arginase decreased. By day seven, both citrulline and NO_x levels continued to drop while ornithine and proline levels began to decrease. These findings are consistent with previous studies on animal models using models of acute and impaired healing.

**Relationship of iNOS and Arginase Activities**

At baseline, arginine, citrulline, ornithine, and proline, were strongly correlated. A very weak but positive relationship was noted between NO* and citrulline as well as arginine. Therefore, an interaction did exist at baseline between iNOS, arginase, and arginine in a positive direction. Although not statistically significant, a positive relationship was noted between ornithine and proline on day seven of V.A.C.® therapy. In contrast, an inverse correlation existed between citrulline and the by-products of arginase. Furthermore, a strong positive relationship existed between arginine and NO_x (p = .002). These findings are consistent with the substrate utilization and reciprocal relationship of iNOS and arginase in wounds.

**Conclusions**

The main research hypotheses for this particular study sample were not statistically significant. However, the by-products of iNOS and arginase are detectable in wound
fluids from patients with pressure ulcers. To date, the metabolism of arginine has not been described in humans with pressure ulcers on V.A.C.® therapy.

The cytotoxic properties of NO• are vital to the inflammatory phase of wound healing. Within seven days of V.A.C.® treatment, NO• levels decreased significantly. This was corroborated by the presence of the pro-inflammatory cytokine, TNF-α. Post-V.A.C.® values at 24 hours, three days, and seven days were found to be significantly different from baseline. This is indicative of a healing wound as previously reported by several investigators. Clearly, the vicious cycle characteristic of chronic wounds was disrupted after V.A.C.® placement. A less cytotoxic environment is created by the V.A.C.®, thereby allowing pressure ulcer wounds to heal.

Recall from aim 1 that post- V.A.C.® levels of NOx at 24 hours and seven days were statistically significant. From aim 2, it was shown that arginine levels before V.A.C.® therapy were significantly different on the 3rd day of V.A.C.® treatment. Both citrulline and NO• levels decreased by day three and continued to drop until day seven. In contrast, proline and ornithine levels peaked at day three and began to decrease by day seven. Hence, the iNOS/citrulline pathway predominated during the first 72 hours of V.A.C.® therapy. Subsequently, the arginase/ornithine pathway dominated the remainder of the therapy. Hence, a pseudo-acute environment is achieved shortly after the V.A.C.® was applied followed by an environment conducive to healing.

Study findings should be cautiously interpreted. First, generalizability is limited to pressure ulcer patients. In addition, a small sample size was used in this study through non-random selection. The Caucasian race is representative of the study sample; therefore, extrapolation to other races would be difficult. Furthermore, there was no
control group. Instead subjects served as their own control due to the repeated measures design of the study.

**Implications for Clinical Practice**

In today’s changing practice, wound health care professionals are bombarded with many new products and technologies. The course of treatment chosen is due to many factors. The V.A.C.®, for example, has been in use for several years. The exact mechanism by which this device accelerates healing is not well understood. Clinical trials are few and the efficacy of this treatment is not well documented (Evans & Land, 2004). It is imperative, therefore, to individualize wound care management and be informed about current research. Knowledge of the physiology of acute wound healing is key to understanding chronic wounds and treatment. This requires periodic literature review and conference attendance. Patient education should include the importance of nutrition, alcohol and smoking cessation, and treatment compliance.

**Recommendations for Further Research**

Limitations of this study include the small sample size and lack of a control group. However, the findings of the study were sufficient to meet the exploratory and descriptive nature of this project. Recommendations for future research include: 1) increasing study sample size to include other race and ethnic backgrounds, 2) randomizing subjects to either conventional treatment or V.A.C.® therapy, 3) increasing the timeframe to quantify healing through wound size measurements and provide a better understanding of arginine metabolism, 4) performing punch biopsies for immunohistochemical studies, 5) creating a research group to recruit subjects and collect data, 6) expanding wound criteria to other types of chronic wounds, and 7) providing incentive to study subjects.
Informed Consent to Participate in Research

You are being asked to take part in a research study. This form provides you with information about the study. The Principal Investigator (the person in charge of this research) or a representative of the Principal Investigator will also describe this study to you and answer all of your questions. Before you decide whether or not to take part, read the information below and ask questions about anything you do not understand. Your participation is entirely voluntary.

1. Name of Participant ("Study Subject")

2. Title of Research Study

   Biochemical analysis of wound fluid from acute and chronic wounds

3. Principal Investigator and Telephone Number(s)

   Joyce K. Stechmiller, PhD, ARNP (352) 273-6370
   Bobbi Langkamp-Henken, PhD (352) 392-1991 x 205
   Beverly Childress, BSN (352) 273-6370
   Tricia Porter (352) 273-6370

4. Source of Funding or Other Material Support

   University of Florida College of Nursing KCI, Inc

5. What is the purpose of this research study?

   You have an acute or chronic wound, which is currently being treated with the
Vacuum Assisted Closure (V.A.C.) device or drainage system as part of standard care. To further evaluate how a wound heals we would like to obtain fluid samples from the suction canister at two-three separate times, depending how long your drainage system is in place. This will occur within 24 hours that the VAC or drainage system is applied and then approximately 23 days and then one week later.

