EFFECT OF LOW LEVEL LASER ON HEALING IN WOUNDED FETAL MOUSE LIMBS

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The purpose of this study was to examine the effects of laser therapy on wound healing processes in vitro. Day 16 Fetal mouse limbs (n=139) were wounded using a standardized full thickness excision, and placed into a serum free organ culture system. All limbs received daily one minute laser treatments for 3 to 7 days at one of the following intensities: 0 (Sham), 0.23, 1.37, 2.75, 3.66, and 4.58 J/cm² from a Gallium Arsenide (Ga-As) 904nm laser. The results showed that laser treatment, at an energy density of 0.23 and 1.37 J/cm², significantly augmented the change in wound size, wound closure rate, and wound appearance in a dose dependent manner. Collagen deposition in the bone and dermis of wounded fetal mouse limbs was greatest in limbs that were treated with certain doses of laser (0.23, 2.75, and 3.66 J/cm²). Administration of higher doses of laser (4.58 J/cm²) was detrimental to both the photographic and histological appearance of the wound. We conclude that laser therapy, when delivered at an appropriate energy density, can directly promote wound healing processes.

Key Words: Laser therapy, Physical Therapy, Wound healing

Introduction

Low level lasers are used clinically in Europe, Asia, and Canada and recent surveys of clinical practice in Northern Ireland⁽¹⁾, Australia⁽²⁾ and Belgium⁽³⁾ would suggest that, at least in some countries, laser is often the modality of choice for therapists when treating chronic wounds.

Previous research performed in both humans and animals suggest that laser can stimulate new tissue formation and promote wound closure. Studies performed in dogs⁽⁴⁾, rabbits^(5,6), rats⁽⁷⁻¹⁰⁾ and mice⁽¹¹⁾ have demonstrated that Ga-As laser increases collagen deposition^(4,11), augments wound tensile strength⁽⁶⁾, increases wound closure rate⁽¹⁰⁾, and improves wound contraction rate⁽¹¹⁾. However, in

these studies, the control group often represented a wound placed in a different location on the same animal. Previous reports have shown that laser treatment can augment wound strength not only in wounds treated with either HeNe or Ga-As laser but also in the untreated control wounds located on the contralateral side of the same animal⁽⁶⁾. Therefore, it is possible that, with this type of experimental design, the lack of effect of laser may be due to chemical mediators that could be released systemically, thus exerting their effects not only on the laser treated wound but also on the control wound located within the same animal.

There are also several animal studies, which fail

to report a benefit of laser on wound healing^(7,9,12). These negative findings tend to occur more commonly in studies where laser treatment regimes would result in the administration of relatively low amounts of laser energy to the wound bed. In addition, it has been suggested that reparative effects of laser may be species specific with negative findings tending to occur more frequently in studies using tight skinned animals⁽¹²⁻¹⁴⁾.

Therefore, although there are numerous experimental studies that suggest laser has a stimulatory effect on the proliferative phase of wound healing, this area of research has been plagued by inconsistent results⁽¹⁵⁾. Variability in results speak to the possibility that the effectiveness of this modality is determined by many factors such as type of laser, wavelength of the light, dosimetry, treatment techniques, and treatment schedules. A clearer understanding of how these factors influence the effects of laser on wound healing is necessary before consistent results can be expected in more complex experimental models such as those used in *in vivo* and clinical studies.

The effects of laser have been examined previously *in vitro* using a monolayer of a single cell type. In these cell culture studies in which several potentially confounding variables can be eliminated and/or controlled, Ga-As laser has been consistently demonstrated to influence several cell processes known to be important in the wound healing cascade. These include increased neutrophil activity⁽¹⁶⁾, collagen and extracellular matrix synthesis⁽¹⁷⁻¹⁹⁾, leucocyte proliferation^(16,22), stimulation of mast cell degranulation⁽²⁰⁾, and promotion of macrophage action⁽²¹⁾. Examination of laser effects *in vitro* allows the study of direct cellular actions of laser. From these *in vitro* studies performed in the absence of systemic or neural

influences, it has been demonstrated that laser can directly influence cell activity by altering DNA synthesis, gene expression, and fundamental intracellular processes such as calcium flux and ATP synthesis⁽²³⁾.

The purpose of the present study was to examine whether there is a dose dependent effect of laser on wound healing processes occurring in an isolated *in vitro* fetal mouse limb organ culture system.

