การใช้ Autologous Stem Cells ที่ได้มาจาก Bone Marrow Aspirate (BMA) สำหรับการรักษาแผลแบบเรื้อรังของรยางค์ขา

นายเกอริด โมลเดอร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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AUTOLOGOUS BONE MARROW ASPIRATE (BMA) DERIVED STEM CELLS FOR CHRONIC WOUNDS OF THE LOWER EXTREMITY

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เกอริด โมลเดอร์: การใช้ Autologous Stem Cells ที่ได้มาจาก Bone Marrow Aspirate (BMA) สำหรับการรักษาแผลแบบเรื้อรังของรยางค์ขา. (AUTOLOGOUS BONE MARROW ASPIRATE (BMA) DERIVED STEM CELLS FOR CHRONIC WOUNDS OF THE LOWER EXTREMITY) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ประวิตร เจนวรรธนะกุล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. ปราณีต เพ็ญศรี, 53 หน้า.

การใช้ stem cells ในการรักษาแผลเรื้อรังที่มีปัญหา เป็นเรื่องที่น่าสนใจ โดยเฉพาะในผู้ป่วยเบาหวาน ซึ่งปัญหาแผลเรื้อรังในผู้ป่วยกลุ่มนี้ ทำให้เกิดการสูญเสียทางสังคมและเศรษฐกิจเป็นอย่างมาก การทำให้แผลปิดและการลดความเสี่ยงต่อการถูกตัดรยางค์เป็นเรื่องที่มีความสำคัญ โดยวิธีการใช้ stem cells ในการรักษาแผลเรื้อรัง อาจช่วยเร่งกระบวนการซ่อมแซมของเนื้อเยื่อได้ ในการศึกษานี้ ได้ทำการศึกษาประวัติของผู้ป่วยแบบย้อนหลัง โดยผู้ป่วยกลุ่มนี้ได้รับการรักษาโดยใช้ autologous bone marrow aspirate stem cells เพื่อประเมินประสิทธิภาพของในการช่วยให้แผลปิด การศึกษานี้เป็นการทบทวนวรรณกรรมในเชิงลึกเกี่ยวกับวิธีการรักษาแผลเรื้อรัง โดยการใช้ autologous mesenchymal stem cells รวมทั้งวิธีการใหม่ๆ ที่ใช้ในการรักษาแผลเรื้อรัง

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

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GERIT MULDER: AUTOLOGOUS BONE MARROW ASPIRATE (BMA) DERIVED STEM CELLS FOR CHRONIC WOUNDS OF THE LOWER EXTREMITY. ADVISOR: ASSOC. PROF. PRAWIT JANWANTANAKUL, Ph.D., CO-ADVISOR: ASST. PROF. PRANEET PENSRI, Ph.D. 53 pp.

The use of stem cells in chronic and problematic wounds is of particular interest as chronic wounds, particularly those of a diabetic nature, are a physical, social and financial drain to both the patient and the medical system. Expediting wound closure and reducing risk of amputation then becomes important. Stem cells in chronic wounds may assist with cell migration, proliferation and the repair process. In this article, we have retrospectively reviewed patients that were treated with autologous bone marrow aspirate (BMA) in an attempt to determine any potential value in expediting wound closure. A comprehensive review of the literature on the treatment of chronic wounds through the use of autologous mesenchymal stem cells was performed and have examined as a novel clinical technique in the treatment of chronic wound.

Field of Study : Biomedical Sciences ..... Student's Signature .....

Academic Year : 2011 ..... Advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

ABMA	=	Autologous bone marrow aspirate
BMA	=	Bone marrow aspirate
MSC	=	Mesenchymal stem cells

CHAPTER I

INTRODUCTION

1.1 Background & Rationale

It is well known that chronic wounds of the lower extremity, particularly diabetic ulcers, can pose a significant burden to society. This burden does not include the physical and mental distress that occurs with the patient. Currently, lower extremity wounds can occur in 4-10 percent of diabetic patients, with a lifetime risk of up to 25% and a 20-80 percent reoccurrence rate. Morbidity is also very high with 25% of all diabetic admissions being the result of foot complications associated with ulcerations and over 100,000 amputations performed annually in the U.S. (1). The cost in US dollars is almost 50,000 per year, not including the cost of secondary problems. There is no financial amount that can be associated with the mental and physical effect on an individual's decreased quality of life and consequence on society in general. Expediting closure of wounds while decreasing associated complications would have a significant impact on quality of life, rate of morbidity and mortality, and cost of care associated with this population.

The key to reducing problems associated with lower extremity ulcers includes early detection, early treatment and prevention. This may prevent up to 85% of the amputations in the diabetic population. While venous disease, arterial disease and other medical conditions may contribute to ulceration, diabetic neuropathy is also a contributor (2). The percent of venous leg ulcers is lower and comprises approximately 1% of ulcers in the world, and comprise almost 70% of leg wounds (excluding the diabetic foot). They also have a high associated cost of care, which can run up to $7000 per year (2-6). Stem cells have been examined as a potential treatment to assist with wound healing. Stem cells have a capacity to assist with tissue repair due to their plasticity and ability to differentiate into different cell types (7-12).

1.2 Chronic Wounds

Any approach to treatment of chronic wounds, which may assist with their rapid closure, may also be expected to decrease potential complications as well as morbidity and mortality. The longer a wound stays open, the higher the risk of bacteria being introduced and infection occurring. Longer duration of presence also correlates with increased costs of prolonged visits and medical care. In the case of the diabetic, this may include but is not limited to hospitalization, antibiotics, off-loading devices, dressings and surgery. Diabetic ulcers are of significant cost and burden to the medical system and would greatly benefit from rapid closure. Expediting closure of lower extremity wounds while decreasing associated complications would significantly impact the individual's quality of life, while decreasing morbidity and mortality and the financial cost to the countries health care system.

Several factors are known to contribute to delayed wound healing including medical status of the patient, medications, and vascular complications. Cell senescence in the diabetic, due to inhibition of fibroblast proliferation results in decreased collagen deposition and cell activity. Cellular senescence is also known to delay the wound closure process (13). There are several factors that contribute to delayed wound healing; macro and microvascular disease, hyperglycemia, infection, pressure, increased inflammatory proteases, and cellular senesence. In chronic wounds, the senescent cells due to the inhibition of fibroblast proliferation are unable to divide and become unresponsive to growth factors. An ulcer with more than 15% of senescent cells is more difficult to heal.5 Ulcers greater than one year's duration have generally proven more difficult to manage and several studies have shown the inverse relationship between duration and healing.1,6 It is not unusual for patients to present to hospitals and wound clinics with ulcers that have been present for greater than one year. Aggressive debridement, in the absence of contra-indicating factors including inadequate vascular flow, may not be sufficient to induce wound closure.

1.3 The Wound Healing Problem

Stem cells in the bone marrow aspirate (BMA) may have promise in the treatment of lower extremity ulcerations. They SMA is easily extracted from the lower extremity (calcaneous being a good source) thereby eliminating the risk of viral and infection transmission when derived in an allogenic fashion. The autogenic aspirate contains both hematopoietic and mesenchymal types of stem cells with the hematopoietic stem cells differentiating into red and while blood cells, platelets and macrophages. The mesenchyal stem cells are of particular importance as they are multipotent and differentiate into multiple cell types involved in tissue repair when placed in the appropriate environment. This cell line may be of benefit in the non-healing wound. (14) The bone marrow is a good source of cells. It consists of various inflammatory cell progenitors, which are known to participate in wound healing, mesenchymal stem cells, which appear to be phenotypically altered and/or senescent in chronic wounds and multipotent stem cells (15-19).

The use of BMA (bone marrow aspirate) in chronic wounds is not without its limitations. The exact method of cell migration and proliferation, matrix deposition and remodeling after application is still unclear. It is also difficult to quantify the number of viable stem cells once the BMA is extracted as well as to determine level of activity once applied to the wound surface. Very little clinical information is available on the chronic wound use and what happens one applied. Furthermore, the chronic wound environment is frequently hostile due to the presence of heavy bacterial burden, biofilm, non-viable tissue, limited blood supply and other factors resulting from the long duration of the wound. These same factors may contribute to amputation in the diabetic patient, delay wound closure and reduce the number of active cells in the wound environment.

Current treatment of chronic wounds has traditionally included conservative and surgical debridement, a variety of topical agents, creams and dressings, and secondary modalities to address additional considerations including compression for venous disease, pressure reduction, and off-loading in the diabetic patient. Topical antimicrobial agents are also routine in many parts of the

world. None of the modalities listed directly address the issue of cell senescence or decreased cell activity. All of the agents and conservative modalities listed simply alter the wound environment to promote the healing process, without directly addressing cellular inactivity.