6. What will be done if you take part in this research study?

If you take part in this research study, your wound fluid will be removed from the suction canister with a sterile syringe at two separate times. This procedure will last approximately 2-3 minutes. We will also review your medical record and obtain general information about you like gender, diagnosis and most recent laboratory findings related to your blood count and blood electrolytes.

7. What are the possible discomforts and risks?

There are no discomforts or risks to you for participating in this study. If you wish to discuss the information above, you may ask questions now or call the Principal Investigator listed on the front page of this form.

8a. What are the possible benefits to you?

There are no direct benefits to you.

8b. What are the possible benefits to others?

There are no direct benefits to others, but allowing us to assess your wound fluid may help nurses and other health providers better understand wound healing in older adults and how we may better meet the health care needs of older people.

9. If you choose to take part in this research study, will it cost you anything?

Participating in the study will not cost you anything. Routine medical care not assigned with the study will be charged to you or your insurance. These costs may not be applicable if you are a veteran and being treated at the North Florida/South Georgia Veteran Health System (NF/SG VFS).

10. Will you receive compensation for taking part in this research study?

You will not receive any money for participating in this study.
11. What if you are injured because of the study?

If you experience an injury that is directly caused by this study, only professional consultative care will be provided without charge. However, hospital expenses will have to be paid by you or your insurance provider. No other compensation is offered. You will not have to pay hospital expenses if you are being treated at the North Florida/South Georgia Veteran Health System (NF/SG VHS) and experience any physical injury during participation in a Veteran's health System-approved study.

12. What other options or treatments are available if you do not want to be in this study?

13. Participation in this study is entirely voluntary.

You are free to refuse to be in the study.

13a. Can you withdraw from this research study?

If you wish to stop your participation in this research study for any reason, you should contact: Joyce Stechmiller, PhD ARNP at (352) 273-6370. You are free to withdraw your consent and stop participation in this research study at any time without penalty or loss of benefits to which you are otherwise entitled. Throughout the study, the researchers will notify you of new information that may become available and that might affect your decision to remain in the study.

In addition, if you have any questions regarding your rights as a research subject, you may phone the Institutional Review Board (IRB) office at (352) 846-1494.

13b. If you withdraw, can information about you still be used and/or collected? No.

13c. Can the Principal Investigator withdraw you from this research study?

You may be withdrawn from the study without your consent for the following reasons: This will not be done.

14. How will your privacy and the confidentiality of your research records be protected?

Authorized persons from the University of Florida, and the Institutional Review Board have the legal right to review your research records and will protect the confidentiality of those records to the extent permitted by law. If the research project is sponsored or if it is being conducted under the authority of the United States Food and Drug Administration (FDA), then the sponsor, the sponsor's agent, and the FDA also have the legal right to review your research records. Otherwise, your research records will not be released without your consent unless required by law or a court order.
15. If the results of this research are published or presented at scientific meetings, your identity will not be disclosed.

16. How will the researcher(s) benefit from your being in this study?

No, the researcher will not benefit from your participation in this study beyond publishing or presenting the results.

Signatures

As a representative of this study, I have explained the purpose, the procedures, the possible benefits, and the risks of this research study; the alternatives to being in the study; and how privacy will be protected:

____________________________________  __________
Signature of Person Obtaining Consent    Date

You have been informed about this study's purpose, procedures, possible benefits, and risks; the alternatives to being in the study; and how your privacy will be protected. You have received a copy of this Form. You have been given the opportunity to ask questions before you sign, and you have been told that you can ask other questions at any time.

You voluntarily agree to participate in this study. By signing this form, you are not waiving any of your legal rights.

____________________________________  __________
Signature of Person Consenting    Date
APPENDIX B
INCLUSION/EXCLUSION CRITERIA

University of Florida
College of Nursing
V.A.C.® Study

Biochemical analysis of wound fluid from pressure ulcers of adults on V.A.C.® therapy

Name: ________________ Study #: _________ M.R.#: __________
Address: ___________________________________________________
Phone: (____)________________  Date/time: ___________________

Inclusion/Exclusion Criteria:

1. YES NO  Patient is ≥ 21 y/o with stage III or IV pressure ulcers.
2. YES NO  Patient requires V.A.C.® therapy on an outpatient or in-patient basis at STH at UF or VAMC, both in Gainesville, FL.
3. YES NO  Pressure ulcer(s):
   is present for > 1 month
   has no previous treatment with dermal skin substitutes
   has received little to NO wound treatment for 1 week prior to V.A.C.® (enzymatic debridement agent)
   has no HBO or warm-up therapy
   has no fistulas, necrotic tissue with eschar, untreated cellulitis or osteomyelitis, connective tissue disorder, no malignancy in the wound
4. YES NO  Patient does NOT have an active systemic infection,
anemia (Hct <26), or immune deficiency diseases.

