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Evaluation of a Surgical Intervention to Experimentally Compare CO2 Laser to Scalpel Incisions, Added Growth Factor, and Suture Material to Reduce Cutaneous Scarring

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EVALUATION OF A SURGICAL INTERVENTION TO EXPERIMENTALLY COMPARE CO_2 LASER TO SCALPEL INCISIONS, ADDED GROWTH FACTOR, AND SUTURE MATERIAL TO REDUCE CUTANEOUS SCARRING

by Rhian E. Davies, D.O.

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ABSTRACT

Evaluation of a Surgical Intervention to Experimentally Compare CO_2 Laser to Scalpel Incisions, Added Growth Factor, and Suture Material to Reduce Cutaneous Scarring

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Introduction

The goal of this study was to determine if the repair of full thickness skin incisions in an animal model could be improved by using a CO_2 laser vs. scalpel, commercial vs. swine intestinal submucosa (SIS) sutures, and addition of exogenous nerve growth factor (NGF).

Materials and Methods

A rat model was used to evaluate the following tissue components: prevalence of mast cell granules, thickness of epidermis, organization of collagen, infiltration of tissue into SIS, neutrophil presence around suture holes, and granulation tissue production around suture holes.

Results

Added NGF led to a significant decrease in the number of granules in mast cells following laser incisions. A significant number of neutrophils were detected in skin following laser incision without added NGF. Added NGF significantly increased the band

of granulation tissue for both types of incision methods however, the laser resulted in a significantly wider band of granulation tissue with or without added NGF. A thicker epithelium was apparent following use of laser as was the level of collagen organization. Added NGF significantly increased incorporation of skin elements into SIS sutures. The use of the laser without NGF resulted in greatest collagen organization, number of mast cell granules, and neutrophils, and significantly greater vascularization.

Discussion

The greater extent and duration of granulation tissue proliferation following laser incision may be attributable to an inappropriately high laser dosage. Collagen organization improves with laser use. Incorporation of tissue into SIS sutures was promoted by adding NGF, but unaffected by surgical technique. Increased vascularity following laser incision suggested blood vessels re-opened or angiogenesis occurred post-surgery. With added NGF, epidermal width following laser incision was even greater in contrast to the scalpel incised group. Depending upon the intent of the surgeon, the use of surgical modality, suture material, or additional exogenous NGFs has to be tailored to the specific patient and desired outcome.

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Chapter 1.

INTRODUCTION

This study was part of a collaborative effort with two other investigators. The overarching goal was to determine if experimental muscle and skin incision repair of a hind leg animal model could be more effectively promoted by: 1. Using a CO_2 laser or a scalpel to produce full thickness skin incisions; 2. Sutures fashioned from swine intestinal submucosa (SIS) as an alternative to commercial sutures to repair cutaneous incisions; 3. Adding nerve growth factor (NGF) in addition to the growth factors already present in SIS.

This study compares the effects resulting from the use of CO_2 laser or a scalpel to perform the cutaneous portion of the incision; the type of suture material used for cutaneous repair; and possible tangential effects on the healing cutaneous wound with the addition of exogenous NGF. Functional evaluations, the effects of the suture material used, as well as the effects of the addition of NGF to the muscle portion of the wound are reported elsewhere.

The degree of scarring associated with wound healing can be affected by infections, surgical methods, or inflammation of tissue.(1,2) Their appearance varies according to tissue type and its location. It has been concluded from a variety of studies that surgical incisions made with a CO_2 laser not only improve the healing outcome at the surgical site but also decrease the risk of infection and scarring.(1,2,3) This study involves a histological light microscopic evaluation that compares scar tissue formation

in healing cutaneous wounds following either CO_2 laser or scalpel surgical incisions, as well as the effects of added NGF.

1.1 The Process of Cutaneous Wound Healing

A wound is the actual disruption of normal anatomical structure and function. Healing is the complex and dynamic process that results in the restoration of anatomical continuity and function.(4)

The healing process can range from weeks to years after injury until complete healing is achieved. Unfortunately, the injured region rarely totally regains its prior function depending upon the severity of the injury itself, as well as the effectiveness of the wound healing process. The process itself includes the following phases: degeneration, inflammation, regeneration, and fibrosis. Scars are an integral part of the wound healing process and consist of fibrous tissue that serves to replace lost or damaged native tissue.

Acute wounds tend to heal in a very organized fashion beginning after the insult has taken place. The injured area undergoes hemostasis (degeneration), followed by inflammation, proliferation (regeneration), and finally remodeling (fibrosis).(4) Hemostasis is achieved immediately upon the interaction of platelets and exposed collagen. Platelets and other blood products spill into the injured site. When platelets aggregate, they release clotting factors, which initiate a fibrin clot that is deposited at the injured site and serves as a basic scaffolding upon which the wound healing

proceeds.(4,5) Platelets also release platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-beta), which aid hemostasis.

The PDGF, which is released by the platelets, initiates the chemotaxis of neutrophils, macrophages, and smooth muscle cells, as well as fibroblasts into the injured area. It also stimulates the mitogenesis of the fibroblasts and smooth muscle cells. (4,5) Macrophages themselves are responsible for releasing additional PDGF and TGF-beta as well as other cytokines including fibroblast growth factor (FGF), tumor necrosis factor alpha (TNF-alpha), and interleukin-1 (IL-1). (4) Macrophages are also accountable for phagocytosing nonfunctional host cells, bacteria-filled neutrophils, damaged matrix, foreign debris, and any remaining bacteria from the site. Macrophages represent the conclusion of the inflammatory phase and the beginning of the proliferative phase. (4)

During the proliferative phase, macrophages and platelets continue to release TGF-beta, which is considered to be the master control signal regulating a host of fibroblast functions (5). It is able to increase the transcription of genes for collagen, proteoglycans, and fibronectin leading to an increase in overall production of matrix proteins and is responsible for decreasing the secretion of proteases that are responsible for breakdown of the matrix by stimulating protease inhibitor. (6)

Neutrophils are seen at roughly the 24 hour mark after an injury has occurred and are the next predominant cell type. The increase in the number of infiltrating phagocytotic neutrophils during the inflammatory phase, results in the removal of damaged tissue, non-functional host cells, and any bacteria or foreign bodies that may have entered the area affected area. (4,5,7,8)

Mast cells also participate in the inflammatory phase by releasing cytoplasmic granules filled with enzymes, vasoactive mediators, including histamine, serine proteases, cytokines, and other active amines. These mediators are responsible for the characteristic signs of inflammation around the wound site. (9) The amines compromise the integrity of blood vessels, which permits the passage of mononuclear cells into the injury site. (4) This effect allows for water to accumulate at the wound site and the characteristic signs of inflammation become evident including rubor (redness), calor (heat), tumor (swelling), and dolor (pain). (4,10-12)

Mast cell activity can be triggered by a variety of environmental and endogenous stimuli, including IgE, neuropeptides, chemokines and physical, chemical and mechanical factors, such as trauma, thermal parameters and ultraviolet light, as well as laser irradiation (10, 13-17). These stimuli can promote degranulation and the release of mediators by directly disturbing the mast cell membrane or indirectly by stimulating local sensory nerve endings in contact with the mast cells. (18) Mast cell mediators include those with specific fibroproliferative activity, such as histamine, tryptase, chymase, fibrogenic cytokines (IL-1, IL-4 and TNF) and growth factors (TGF and basic-FGF) that stimulate chemotaxis, migration, phenotype differentiation and biosynthesis activity by the fibroblasts. (18-27)

During the remodeling phase, mast cell degranulation also plays an important role in the deposition and formation of connective matrix of which collagen is the main component. (10,18) Of all the mast cell granule contents, chymase appears to be the most relevant to tissue matrix remodeling by activating procollagenase and the direct processing of pro-collagen into collagen fibrils. (28,29)

After the site has been cleared of damaged tissue, fibroblasts migrate into the area and initiate the proliferative phase by depositing a new extracellular matrix composed of collagen that subsequently becomes cross-linked and provides strength and integrity, thus facilitating the final phase of the remodeling process. (4) There are several alternative roles related to the deposition of collagen in the healing process: a.) a normal repair characterized by an equilibrium between scar formation and scar remodeling, b.) a pathological response resulting in fibrosis, strictures, adhesions, and contractures, as a consequence of inappropriately large amounts of collagen incorporated into the wound that results in restricted function, c.) alternatively, when an insufficient quantity of collagen is incorporated into the site a weaker wound repair results.

Epithelization begins and is stimulated by the presence of epidermal growth factor (EGF) and TGF-alpha. Both of which are produced by macrophages, platelets, and keratinocytes. (30-32) Final epithelization of the cutaneous wound is achieved by the loss of contact inhibition of epithelial cells derived from the margins of the wound, adjacent hair follicles, and sebaceous glands, which move by epibolic migration into the wound area (6). The process continues with the proliferation of new cells derived from the basal layer of the remodeling epithelium.

There is a heightened metabolic state within the wounded area resulting in an increased demand for oxygen and nutrients. The presence of a low pH, reduced oxygen tension, and increased lactate production provide the stimulus for angiogenesis mediated by vascular endothelial cell growth factor (VEGF), bFGF, and TGF-beta. (4,33)

Most surgically induced, acute, cutaneous wounds heal by ‘primary intention’. Primary intention occurs within hours following the approximation of the wound edges. The close contact is maintained through closure of the incision using sutures, staples or adhesive tape. These reparative techniques stabilize the site and provide the conditions necessary for the formation of a connective tissue matrix. The union of the tissue is followed by an orderly process by which collagen, proteoglycans, and attachment proteins work together in the creation of a new extracellular matrix. (4) Alternatively, wounds can heal by secondary intention when the wound edges are not approximated and they heal with formation of granulation tissue, contraction and eventual spontaneous migration of epithelial cells. Fibroblastic differentiation into myofibroblasts, which resemble contractile smooth muscle, is believed to contribute to wound contraction.(34) Healing by third intention occurs when a wound is left open for observation for several days and then closed via a primary closure.(35) Such wounds are left open initially because of gross contamination.(35)

1.2 Carbon Dioxide Surgical Laser Incisions

The CO_2 laser is a precise surgical instrument that can be an alternative to traditional scalpel surgical applications because it has a high degree of absorption in soft tissue with limited lateral damage. The CO_2 laser is able to control bleeding and provide the necessary precision to weld arteries for microsurgery, as well as anastomose small vessels and nerves. The depth of the incision is determined by the power density and the

duration of application (the sweep speed). The feel for controlling the depth of the incision is developed by practice.(36)

The CO_2 laser incisions tend to have reduced blood accumulation in the surgical field due to their coagulative properties, which seal small blood vessels. The black char that forms at the edges of the incisions is entirely superficial and can be irrigated away. The lateral zone of damage for the CO_2 laser extends less than 0.5mm from the incision. The surrounding tissue will demonstrate minimal edema, scarring, or stenosis.(36)

The CO_2 lasers beam can be manipulated by the power setting and the spot size. It performs by cutting, evaporating, and coagulating. When it is used to cut or evaporate tissue, the best approach is to employ the highest power one can safely control. The use of high power localizes the thermal damage to the impact site, thus minimizing the effect on the surrounding tissues.(36)

The spot size of the CO_2 laser beam is adjusted by moving the lens farther away or closer to the tissue so that it is able to produce a very circumscribed and defined incision. When the beam is used for cutting, the spot is focused on the tissue. With the laser beam in the focused mode, its effect is precise, and damage is localized.(36)

The mechanism of action of the CO_2 laser is to destroy tissue by rapidly heating and vaporizing intracellular water. (4,6,37) The CO_2 laser is also able to successfully seal small nerve endings, leading to a reduction in pain post-operatively (38). In addition, edema can be reduced post-operatively because of the lasers ability to seal off lymphatics and bleeding in the surgical area allowing for a much more efficient and visible surgery.(4,39,40)

It is important to consider the thermal response of the tissue when using a CO_2 laser, in order to select the proper mode of delivery (continuous or pulsed) wavelength, irradiance and dose necessary to achieve an appropriate surgical incision. CO_2 lasers can increase the risk of thermal damage and this can be difficult to control without adequate practice. The thermal damage can be due for example, to excessive heat diffusion resulting from a continuous beam leading to the development of undesirable thermal necrosis in the surrounding tissues.(4,38)

It has been demonstrated that a pulsed beam is superior to a continuous beam for cutaneous incisions with respect to scar tissue formation because it decreases the zone of thermal damage (4,38). Prior knowledge of the interaction between different tissues and lasers, has lead to a minimization of thermal damage.