Methods

Animals

A total of 139 fetal limbs were obtained from 12 time dated CD1 pregnant mice on Day 16 of gestation (Charles River Laboratories, LaChine, Quebec). Prior to surgery, the pregnant mice were housed in groups in an environmentally controlled room with standard temperature and lighting, and fed a standard rodent chow and water *ad libitum*. On Day 16 of gestation (term = 20 days), the pregnant mice were sacrificed using an overdose of carbon dioxide. Using aseptic techniques, the fetuses were removed within the entire uterus through a large abdominal incision and placed in ice cooled Hanks balanced salt solution (Sigma-Aldrich Canada Ltd., Oakville, Ontario). Using microsurgical techniques within a tissue culture hood, each fetus was removed from the uterus at regular intervals and a standardized full thickness incision was placed into each limb using a 15-degree microsurgical blade (Feather Safety Razor Co., Ltd, Japan). Following wounding, the limbs were amputated and placed into individual organ culture dishes. All study protocols were in compliance with institutional and Canadian Council on Animal Care guidelines (CCAC) for the humane care of animals.

Fetal Mouse Limb Organ Culture System

The fetal limbs were grown in a serum free organ culture system as described in detail elsewhere⁽²⁴⁾. Briefly, the limbs were maintained in organ culture dishes (Falcon, Lincoln Park, NJ) on stainless steel wire mesh triangles (Industrial Wire Products, Los Angeles, CA) partially submerged in a chemically defined serum free medium (BGJb - Fitton-Jackson Modification; Life Technologies Inc, Rockville, MD supplier). The medium was supplemented with antibiotics (Penicillin G 100 U/ml, Streptomycin 0.1mg/ml, Amphotericin B 0.25 mg/ml) and ascorbic acid (1 μ g/ml). The media was changed on alternate days. Limbs were grown in this serum free culture system at 37°C in humidified air with 5% CO₂ for up to seven days.

Laser Treatment

Organ culture dishes containing wounded limbs were exposed to laser irradiation for one minute each day for up to seven days beginning on the day following wounding (Day 1). Laser treatments were delivered via an apparatus that was assembled in a tissue culture hood at room temperature using a Ga-As laser Model IMLS MD5; 904 nm, spot size = 0.002 cm², pulse duration = 200 nanosecond, peak power = 60 mW, pulse frequency = 10000 Hz (supplied by Theralase Inc., Mississauga, Ontario, Canada). Limbs were randomly assigned to receive laser treatment at one of the following energy densities: 0.23 J/cm², 1.37 J/cm², 2.75 J/cm², 3.66 J/cm², 4.58 J/cm² as outlined in Table 1. The laser treatment was delivered to a single point perpendicular to the limb using a non-contact technique at a distance of 1 cm from the surface of the limb. Limbs that were randomly placed into the laser sham group were treated identically to the laser treated limbs, except the machine was not turned on.

Determination of Wound Appearance

Images of the wounds located in the fetal limbs were obtained prior to the onset of treatment (Day 1) and on three and seven days post wounding using a Wild Leitz Stereo microscope at 16X magnification under consistent illumination conditions and digitized using a Sony DXC930 3CCD RGB camera.

Each limb image was coded so as to conceal the laser treatment protocol and the number of days post wounding. Two experimentally blinded observers rated the wound appearance of each using the criteria outlined in Table 2. Using this descriptive tool, higher scores were assigned to limbs that possessed visual evidence of wound healing such as an advancing border of epithelium, indistinct wound borders, or the apposition of wound edges. Examination of a preselected series of photographic images of wounded limbs by the same observer on two separate occasions and a repeated measures ANOVA revealed this semi-quantitative scale of wound appearance had an excellent intrarater reliability (ICC = .93). In addition, calculation of the inter class coefficients (ICC) for images rated by both observers revealed that this tool also yielded excellent interrater reliability (ICC = .85).

Determination of Wound Size

The total wound surface area, perimeter, length and width were determined from photographic images of wounded limbs via a computer image analysis system (Empix Imaging, Mississauga, Ontario, Canada), using a distance measuring tool previously calibrated to 1 mm and having a resolution of 1 μ m. The use of this computerized system to assess morphometric changes in limb images has been previously validated⁽²⁵⁾. In order to account for variations in initial wound size, the change in wound dimensions

Table 1: Laser Treatment

Energy Density (J/cm ²) ^a	Number of Limbs Limbs	Frequency (Hz)	Average Power Output(mW)
0 (Sham Control)	20	0	0
0.23	25	500	3
1.37	25	3000	18
2.75	25	6000	36
3.66	32	8000	48
4.58	24	10000	60

^aThe Energy Density (J/cm²) represents the therapeutic dose incident on the target tissue. Calculation of energy density assumes an effective radiating area of 1 cm².