1.4 The Approach

The approach to "Bone Marrow Aspirate (BMA) Derived Stem Cells for chronic Wounds of the Lower Extremity" is to address the following concerns:

1.4.1 Determine if any past clinical trials or work have demonstrated efficacy

1.4.2 Determine if a randomized trial will help demonstrate efficacy

1.4.3 Provide healthcare providers with a template for a clinical protocol by actually conducting a clinical trial

1.4.4 Create a comparative study with a non treated control.

1.4.5 Provide physician and health care provider with further information on stem cell use in chronic wounds.

1.5 The Proposal

The proposal for this PhD program is to illustrate and describe in 2 (two) manuscripts/papers on the topic of bone marrow aspirate (BMA) derived stem cells for chronic wounds of the lower extremity.

These manuscripts/papers will be submitted for publication and for fulfillment of the requirement for the PhD program.

1.5.1 Establish a retrospective review of patients treated in the past with bone marrow-derived stem cells

1.5.2 Establish a randomized control trial with bone marrow aspirate derived stem cells and compression versus compression alone.

1.5.3 Extensively review past literature on the topic.

1.5.4 Program Description

1.5.4.1 Chronic wounds are a painful, long term and expensive treatment problem resulting in significant morbidity, mortality and quality of life related issues.

1.5.4.2 Mission Statement: To determine the effect if any, of bone marrow aspirate derived stem cells for chronic wounds, of venous etiology.

1.5.4.3 Vision: To determine if stem cell therapy is of value in the treatment of venous ulcers of the lower extremity.

1.6 Research Questions

1.6.1 Do past studies and literature support the use of bone marrow aspirate derived stem cells in the treatment of venous ulcers?

1.6.2 Would a randomized controlled trial of the treatment of lower extremity venous ulcers establish a suggested benefit to the use of stem cells in the treatment of lower extremity venous ulcers?

1.6.3 Does an extensive review of past literature support the study thesis?

1.7 Objectives of the study

1.7.1 To review past patients treated with bone marrow aspirate derived stem cells at the University of California San Diego Wound Treatment and Research Center.

1.7.2 To identify benefit of bone marrow aspirate derived stem cells through a randomized controlled trial for the treatment of chronic lower extremity ulcers of venous etiology.

1.7.3 To conduct an extensive literature review of currently available data on the treatment of chronic wounds of venous and other etiologies with stem cell therapy.

1.8 Hypotheses of the study

1.8.1 Minimal success has been shown for the treatment of chronic wounds of venous and other etiologies, with stem cells.

1.8.2 A well designed randomized trial is needed to determine if stem cells of bone marrow origin may be of benefit in the treatment of venous leg ulcers.

1.8.3 A comprehensive review of past literature is needed to provide clinicians with extensive background on the treatment of problematic lower extremity wounds of venous etiology with bone marrow aspirate derived stem cells.

1.9 Conceptual framework

1.9.1 Conduct a brief retrospective review of use of stem cells in the treatment of chronic wounds.

1.9.2 Conduct a randomized controlled of venous ulcers of the lower extremity with bone marrow aspirate derived stem cells

1.9.3 Collect data on retrospective studies and literature.

1.9.4 Analyze data

1.9.5 Draft manuscripts

1.9.6 Submit manuscripts for publication to Journal

1.10 Research design

Comprehensive review of published literature and protocols on bone marrow derived stem cells.

1.11 Research Methodology

1.11.1 Comprehensive review of published literature and protocols on worldwide treatment with bone marrow aspirate derived stem cells of chronic wounds.

1.11.2 Retrospective review of patients with chronic wounds with bone marrow aspirate derived stem cells.

1.11.3 Conduct a randomized control trial of venous ulcers of the lower extremity treated with stem cells versus no stem cells of bone marrow aspirate derived origin. Materials and Methods: Randomized, controlled prospective single site

Enroll up to 40 evaluable patients to provide a proof of concept

12 week follow up, Randomized 1:1 ratio to BMA with or without matrix. All patients get same wound covering/xenograft. All patients receive same compression modality. Proportion of wounds attaining complete closure by 12 weeks, Time to wound closure, Percent of wound size reduction at 12 weeks. Inclusion: A venous leg ulcer confirmed by duplex ultrasound, The ulcer is greater than 3 months duration, Post debridement, the ulcer size must be $\geq$2.0 sq cm and $\leq$25 sq sq cm, Between ages of 18 and 60, ABI between 0.7 and 1.2 or Tc PO2 > 30 mmHg at the ankle or a Doppler waveform consistent with biphasic or triphasic flow. Patient is already scheduled for a surgery prior to screening and enrollment. Patient is willing and able to sign a voluntary informed consent. Patient must have 3 or fewer ulcers separated by >3.0 cm distance. Patient must be able and willing to attend follow up visits and study examinations. Patient willing to wear compression. Exclusion: Clinical infection at the study site e.g. cellulitis or ostemyelitis, Ulcer is not of a confirmed venous etiology Peripheral arterial disease (as defined in inclusion criteria), Phlebitis or deep leg vein thrombosis in the past 30 days, Arterial bypass in previous 30 days

Severe anemia (Hgb<8), Serum albumin <3.0, Renal failure with creatinine >2.5 mg/dl, Rheumatoid arthritis or other collagen vascular disease), vasculitis, sickle cell disease or HIV, Severe liver disease as defined by treating physician

Uncontrolled diabetes mellitus as determined by the treating physician, Malignancy at or near the ulcer site, Any condition judged by the PI that would cause the study to be detrimental to the patient, Known allergy to equine derived tissue, Received another investigational device or drug within 30 days of Day 0

History of radiation therapy at wound site, Chemotherapy or immunosuppressive therapy within 30 days of enrollment, Received another graft, allograft or xenograft within 30 days of the study, Pregnant or nursing woman, Risks and benefits of surgery discussed with all patients undergoing surgery

Patients were not enrolled in any other study at the time of surgery and treatment wit BMA, Patients were not receiving any other experimental device

This data collected is retrospective, Investigational Review Board permission obtained to review charts. Charts were reviewed from initial visit to final treatment and progress note. All statistics, previous treatments, co-morbidities, surgeries, wound treatments and results were recorded. Complete wound assessment were performed. Surgical treatment plan was not altered based on decision to apply BMA. Standard surgery procedures were followed

All ulcers underwent sharp surgical excisional debridement. BMA was extracted through a lateral hind foot app approach using a trephine to harvest marrow from the ipsilateral calcaneal bone. Minimum of 5 ml of bone marrow aspirate will be collected depending on the wound size, the amount will be controlled via the aspirating syringe. (same size used for every procedure). Aspirate is applied immediately directly over the entire wound surface in an even layer. All wounds were covered with xenograft. All dressings were changed at 7 ± 2 days for 12 weeks or until complete wound closure. If no closure was obtained, new treatments were considered.

1.12 Mission Statement

To determine the effect if any, of bone marrow aspirate derived stem cells for chronic wounds, of venous etiology.

1.13 Vision

To determine if stem cell therapy is of value in the treatment of venous ulcers of the lower extremity.

1.14 Benefit of the study

To provide foundation findings to one of the most novel techniques of our times, using bone marrow derived stem cells for the healing for our patients suffering with diabetes and chronic wounds.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction: Analyses of Need

A precursory review of the literature indicates minimal data is available on the treatment of chronic wounds of a venous etiology, particularly with stem cell therapy. A need exists to further investigate this area of potential benefit in the treatment of problematic wounds.

2.2 Bone marrow derived stem cells

These are newly discovered techniques tools in the wound healing. There have been little to few human and clinical studies performed and published. The basic science of the stem cell potential in wound healing is vast and promising.

2.3 Preliminary studies

Myriad recent evidence suggests that autologous stem cells derived from bone marrow have potential to treat many disorders given their plasticity and ability to differentiate into various types of tissues, including endothelium, liver, muscle, skin, bone, cartilage, brain, fibroblasts and keratinocytes. They are known to assist with the tissue repair process by secreting large amounts of growth factors and cytokines.7-10

Upon initial application of mesenchymal stem cells (MSCs) into a chronic wound environment, they work primarily to amplify the signals of surrounding cells via mRNA production and release.8 MSCs are very resilient and persist in wounds for extended periods acting as only modulators of cellular

signaling initially.10 Full differentiation into keratinocytes, myofibroblasts11 and epidermal stem cells is seen; however this is not until later in the wound healing process.8,9 MSCs furthermore play a large role in angiogenesis when applied or injected locally. Secreted chemokines attract pericytes which potentially differentiate into fibroblasts, smooth muscle, or marcophages and regulate endothelial microvasculature at the capillary level.12 A large down regulation of wound degrading matrix metalloproteinases is also observed following MSC introduction into a wound.13

CHAPTER III

MATERIALS AND METHODS

3.1 Patient selection: All patients were selected from the University database without regard to their race, age, gender, ethnicity or economic status. All patients had been treated directly by the one or both of the authors who were familiar with the medical background and surgical treatment of the patients. The small number of patients identified is due directly to the limited number of patients who underwent this treatment or that had consented to the procedure.