Research conducted by Shang et al., has shown that laser irradiation promotes the infiltration of mast cells into the dermis and with respect to mast cell degranulation, the number of degranulated mast cells observed in the irradiated specimen was significantly higher than that in the control group. (18) This increase suggests that dermal mast cells are involved in the inflammatory process, because preformed mediators released after degranulation of mast cells can serve to promote inflammation via a multiplicity of actions. (18) They also found that during the proliferative and remodeling phases, the total number of mast cells in the experimental group was statistically higher than in the controls.(18) Therefore, the increase in mast cell number after laser treatment effects the later phases of wound healing by stimulating wound repair. The released mediators attract an influx of fibroblasts, collagen, and various other cells essential to the wound healing process.

1.3 Swine Intestine Submucosa (SIS)

SIS was chosen as a suture material because of its ability to provide functional integrity to the tissue, serve as a scaffold promoting host tissue ingrowth, and appears promising in the management of tears and defects in muscles and tendons. (41-43) It also is able to help the rate and quality of tissue repair after an injury has occurred to a tendon or muscle.(44,45)

SIS is naturally derived and consists of acellular collagen. It contains endogenous growth factors including FGF-2, TGF related protein, and VEGF. SIS is able to provide a rich environment that signals surrounding tissue to grow into and around it, gradually replacing the SIS with native tissue that has the necessary properties to continue the repair.

The present study is an expansion upon the research conducted by Benquista et al which examined superficial scar tissue formation over a lacerated skeletal muscle.(41) The injury was repaired with either 7-0 prolene suture material or suture material made of SIS. They showed that the superficial scar repaired with SIS appeared organized without insult to the adjacent muscle tissue, while the muscle repaired with prolene resulted in increased formation of fibroblastic tissue that was not only disorganized but appeared to disrupt the adjacent muscle tissue.(41)

1.4 Goals and Specific Aims

This research project was designed to determine the differences in healing and scar tissue formation between: Scalpel or Sharplan 1041S surgical carbon dioxide laser (Lumenis; Dreieich, Germany) induced full thickness cutaneous incisions overlaying the gastrocnemius muscle in an experimental rat model and the use of either commercial or SIS sutures to repair the incisions with or without nerve growth factor (NGF) injections.

To achieve these goals, specific aims were established:

1. To incise and repair the cutaneous tissue overlying the gastrocnemius muscle in a rat model to determine which surgical modality, CO_2 laser or scalpel, promotes better healing and restored histological integrity.
2. To determine if the addition of NGF in association with SIS or commercial suture promotes better healing and restored histological integrity
3. To evaluate differences in treatments histologically using light microscopy morphometric techniques and statistical analysis to conclude which was the best treatment.

Chapter 2.

MATERIALS AND METHODS

2.1 Preparation of SIS Sutures

Porcine small intestine was obtained from a USDA approved vendor in the fresh state. The jejunum was identified and immediately separated from the rest of the small intestine, keeping a remnant of the mesentery intact to distinguish orientation. A segment of jejunum was transected and cut longitudinally along the remnant of the mesentery to form a sheet. The sheet was placed serosal side up, then starting at the edge, the serosa and muscularis layers were peeled away from the submucosa and discarded. The sheet was then reoriented to expose the mucosal surface and the mucosal layer was denuded from the SIS sheet. The SIS sheet was further subdivided into thin strips to be used as suture material and stored in a sterilizing 10% gentamicin/physiological saline solution. The final dimension of the suture material was comparable to 7-0 commercial suture. Sutures were threaded to a C-3 reverse cutting needle the day before surgery and returned to the sterilizing solution.

2.2 Operative Model

Thirty-two male Sprague-Dawley rats, initially weighing between 310 and 365grams were obtained from a commerical vender (Charles River Braintree, MA). The animals were stabilized for 24 hours upon arrival and confirmed to be in good health and maintained on a diet of normal rat chow over the length of study. The rats were randomly assigned to one of four groups: Group A = laser without NGF; Group B = laser

with NGF; Group C = scalpel without NGF; Group D = scalpel with NGF. This study was approved by the Philadelphia College of Osteopathic Medicine Institutional Animal Care and Use Committee (IACUC).

2.3 Surgical Protocol

1. All animals were induced to a surgical plane of anesthesia with an intramuscular (IM) injection of Ketamine 40mg/kg and Xylazine 5mg/kg. Mezlocillin sodium 75mg/kg IM was administered both pre & post-operatively.
2. A sterile field was established and the animal was draped in the appropriate fashion. Aseptic conditions were maintained throughout surgery.
3. A posterior longitudinal skin incision was made along the calf of the left posterior leg to expose the surgical field using either the Sharplan 1041S surgical carbon dioxide laser at 5 watts continuously over a period of 1 second, two times or the Scalpel.
4. The cutaneous wound was closed with either SIS or 3-0 vicryl (Ethicon; Cincinnati, Ohio) with or without added NGF using a simple interrupted suturing technique. The entire length of each incision in Group A animals were closed using simple interrupted SIS sutures. However, incisions were closed using 3-0 vicryl and one SIS suture at the distal end of the incisional site in Groups B, C, and D.
5. The incision was washed and dried aseptically and Neosporin® ointment was applied.

2.4 Post-Operative Protocol

1. An initial dose of Butorphanol 2.0mg/kg was administered subcutaneously (SQ) at least 30 minutes prior to the emergence from anesthesia, and a second dose at 4 hours post-operatively.
2. The animals were visually monitored until they were able to resume dorsal recumbent position and stabilization of vital signs. There after, they were monitored on a daily basis. The incision was inspected for cleanliness, dryness, and closure, and their feeding habits and activity were noted.
3. Animals in Groups B and D received an additional 0.1ml of a solution containing 1000ng of NGF per 1ml of normal saline on days 1,3,5, and 10 post-operatively.

2.5 Tissue Recovery and Preparation

1. The animals were euthanized on Day 35 post surgery by CO_2 inhalation in a closed gasketed chamber.
2. An area of cutaneous tissue encompassing the operative site was removed and placed in formalin, paraffin embedded, and sectioned according to standard histological techniques.
3. The sections of cutaneous tissue were stained using Toren's Method for mast cell granules, collagen and cartilage, muscle and elastic fibers, fibrin, bone, colloid, keratin and erythrocytes (Electron Microscopy Sciences, Hatfield, PA).
4. A Nikon Eclipse 50i Microscope (Philadelphia, PA) was used to visualize the tissue and an image analysis program (NIS-elements AR 3.0; Philadelphia, PA) was used to quantitate the parameters of interest.

5. The following tissue components were evaluated:
    a. Prevalence of mast cell granules
    b. Thickness of epidermis
    c. Organization of the collagen
    d. Native tissue infiltration into the SIS
    e. Neutrophils present around the suture holes
    f. Production of granulation tissue around the suture holes

2.6 Tissue Analysis

1. Mast cells: the number of granules in each cell was rated on a scale of +1,+2,+3 for minimal, moderate, or large numbers respectively at 40X magnification.
2. Neutrophils: the number of cells in the inflammatory tissue surrounding the suture holes were rated on a scale of +1,+2,+3 for minimal, moderate, or large numbers respectively at 40X magnification.
3. Epidermis: the width was measured in pixels at 40X magnification and then converted to and reported in microns.
4. Granulation tissue: the width was measured in pixels at 40X magnification and then converted and reported in microns.
5. Collagen: the organized/disorganized appearance was visualized at 20X and was rated as ‘1’ for organized and ‘0’ disorganized.
6. Native tissue infiltration into SIS: the extent of infiltration was rated as ‘1’ for infiltrated and ‘0’ for no infiltration.
7. Blood Vessels: the number in each 20X field was counted and recorded.

In order to analyze each slide appropriately, a master key was designed. This provided a sample image for each item being evaluated such as the prevalence of mast cell granules, thickness of the epidermis, organization of the collagen, native tissue infiltration into the SIS, neutrophils present around the suture holes and production of granulation tissue around the suture holes.

2.7 Laser Calibration Data

The Sharplan CO_2 laser is a class I type B laser that operates at 10.6um wavelength infrared in a Gaussian (TEMoo) mode. The Sharplan laser is continuously adjustable between 2 and 40W in one-watt increments and between 100 and 1,000 mW. The beam is delivered through a spring-balanced articulated arm and a hand-held manipulator. The impact size of the focused beam at 5W was determined by a modification of the method of Kiang and Lang, by burning isolated lines of single laser spots with different focal adjustments near the damage threshold of thermally sensitive paper to determine the exact focal point. Impact size was evaluated microscopically.(46) A 400mm focal length lens was used. The impact diameter of the laser was measured at 2 mm with 5W of power measured at the target plane with a Diamond Ophier power meter giving a power density (irradiance) of 159 W/cm^2 resulting in a fluence of 318J/cm^2.

2.8 Statistical Analysis

A univariate analysis was performed for the variables: surgical method, use of growth factor (GF), and by parameters comparing the production of granulation tissue, amount of mast cell granules produced, presence of neutrophils, width of the epithelium post injury, and infiltration of native tissue into the swine intestine submucosa (SIS) sutures.

A one-factor analysis of variance was performed to evaluate the CO_2 laser versus the scalpel experimental groups with and without the addition of GF for the following parameters; production of granulation tissue, number of mast cell granules, number of neutrophils, infiltration of native tissue into the SIS, and width of the epithelium to help define the extent of wound-healing related to each of the variables being studied in this experiment. One-factor analyses of variance were then preformed based on a.) the intra-animal arithmetic average and b.) the intra-animal median value to evaluate the granulation tissue production and width of the epithelium. The following comparisons were made: surgical methods with and without GF, surgical methods with and without GF combined, laser and scalpel groups combined with and without GF. A probability matrix was constructed to compare the intra-animal arithmetic averages among the groups for the production of granulation tissue and the width of the epithelium. A Student-Newman-Keuls test was preformed based on the one-factor analyses of variance for granulation tissue production and width of the epithelium.