Table 2: Criteria for Wound Appearance Scores

- 1 = open wound, frayed edges, no sign of healing
- 2 = open wound, round edges, some evidence of closing
- 3 = open wound, edges opposed, not completely healed over
- 4 = closed wound, scar evident
- 5 = closed wound, no wound distinguishable

that occurred during the time in culture was calculated for each limb. The initial wound size was considered to be that measurement taken on Day 1 post wounding prior to beginning laser treatment (Day 1). The Mean $\pm$ SEM of the change in wound dimensions that occurred from initial wound size to Days 3 and 7 postwounding was determined for limbs in each of the laser treatment groups.

Tissue Processing

Limbs were harvested from culture at seven days post wounding and placed in 10% PBS buffered formalin, processed through graded alcohols, and then embedded in paraffin wax. The limbs were placed in paraffin wax blocks that were numerically coded to conceal the treatment protocol to the blinded observer. Serial cross-sections were cut at 5 μ m thicknesses and placed on microscope

slides. The sections were stained using classical histological methods for Hematoxylin & Eosin, Alcian Blue, and Masson Trichrome staining techniques. In addition, serial cross-sections were also stained simultaneously with Sirius Red F3BA and Fast Green FCF dyes (Rowley Biochemical, Rowley, MA) in saturated picric acid. Sirius red dye binds selectively to collagen while the fast green dye binds to all other non collagenous proteins in the tissue sections. This Picrosirius red and fast green dye technique has been used previously to localize and quantify collagen deposition within tissue sections taken from fetal mouse limbs⁽²⁶⁾.

Histological Analysis of wound healing

A complete histological analysis of the organization and composition of the epidermis, dermis and bone of the wounded fetal limbs was performed using microscopic examination of serial cross sections that were stained using all four different staining techniques (Alcian Blue, Picrosirius Red/Fast Green, Masson's Trichrome, and H&E). An initial scan of all serial cross sections for each limb identified the wounded region of each limb. A standardized histological analysis of the wounded regions of each forelimb was then performed using previously determined criteria that are outlined in Table 3. These criteria were based on previous studies performed in this laboratory, which documents tissue repair processes occurring in the wounded limbs maintained in this organ culture system^(24, 27). This semi-quantitative histological description yielded a numerical score between 1 and 3 for the epidermal closure and thickness, collagen and cell distribution within the dermis and bones. In addition, the total score for each limb section examined was also calculated using the sum of the scores from each of the seven domains of the wound assessment tool. Two observers that were blinded as to laser treat-

ment protocol received by each limb, performed the histological analysis of the tissue sections of the wounded fetal mouse limbs. After the histological analysis was completed on all the limbs, the code was then revealed to permit calculation of Mean $\pm$ SEM of the scores for limbs receiving daily laser treatments at various energy densities.

Calculation of the ICC for histological analysis of sections wounded fetal mouse limbs revealed that the same set of images rated on two separate occasions by the same observer has excellent intrarater reliability (ICC = 0.81). In addition the ICC for intrarater scores for each of 12 separate domains included in the histological assessment tool were all above 0.75. Since the ICC of the histological scores obtained by two separate observers was less than 0.75, the interrater reliability of the histological assessment tool was not considered acceptable. As a result, only the histological scores obtained from a single observer were used in the data analysis.

Determination of Relative Amount of Collagen Deposition

The limb sections stained with picrosirius red and fast green dyes were analyzed using a color image analysis system (Northern Exposure, Empix Imaging, Mississauga, Ontario) to determine the distribution of collagen within the dermis and bone of the wounded regions of each limb. This was accomplished by digitizing a microscopic image of each limb section stained with picrosirius red and fast green dyes using a Sony DXC930 3CCD RGB camera connected to a Leitz Laborlux microscope at 400X magnification under consistent illumination conditions. Using this computer image analysis technique that has been described and validated previously^(25, 28), the color density of red stained collagen fibers and green stained non-collagenous proteins was ob-

tained, and the relative amount of collagen deposition was expressed as the collagen/protein ratio. Three different locations were analyzed within the dermis and bone tissue from the wounded region of each limb. The Mean $\pm$ SEM of the collagen/protein ratio determined in wounded regions of limbs in each of the 5 laser treatment groups (0.23 J/cm², 1.37 J/cm², 2.75 J/cm², 3.66 J/cm², and 4.58 J/cm²) and in those in the sham control group.