3.2 This study is a retrospective review; so all treatments rendered were not a part of any study but were considered the most appropriate care for the patient at the time of surgery. All patients that were treated were included in this retrospective review unless insufficient data for analysis was found to exist. All patients also had non-healing ulcers of the extremities which were present for at least one year or more and had not responded to standard approaches to wound care which might include but were not limited to topical dressings, various off-loading devices, debridement, topical ointments, creams and enzymatic agents as well as skin substitutes. The patients all underwent standard preoperative measures, which routinely include a complete history and physical examination, imaging studies when applicable as well as education and counseling by the anesthesia staff. All risks and benefits inherent to the procedure which they were undergoing, was explained to the patient prior to obtaining consent as this is standard practice in our hospital setting. At the time of treatment, the patients were not enrolled in a clinical study or trial and were not being treated with any unapproved or experimental drug, dressing or deice. Although this study is a retrospective review and did not involve any patient enrollment, consent or other approval, IRB approval was obtained to review charts of patients that had undergone this treatment, for academic, scientific and clinical care purposes. No identifying information was used in any way in compiling and presenting the data. It should be noted that none of the patients reviewed had any severe infection, osteomyelitis, Charcot

neuroarthropathy or significant vascular disease. Patients with significantly abnormal lab values including significantly abnormal albumin, creatinine levels or malignancy as well as woman that are pregnant or nursing are not treated, as standard practice, with BMA. It is standard surgical practice to control those factors that might affect the patient's well being or negatively affect surgical outcomes. It is important to stress that all the patients reviewed were undergoing an approved surgery and treatment that was in no way, a clinical study.

3.3 Evaluation and procedure: The retrospective study was conducted by reviewing all of the patient's clinical data which included their medical chart from the time of initial visit until the last available clinical note. All co morbidities, body mass index, age, previous treatments, surgeries, wounds, wound etiologies and data were recorded. Complete wound assessments were available and were based on recorded data, which included size, location, characteristics, past clinical treatments, and treatment outcomes. Surgical treatment is not significantly altered when BMA is added to the surgical procedure. Standard surgical procedure at our institution consists of a preoperative history and physical and anesthesia evaluation, preoperative preparation and administration of anesthesia, aggressive wound debridement to remove any nonviable tissue and bacteria from the wound bed and to prepare the wound bed for application of the BMA. Standard surgical techniques are used for wound bed preparation that was no different for patient receiving BMA than those who have not received BMA but have undergone similar surgical procedures. A clean wound base is imperative for successful application and retention of applied cells.

The technique for BMA extraction entails a lateral hind foot approach, utilizing a bone trephine to harvest the marrow from the ipsilateral calcaneal bone. The usual amount extracted ranges from 3-5 cc of bone marrow aspirate and depends on the wound size to be treated. The aspirate is then immediately applied to the wound base using a syringe for better distribution of the BMA over the wound surface. Once treated, the wound is then covered with a xenograft. In all reviewed patients, a xenograft (Unite®, Synovis, Irvine, CA) was used to cover the wound. Current medical data does not support the use of one type of xenograft or allograft over another when used as a biological covering

for chronic wounds. The particular xenograft used in our clinical setting was based on qualitative features such as cross-linking, durability, sterility, handling properties, and physician experience. All patients had received the same xenograft. Xenografts have been historically used on chronic wounds as they usually consist of a one-time application and are left intact until they are displaced once the one has progressed to closure or close to complete closure. Data is available in the literature on use of xenografts and healing of chronic wounds. Thus any significant difference in complete wound closure in the patients reviewed might logically be attributed to the one difference in care: the application of BMA. Photographs were taken to illustrate the pre and post debridement appearance of the wound, BMA extraction, and application of the BMA followed by coverage with a xenograft. One surgery is completed, surgical sites are covered with non-adherent wound contact dressings, gauze and cotton gauze wraps. Patients are put in off-loading devices depending on the location of the wound. Dressings are left intact for approximately 7 days from the time of surgery, plus or minus two days. Patients are normally seen in the wound clinic weekly until healing occurs. Clinic visits consist of dressing changes, wound examination and wound documentation. The rate of closure based on wound size is also documented during the clinical visit only if and when the xenograft is displaced, as the wound cannot be visualized underneath a xenograft. Other treatment therapies are considered when grafts are displaced and the wound does not demonstrate a significant decrease in size in the subsequent 6 weeks.

3.4 A comprehensive review of the literature available was performed to review and discuss the comprehensive relevance of the clinical application of autologous mesenchymal stem cells in the treatment of chronic wounds and diabetic bone healing.

CHAPTER IV

RESULTS

4.1 There were a total of 8 patients that could be identified and were eligible to be reviewed for this retrospective study.

4.2 The wound etiologies included past burns, vasculitic ulcers and wounds related to venous disease. Secondary diagnoses were variable and included trauma, lupus, pyoderma gangrenosum and/or lymphedema. All the co morbidities of the patients reviewed are included in Table 1 along with the patient age, established wound etiology, wound characteristics, treatments and sizes. Previous surgeries have also been listed. All patients had received wound dressings prior to the surgery as well as post surgical wound dressings directly on the wound in cases of xenograft displacement.

4.3 A total of three patients showed a continued and gradual decrease in the wound size over the following months. One of the latter three patients had a left saphenous vein radiofrequency ablation, which was performed, based on the vascular surgeons recommendations, three months post BMA application. This recommendation was not based on the presence or absence of a wound but rather on the patient's continued varicosities and vascular disease. Two of the total patients did show a progressive increase in the size of the wounds over several subsequent months. This may be due to various factors including but not limited to poor patient compliance with post operative instructions, re-injury to the area or poor medical control of the underlying condition contributing to ulcer development. The remaining three patients did not show a significant improvement or reduction in wound size after size weeks although there was no apparent further deterioration. These patients went on to use other forms of wound modalities. Two of these patients did get treated with living

skin equivalents to assist with wound closure and one patient had a split-thickness graft over their ulcers along with hyperbaric oxygen therapy.

4.4 A very limited number of publications relevant to autologous mesenchymal stem cells in the treatment of chronic wounds were found. These are all listed in the appendix. The limited data underscores the newness of this novel treatment and stresses the importance of our research in encouraging further studies and clinical trials.

4.5 Statistical analysis

All descriptive and inferential statistical analyses will be performed using the SPSS Statistical, Versions 15 and 17 (Chicago, IL). When appropriate, continuously distributed numerical outcome data will be analyzed using Student's Independent Groups T-Tests and One and Two Factor Analysis of Variance. Categorical outcome variables will be analyzed using Person's $\chi 2$ Test of Association. All results will be considered statistically significant when $p<=.05$. When appropriate, post hoc testing will be performed on the numerical data using the Bonferroni correction method for holding the experiment wise alpha=.05.

CHAPTER V

DISCUSSION

5.1 Cellular perspective

The method of action of autologous bone marrow-derived stem cells is believed to be due to the assistance provided by the cells for tissue repair. This occurs through the secretion of large amounts of cytokines and growth factors integral to healing. The cells are also capable of differentiating into multiple cell types which ma include endothelium (especially relevant to the wound repair process), liver, muscle, skin, bone, cartilage, brain, fibroblasts and keratinocytes (20). Deng et al (21) was able to demonstrate through the use of a mouse model, that fluorescent labeled mesenchymal cells in mice gave rise to stem cells in the skin.

5.2 Clinical observation

Rogers et al (22) demonstrated in 2008 that injected bone marrow aspirate into wound borders may be a safe and useful adjunct to healing. The weakness of this study is that only three patients with wounds of varying etiology were examined. These ulcers healed at 47, 50 and 60 days respectively, however little data can be established based on the population number reviewed. Badavias et al had similar results with 3 patients with ulcers present for more than one year (23). BMA and cultured cells were used to attain complete healing in 3 months. It should be noted that one patient also received a bioengineered tissue that could have significantly affected the results. The same author conducted another study in 2007, wherein4 subjects were examined with only one attaining complete closure (24). Again, this data is of minimal value due to the patient size and the single patient attaining closure.

5.3 Clinical findings

Our study was somewhat larger with eight patients, although still small in total number. Three of the eight patients did attain closure in the 12 week period after having had the wounds for greater than one year. The patients had routine follow up visits for up to six months. When no improvement in wound size is seen, patients are treated with different modalities in the hope of stimulating additional wound closure. Two of the patients did receive multiple applications of living skin equivalents when their wounds had not responded to the surgery. As patients were retrospectively reviewed and this was not a prospective study, the post surgical treatment in all patients varied depending on the condition of their wounds and their medical status .