A two-factor analysis of variance including the interaction term was performed for the laser or the scalpel with and without GF to compare the production of granulation

tissue, number of mast cell granules, number of neutrophils, infiltration of native tissue into the SIS, and width of the epithelium.

An additional one-factor analyses of variance was preformed based on the ranked intra-animal arithmetic average values to evaluate the effects of laser versus scalpel with and without the addition of GF on the organization or disorganization of the collagen, number of mast cell granules, number of neutrophils, infiltration of SIS, and number of blood vessels visible in each randomly surveyed 20X field. A probability matrix comparing the intra-animal arithmetic averages among the groups from the one-factor analyses of variance tests based on the ranked intra-animal arithmetic average values by parameter (organization or disorganization of the collagen, number of mast cell granules, number of neutrophils, infiltration of native tissue into the SIS, and number of blood vessels visible in each randomly surveyed 20X image). A student-Newman-Keuls test from the one-factor (group) analysis of variance tests based on the ranked intra-animal arithmetic average values by parameter (organization or disorganization of the collagen, number of mast cell granules, number of neutrophils, infiltration of native tissue into the SIS, and number of blood vessels visible in each randomly surveyed 20X image) was also completed.

A chi-square test was performed to compare the differences between the outcomes of a CO_2 laser incision versus a scalpel incision with and without GF for the parameters of the organization or disorganization of the collagen, infiltration of native tissue into the SIS, and presence of blood vessels in each randomly surveyed 20X field.

Chapter 3.

RESULTS

3.1 Mast Cells

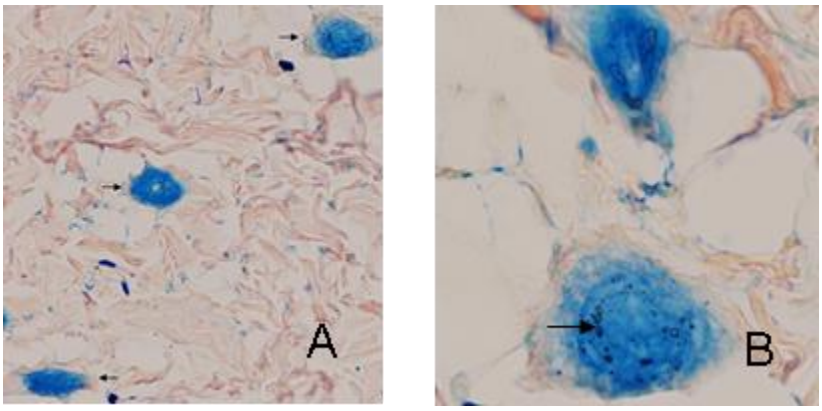


Figure 1(A). Light micrograph illustrating three different mast cells (laser, no added growth factor; 20X).

Figure 1(B). Mast cell (laser, no added growth factor; 40X)

The variation in staining intensity of the mast cells and the appearance of the central region can be attributed to differences in reactivity of the tissue to processing and staining. Mast cells contain a variable number of granules depending upon their content of histamine, heparin, etc. Scalpel and laser incisions with and without added growth factor were compared at Day 35 post operatively to quantitate the number of granules contained within the mast cells found in close proximity to the incisions as an indicator of the amount of pro-inflammatory substances released into the tissue under these conditions. The number of granules were estimated in each mast cells and then rated as containing minimal, moderate or large numbers of granules as +1, +2,+3 respectively.

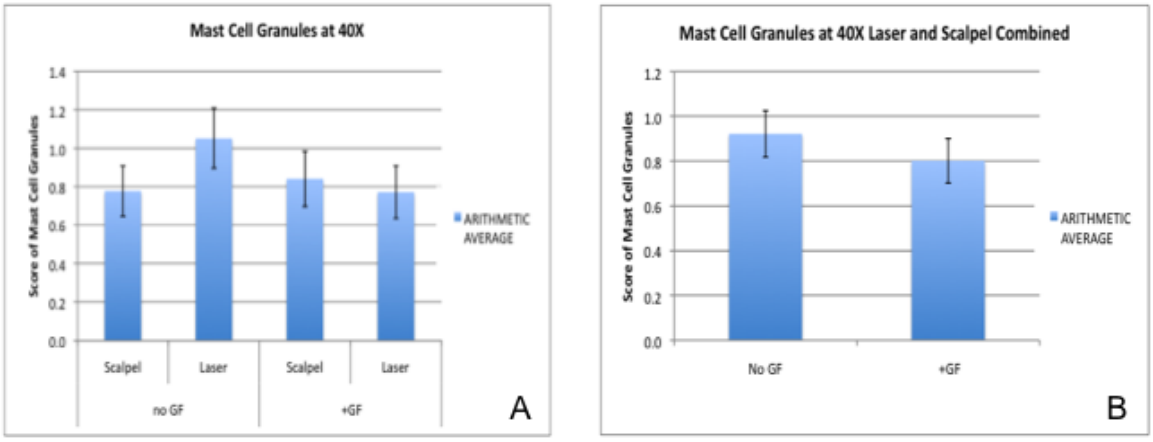


Figure 2(A). Without growth factor, the laser incision resulted in a significantly greater number of granules in the mast cells than the scalpel incision ($p<0.01$). When growth factor was added, the laser incised skin resulted in a significantly decreased number of granules in the mast cells versus the scalpel incision ($p<0.02$).

Figure 2(B). Combined laser and scalpel incisions without added growth factor showed a significantly greater number of granules in the mast cells when no growth factor was added ($p<0.01$) versus with growth factor.

When the laser and scalpel incisions without added growth factor (n=8; n=7, respectively) were compared, the mast cells in the skin undergoing laser incision contained a significantly greater number of granules than those from the scalpel incised skin ($p<0.01$). The result of added growth factor with both laser and scalpel incisions led to a significantly greater decrease in the number of granules in the mast cells of the laser group compared to the scalpel group ($p<0.02$), (Figure 2A &B).

Regardless of which surgical technique was used, a significantly decreased number of mast cell granules ($p<0.01$) were found in the groups with growth factor added (n=15) versus the groups without growth factor (n=16).

3.2 Neutrophils

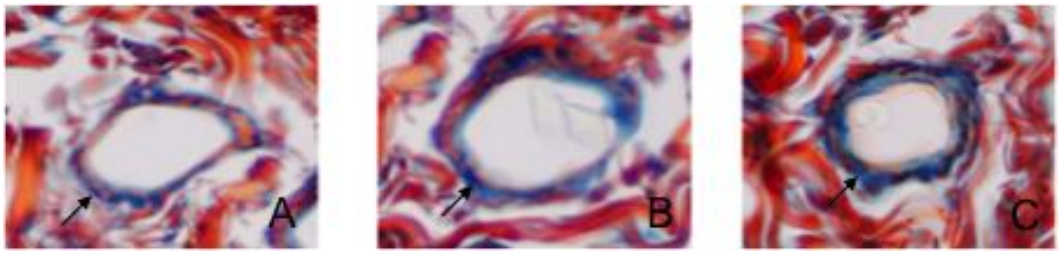


Figure 3(A,B,C). Light micrograph illustrating the appearance and contrasting the relative number of neutrophils within the granulation tissue surrounding three different suture holes (laser, no added growth factor group; 40X).

Since neutrophils are the first white blood cell to engage in the wound repair process and the first to become absent, this analysis was used to gauge the responsiveness of the skin reparative processes to the suture wounds and to determine how far the healing process had progressed by Day 35 post operatively. The number of neutrophils were estimated within the granulation tissue surrounding the suture holes and then rated as containing minimal, moderate or large numbers of granules as +1,+2,+3 respectively.

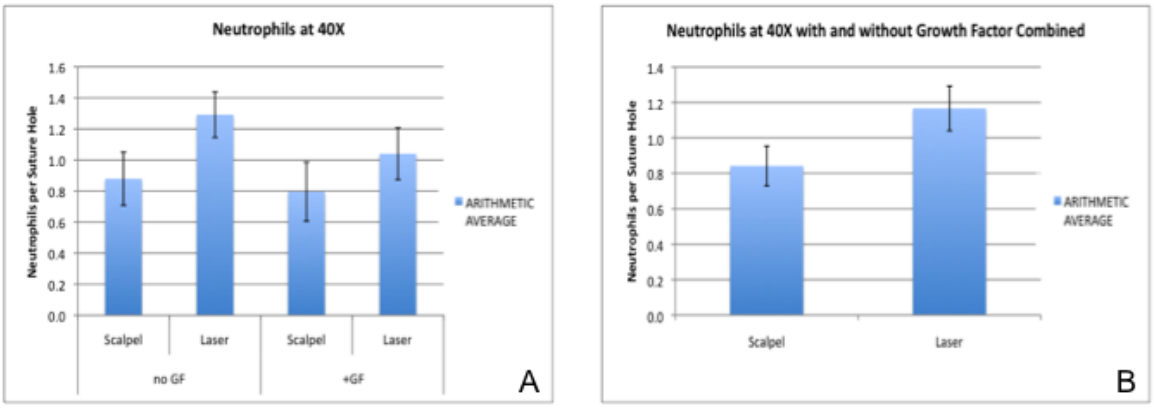


Figure 4(A). The laser incision repair resulted in a significantly greater number of neutrophils in the granulation tissue surrounding the suture holes (n=8) versus the number of neutrophils in the scalpel incised (n=7) skin ($p<0.001$). Similar comparisons with added growth factor showed a trend towards significance ($p=0.057$) for the laser group.

Figure 4(B). When with and without growth factor were combined, the number of neutrophils within the granulation tissue for the laser groups (n=16) was significantly greater than the number of neutrophils in the scalpel groups (n=15), ($p<0.001$).

When the number of neutrophils in the granulation tissue surrounding the suture holes without added growth factor were compared, the skin incised with laser (n=8) demonstrated a significantly greater number of neutrophils than the scalpel incised skin (n=7) ($p<0.001$). The result of added growth factor in these two groups demonstrated a trend towards significance ($p=0.057$), (Figure 4A). Combining results with and without growth factor, the laser groups (n=16), demonstrated a significantly greater number of neutrophils in the granulation tissue around the suture holes ($p<0.001$) versus the scalpel groups (n=15) (Figure 4B).

3.3 Granulation Tissue

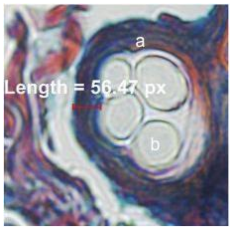
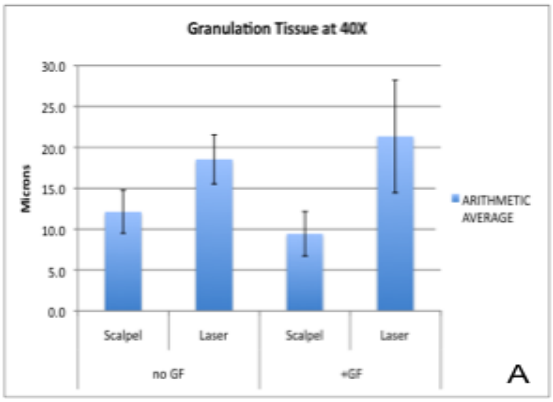


Figure 5. Light micrograph illustrating the appearance of the granulation layer surrounding a suture hole containing remnants of 3-0 vicryl suture material (laser, no added growth factor; 40X).