Statistical Analysis

The intrarater and interrater reliability were assessed for the semi-quantitative scores of wound appearance (Table 2) and histological appearance (Table 3) by calculating inter class coefficients (ICC_(2,1)). The ICC_{S(2,1)} were calculated for the data after a repeated measures ANOVA was performed. ICC values greater than 0.75 were considered to indicate good reliability.

A One Way Analysis of Variance (ANOVA) for non parametric data (Kruskal's Wallis One way ANOVA on Ranks) was used to compare the Mean $\pm$ SEM of the scores for the photographic appearance and histological appearance of wounded limbs treated with various doses of laser (0 J/cm², 0.23 J/cm², 1.37 J/cm², 2.75 J/cm², 3.66 J/cm², and 4.58 J/cm²). A One Way ANOVA for parametric data was used to determine if statistically significant differences existed in the change in wound size, and in collagen/protein ratios determined between the laser treatment groups. Multiple comparisons of the data obtained from wounded limbs receiving laser at each of the five intensities were compared to those in the sham control group, using a Dunn's Post Hoc Test. $P \leq 0.05$ was considered to be statistically significant.

Results

Wound Appearance

Examination of photographic images taken of the wounded limbs placed into the organ culture system revealed that the limbs gradually closed over the 7-day period in all treatment groups (Figure 1). However the number of wounded limbs that closed within a given treatment group were markedly different (refer to Figure 2). By Day 1 after wounding, which is prior to the commencement of any laser treatments, some wound edges had reapproximated following surgical incision. This occurred slightly more frequently in limbs assigned to receive laser at a dose of 1.37 J/cm². Following three days there was a greater percentage of the wounded limbs that had closed in the laser treatment groups compared to those in the sham control groups. This is particularly true in the case of limbs that had been receiving daily treatments of laser at an intensity of 1.37 J/cm², where more than half of the wounded limbs in this group were completely closed. Limbs in this treatment group (1.37 J/cm²) continued to heal remarkably well in the next four days, where by Day 7 post wounding all of the limbs in this group were closed. Also by Day 7, over half of the wounded limbs receiving lower amounts of laser (0.23 J/cm²) and in the sham control group had closed. Limbs treated for seven days with higher doses of laser had much lower percentage of wounded limbs that were closed.

Examination of the wound appearance scores for each laser treatment group revealed that by three days postwounding there was greater visual evidence of wound healing in limbs that received laser treatment (0.23 J/cm²) compared with sham controls (Figure 3). Following seven days of treatment, the higher doses of laser (2.75, 3.66,

Table 3: Criteria for Qualitative Histological Analysis of Tissue Healing in Wounded Fetal Mouse Limbs

Indicators of Wound Healing	Predetermined criteria and assigned scores		
	1	2	3
Wound	definitely distinguishable very subtle	not as obvious	no wound site evident
Epidermal Closure	open wound; > 2 cm gap > 1 cm gap	signs of wound closure small gap < 1 cm	closed wound
Epidermal Thickness	no epidermis present	few layers of epidermal cells; thinner than surrounding epidermis	epidermis thickness equal to adjacent unwounded region
Dermal Cells	few cells present in wound site	some cells present in wound site; fewer than unwounded edges	many cells in wound site; greater or equal to unwounded edges
Dermal Collagen Deposition	none present in wound site	small pockets of collagen deposited in wound site	even distribution of collagen across wounded region
Bone Cells	no viable cells present in bone tissue	few large bone cells present in bone	numerous small cells throughout bone
Bone Collagen	No collagen present	few areas of diffuse collagen staining in bones	collagen present throughout bone; intense red staining within bone tissue

Figure 1: Wound placed into Day 16 fetal mouse limb at one day postwounding and same limb photographed after seven days of laser treatment. Stereomicroscope, 10X magnification.

Wound Closure Rate

Figure 2: Percentage of the total number of limbs in which the wounds had completely closed following one (open bars), four (slashed bars), and seven (closed bars) days of treatment with laser at various energy densities.

4.58 J/cm²) no longer had better wound appearance scores. Rather, wounded limbs receiving seven days of treatment with the lower doses of laser (0.23 and 1.37 J/cm²) had significantly higher wound appearance scores than wounded limbs treated with higher doses of laser (3.66 and 4.58 J/cm²; $P \leq 0.05$).