5.4 Literature findings

While data suggests that mesenchymal stem cells may assist with the wound repair process, there is still insufficient data to clinically encourage or support its use. The largest mesenchymal cell study to date was conducted by Yoshikawa et al in 2008 (25). A total of 20 subjects with wounds of varying etiology were treated with 18 patients attaining complete healing. Histological examination demonstrated dermal rebuilding, increased wound vascularity, tissue remodeling and reduced presence of fibrosis. Falanga et al reported the use of autologous culture expanded mesenchymal cells in conjunction with fibrin glue, on both chronic and acute wounds (26). The acute wounds were the result of excision of melanomas with resulting chronic wounds. These required 8 weeks to heal. The chronic wounds, which were present for more than one year, healed or significantly decreased in size in the 16-20 weeks post application. The healing time listed is not significantly or impressively different for healing times of similar types of wounds treated with standard dressings or more universally used topical treatments. The most significant value of this study was its ability to help quantify the number of mesenchymal stem cells per square centimeter of wound surface area and its correlation with reduction in wound size. In our study, due to the retrospective nature, we were not able to quantify the number of active cells in the applied solution. The use of fibrin glue in the

previous study was of importance as it helped to anchor the stem cells (26). We attempted to anchor our cells through coverage with the aforementioned xenograft. Fibrin glue has been used with adipose tissue derived mesenchymal stem cells injected into the fistula tract of 25 patients with a resulting healing rate of 71 percent and recurrence rate of 17.6 percent (27) Our study suggests that topically applied fresh autologous bone marrow aspirate may not necessarily contribute to a significant difference in healing compared to the same surgical procedures without the bone marrow aspirate. A larger study is warranted to further explore any value to the use of this technique to expedite wound closure.

5.5 Literature review

As the results of the search are from a review of the literature, a discussion of the clinical data from the publications data base rather than a study conclusion is presented. Several published case reports indicate the clinical effectiveness of BMA or MSC for healing chronic wounds. Humpert5 applied BMA topically on a neuroischemic chronic DM wound and showed reduced wound size, increased wound vascularity without any systemically observed effects within 7 days of application. Although this was a single case presentation, the result should be considered for future study designs. The weakness is that of any single case presentation. Vojtassak14 reported complete wound resolution of a 25 year open wound within 4 weeks of application marrow derived MSCs and fibroblast collagen membrane. As with the other case studies, the single patient report is limited in validity. Ichioka15 reported complete wound closure after stem cell therapy with > 1 year open wound. The Ichioka study was based on mouse models. While this data is encouraging, animal studies are very limited in their ability to mimic the complexity of a diabetic, venous or other chronic wound. Kirana16 reported a positive result with topical application on a neuroishemic diabetic ulceration. Again, the presentation of is of a single patient. However, of note is the result of bone marrow mononuclear cells when applied in an ischemic environment. Lataillade and colleagues17 have shown promise in conservatively treating severe radiation burns with MSCs. A single patient was presented with notable results. As with chronic wound studies, larger randomized trials are needed in the bone

population. In 2008, Rogers18 injected bone marrow aspirate topically into the wound periphery in 3 patients with differing etiologies and suggested this procedure as useful and safe adjunct to wound closure. These ulcers healed in 47, 50, 60 days respectively. Safety is supported but not significantly confirmed with the small number of patients were reviewed. Similar results were achieved by Badiavas and Falanga19 in which, 3 patients had complete closure of their year long ulcers with use of bone marrow aspirate and cultured cells. The purpose of this study was to determine effects of dermal rebuilding. This was seen in all the patients thereby providing insite into possible effects of bone marrow-derived cells. All healed within 3 months, although 1 patient required additional application of bioengineered skin. In 2007, Badiavas20 conducted a randomized trial applying cultured MSCs vs autologous bone marrow aspirate (ABMA) into the wounds of 4 subjects, only one healed completely, but a positive clinical response was seen in all patients. As with the previous study, the numbers analyzed were small.

Falanga21 applied up to three applications of autologous culture-expanded mesenchymal stem cells with a fibrin glue system to acute and chronic wounds. This delivery system was proven to allow MSC persistence to stimulate wound healing for prolonged periods of time. The acute wounds secondary to excision of non-melanoma skin cancers healed within 8 weeks. The chronic year long lower extremity wounds significantly decreased or healed in 16 to 20 weeks. This study showed a strong correlation between the number of mesenchymal stem cells per square centimeter surface area and reduction in ulcer size. The study was larger than most trials yet was non-randomized with a small patient population. The value of the results are in their similarity in positive outcomes compared to other small trials, as well as the data on cell concentration and its affect on outcomes. The comparison with mouse models lends credibility to other animal studies using stem cells. In 2008 Yoshikawa23 performed a larger study which included 20 subjects with nonhealing wounds of various etiologies. The authors reported nearly complete healing in 18 patients and showed fibrous and vascular regeneration of native tissue by immunohistolochemical examination. In our institution, (ref) we reviewed 8 patients with chronic wounds of several etiologies who received ABMA collected from the ipsilateral calcaneal bone and xenograft application. Out of the eight patients, only 3 showed

significant wound size reduction during the 12 week follow up. The low numbers attaining significant results may have been due to the small population number, the non equivalent demographics and the retrospective nature of the review. The largest study to date using bone marrow-derived mesenchymal stem cells in extremity based wounds was published by Dash23. In this level 1 randomized controlled trial the MSC therapy significantly reduced wound size and increased several clinical parameters as compared to controls. MSC treatment groups were also shown to lack any ill effects of the body's normal biochemical parameters. To date, this is the largest trial conducted with MSC lending support to the use of the cells to expedite wound closure.

5.6 What is the currently known

This review is a limited retrospective one, designed to exam available evidence to support proof of concept in use of BMA for the treatment of chronic wounds of the lower extremity. Wounds that failed standard approaches to care are of specific interest as most chronic wounds will respond to traditional modalities and would not be undergoing aggressive or surgical treatments. The goal of the review was to determine if autologous bone marrow stem cell aspirate had the potential to assist non-healing lower extremity ulcers, which had been unresponsive to the traditional treatments, and to determine if it would expedite wound closure. This review was designed with the same intent as a Phase 1 clinical trial, determining whether there is any evidence to support clinical efficacy and future clinical trials.

5.7 What is known about stem cells

The area of clinical use of mesenchymal stem cells is relatively new, particularly in the clinical setting and the treatment of chronic wounds. Therefore, limited data is available. The data that can be found relevant to clinical use is limited to small studies with relatively low numbers of patients reviewed. In an attempt to find all publications, all major search engines were used. These included

but are not limited to Pubmed, Medscape, as well as standard searches through google, journals and all other sources dedicated to the publication of medical information

The comprehensive review presented is similar in some ways, to a Cochran review, in that data researched and included was analyzed in a scientific fashion. The opinions of the authors, is also included.

5.8 Strengths and Limitations of this study and suggestions for further study

There are clear obstacle and strategies to solve these projects. These studies we would like to present through corresponding papers for publication. They are extensive and difficult to analyze and to achieve a meaningful outcome. The authors are well aware of the limitations of this retrospective review resulting from the small population examined. As the technique is relatively new and unexplored in the treatment of chronic wounds, only a small number of previously treated patients were available for review. One cannot reasonable expect to find higher numbers of treated patients based on the time that BMA was first introduced into the surgical treatment regimen. Further limitations in a retrospective review result from unequal demographics, wound etiologies and lack of standardization of treatment prior to surgery. This also is inevitable due to the retrospective nature of this study. The small numbers do not however detract from the value of such a retrospective review.

5.9 There are other clinical barriers for Stem Cell Application

Operative intervention, careful patient preparation, poor native/host bone condition, high levels of matrix metalloproteases, poor control of underlying disease state such as arterial disease, venous return and diabetes, poor patient nutrition / health status, immunosuppression in select patients.

CHAPTER VI

CONCLUSION

6.1 This finding provides valuable insight into a new technique for treatment of unresponsive or chronic wounds, and encourages physicians to further explore its potential use in the clinical and surgical setting. A prospective trial, which randomizes and controls treatment, is highly encouraged although it will require large amounts of time and financial support.

6.2 This manuscript was a retrospective review of the publications relevant to the clinical application of autologous mesenchymal stem cells in the treatment of chronic wounds and diabetic bone healing. The manuscript supported the lack of published information relevant to the use of stem cells in the clinical setting. After an extensive review, one may conclude that this promising new approach to clinical care of chronic wounds and diabetic patients warrants more extensive research and studies.

6.3 Due to the lack of extensive credible material on the treatment of venous ulcers of a chronic nature, treated with bone marrow aspirate derived stem cells, the result cannot be determined until the randomized control trial is completed and supported by available evidence.

6.4 Based on the information provide and the study parameters, we expect the results to reveal: Improved wound healing, Improved tissue repair and regeneration, Improved clinical outcome in the stem cell group (BMA) than the non-stem cell group.

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APPENDICES

APPENDIX A

Paper #1

Title: Autologous Bone Marrow-Derived Stem Cells for Chronic Wounds of the Lower Extremity: A Retrospective Study Surgery

Journal Published: Wounds Journal, WOUNDS 2010; 22: 219–225

APPENDIX B

Paper #2

Title: Comprehensive review of the clinical application of autologous mesenchymal stem cells in the treatment of chronic wounds and diabetic bone healing

Journal Published: International Wound Journal, (*in press*).