Sutures *per se* are perceived as foreign bodies by skin and are anticipated to provoke a local, immunologically mediated response reflected by the development of granulation tissue; a part of the normal wound healing process. The width of the

granulation layer was measured in this analysis as being reflective of the balance between the extent of injury and the vigorousness of the reparative process.



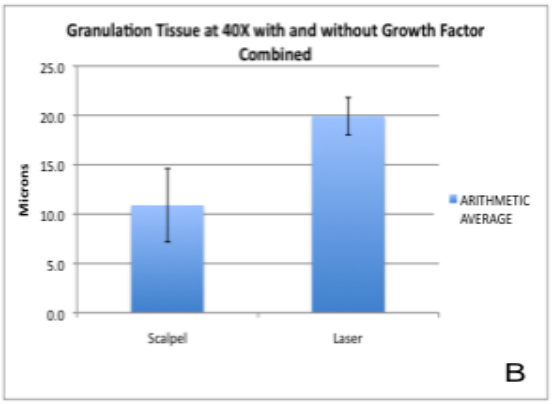


Figure 6(A). Without growth factor, the laser incision repair resulted in a significantly greater width of the granulation layer surrounding the suture holes (n=8) than the scalpel (n=7) incision (p<0.002). Added growth factor also showed the laser incision repair resulted in a significantly greater width than the scalpel incision (p = 0.005).

Figure 6(B). With and without growth factor combined demonstrated the width of the granulation tissue for the laser (n=16) being significantly greater (p<0.001) than the scalpel (n=15) width of the granulation tissue.

When the width of the granulation tissue surrounding the suture holes in the repair of skin incised with laser or scalpel without added growth factor (n=8; n=7, respectively) was compared, the granulation tissue layer in the laser group (n=8) was significantly wider than the scalpel group (n=7) (p<0.002). The result of added growth factor also resulted in a significantly wider band of granulation tissue (p=0.005) in the laser group (Figure 6A). Combining with and without growth factor results, the width of the granulation layer for the laser groups (n=16) was demonstrated to have a significantly wider band of granulation tissue versus the scalpel groups (n=15) (Figure 6B).

3.4 Width of the Epithelium

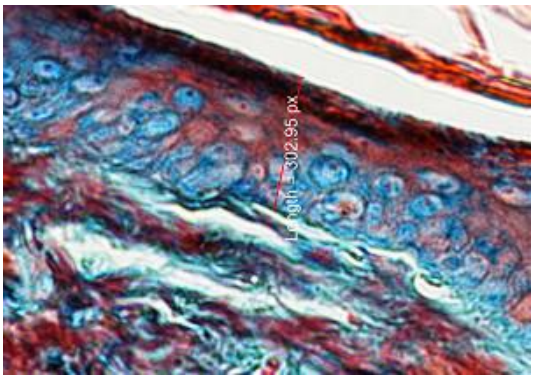
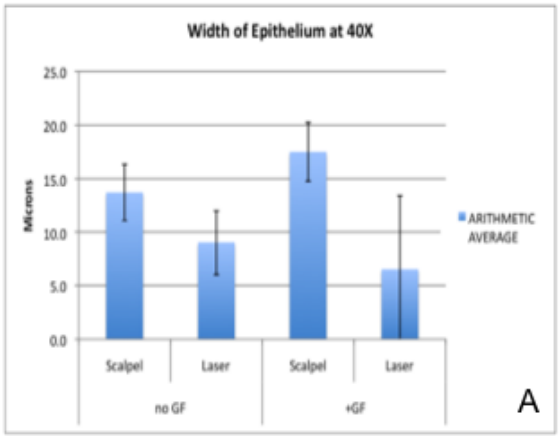


Figure 7. Light micrograph of epidermal layer with superimposed calibration line (laser, no added growth factor; 40X).

Epidermal layer width was measured from beneath the keratin layer to the beginning of the dermal layer to assess epidermal remodeling at the site of the incision in locations between sutures at Day 35 post operatively.



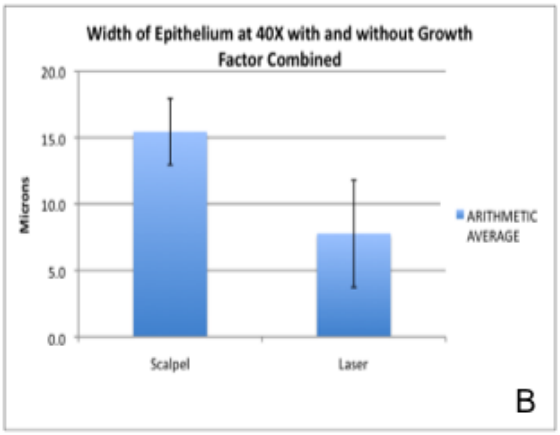


Figure 8(A). Without added growth factor, the incision repair trended towards a greater width of the epidermal layer (n=7) with the scalpel versus the laser incision groups (n=8) (p<0.084). With the addition of growth factor, the scalpel incised group also had a significantly greater width of the epidermal layer when compared to the laser incised group (p = 0.005).

Figure 8(B). When the with and without growth factor groups were combined, the width of the epidermal tissue for the scalpel (n=15), was significantly greater than the width of the epidermal tissue for the laser (n=16) (p<0.001).

When the width of the epidermal tissue in the repair of skin incised with laser or scalpel without added growth factor (n=8; n=7, respectively) was compared, the epidermal tissue layer in the scalpel group trended towards a greater width than that in the laser group (p<0.084). However, a similar outcome was demonstrated with the addition of growth factor to these two groups, which resulted in a significantly wider band of epidermal tissue in the scalpel group (p=0.005) versus the laser group (Figure 8A). Combining groups with and without growth factor (n=16), and comparing the width of the epidermal layer from the laser to that of the scalpel (n=15) still demonstrated a significantly wider band of epidermal tissue for the scalpel groups (p<0.001), (Figure 8B).

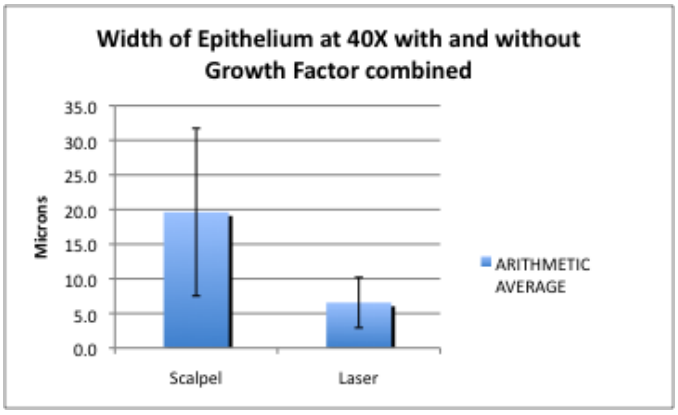


Figure 9: The use of the CO_2 laser with and without GF combined demonstrated a statistically significant difference (p=0.034) when compared to the scalpel for the width of the epithelium at post-operative Day 35, with the scalpel group having a wider epithelium.

Based on each animal as the sampling unit and comparing the response among all 4 groups, independent of all other factors, a statistically significant difference (p=0.034)

was detected for the width of the epithelium when measured at 40X for the scalpel versus the CO_2 laser (Reference Table 4.1).

		LASER			SCALPEL		
GROWTH FACTOR	**PARAMETER**	ARITHMETIC AVERAGE	STANDARD DEVIATION	MEDIAN	ARITHMETIC AVERAGE	STANDARD DEVIATION	MEDIAN
NO GF	WIDTH OF GRANULATION TISSUE- 40X	15.794	7.156	16.273	15.504	8.420	14.000
NO GF	WIDTH OF EPITHELIUM - 40X	8.053	7.084	6.683	17.843	18.156	14.276
YES GF	WIDTH OF GRANULATION TISSUE- 40X	23.072	19.387	20.191	10.857	8.506	7.649
YES GF	WIDTH OF EPITHELIUM - 40X	5.074	5.532	3.814	20.958	23.992	12.632

Table 1: The univariate analysis by surgical method based on the intra-animal arithmetic average demonstrated that both with and without the GF, differences between the laser and scalpel were observed in the width of epithelium measurements with the mean values being generally wider in the animals where the scalpel was used. The granulation tissue production also was increased in the animals where the laser and GF was used, compared to the scalpel.

	NO GROWTH FACTOR				GROWTH FACTOR			
PARAMETER	N	ARITHMETIC AVERAGE	STANDARD DEVIATION	MEDIAN	N	ARITHMETIC AVERAGE	STANDARD DEVIATION	MEDIAN
WIDTH OF GRANULATION TISSUE- 40X	13	10.774	11.411	9.756	15	6.179	8.794	0.000
WIDTH OF EPITHELIUM - 40X	13	6.124	15.208	0.000	15	6.315	16.706	0.000

Table 2: The univariate analysis with and without GF combined for granulation tissue production and width of epithelium, independent of the surgical method used, based on the median intra-animal measurements, demonstrated that the groups without the addition of GF had greater granulation tissue production than animals receiving GF.

		GROUP			
PARAMETER	**SOURCE**	A	B	C	D
WIDTH OF GRANULATION TISSUE- 40X	A	_	0.2655	0.9656	0.432
WIDTH OF GRANULATION TISSUE- 40X	B	0.2655	_	0.266	0.060
WIDTH OF GRANULATION TISSUE- 40X	C	0.9656	0.266	_	0.478

WIDTH OF GRANULATION TISSUE- 40X	D	0.4323	0.0597	0.4782	_
WIDTH OF EPITHELIUM - 40X	A	_	0.731	0.283	0.133
WIDTH OF EPITHELIUM - 40X	B	0.731	_	0.1649	0.067
WIDTH OF EPITHELIUM - 40X	C	0.283	0.1649	_	0.722
WIDTH OF EPITHELIUM - 40X	D	0.1328	0.0674	0.722	_

Table 3: Probability matrix comparing the intra-animal arithmetic average measurements among the groups by parameter demonstrates that a statistically significant difference was seen when comparing groups B and D for both the width of the granulation tissue production and the width of the epithelium, ($p<0.06$ and $p<0.07$ respectively). Therefore, more granulation tissue production occurred with the use of the CO_2 laser and GF versus the scalpel and GF.