Changes in Wound Size

At three days post wounding, the decrease in wound surface area was significantly greater in limbs treated with laser ($P \leq 0.05$) compared to sham controls ($1300 \pm 200 \mu\text{m}^2$) with the greatest effect observed at the highest intensity of laser that was administered ($4.58 \text{ J/cm}^2 = 7700 \pm 2200 \mu\text{m}^2$). However, following seven days, only the limbs treated with lower doses of laser exhibited a further decrease in wound size whereas the limbs treated with higher doses of laser did

not show continued wound closure (Figure 4). In fact, in wounded limbs treated with the highest dose of laser (4.58 J/cm²), the initial improvements in wound surface area observed at Day 3 were reversed when treatments at this high dose continued for a further four days (until Day 7 post wounding).

Changes in Histological Appearance

Assessment of the histological quality of tissue sections taken from wounded regions of limbs using the semi-quantitative scoring system revealed that there was a dose dependent increase in the histological scores assigned to wounded limbs following seven days of laser treatment (Figure 5). The total histological score for wounded limbs treated for seven days with a laser intensity of 4.58 J/cm² was significantly higher than scores assigned to wounded limbs in the

Figure 3: Mean $\pm$ SEM of the scores for wound appearance that were obtained from images of wounded limbs following three (Day 3) or seven (Day 7) days of laser treatment at bargraph measurements left to right as indicated above: 0 (sham), 0.23, 1.37, 2.75, 3.66, and 4.58 J/cm². The wound appearance scores were assessed by two observers that were blinded to the laser treatment groups (ICC > 0.75). Asterisks indicate statistically significant differences between laser treatment group and sham controls (P < 0.05).

Figure 4: The change from initial wound size (Day 1) that occurred by three (Day 3) or 7 (Day 7) following treatment with laser at bargraph measurements left to right as indicated above: 0 (sham), 0.23, 1.37, 2.75, 3.66, and 4.58 J/cm². The change in wound size was determined by subtracting estimates of wound surface area on Day 3 and Day 7 from the initial wound surface area determined on Day 1 postwounding prior to the commencement of laser treatments. Bars represent Mean $\pm$ Standard Error of the Mean of the measurements for each laser treatment group. Asterisks indicate statistically significant differences between laser treatment group and sham controls (P < 0.05).

Figure 5: Mean $\pm$ SEM of the scores for histological appearance that were obtained from tissue sections taken from wounded limbs following three (Day 3) or seven (Day 7) days of laser treatment at bargraph measurements left to right as indicated above: 0 (sham), 0.23, 1.37, 2.75, 3.66, and 4.58 J/cm². The histological appearance scores were assessed by the same observer that was blinded to the laser treatment groups (ICC = 0.81). Asterisks indicate statistically significant differences between laser treatment group and sham controls ($P < 0.05$).

sham control group (Figure 5; $P \leq 0.05$). These higher scores were reflective of the thicker epidermis, greater cellular infiltrate, and the presence of numerous thick collagen fibers in both the dermis and bone of these injured tissues that had been exposed to daily laser treatments. No statistical differences were detected in the scores assigned to histological sections taken from wounded limbs following 3 days of treatment, however wounded limbs treated with higher doses of laser (3.66 and 4.58 J/cm²) tended to have higher histological scores than sham controls.

Collagen Deposition

Collagen deposition in the dermis of the wounded limbs was elevated above sham controls following three days of laser treatment at the lowest intensity of laser that was administered (0.23 J/cm², Figure 6). On Day 3 postwounding, higher doses of laser treatment did not augment collagen deposition in the dermis of wounded fetal

mouse limbs above that which occurred in sham control limbs. However, greater amounts of collagen were measured in the bones of wounded limbs treated for three days with several doses of laser (0.23, 2.75, 3.66, and 4.58 J/cm²). The collagen/protein ratios measured in the bone region of wounded limbs were statistically greater than sham controls following three days of treatment with laser at an intensity of 3.66 J/cm² (Figure 6; $P \leq 0.05$).

Following seven days of laser treatment, collagen deposition in both the dermis and bone was significantly increased above sham controls in wounded limbs treated with laser at certain intensities (0.23, 2.75, and 3.66 J/cm²; Figure 6; $P \leq 0.05$). Elevated collagen deposition observed following administration of lower doses of laser was eliminated when the intensity of the laser was increased to 4.58 J/cm². Seven days of laser treatments at an intensity of 1.37 J/cm² did not appear

to have a significant effect on collagen deposition in either the bone or dermis regions of wounded fetal mouse limbs.