APPENDIX C

PAPER #3

Title: Limb salvage surgery and wound treatment in the establishment of globally standardized diabetes, amputation, and limb salvage centers to address lower extremity morbidity and mortality in Thailand

Journal Published: Journal of the American College of Certified Wound Specialists, 2010; 2: 32-36.

APPENDIX D

Tables: Table 1 Patient Profiles

Age/sex	Etiology	Wound	Previous treatment	Previous surgery
32/M	Burn Injury, Trauma	**R lower leg** 7/30/08: 1.5x2x0.1cm 12/22/08: 2.7x1.5x0.1cm 2/10/10: 2.5x2x0.2cm 3/31/10: 2.2x1.7x0.1cm 4/21/10: 2x1.3x0.1cm	7/08: Panafil, Santyl 3/10: Fibracol, MediHoney Restore Silver	2/18/10 Debridement, BMA & Xenograft 3/31/10: Dermagraft 4/21/10: Dermagraft
35/M	Venous Dz Lupus, RA Lymphedema	**L lateral leg** 7/27/09: 4.2x4.5x0.3 10/6/09: 4.5x4.5x0.1 2/24/10: 4.5x3.5x0.1 3/3/10: 4.2x3.5x0.1 6/2/10: 2.5x3.0x0.1	7/09: Santyl Unna Boot 10/09: Silvadene, MediHoney 4/10: MeSalt 5/10: XCell	8/17/09: Xenograft 11/16/09: Xenograft 2/1/10: Dermagraft 2/18/10: Apligraf 3/8/10 Debridement, BMA, and Xenograft
60/M	PVD Hep C Spondylarthrosis	**R lateral leg** 2/10/09: 0.8x1.0x0.1 6/24/09: 1.0x1.0x0.1 11/30/09: 2.0x2.2x0.1 1/4/10: 2.5x2.5x0.1 3/11/10: 3.0x2.6x0.1 4/19/10: 3.5x2.5x0.1 5/25/10: 2.5x2.2x0.1 6/8/10: 2.4x2.0x0.1	2/09: Acticoat, Santyl 7/09: CoDa study 10/09: Regranex, Silvadene 1/10: Acticoat 3/10: Fibracol, MediHoney 4/10: Prisma	6/9/09: Apligraf 6/24/09: Xenograft 1/11/10 Debridement, BMA, and Xenograft 4/5/10: Dermagraft 4/12, 4/19/10: Apligraf 5/18, 5/25/10: Apligraf 6/1/10: Dermagraft
76/M	Trauma Hx R ankle ORIF s/p MVA Dermatitis	**L medial leg** 8/13/08: 3.1x2.1x0.1 2/11/09: 0.2x0.2x0.1 5/26/09: 1.6x1.5x0.1 12/15/09: 3.5x3.5x0.1 2/10/10: 3.4x2.6x0.1 4/5/10: 4.3x5.0x0.1 5/12/10: 5.1x4.4x0.1 6/8/10: 4.9x4.4x0.1	8/08: Panafil, Restore Silver 12/08: Fibracol, Unna Boot, CoDa study 11/09: Prisma 12/09: Fibracol 4/10: MediHoney 5/10: Fibracol	1/11/10 Debridement, BMA, Xenograft
78/F	Venous Dz PAD	**L medial ankle** 1/27/09: 0.8x1.0x0.1 3/10/09: 2.5x2.0x0.1 11/18/09: 1.8x1.8x0.1 1/13/10: 2.4x1.5x0.1 3/3/10: 1.5x1.5x0.1 4/14/10: 1.5x1.4x0.1 6/1/10: 1.5x1.1x0.1	4/09: CoDa study 9/09: Altrazeal 1/10: Fibracol 2/10: XCell, Allevyn, Regranex 4/10: Mist Therapy	1/12, 2/10, 2/24, 3/10/09: Apligraf 10/20/09: MediHoney 11/18, 12/3/09: Apligraf 1/21/10 Debridement, BMA, and Xenograft OA
79/M	PG PVD	**R posterior leg** 3/4/08: 8.5x12.0x0.1 7/27/09: 3.5x2x0.1 1/26/10: 5.0x2.5x0.1 2/10/10: 4.5x1.2x0.1 3/17/10: 3.5x2.5x0.1 5/18/10: 6.7x4.3x0.5	3/08: Aquacel Ag 2/10: Santyl 3/10: Acticoat, MediHoney, Unna Boot 5/10: Fibracol	1/06/10: Xenograft 2/18/10 Debridement, BMA, Xenograft 4/12-19/10: HBO 6/14/10: Xenograft 6/17/10: Debridement, STSG 6/18-22/10: HBO
92/F	PVD Varicosities	**L medial ankle** 1/27/10: 2.5x1.5x0.1 2/8/10: 2.4x1.8x0.1 3/31/10: 1.5x1.5x0.1 4/21/10: 2.1x1.3x0.1 5/11/10: 2.4x0.7x0.1 6/7/10: 1.6x1.0x0.1	1/10: Santyl 2/10: Fibracol, Acticoat, Prisma	2/24/10: Debridement, BMA, and Xenograft 5/10: L RFA SSV
96/F	PVD PAD Trauma	**R lower leg** 2/17/09: 1.5x0.7x0.1 9/09: nearly closed 11/3/09: 4.2x2.0x0.2 1/6/10: 6x2.5x0.1 3/22/10: 5.7x1.9x0.1 5/18/10: 5.6x3.3x0.1 6/9/10: 6.1x3.6x0.2	2/09: Acticoat 5/09: MediHoney 1/10: Mepilex 2/10: Acticoat 3/10: Fibracol, Regranex 5/10: Prisma	3/09: Dermagraft 10/15,27: Apligraf 11/3, 12: Apligraf 12/1: Apligraf 2/4/10: Debridement, BMA, and Xenograft 5/10: Angiogram, angioplasty, stent R SFA

APPENDIX E

Photos (Figures 1-4)

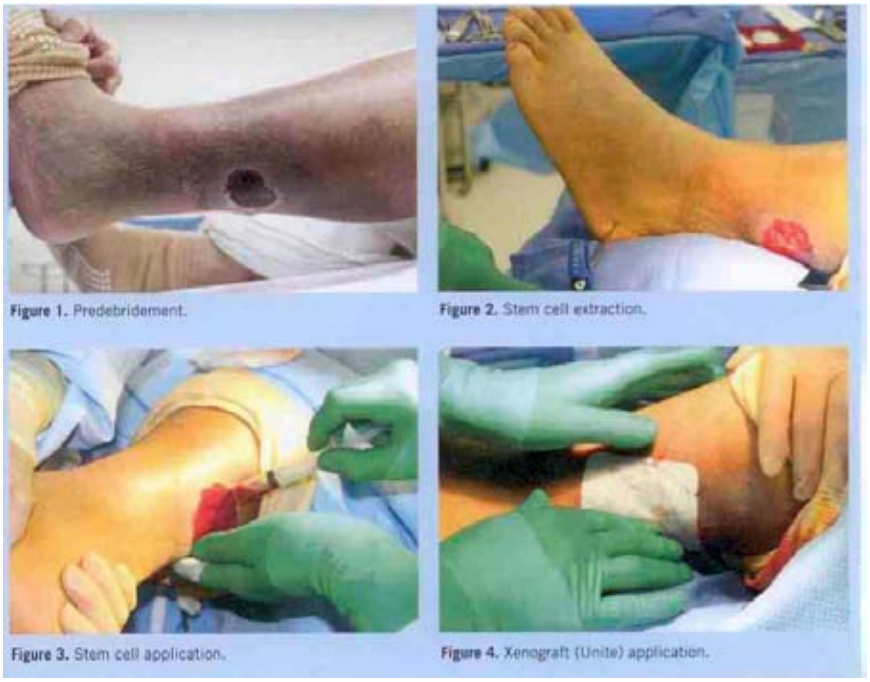


Figure 1. Predebridement.

Figure 2. Stem cell extraction.

Figure 3. Stem cell application.

Figure 4. Xenograft (Unite) application.

APPENDIX F

Table 2. Current clinical studies published using MSC therapy in chronic wound care

	NUMBER OF PATIENTS	CLINICAL LEVEL OF EVIDENCE	STUDY DESIGN
Badiavas et al 2007 [20]	4	2	Randomized Controlled Trial
Badiavas and Falanga 2003 [19]	3	4	Case Series
Dash et al 2009 [23]	24	1	Randomized Controlled Trial
Falanga et al 2007 [21]	13	3	Case Control
Humpert et al 2005 [5]	1	5	Case Report
lataillade et al 2007 [17]	1	5	Case Report
Ichioka et al 2005 [15]	1	5	Case Report
Kirana et al 2007 [16]	1	5	Case Report
Mulder et al	8	4	Case Series
Rogers et al 2008 [18]	3	4	Case Series
Vojtassak et al 2006 [14]	1	5	Case Report
Yoshikawa et al 2008 [22]	20	3	Case Control

APPENDIX G

PROTOCOL

STUDY PURPOSE

The purpose of this study is to assess the effect of autologous bone marrow aspirate (ABMA) when used with equine xenograft (Unite® Biomatrix) and compression for the treatment of venous leg ulcers (VLU), and compare its performance with those treated with equine xenograft and compression alone.