3.5 Incorporation of SIS into native tissue

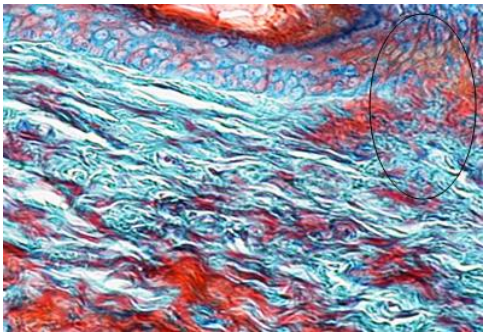


Figure 10. Light micrograph illustrating the histologic appearance of an area of integration of SIS suture into the adjacent native skin (oval) in the region of the remodeling incision (laser, no added growth factor; 20X).

Each specimen was randomly surveyed for evidence of the infiltration of specific constituents of skin tissue into adjacent SIS suture and scored ‘1’ if present or ‘0’ if no evidence was demonstrated.

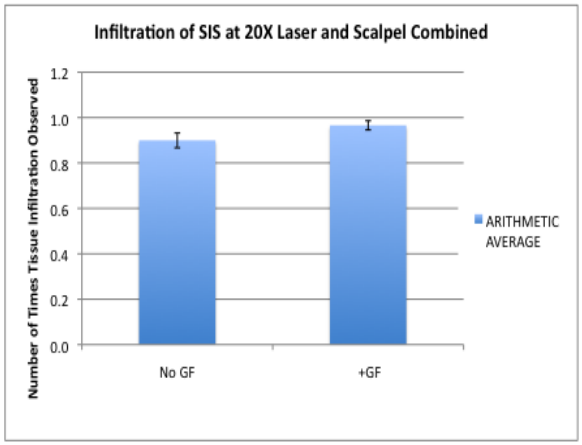


Figure 11. When the laser and scalpel were combined with added growth factor (n=15), a significant difference in the amount of SIS remodeling was demonstrated ($p<0.001$) versus the groups without added growth factor (n=16).

When the evidence of skin remodeling in association with the SIS sutures was observed in a random survey field a scoring of '1' was assigned, and in the absence of such remodeling the field was assigned a score of '0'. A significantly greater incorporation of skin elements into the SIS was demonstrated in the presence of added growth factor ($p<0.001$) (Figure10).

3.6 Collagen Organization

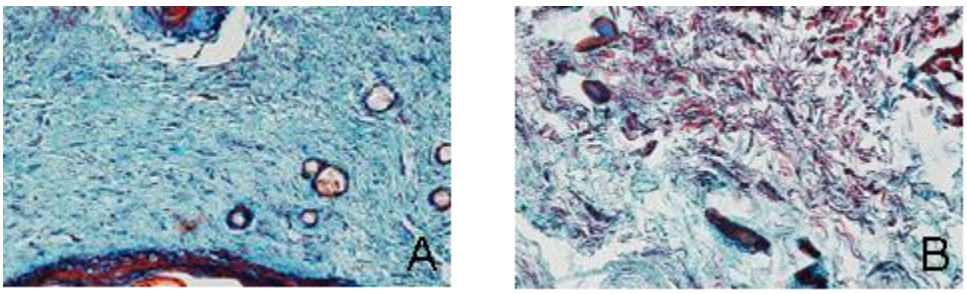


Figure 12. Light micrograph illustrating the appearance of organized collagen (A) and disorganized collagen (B) in the region of the incision at Day 35 post operatively (laser, no added growth factor; 20X).

A well-developed collagen matrix is essential for normal skin remodeling and since it is the largest component of the supporting extracellular matrix, each specimen was randomly surveyed to determine the characteristic of the collagen fibers at the site of the incision through the skin. At day 35, Figure 12A is illustrative of the appearance of well organized collagen at the site of the incision (20X, laser incision with no added growth factor) and Figure 12B illustrates the appearance of disorganized collagen at the site of the skin incision (20X, scalpel incision with no added growth factor).

PARAMETER	RANK	MEAN	GROUP	GROUP DESCRIPTION
COLLAGEN - 20X - Y/N	1	21.5	A	Laser
COLLAGEN - 20X - Y/N	1	21.5	B	Laser and GF
COLLAGEN - 20X - Y/N	2	7.5	C	Scalpel
COLLAGEN - 20X - Y/N	2	7.5	D	Scalpel and GF
MAST CELLS - 40X	1	17.5	A	Laser
MAST CELLS - 40X	1	15.25	C	Scalpel
MAST CELLS - 40X	1	14.75	D	Scalpel and GF
MAST CELLS - 40X	1	10.57	B	Laser and GF
NEUTROPHILS - 40X	1	18.43	A	Laser
NEUTROPHILS - 40X	1	14.83	C	Scalpel
NEUTROPHILS - 40X	1	12.71	B	Laser and GF
NEUTROPHILS - 40X	1	12.38	D	Scalpel and GF
SIS - 20X	1	15	C	Scalpel
SIS - 20X	1	15	B	Laser and GF
SIS - 20X	1	15	D	Scalpel and GF
SIS - 20X	1	13	A	Laser
VASCULAR - 20X - Y/N	1	17.5	A	Laser
VASCULAR - 20X - Y/N	1	13.5	B	Laser and GF
VASCULAR - 20X - Y/N	1	13.5	C	Scalpel
VASCULAR - 20X - Y/N	1	13.5	D	Scalpel and GF

Table 4: The use of the CO_2 laser resulted in significantly more organized collagen ($p<0.0001$) when compared with the use of the scalpel. (Reference Table 3.3)

The Student-Newman-Keuls test based on the one-factor (group) analysis of variance tests for the ranked intra-animal arithmetic average values by parameter demonstrated that the level of organization of the collagen in animals in the laser group was significantly greater than for animals in the scalpel group. Consistently, animals in the laser group without growth factor had the most organized collagen, number of mast cell granules, number of neutrophils, and number of blood vessels. Animals in the laser group without growth factor also had the lowest reported infiltration of native tissue into the SIS.

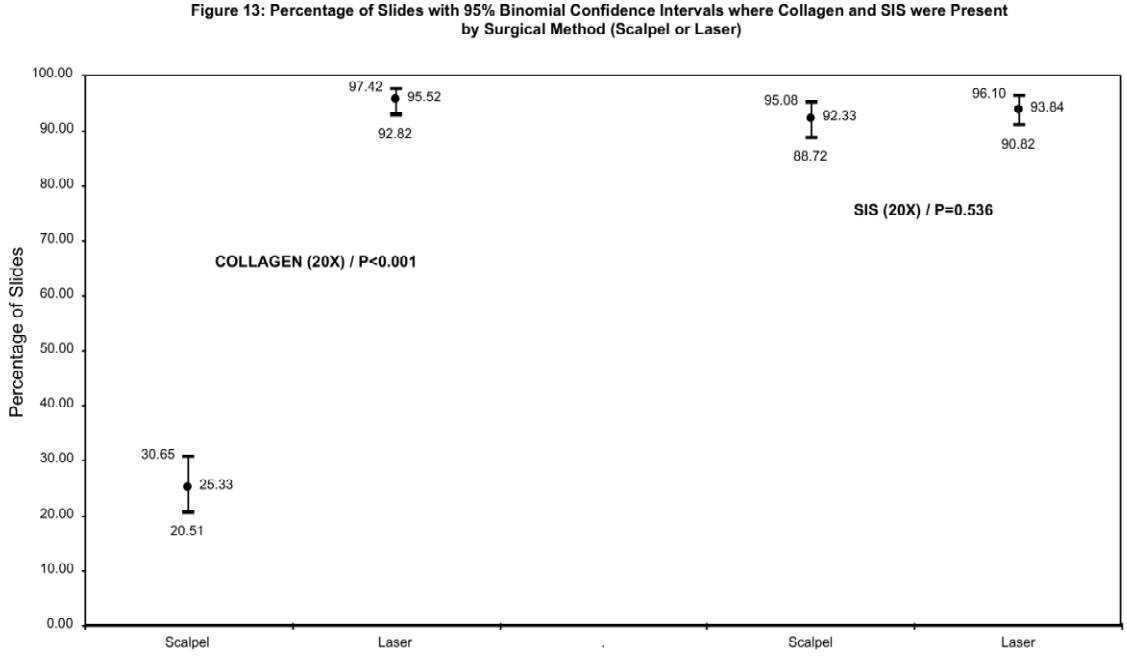


Figure 13: Represents the mean and associated confidence intervals within the percentage of slides seen when evaluating Collagen at 20X. As demonstrated, the CO_2 laser results with a statistically significant organization of collagen ($p<0.001$) when compared to the scalpel. However, the native tissue infiltration into the SIS showed no difference between the surgical methods.

3.7 Vascularization

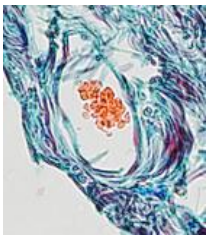


Figure 14. The vascularization in proximity to the incision sites was quantitated in random fields by counting the actual number of blood vessels present in the remodeling cutaneous layers (scalpel, no added growth factor; 20X).

Angiogenesis is an important component of successful wound healing since blood vessels are essential in carrying oxygen and nutrients necessary to sustain cell metabolism. The number of blood vessels in each randomly selected field at the site of the skin incision was quantitated and comparisons made between each experimental group (Figure 14).

		GROUP			
PARAMETER	**SOURCE**	**A**	**B**	**C**	**D**
VASCULAR (20X)	A	_	0.0384	0.046	0.033
VASCULAR (20X)	B	0.0384	_	1	1.000
VASCULAR (20X)	C	0.046	1	_	1.000
VASCULAR (20X)	D	0.033	1	1	_

Table 5: Probability matrix comparing the arithmetic average intra-animal among the groups by parameter from the one-factor (group) analysis of variance tests based on the ranked intra-animal arithmetic average values by parameter

In individual pair-wise comparisons, Group A differed from Groups B, C, and D in the amount of blood vessels observed at 20X, ($p<0.04$, $p<0.05$, p,0.03, respectively).

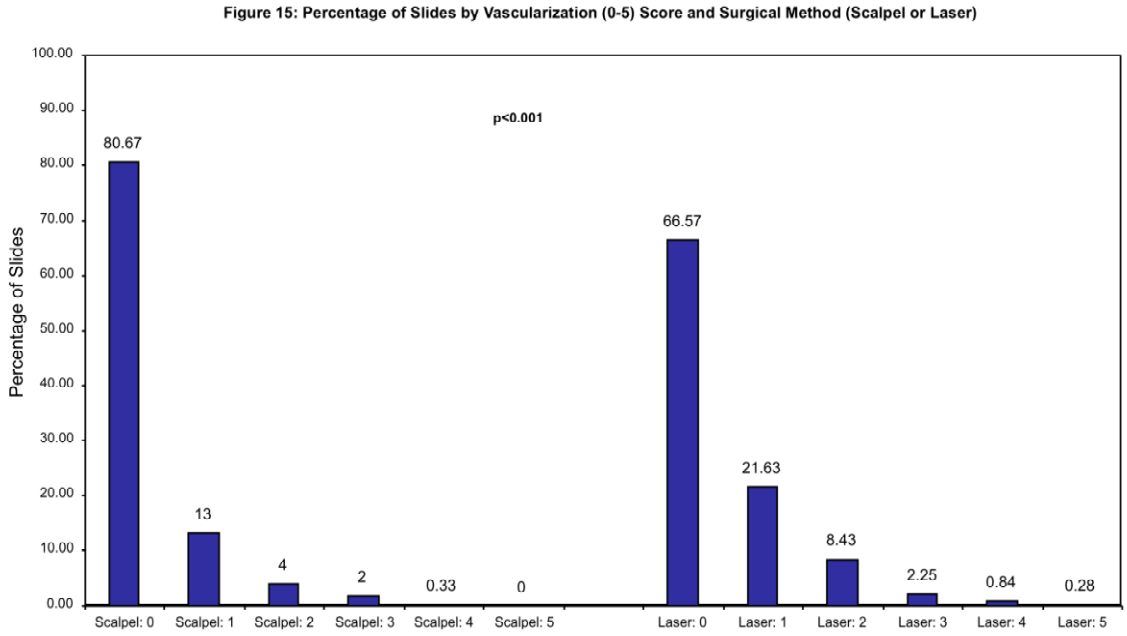


Figure 15: Represents the amount of vascularization seen at 20X in randomly surveyed images which were analyzed. The histogram demonstrates that the laser resulted in significantly more vascularization compared to the scalpel.