5. YES NO Patient has STOPPED smoking within the past 6 months.

6. YES NO Patient is currently NOT receiving steroids, immuno-suppressive or cytotoxic medications.

7. YES NO Informed consent has been obtained and copies given to patient/surrogate/durable power of attorney/proxy.
APPENDIX C
DEMOGRAPHIC INFORMATION

University of Florida
College of Nursing
V.A.C.® Study

Biochemical analysis of wound fluid from pressure ulcers of adults on V.A.C.® therapy

Section I: General Info

Name:   M.R.#:     Study #: 
DOB:    Age:  Sex: M F  Wt: Ht: Race:  
Date enrolled:    Dates of Fluid Collection:  

Section II: Pertinent H & P

HPI:  

PMH:   DM, CAD, PVD, HTN, DVT, venous insufficiency, clots/coagulopathies,
Medications:

<table>
<thead>
<tr>
<th>Rx &amp; OTC</th>
<th>Dose</th>
<th>Frequency</th>
</tr>
</thead>
</table>

SH:

- smoker: Y N
- living situation: home care, nursing home, lives alone, family support
- activity: ambulates, moves all extremities, non-mobile, chair bound
- ADL
- nutritional status – BMI (standard chart)
- hygiene: incontinent, clean/dry skin, bathes daily
- previous ulcer? Treatment?

FH:
ROS: (general survey & other pertinent data)

PE: (general appearance, VS, and other pertinent data)

Section III: Labs & Pertinent Diagnostic Tests

CMP/date:

Albumin/date:
APPENDIX D
WOUND ASSESSMENT

University of Florida
College of Nursing
V.A.C.® Study

Biochemical analysis of wound fluid from pressure ulcers of adults on V.A.C.® therapy

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<th>Post-V.A.C.®</th>
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<tr>
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<tr>
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<tr>
<td>Sinus/tunneling</td>
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<td>Bacterial loading</td>
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Note: if undermining and sinus/tunneling exist, then use clock method.
Biochemical analysis of wound fluid from pressure ulcers of adults on V.A.C.® therapy

<table>
<thead>
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<th>TIME</th>
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LIST OF REFERENCES


Davis, J., Childress, B.B., & Stechmiller, J.K. (April, 2004). Potential nitrate/nitrite (NO\textsubscript{x}) contaminant analysis. Poster presented at the 2\textsuperscript{nd} Annual College of Nursing Research Day, Gainesville, FL.


BIOGRAPHICAL SKETCH

Beverly Bibera Childress was born in Hilongos, Philippines. She grew up with her grandmother while her mother worked abroad. At the age of 12, she came to the United States and lived with her mother and stepfather. Even though she spoke little English, Beverly was not held back in grade school. Instead, she was enrolled in junior high school and took several courses in English as a second language class. Beverly graduated with high honors from Port St. Lucie High School. She was ranked eleventh out of over 300 students. After entering the University of Florida in 1993, she received her Bachelor of Science in Nursing with honors. She began her nursing career on a cardiothoracic with telemetry unit at Shands Hospital at UF. As a preceptor for students, she realized her passion for teaching. Consequently, she enrolled in the accelerated BSN to PhD program in 2000 on a two-year Nursing Traineeship. While in the program, Beverly worked as a research assistant for Drs. Stechmiller and Yucha. As a teaching assistant to an undergraduate pharmacology class, she gave lectures and prepared exam questions. Additionally, she maintained her RN position while attending graduate school full-time. On her spare time, Beverly served as president of the Doctoral Student Council as well as mentored graduate and undergraduate honors students. In 2003, the Florida Nurses Foundation and Sigma Theta Tau, Alpha Theta Chapter awarded Beverly research grants.

Teaching is no longer the primary focus of Beverly’s educational career, but also research. As a nurse scientist, Beverly realizes how she may contribute to the greater good of the society through scientific research. She believes that dissemination of
knowledge through research is one of the ways to provide evidence-based practice. Furthermore, it enables the nursing profession to develop and advance. By sharing research findings with other investigators, current knowledge is confirmed, modified, or discarded. In addition, ideas are born and shaped through these collegial interactions. Consequently, she plans to continue her research program on nitric oxide and its role in wound healing. Additionally, she plans to teach students at the university level and practice as an Advanced Nurse Practitioner.