4.1 Study Materials

The study materials include Autologous Bone Marrow Aspirate collected from the ipsilateral calcaneal bone and equine xenograft, which is a collagen based, decellularized equine pericardial dressing for local management of moderately to heavy skin exuding wounds including the venous leg ulcer. The equine xeonograft is a FDA cleared device.

5. STUDY ENDPOINTS

The study endpoints include:

1) The proportion of wounds attaining complete closure by 12 weeks
2) Time to wound closure
3) Percent of wound sixe reduction at 12 weeks

6. STUDY RATIONALE

Autologous bone marrow aspirate has been studied in wounds. Although there is strong evidence that mesenchymal stem cells can assist in wound healing, there are insufficient well-designed human studies with adequate number of subjects to prove the validity and efficacy. We believe this study will provide a proof of concept of the treatment effect with ABMA in patients with VLUs that fail other treatment and are scheduled for surgical procedure.

Autologous products are not FDA regulated. The equine xenograft, and multi-layered compressions have been FDA cleared for use in venous leg ulcer.

7. STUDY DESIGN

This is a prospective, randomized, controlled, single-center physician-initiated study for patients undergoing venous ulcer treatment. Patients will be randomly assigned (1:1) to 2 groups; test group (ABMA and equine xenograft) and control group (equine xenograft alone). After the surgery, patients in both groups will have multi-layered compressions

It is expected that each patient will participate in this study for the duration of 12 weeks from the time of initial surgery (Day 0).

8. STUDY POPULATION

Up to 40 patients with VLU will be considered in this study if they sign the informed consent, and meet all inclusion criteria and none of the exclusion criteria.

Patients will be considered enrolled for study participation at the time the patient signs the informed consent. Patients excluded from the study will be identified and the reason for exclusion will be indicated.

8.1. Inclusion criteria

1) A venous leg ulcer confirmed with duplex ultrasound
2) The ulcer is greater than 3 months duration
3) Post-debridement, the ulcer size must be greater than 2 and less than 25 cm^2
4) At least 18 and not more than 84 years old
5) Ankle / brachial index is between 0.7 to 1.2 and or one of the following:
    a. transcutaneous partial pressure oxygen (TcPO2) > 30 mmHg at the ankle
    b. a Doppler waveform consistent with adequate flow in the foot (biphasic or triphasic)

6) Patient is scheduled for surgical procedure prior to study screening.
7) Able and willing to provide a voluntary written informed consent
8) Three or fewer ulcers separated by > 3.0 cm distance
9) Able and willing to attend scheduled follow-up visits and study related exams
10) Patients are already scheduled for a surgical intervention for the ulcer at the time of evaluation.

8.2. Exclusion criteria

1) Ulcer with exposed bone
2) Clinical infection at the studied ulcer site including cellulitis and osteomyelitis
3) Ulcer of a non-venous insufficiency etiology (e.g., rheumatoid, radiation-related, diabetic and vasculitis related ulcers).
4) Significant Peripheral arterial disease (PAD) as determined by the clinician
5) Phlebitis or deep leg vein thrombosis in the past 30 days
6) Arterial bypass in previous 30 days
7) Severe anemia (Hgb < 8)
8) Serum albumin < 3.0
9) Uncompensated congestive heart failure
10) Renal failure with Creatinine > 2.5 mg/dl
11) Rheumatoid arthritis (and other collagen vascular disease), vasculitis, sickle cell disease, HIV
12) Severe liver disease as defined by treating physician
13) Uncontrolled diabetes mellitus determined by the treating physician
14) Malignancy at or near the ulcer site
15) Any condition judged by the PI that would cause the study to be detrimental to the patient
16) Known allergy to equine derived tissue
17) Received another investigational device or drug within 30 days of Day 0
18) Radiation therapy at the wound site
19) Chemotherapy or immunosuppressive therapy within 30 days of enrollment
20) Received another allograft, autograft or xenograft within 30 days of the study

21) Pregnant or nursing women

8.3. Sample Size

This study will enroll up to 40 patients. This number is sufficient to provide a proof of concept needed for a pivotal study.

8.4. Patient Numbering

Upon consenting to participate in the study, patients will be assigned a 3-character enrollment number representing the patient number (e.g. 007 would be the seventh consented Patient).

8.5. Patient Confidentiality

All information and data concerning patients or patient participation in this investigation will be considered confidential. Patients participating in this study are identified on all documentation by their identification number only. Documents are kept in strict confidence by the investigator. Only authorized UCSD personnel will have access to these confidential files. All data used in the analysis and reporting of this investigation will be without identifiable data as stated under the Health Insurance Portability and Accountability Act (HIPAA).

9. STUDY MATERIALS

9.1. ABMA

3-5 cc of the Autologous Bone Marrow Aspirate will be collected from the ipsilateral calcaneal bone of patients in the treatment group as shown below.

Figure 1- ABMA Collection Methods

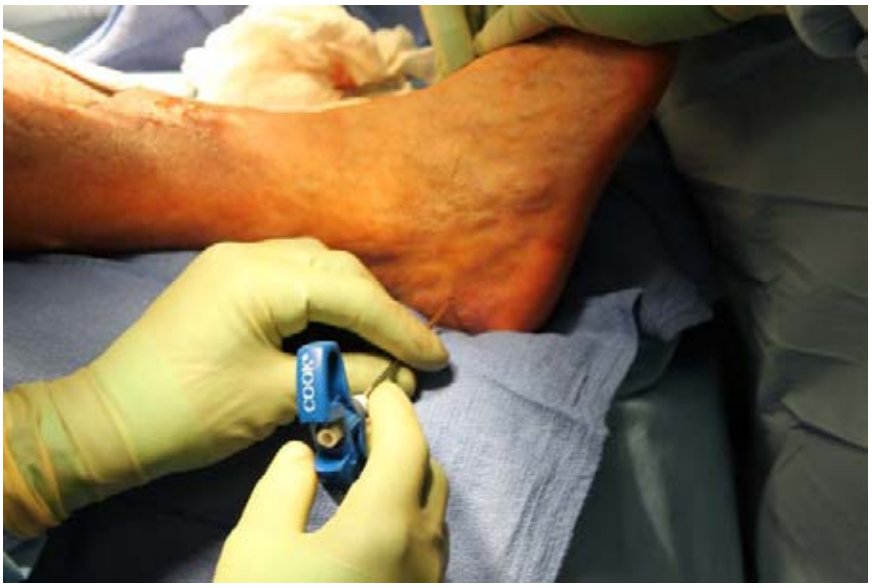
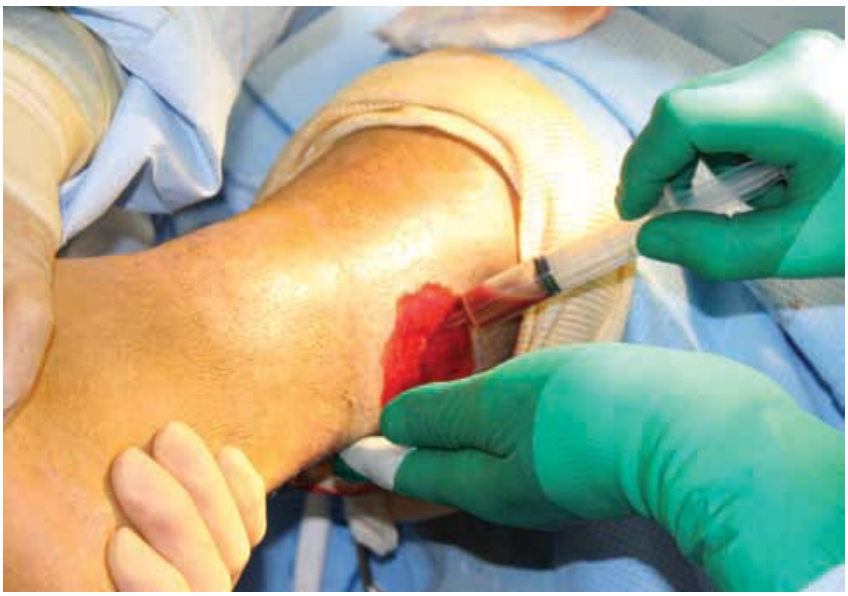


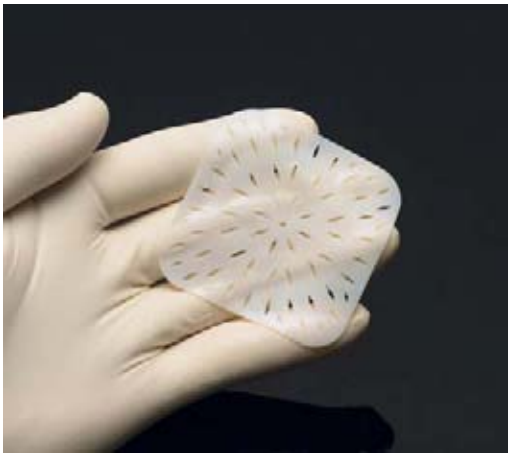
Figure 2- ABMA Application



9.2. Unite Biomatirx

The Unite® Biomatrix is a collagen based, decellularized equine pericardial dressing for skin wounds. It is supplied in various sizes and configurations. Refer to the Instruction for Use for further details.