3.8 All Results

TABLE 1: UNIVARIATE ANALYSIS BY SURGICAL METHOD, USE OF GROWTH FACTORS, AND PARAMETER

SURGICAL PROCEDURE	GROWTH FACTOR	PARAMETER	N	ARITHMETIC AVERAGE	STANDARD DEVIATION
LASER	NO	GRANULATION WIDTH - 40X	179	18.533	20.310
LASER	NO	MAST CELLS - 40X	175	1.051	1.046
LASER	NO	NEUTROPHILS - 40X	175	1.291	0.983
LASER	NO	SIS - 20X	180	0.911	0.285
LASER	NO	WIDTH OF EPITHELIUM - 40X	179	9.005	25.641
LASER	YES	GRANULATION WIDTH - 40X	175	21.339	46.204
LASER	YES	MAST CELLS - 40X	175	0.771	0.912
LASER	YES	NEUTROPHILS - 40X	175	1.040	1.116
LASER	YES	SIS - 20X	177	0.966	0.181
LASER	YES	WIDTH OF EPITHELIUM - 40X	175	6.513	21.938
SCALPEL	NO	GRANULATION WIDTH - 40X	157	12.132	16.713
SCALPEL	NO	MAST CELLS - 40X	157	0.777	0.829
SCALPEL	NO	NEUTROPHILS - 40X	157	0.879	1.082

SCALPEL	NO	SIS - 20X	158	0.886	0.319
SCALPEL	NO	WIDTH OF EPITHELIUM - 40X	157	13.714	23.834
SCALPEL	YES	GRANULATION WIDTH - 40X	132	9.425	15.817
SCALPEL	YES	MAST CELLS - 40X	132	0.841	0.836
SCALPEL	YES	NEUTROPHILS - 40X	132	0.795	1.103
SCALPEL	YES	SIS - 20X	142	0.965	0.185
SCALPEL	YES	WIDTH OF EPITHELIUM - 40X	132	17.500	44.534

TABLE 1.1: UNIVARIATE ANALYSIS BY SURGICAL METHOD AND PARAMETER

SURGICAL PROCEDURE	PARAMETER	N	ARITHMETIC AVERAGE	STANDARD DEVIATION
LASER	GRANULATION WIDTH - 40X	354	19.920	35.528
LASER	MAST CELLS - 40X	350	0.911	0.990
LASER	NEUTROPHILS - 40X	350	1.166	1.058
LASER	SIS - 20X	357	0.938	0.241
LASER	WIDTH OF EPITHELIUM - 40X	354	7.773	23.881
SCALPEL	GRANULATION WIDTH - 40X	289	10.896	16.338
SCALPEL	MAST CELLS - 40X	289	0.806	0.832
SCALPEL	NEUTROPHILS - 40X	289	0.841	1.091
SCALPEL	SIS - 20X	300	0.923	0.267
SCALPEL	WIDTH OF EPITHELIUM - 40X	289	15.443	34.834

TABLE 1.2: UNIVARIATE ANALYSIS BY USE OF GROWTH FACTORS, AND PARAMETER

GROWTH FACTOR	PARAMETER	N	ARITHMETIC AVERAGE	STANDARD DEVIATION
No GF added	GRANULATION WIDTH - 40X	336	15.542	18.960
No GF added	MAST CELLS - 40X	332	0.922	0.958
No GF added	NEUTROPHILS - 40X	332	1.096	1.050
No GF added	SIS - 20X	338	0.899	0.301
No GF added	WIDTH OF EPITHELIUM - 40X	336	11.205	24.888
GF added	GRANULATION WIDTH - 40X	307	16.217	36.823
GF added	MAST CELLS - 40X	307	0.801	0.880
GF added	NEUTROPHILS - 40X	307	0.935	1.115
GF added	SIS - 20X	319	0.966	0.183
GF added	WIDTH OF EPITHELIUM - 40X	307	11.237	33.947

TABLE 2: TWO-FACTOR ANALYSIS OF VARIANCE INCLUDING THE INTERACTION TERM BY PARAMETER

SOURCE	PARAMETER	PROBABILITY VALUE
GF	GRANULATION WIDTH - 40X	0.983
SCALPEL	GRANULATION WIDTH - 40X	0.000
SCALPEL*GF	GRANULATION WIDTH - 40X	0.225
GF	MAST CELLS - 40X	0.140
SCALPEL	MAST CELLS - 40X	0.161
SCALPEL*GF	MAST CELLS - 40X	0.019
GF	NEUTROPHILS - 40X	0.050
SCALPEL	NEUTROPHILS - 40X	0.000

SCALPEL*GF	NEUTROPHILS - 40X	0.325
GF	SIS - 20X	0.001
SCALPEL	SIS - 20X	0.503
SCALPEL*GF	SIS - 20X	0.547
GF	WIDTH OF EPITHELIUM - 40X	0.781
SCALPEL	WIDTH OF EPITHELIUM - 40X	0.001
SCALPEL*GF	WIDTH OF EPITHELIUM - 40X	0.178

TABLE 3: ONE-FACTOR ANALYSIS OF VARIANCE COMPARING THE LASER TO THE SCALPEL BY PARAMETER

PARAMETER	PROBABILITY VALUE
GRANULATION WIDTH - 40X	0.000
MAST CELLS - 40X	0.152
NEUTROPHILS - 40X	0.000
SIS - 20X	0.448
WIDTH OF EPITHELIUM - 40X	0.001

TABLE 4: ONE-FACTOR ANALYSIS OF VARIANCE RESULTS WITH THE GROWTH FACTOR, COMPARING THE LASER TO THE SCALPEL BY PARAMETER

PARAMETER	PROBABILITY VALUE
GRANULATION WIDTH - 40X	0.005
MAST CELLS - 40X	0.494
NEUTROPHILS - 40X	0.057
SIS - 20X	0.949
WIDTH OF EPITHELIUM - 40X	0.005

TABLE 5: ONE-FACTOR ANALYSIS OF VARIANCE RESULTS WITHOUT THE GROWTH FACTOR, COMPARING THE LASER TO THE SCALPEL BY PARAMETER

PARAMETER	PROBABILITY VALUE
GRANULATION WIDTH - 40X	0.002
MAST CELLS - 40X	0.009
NEUTROPHILS - 40X	0.000
SIS - 20X	0.447
WIDTH OF EPITHELIUM - 40X	0.084

TABLE 6.1: UNIVARIATE ANALYSIS BY SURGICAL METHOD, USE OF GROWTH FACTORS, AND PARAMETER BASED ON THE ARITHMETIC AVERAGE INTRA-ANIMAL MEASUREMENTS

SURGICAL PROCEDURE	GROWTH FACTOR	PARAMETER	N	ARITHMETIC AVERAGE	STANDARD DEVIATION
LASER	NO	GRANULATION WIDTH - 40X	7	15.794	7.156
LASER	NO	WIDTH OF EPITHELIUM - 40X	7	8.053	7.084
LASER	YES	GRANULATION WIDTH - 40X	7	23.072	19.387
LASER	YES	WIDTH OF EPITHELIUM - 40X	7	5.074	5.532
SCALPEL	NO	GRANULATION WIDTH - 40X	6	15.504	8.420

SCALPEL	NO	WIDTH OF EPITHELIUM - 40X	6	17.843	18.156
SCALPEL	YES	GRANULATION WIDTH - 40X	8	10.857	8.506
SCALPEL	YES	WIDTH OF EPITHELIUM - 40X	8	20.958	23.992

TABLE 6.2: UNIVARIATE ANALYSIS BY SURGICAL METHOD AND PARAMETER BASED ON THE ARITHMETIC AVERAGE INTRA-ANIMAL MEASUREMENTS (INDEPENDENT OF THE USE OF GROWTH FACTORS)

SURGICAL PROCEDURE	PARAMETER	N	ARITHMETIC AVERAGE	STANDARD DEVIATION
LASER	GRANULATION WIDTH - 40X	14	19.433	14.538
LASER	WIDTH OF EPITHELIUM - 40X	14	6.563	6.299
SCALPEL	GRANULATION WIDTH - 40X	14	12.849	8.480
SCALPEL	WIDTH OF EPITHELIUM - 40X	14	19.623	20.959

TABLE 6.3: UNIVARIATE ANALYSIS BY USE OF GROWTH FACTORS AND PARAMETER, INDEPENDENT OF THE SURGICAL METHOD, BASED ON THE ARITHMETIC AVERAGE INTRA-ANIMAL MEASUREMENTS

GROWTH FACTOR	PARAMETER	N	ARITHMETIC AVERAGE	STANDARD DEVIATION
NO	GRANULATION WIDTH - 40X	13	15.660	7.427
NO	WIDTH OF EPITHELIUM - 40X	13	12.571	13.720
YES	GRANULATION WIDTH - 40X	15	16.557	15.396
YES	WIDTH OF EPITHELIUM - 40X	15	13.545	19.189

TABLE 6.4: UNIVARIATE ANALYSIS BY SURGICAL METHOD, USE OF GROWTH FACTORS, AND PARAMETER BASED ON THE MEDIAN INTRA-ANIMAL MEASUREMENTS

SURGICAL PROCEDURE	GROWTH FACTOR	PARAMETER	N	STANDARD DEVIATION	MEDIAN
LASER	NO	GRANULATION WIDTH - 40X	7	10.061	14.319
LASER	NO	WIDTH OF EPITHELIUM - 40X	7	0.000	0.000
LASER	YES	GRANULATION WIDTH - 40X	7	8.289	5.556
LASER	YES	WIDTH OF EPITHELIUM - 40X	7	0.000	0.000
SCALPEL	NO	GRANULATION WIDTH - 40X	6	13.468	0.000
SCALPEL	NO	WIDTH OF EPITHELIUM - 40X	6	21.007	0.000
SCALPEL	YES	GRANULATION WIDTH - 40X	8	9.780	0.000
SCALPEL	YES	WIDTH OF EPITHELIUM - 40X	8	21.986	0.000