Figure 2- Unite Biomatrix



10.STUDY PROCEDURES

All patients will be assessed according to the following schedule:

- Screening/ Baseline (within 5 weeks of the surgery date)
- Treatment (Day 0)
- Weekly Post Day 0: until week 12 (+/- 2 days) or wound closure.
- Exit visit on Week 12 if the wound is not healed. Patient with non-healed wound will be followed as per standard of care.

A tabular summary of all the study procedures to be evaluated during this clinical study is provided in section 2 (Study Procedures Outline).

The investigator will make every attempt to follow the patients and will document the information gathered during the follow-up visits on the case report forms (CRFs). The patients will be informed of the importance of returning for scheduled follow-up visits even if they are not having any problems.

10.1. Baseline/ Screening Procedures

The investigator will determine and document whether each patient meets the selection criteria previously outlined before enrollment into the study. The investigator or designee will also obtain an IRB approved informed consent for study participation from each patient prior to randomization. A patient identification number will be assigned to each patient who signs a consent form.

As noted in table 1, the investigator will determine and record each patient's demographics (date of birth, sex), height, weight, brief medical history and wound assessment. Hematology and chemistry are performed if clinically indicated to confirm exclusion criteria (blood drawn within 30 days of procedure is acceptable if no change in clinical condition).

Table 1: Baseline / Screening Evaluation

Clinical information	Wound Assessment
• Date of Birth • Sex • Height (in) / Weight (lb) • Medical conditions (Med. History/brief physical exam) • *Labs (hematology, chemistry) • Vascular study (ABPI, TcPO2 or Doppler) • Concomitant Medication • Peri-wound skin status	• Location • Etiology • Duration • Size (length, width, depth) • Exudates qualification and quantification • Previous treatment • Pain • Photo

** Lab is done to confirm the exclusion criteria.*

10.2. Treatment (Operative) Procedures (Day 0)

At this visit, if the patient continues to meet all inclusions and none of the exclusion criteria, the patient will be randomized to either ABMA and equine xenograft (treatment group) or equine xenograft alone (control group). If the patient exhibits clinical infection prior to randomization, he or she may be treated with appropriate antibiotics and

randomized one week later, provided the investigator has determined that clinical signs and symptoms of infection are no longer present. The investigator will record the Treatment (Operative) information as listed in table 2.

Table 2: Treatment (Operative) Evaluation

Clinical information	
• Date of treatment • Wound bed preparation and debridement • Wound size (Length, width and depth) • Randomization • Pain assessment	• ABMA & xenograft / or xenograft alone • Intra-operative complications • Photos prior and after debridement • Peri-wound skin status

10.2.1. Wound Treatment

During the procedure, basic surgical procedures are not altered. The patients are taken to the operating room and placed under general or monitored local anesthesia. An appropriate lower extremity block may be administered as needed following counseling of the patient by the anesthesia team. The ulcers are surgically debrided to ensure a clean base with no eschar or fibrotic tissue.

In the Treatment Group:
Through a lateral hindfoot approach, a trephine is utilized to harvest the marrow from the ipsilateral calcaneal bone as shown in Figure 1. Approximately 3 cc–5 cc of bone marrow aspirate will be collected, depending on the ulcer size. The aspirate is then immediately and directly applied to the wound bed so that the entire wound surface is coated with an even layer of the aspirate and then the xenograft will be applied per standard protocol.

In the Control Group:
The wound surface is dressed with equine xenograft directly without ABMA.

In Both Groups: Equine xenograft is applied into the underlying wound surface making sure it is free of underlying air bubbles or folds in the material. 1.5-2.0 cm is allowed to overlap with intact wound margins. Selection of retention materials is based on the presentation of the peri-wound tissue. Staples or sutures are used to secure the xenograft. In cases when severe inflamed or fragile peri-wound tissue, application of a Tincture of Benzoin® and Steri-Strips® may be less traumatic when anchoring the material, with the exception of tissue subject to repetitive pressure or trauma. After equine xenograft application, Restore AG is then applied and followed by bolstered gauze, gauze wrap and a multi-layer compression.

Dressings Change: While dressing may be changed as needed, the xenograft is left intact. With low exudates wounds, outer dressings can be left out for 7 days. The xenograft margins can be trimmed as they desiccate and lift from re-epithelialized wound margins.

Compressions are changed once a week or more frequently if clinically needed.

10.3. Postoperative Procedures

10.3.1. Discharge

At discharge, the investigator will record the date of the patient's discharge from the hospital, prescribed medication, and complications/unanticipated adverse events.

10.3.2. Postoperative Follow-up Visits

Postoperative follow-up visits are required weekly until week 12 post surgery (Day 0) or until wound is closed. See table 3 for data collection.

At each follow up, the investigator will record wound assessment including the healing status, dimensions, the graft status (whether incorporated, intact or fell off, dry or moist), and the percentage of granulation, fibrosis and necrosis in the wound bed in cases where the wound bed is visible.. Restore Ag, gauzes and Multi-layer compression system are applied once a week or more frequently if clinically needed in both groups.

Table 3: Postoperative Evaluation at Discharge

Clinical information	
• Date of follow up • Prescribed Medication • Complications • Wound assessment (closure, size, Graft status, % of granulation, fibrosis, and necrosis)	• Pain assessment • Compression system • Wound Photo • Peri-wound skin status

At each postoperative assessment, the investigator will determine the patient's availability for future follow-up.

10.4. Patient Completion

Patients are considered to have completed the study if they have completed all follow-up visits through 12 weeks. A Study Conclusion Form must be completed for all enrolled patients who either completed study follow-up visits or are discontinued from the study.

10.4.1. Patient Withdrawal

The investigator will make every attempt to follow the patient at each of the required assessment periods. Patients may be discontinued from the study in the event of a condition that may cause them harm if participation were to be continued. Patients may withdraw from the study without penalty or loss of benefits to which they are otherwise entitled.

10.4.2. Lost to Follow-Up

If a patient cannot be reached for a follow-up visit, the investigator will document on the CRF the effort(s) he/ she made to contact that patient or the patient's primary health care provider.

Patients who do not return to their follow-up visits and cannot be contacted within a reasonable timeframe via letter or telephone will be considered lost to follow-up.

11. CLINICAL ADVERSE EVENT

11.1. Overview and Definitions

Adverse Events associated with the device or the procedure will be recorded on the Adverse Event Case Report Form and analyzed.

Consideration of Adverse Events will hereafter consist of Adverse Events and Adverse Device Effects, including Serious Adverse Events, and Unanticipated Adverse Device Effects.

Adverse Event (AE): any unfavorable and/or unintended sign, symptom, or disease temporarily associated with the use of a device product, whether or not considered related to the device product.

A Serious Adverse Event (SAE) includes any of the following events that may or may not be considered related to the device:

- Death due to any cause.
- Life-threatening or permanently disabling events.
- Any event resulting in additional treatment (intervention to prevent permanent impairment/ damage), hospitalization or prolonged hospitalization.

Unanticipated Adverse Device Effects (UADE): Any serious adverse effects on health or safety or any life threatening problem or death caused by, or associated with, a device, if that effect, problem, or death was not previously identified in nature, severity or degree of incidence in the protocol or application (including a supplementary plan or application) or any other unanticipated serious problem associated with a device that relates to the rights, safety or welfare of Subjects.

11.2. Adverse Experience Reporting

The investigator will report any serious, unexpected adverse device event occurring during the investigation to the USCD IRB within 10 calendar days from the time he or she learned of the event.

After review with the patient by the study site personnel, all adverse events will be documented in the patient's records. The following attributes must be recorded on the Adverse Event Form:

1. Description of event.
2. Date of onset.
3. Date of resolution.
4. Severity.
5. Relationship to the study device and/or procedure.
6. Action(s) taken.
7. Outcome(s).
8. Attending physician who is treating the event.

If the adverse event is of such severity in the Investigator's judgment that it warrants withdrawal from the study, the patient should be withdrawn from treatment and a termination assessment performed. The patient should be given appropriate care under medical supervision until symptoms resolve.

Adverse events are described as mild, moderate or severe. The severity of adverse events will be assessed on the following severity index scale:

Mild

The adverse event is transient, requires no treatment, and does not interfere with the patient's daily activity.

Moderate

The adverse event introduces a low level of inconvenience or concern to the study Subject and may interfere with daily activities, but is usually ameliorated by simple therapeutic measures.

Severe

The adverse event interrupts the patient's usual daily activity and requires systematic therapy or other treatment.

Severe is defined as a measure of the intensity of a reaction, effect or experience. The measurement(s) are described as mild, moderate or severe. The event itself, however, may be of relative minor medical significance.

12. RISKS AND BENEFITS

There is no known risk to using ABMA. Equine xenograft does not impose a significant risk on human subjects. To date, there has been no report of significant product complaint. However, there is a possibility that patients may develop an allergic reaction to the implant materials. Allergic reactions vary widely from a simple skin rash and itchiness to a more serious event such as anaphylactic shock.

The surgical risks to patients who participate in this study are the same as for any patient who undergoes wound surgical debridement and treatment for a venous ulcer and may include but is not limited to wound breakdown, infection, pain, failure of procedure and need for surgical intervention. These risks include infection, cellulitis, osteomyelitis, and this could lead to limb amputation and death.

Complications have been reported in the literature when using xenograft to treat the venous leg ulcer. Among these complications, infection was reported at a rate of 1.6% with SIS Wound Matrix (OASIS), 8.6% with compression treatment alone. Mostow et al reported a new ulcer due to compression therapy in one patient (4.16%).[15]

12.1. Benefits

Potential benefit to the patient may include, but are not limited to the following:

1. Reduce the time to wound healing
2. Healing of recalcitrant wounds
3. Provide the wound research community information that possibly help in future treatments

13. DATA ANALYSIS METHODS

13.1. Randomization

After informed consent is received, and study eligibility criteria are met, patients will be randomized on the day of surgery on a 1:1 basis.

Patients will be randomized according to a computer-generated randomization scheme by opening sequential randomization envelopes.

13.2. Analysis Populations

Treatment Population:	All patients who are randomized and treated. Primary Analysis Population.
Per-Protocol Population:	All Treatment Population patients who have no protocol deviations.

13.3. Patient Disposition

The number of patients who and are enrolled and treated will be presented by treatment group. The primary reasons for discontinuation will be summarized for all patients not completing the Screening or Treatment Phase.

13.4. Subject Demographic and Baseline Characteristics

Patient demographic and baseline characteristics will be summarized by treatment group. Descriptive summaries will include number of Subjects, mean, standard deviation, median, minimum and maximum for continuous parameters, frequencies and percentages for categorical parameters.

13.5. Descriptive Statistics

Descriptive statistics for all endpoints will include number of Subjects, mean, standard deviation, median, minimum and maximum for continuous variables, and frequencies and percentages for categorical variables.

13.6. Primary Analysis

The endpoints of the study are:

1. Percent of wounds healed by 12 weeks will be compared between groups using Fisher's exact test, as a two-tailed test, with significance judged at $\alpha = 0.05$.
2. Time to healing. Censored events will include withdrawals, explants for reasons other than infection and deaths.
3. Mean and median wound size change at Week 12 will be calculated and compared between both groups using.

13.7. Secondary Analyses

1. Incidence of device or procedure related adverse events.

13.8. Safety Analyses

Descriptive statistics for adverse events, and medications, will include number of Subjects, mean, standard deviation, and frequencies and percentages.

13.9. General Methodology

For all categorical variables, frequencies will be provided. For any binary outcomes, confidence intervals will be computed using the exact binomial distribution. When applicable, comparisons of groups will be performed using Fisher's exact test; odds ratios, risk differences, and relative risk may also be presented as appropriate.

For continuous variables, descriptive statistics (e.g. means, standard deviations, median, minimum, and maximum) will be provided. In addition to descriptive statistics, confidence intervals will be computed based on the *t*-distribution.

Actuarial analysis will be performed for time dependent binary variables using the Kaplan-Meier method. Cochran test, the Kruskal-Wallis and log-rank test will be used to compare differences in means, medians and distributions, respectively.

Unless otherwise specified, confidence intervals and group comparisons will be two-sided. The value α =5% will be used to determine significance; each analysis will be

reported separately with no adjustment for multiple comparisons. Confidence intervals will be 95% intervals.

Unless otherwise specified the exact form of each algorithm will be the default of SAS®, version 9.1.

13.10. Data Considerations

13.10.1. Protocol Violations

Protocol violations include anything performed outside of the protocol guidelines, as well as, anything stipulated in the protocol that is not carried out.

14. ETHICAL AND REGULATORY CONSIDERATIONS

Informed Consent and IRB/Ethical Review Committee

For purposes of this study, a written informed consent will be obtained from all patients. The patient must be adequately informed of his/her participation in the clinical study and what will be required of him/her in order to comply with the protocol. In addition, informed consent is required to allow appropriate data monitoring including access to medical records by regulatory agencies.

An IRB or ethical review committee for an institution must approve the informed consent and protocol for use at its institution. A written statement by the IRB / ethics committee indicating approval of the informed consent and protocol must be obtained prior to study initiation.

Case Report Forms

Paper CRFs will be used to collect patient information. The principal investigator or designee will keep a separate log of patient names and current addresses to facilitate record keeping and the ability to contact patients for future follow-up.

CRFs are used to record study data and are an integral part of the study and subsequent reports. Therefore, the reports must be legible and complete. All forms should be filled out using a black ballpoint pen. Errors should be lined out but not

obliterated and the correction inserted, initialed and dated by the investigator. Copies of the changed form must be retained in the patient study file.

A CRF must be completed and signed by the principal investigator or co-investigator (paper or electronic) for each subject enrolled in the study, including subjects withdrawn from the study for any reason. The reason for withdrawal must be noted on the CRF by the investigator for each subject.

Since there is a potential for errors, inaccuracies, and illegibility in transcribing data onto CRFs, originals or photocopies of all relevant operative records and reports, postoperative examinations, laboratory and other test results must be kept on file. CRFs must be kept current to reflect patient status at each phase during the course of the study.

14.1. Record Retention

Study files will be maintained in accordance with the UCSD policy.

14.2. Study Responsibilities

14.2.1. Investigator Responsibilities

The principal investigator is responsible for obtaining IRB/Ethics Committee approval for the study at his/her institution.

Study records including CRFs, signed Agreement, signed informed consents, a copy of the implant data card, IRB/Ethics review committee approval letters, the log of IRB/Ethics review committee submissions, and other documents pertaining to the conduct of the study must be kept on file by the investigator.

The responsibilities of the investigator(s) comply with the requirements set forth in the US Code of Federal Regulations.

- 21 CFR part 50: Protection of Human Subjects
- 21 CFR part 56: Institutional Review Boards
- Declaration of Helsinki

- The local laws of the country

The investigator(s) will adhere to the regulations that provide the greatest protection to the patient.

Any protocol deviations must be fully documented and explained on the CRF.

Any unusual or unanticipated complications will be reported immediately, if applicable, to the Institutional Review Board (IRB)/ethical review committee. If deemed necessary by the investigator, the IRB/ethical review committee, or the investigation may be suspended pending a thorough study of the incident.

If the investigator wishes to assign the files to someone else or remove them to another location, he or she should consult with the sponsor in writing as to this change. If there is a change or addition of co-investigator, an amended Investigator's Statement and Agreement must be completed promptly. Any other personnel changes must be reported immediately to the IRB.

14.3. Study Changes

Changes in the protocol may be made only by written amendment and must be approved by the IRB or ethical review committee.

BIOGRAPHY

Gerit Mulder obtained his Bachelor of Science degree from the University of Redlands in Southern California in 1971 as well as a Bachelor of Science in Premedical work from the California College of Podiatric Medicine in San Francisco in 1982. In addition to the BS degree, he also obtained a Master of Science with a focus on Neurophysiology from the California State University in San Bernardino in 1978. He obtained his medical degree as a Doctor of Podiatric Medicine at the California College of Podiatric Medicine in San Francisco in 1982 following which he completed a surgical podiatric residency at the University of Colorado and Veterans Administration Hospitals in Denver, Colorado. In addition opening the first Wound Clinic at the Veterans Administration Hospital in Denver in 1984, which he directed for 10 years, he also opened the first full time Wound Clinic (Wound Healing Institute) in Denver from 1984-1994. This was a first of its kind clinic offering treatment for all types of chronic wounds as well as serving as a research center. Dr. Mulder also worked for 3 years in the Medical Industry as Medical and Regulatory Director and assisted in the research and development of tissue engineered products and medical devices. During the last 12 years he has directed the Wound Treatment and Research Center in the Division of Trauma, Department of Surgery at the University of California San Diego and is currently a Professor of Surgery and Orthopedics at the University. He has published over 200 articles, manuscripts, abstracts, chapters and texts as well as being involved in over 100 research projects. He is an internationally recognized speaker, teacher and academician and is currently assisting the government of various countries with the design, development and implementation of Diabetic Centers of Excellence for amputation prevention. He has been nominated as one of the most influential physicians in his field of Wound Care and has received numerous national and international recognition awards.