TABLE 6.5: UNIVARIATE ANALYSIS BY SURGICAL METHOD AND PARAMETER BASED ON THE MEDIAN INTRA-ANIMAL MEASUREMENTS (INDEPENDENT OF THE USE OF GROWTH FACTORS)

SURGICAL PROCEDURE	PARAMETER	N	STANDARD DEVIATION	MEDIAN
LASER	GRANULATION WIDTH - 40X	14	9.404	8.244
LASER	WIDTH OF EPITHELIUM - 40X	14	0.000	0.000

SCALPEL	GRANULATION WIDTH - 40X	14	11.103	0.000
SCALPEL	WIDTH OF EPITHELIUM - 40X	14	20.750	0.000

TABLE 6.6: UNIVARIATE ANALYSIS BY USE OF GROWTH FACTORS AND PARAMETER, INDEPENDENT OF THE SURGICAL METHOD, BASED ON THE MEDIAN INTRA-ANIMAL MEASUREMENTS

GROWTH FACTOR	PARAMETER	N	STANDARD DEVIATION	MEDIAN
NO	GRANULATION WIDTH - 40X	13	11.411	9.756
NO	WIDTH OF EPITHELIUM - 40X	13	15.208	0.000
YES	GRANULATION WIDTH - 40X	15	8.794	0.000
YES	WIDTH OF EPITHELIUM - 40X	15	16.706	0.000

TABLE 7.1: ONE-FACTOR (GROUP) ANALYSIS OF VARIANCE TESTS BASED ON THE INTRA-ANIMAL ARITHMETIC AVERAGE VALUES BY PARAMETER

PARAMETER	PROBABILITY VALUE
GRANULATION WIDTH - 40X	0.292
WIDTH OF EPITHELIUM - 40X	0.208

TABLE 7.2: PROBABILITY MATRIX COMPARING THE ARITHMETIC AVERAGE INTRA-PATIENT MEASUREMENTS AMONG THE GROUPS BY PARAMETER

		GROUP			
PARAMETER	SOURCE	A	B	C	D
GRANULATION WIDTH - 40X	A	_	0.2655	0.9656	0.432
GRANULATION WIDTH - 40X	B	0.2655	_	0.266	0.060
GRANULATION WIDTH - 40X	C	0.9656	0.266	_	0.478
GRANULATION WIDTH - 40X	D	0.4323	0.0597	0.4782	_
WIDTH OF EPITHELIUM - 40X	A	_	0.731	0.283	0.133
WIDTH OF EPITHELIUM - 40X	B	0.731	_	0.1649	0.067
WIDTH OF EPITHELIUM - 40X	C	0.283	0.1649	_	0.722
WIDTH OF EPITHELIUM - 40X	D	0.1328	0.0674	0.722	_

TABLE 7.3: STUDENT-NEWMAN-KEULS TEST FROM THE 1-FACTOR ANALYSIS OF VARIANCE TEST BY PARAMETER

PARAMETER	RANK	MEAN	N	GROUP
GRANULATION WIDTH - 40X	A	23.07	7	B
GRANULATION WIDTH - 40X	A	15.79	7	A
GRANULATION WIDTH - 40X	A	15.5	6	C
GRANULATION WIDTH - 40X	A	10.86	8	D
WIDTH OF EPITHELIUM - 40X	A	20.96	8	D
WIDTH OF EPITHELIUM - 40X	A	17.84	6	C
WIDTH OF EPITHELIUM - 40X	A	8.05	7	A
WIDTH OF EPITHELIUM - 40X	A	5.07	7	B

TABLE 8.1: ONE-FACTOR (GROUP) ANALYSIS OF VARIANCE TESTS BASED ON THE RANKED INTRA-ANIMAL ARITHMETIC AVERAGE VALUES BY PARAMETER

PARAMETER	PROBABILITY VALUE
COLLAGEN - 20X	0.000
MAST CELLS – 40X	0.337
NEUTROPHILS – 40X	0.437
SIS - 20X	0.410
VASCULAR - 20X	0.093

TABLE 8.2: PROBABILITY MATRIX COMPARING THE ARITHMETIC AVERAGE INTRA-PATIENT MEASUREMENTS AMONG THE GROUPS BY PARAMETER FROM THE ONE-FACTOR (GROUP) ANALYSIS OF VARIANCE TESTS BASED ON THE RANKED INTRA-ANIMAL ARITHMETIC AVERAGE VALUES BY PARAMETER

		GROUP			
PARAMETER	SOURCE	A	B	C	D
COLLAGEN - 20X	A	_		<.0001	<.0001
COLLAGEN - 20X	B		_	<.0001	<.0001
COLLAGEN - 20X	C	<.0001	<.0001	_	
COLLAGEN - 20X	D	<.0001	<.0001		_
MAST CELLS - 40X	A	_	0.0773	0.57	0.457
MAST CELLS - 40X	B	0.0773	_	0.2428	0.262
MAST CELLS - 40X	C	0.57	0.2428	_	0.896
MAST CELLS - 40X	D	0.4566	0.2616	0.8962	_
NEUTROPHILS – 40X	A	_	0.1776	0.4094	0.142
NEUTROPHILS – 40X	B	0.1776	_	0.6252	0.933
NEUTROPHILS – 40X	C	0.4094	0.6252	_	0.560
NEUTROPHILS – 40X	D	0.1417	0.9328	0.5598	_
SIS - 20X	A	_	0.1701	0.1869	0.157
SIS - 20X	B	0.1701	_	1	1.000
SIS - 20X	C	0.1869	1	_	1.000
SIS - 20X	D	0.1571	1	1	_
VASCULAR - 20X	A	_	0.0384	0.046	0.033
VASCULAR - 20X	B	0.0384	_	1	1.000
VASCULAR - 20X	C	0.046	1	_	1.000
VASCULAR - 20X	D	0.033	1	1	_

TABLE 8.3: STUDENT-NEWMAN-KEULS TEST FROM THE ONE-FACTOR (GROUP) ANALYSIS OF VARIANCE TESTS BASED ON THE RANKED INTRA-ANIMAL ARITHMETIC AVERAGE VALUES BY PARAMETER

PARAMETER	RANK	MEAN	N	GROUP
COLLAGEN - 20X	A	21.5	7	A
COLLAGEN - 20X	A	21.5	7	B
COLLAGEN - 20X	B	7.5	6	C
COLLAGEN – 20X	B	7.5	8	D
MAST CELLS - 40X	A	17.5	7	A
MAST CELLS - 40X	A	15.25	6	C
MAST CELLS - 40X	A	14.75	8	D
MAST CELLS - 40X	A	10.57	7	B

NEUTROPHILS - 40X	A	18.43	7	A
NEUTROPHILS - 40X	A	14.83	6	C
NEUTROPHILS - 40X	A	12.71	7	B
NEUTROPHILS - 40X	A	12.38	8	D
SIS - 20X	A	15	6	C
SIS - 20X	A	15	7	B
SIS - 20X	A	15	8	D
SIS - 20X	A	13	7	A
VASCULAR - 20X	A	17.5	7	A
VASCULAR - 20X	A	13.5	7	B
VASCULAR - 20X	A	13.5	6	C
VASCULAR - 20X	A	13.5	8	D

TABLE 9.1: ONE-FACTOR (SCALPEL VS. LASER) ANALYSIS OF VARIANCE TESTS BASED ON THE INTRA-ANIMAL ARITHMETIC AVERAGE VALUES BY PARAMETER

PARAMETER	**PROBABILITY VALUE**
GRANULATION WIDTH - 40X	0.155
WIDTH OF EPITHELIUM - 40X	0.034

Chapter 4.

DISCUSSION

The goal of this study was to develop improved techniques for cutaneous surgery that can be applied to various situations. The objectives of this study focus on four different aspects of this problem: The consequences of using a scalpel or carbon dioxide laser for cutaneous incisions, whether the addition of nerve growth factor improves incisional wound healing regardless of which surgical modality is used, and whether a difference can be observed in the healing process when SIS sutures are substituted for commercial vicryl sutures.

4.1 Granulation Tissue

It has been determined that a surgical wound can never attain the same cutaneous tensile strength as uncut skin. However, 2 weeks after suturing, 3-5% of original strength will be achieved. By the end of the 3rd week, 20% of the strength is achieved. Then by one month, only 50% of the wound strength is attained. (47) Peak inflammatory responses in the host dermis are seen between the 2nd and 7th day with the abundance of PMN leukocytes, lymphocytes, and large monocytes (48). Between the 3rd-8th days, the epithelial cells deeply invade the suture tracts. (47)

Sutures can provoke foreign body reactions in cutaneous tissue, and when this occurs it is anticipated that they will provoke a local, immunologically mediated response that is reflected in the development of granulation tissue; a part of the normal wound

healing process. However a severe and prolonged inflammatory response with a proliferation of granulation tissue can interfere with the normal wound healing process allowing the wound to become more susceptible to infection. (49,50) The width of the granulation tissue surrounding the suture holes was measured and employed as a method for evaluating the balance between the extent of injury induced and the vigorousness of the reparative process. The measurements were performed on images of histological sections obtained from random fields of tissue surrounding the suture holes in each of the experimental groups; laser alone 179 random fields (n=8), laser with growth factor 175 random fields (n=7), scalpel alone 157 random fields (n=8), scalpel with growth factor 132 random fields (n=8). Analysis of these comparisons indicated that the laser incisions when repaired with vicryl sutures, both with and without added growth factor produced a significantly greater amount of granulation tissue. However, it was not possible to compare the effects of vicryl sutures to SIS sutures, as only one SIS suture was placed across each incision, except in group A; the remaining closure of each incision was completed using vicryl suture material. This was done as a precaution because there is a lack of information concerning the strength of SIS sutures when used to close to cutaneous incisions.

Absorbable 3-0 vicryl sutures were selected to be used in this research experiment because they are known to maintain strength over the incisional site for at least 14-21 days and absorbed by 90 days or more (47). The absorbable sutures were also selected to close the cutaneous layer so as to not introduce another type of suture material into the incisional area since absorbable was necessary for closer of the muscular incision.

Ending the research at 35 days, allowed suture material to remain present and avoided the necessity of removing non-absorbable sutures.

Absorbable sutures do increase the amount of enzymatic reactions occurring at the incisional site, which may or may not increase the granulation tissue production. Plain gut and SIS, can be used in superficial healing, but they only allow 3-4 days of maximal tension, which would be inadequate for healing time followed in this protocol. Even the addition of chromic salts added to the plain gut, would only increase the integrity of the sutures for approximately 10-14 days. (47) It is recommended in future studies to use the SIS suture material in between vicryl sutures in order to determine a difference in healing strength and cosmetic outcomes of the incisional area. Alternatively, vicryl sutures could be embedded in SIS sutures and used to close cutaneous incisions in order to determine if there is a decrease in healing time, better cosmetic outcome, or any other added benefits. Further investigations are necessary in order to evaluate the tensile strength of SIS sutures for repair of cutaneous incisions. This was not a focus of this study.

These results suggest that the greater proliferation of granulation tissue produced with use of the laser may have resulted from an inappropriate dose of laser energy as a result of the photothermal effects produced by the use of continuous mode and repeated passes that were employed in this study which have been avoided by using a lower dose to achieve the cutaneous incisions. Future studies should be aimed at titrating the dose to achieve cutaneous incisions separately from the dose to achieve gastrocnemius muscle dissection. Papadavid et al discovered there are several effective techniques for

employing lasers to create cutaneous incisions but they all have their drawbacks due to a lack of precise depth control and unwanted damage to the lower layers of the dermis (51). It was also reported that by delivering rapidly overlapping pulses and scans, residual thermal damage and cell death depth were increased as much as 100% over areas without immediate overlap of laser impacts (52). In this study, the laser cutaneous incisions were created using a "four-pass" technique.

Although practice was performed on cadaver chicken prior to this study, the chicken skin is not an exact replica of the cutaneous properties of the rat and therefore injury may have occurred secondary to an inappropriate carbon dioxide laser dose (fluency).(53,54) This was a proof of concept study with a limited number of animals; it was impractical to do titration studies in order to optimize the laser dose for each of the tissues incised and the selection of dose was based upon best estimates from accounts in the literature.

The results also suggest that the addition of growth factor may have promoted an increased development of granulation tissue post-operatively in association with laser irradiation. It has been reported by Ross et al, that an increase in granulation tissue will result secondary to not allowing enough time for tissues to cool prior to passing over them again because of the additive effects (52). Although laser irradiation at surgical intensities tends to depress fibroblastic activity, nerve growth factor has been shown to enhance the response of pro-inflammatory cells (55).

A temperature controlled fiberoptic laser-soldering system was used in research conducted by Simhon et al., whereby they were able to determine the temperature of the

tissue as they were using the laser (56). This aided them in reducing the amount of excess damage inflicted on the tissue as a result of to great of a temperature being produced during their experiment. A similar approach could be adapted to the protocol implemented in our study.

4.2 Mast Cells

Mast cells play a key role in the initiation of the inflammatory response. Their cytoplasm contains granules filled with various mediators of inflammation such as histamine, heparin, and bradykinin.(57) The release of the content of these granules is associated with various components of the inflammatory response. In this study, the number of granules contained in the mast cells found in close proximity to the incisions were quantitated as an indicator of the intensity of the inflammatory response with the assumption that a lesser number of granules indicated a greater release of pro-inflammatory substances. A rating was applied to reflect the relative number of granules observed within each mast cell; minimal, moderate, large number of granules were scored as +1, +2, +3, respectively. The use of the laser to incise the cutaneous layer resulted in a significantly greater number of granules within the mast cells than seen in mast cells from similar tissue incised with the scalpel. The effect of added growth factor was seen to decrease the number of granules observed within the mast cells associated with laser incisions to a point where this decrease in granule number significantly exceeded the decreased number observed in association with scalpel incisions. This disproportionate effect on the number of granules in the laser incised skin may be related to the

inappropriately large laser dose employed acting to enhance the pro-inflammatory properties of nerve growth factor (58). It is well established that certain nociceptor responses in skin such as vasodilation are reduced when NGF activity is compromised. In contrast when in the presence of an inflammatory response, increased amounts of NGF may produce a state of hyperalgesia in part by mediating the release of histamine from mast cells.(59)

4.3 Neutrophils

The number of neutrophils present in each random histologic slide was used as another gauge of the responsiveness of the cutaneous reparative processes to the suture hole wounds induced in each of the four experimental groups during the inflammatory phase of wound healing. This also was a marker to determine how far the healing process had progressed by Day 35 post operatively, since neutrophils are the first white blood cell to engage in the wound repair process and the first to disappear.

Neutrophils were quantified using a scale of +1, +2, and +3 depending on the number present within the granulation tissue. Therefore it was of no surprise to see a similar trend as was seen in the analysis of granulation tissue. The neutrophil numbers were higher for the laser regardless if the growth factor was or was not used. However, a decrease in number was observed with both the scalpel and laser incisions when growth factor was added, which supports the idea that healing was faster with the addition of growth factor.

The increase of neutrophils seen with the use of the laser may be a result of the tissue being in an earlier phase of healing and therefore still being acted upon by initial healing cascades, or it may be a result of neutrophils being present in different quantities secondary to the natural progression of healing, which is variable for each animal. As stated following research conducted by Min Wu et al., "It is difficult to assess the importance of increased neutrophils, rather, their actions are the more important focus" (60). Therefore, in future research, it is recommended that neutrophils should be studied at various times during the healing process with a focus on the oxidative burst. This can be done using the nitroblue tetrazolium test, which is sensitive to the presence of alkaline phosphatase and can therefore determine the amount of phagocytosis taking place by the neutrophils.(60,61)

4.4 Width of epithelium

Another aspect of this study focused on an evaluation of the width of the epidermis at post-operative Day 35. Measurements extended from immediately under the keratin through the layers of the epidermis, which include the stratum corneum, lucidum, granulosum, spinosum, and basale, stopping at the dermis. When the width of the epidermal tissue in the repair of skin incised with laser or scalpel without added growth factor were compared, the epidermal tissue layer in the scalpel group trended towards a greater width than that in the laser group. However, adding growth factor to these two groups resulted in a significantly wider band of epidermal tissue in the scalpel group. Combining the laser groups with and without growth factor, and comparing the width of

the epidermal layer to that of the combined groups scalpel with no growth factor, and scalpel with added growth factor still demonstrated a significantly wider band of epidermal tissue for the scalpel groups. This supports the idea that the extent and duration of the normal proliferative stages in rats incised with the laser was altered as a result of increased trauma secondary to an inappropriate laser dose to the tissue. With added growth factor, the epidermal width for the animals incised with the laser was even more attenuated in contrast to that of the scalpel incised group which demonstrated a marked increase in the width of the epidermis.

It has also been documented in the literature that the laser does decrease wound strength which may be secondary to the development of a thinner epidermis post-operatively. Despite its 'normal' appearance in that it contains all the expected cutaneous components, repaired skin following laser surgery achieves only 70-80% of its original tensile strength (62). This characteristic of laser surgery also was discussed by Ben-Baruch et al, when looking at the difference between scalpel, pulsed CO_2 and Continuous wave CO_2 incisions (63). They reported that CO_2 laser scars were less mature and contained cellular granulation tissue without visible collagen fibers. Laser wounds were weaker than the scalpel wounds, which were measured with a tensiometer (63).

4.5 Collagen Organization

The appearance of the collagen also was a focus of this study. A well developed collagen matrix is essential for normal skin remodeling and since it is the largest component of the supporting extracellular matrix, each specimen was randomly surveyed

to characterize its appearance at Day 35 post-operatively, and was judged to be organized or disorganized and then rated, “1” or “0” respectively.

Alignment of the tissues during the embedding procedures may have contributed some variability although attempts were made to preserve alignment in the horizontal plane. With respect to improved methods for analyzing the collagen sheets a method has been reported by Noorlander et al. whereby the use of picrosirius red-stained cryostat sections allow for collagen fibers to reflect light strongly when epipolarization microscopy is used. Digital images could then be analyzed quantitatively on the basis of the length of the collagen fibers in the plane of the section as a measure for the orientation of the fibers.(64)

It was frequently difficult to determine the exact site of the incision in well-healed areas. This difficulty might be addressed by the use of tattooing or permanently marking the skin at either end of the longitudinal incision.

4.6 Incorporation of Swine Intestine Submucosa into Native Tissue

No significant differences were found in the incorporation of native skin components into the SIS when comparisons were made between the scalpel and laser incisions alone. However, the addition of growth factor significantly increased the incorporation of the cutaneous component elements into the SIS sutures. Shell has reported that SIS induces host tissue remodeling and has shown a decreased neointimal response to infection (65). Others have demonstrated that glycol-proteins such a fibronectin, a general adhesion molecule that promotes basement membrane assembly and attachment of epidermal cells, fibroblasts, endothelial cells, and laminin, a linker

molecule that joins collagen to proteoglycans and promotes endothelial cell adhesion and growth, have been isolated from SIS (66-70). In addition, glycosaminoglycans (GAGs) also have been shown to be incorporated in the SIS (71). GAGs have functional roles, such as organizing collagen deposition, stimulating angiogenesis and initiating cell differentiation (71). Examples of GAGs found in SIS are heparin, which stimulates angiogenesis, potentiates both epidermal growth factor (EGF) and platelet derived growth factor (PDGF)-induced fibroblast differentiation, hylauronic acid which sequesters transforming growth factor beta (TGF-beta) into the extracellular matrix, chondroitin sulfate that works to increase proteoglycan synthesis, and dermatan which interacts with TGF-beta1 and may control tissue remodeling (72).

SIS has been reported to cause only minimally detectible experimental immune reaction when implanted (73). Firstly, it is avascular, so hyper-acute rejection cannot occur. It is acellular, so it has a paucity of antigens that might cause hypersensitivity. In fact, experimental studies have shown no clinical or histological evidence of immediate or delayed rejection to SIS (73). Rabbits implanted with SIS show no signs of antibody production to their major components (74). Even complement activation, which is a very nonspecific immune response, is absent (73). Some researchers have described a self-limited early acute inflammatory response, which is largely resolved by day 10 consisting of polymorphonucleocyte (PMN) infiltration followed by modest monocyte infiltration (73). Interestingly, there is evidence that unknown factors in SIS actually inhibit local immune response by suppressing Helper T cells through interfering with local interferon-gamma expression (73).

4.7 Vascularization

The CO_2 laser is known to seal blood vessels during surgery, thereby allowing for a cleaner and more visible field. In the short term, a decrease in blood flow has been seen in research conducted by Lofti et al., but in the long term, greater than 7 weeks, reperfusion of once sealed vessels has been seen as well as growth of new blood vessels (75,76).

Angiogenesis is an important component of successful wound healing since blood vessels are essential in carrying oxygen and nutrients necessary to sustain cell metabolism. The number of blood vessels in each randomly selected field at the site of the cutaneous incision was quantified and comparisons made between each experimental group.

Vascularization was seen to increase in the animals incised with the CO_2 laser compared to those incised with the scalpel. This demonstrates that even though blood vessels were sealed during the surgery, aiding in the visibility of the surgical field, they were able to either re-open or undergo angiogenesis at the surgical site.

4.8 Future Studies

The research conducted herein is part of a larger collaborative effort involving two other student colleagues. One study is focused on the effects produced with a scalpel incision versus that of the CO_2 laser in the healing process of the muscle. The other study

is focused on the kinesthetic changes post-operatively resulting from skin and muscle limb injury produced with the scalpel versus the CO_2 laser. All three are examining the added effects of the nerve growth factor on the surgical modalities. This combined effort will hopefully lead to improvements in the treatment of incisions by surgeons, as well as emergency physicians, and various other specialists who wrestle with decisions on how to best manage incisions and their subsequent healing to the greatest benefit of their patients. In order to expand upon this research and make it more clinically relevant it is recommended that future investigations be performed using an animal model larger than the rat with skin characteristics more closely related to humans such as guinea pigs or domestic swine. It is hoped that the evidence provided by this study serves as an incentive and the foundation upon which to continue investigations into techniques that lead to more effective use of these modalities in the clinical setting.

Chapter 5.

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