Discussion

Results from the present study have demonstrated that wound size, wound appearance, and the percentage of wounded limbs that were closed improved progressively over the 7-day treatment period in sham control limbs, and was significantly better than the sham controls for wounded limb treated with lower doses of laser (0.23 J/cm^2 , 1.37 J/cm^2). These data suggest that laser therapy is capable of photostimulating injured tissue to produce positive effects on wound healing. Our results are consistent with numerous previous reports of cell culture studies that have examined the effects of laser therapy on reparative processes of several specific cell types⁽¹⁶⁻²²⁾. These data also corroborate results from animal studies in which Ga-As laser has been shown to accelerate collagen deposition^(4,11), augment wound closure rates⁽¹⁰⁾, improve wound breaking strength⁽⁶⁾, and increase wound contraction⁽¹¹⁾.

We have shown, previously, that wounds placed into late gestational fetal mouse limbs heal in a manner similar to that occurring in adult animals and result in the production of collagenous scar formation^(24,26). This wound healing model has the advantage of eliminating the myriad of confounding factors and systemic effects that are capable of altering the response to laser, while maintaining cells in their natural three dimensional structures. This organ culture model also permits interaction of several different cell types with their normal extracellular environment. This is of importance since recent reports suggest that the function of cells can be greatly influenced by the surrounding

extracellular matrix⁽²⁹⁾. Examination of the effects of several different doses of laser within the same experiment is often not practical in studies using animal model, and is almost impossible in clinical studies. This is another advantage of an *in vitro* system, in which several individual and isolated wounds can be easily examined under identical experimental conditions.

Since we observed photostimulatory effects of laser on healing of wounds placed in isolated fetal mouse limbs that lack neural or circulatory connections, we may conclude that these changes in wound healing occurred via direct effects on cellular components present within the local wound environment, and do not require systemic or neural changes.

We found in the present study that both the photographic and histological changes occurring in wounded fetal mouse limbs treated with laser were dose dependent. Compared to sham controls, limbs receiving laser treatments at lower energy densities for seven days had higher wound closure rates, greater decrease in wound size, better wound appearance scores, and more collagen deposition; whereas wounded limbs treated with the highest dose of laser for seven days (4.58 J/cm^2) were not different from sham controls. Although it is generally accepted that the effects of laser are dependent upon the amount of laser energy delivered to the tissue (laser energy density), very few studies have examined the effects of different doses of laser. In most of these *in vitro*^(16,30-32) and *in vivo* studies^(33,34) in which there were relatively few doses of laser examined, either no significant effect of either dose of laser was found^(30,32) or both doses demonstrated similar effects^(31,33,34).

Figure 6: Mean $\pm$ SEM of the collagen/protein ratios determined from that were obtained in the Bone (top) and Dermis (bottom) regions of tissue sections taken from wounded limbs following three (Day 3) or seven (Day 7) days of laser treatment at bargraph measurements left to right as indicated above: 0 (sham), 0.23, 1.37, 2.75, 3.66, and 4.58 J/cm². The collagen/protein ratios were obtained using color image analysis system (Northern Exposure, Empix Imaging) on wounded limb sections stained with Picrosirius Red/Fast Green dye staining technique. Asterisks indicate statistically significant differences between laser treatment group and sham controls ($P < 0.05$).

Initial improvements in wound size observed following three days of treatment with higher doses of laser were reversed when higher doses of laser continued for a further 4-day period. This response supports the general notion that low levels of laser can stimulate wound healing processes, whereas the same cell processes can be inhibited when exposed to excessive amounts of laser energy. Furthermore, our results suggest that successive laser treatments can accumulate in the tissue, and result in bioinhibitory effects. Therefore, treatment schedules and the time interval between laser treatments may represent yet another important factor in determining tissue responses to laser. This bimodal response to laser may also explain why previous research on the effects of laser has yielded conflicting results.

In the present study a Ga-As laser was used in the pulsed mode and changes in energy density were accomplished by progressively increasing pulse frequency. Therefore it is possible that differences observed between laser treatment groups may be due to a physiological response to higher pulse frequencies. There is limited information on the effects of laser pulse frequency on wound healing. Previous reports have demonstrated that the effects of laser on wound healing processes were significantly altered when laser was delivered at different pulse rates^(10,11,20). Further research is needed to determine if changing pulse frequency alone, without altering the energy density delivered, can produce different cellular responses to laser.

We also examined the histological properties of wounded tissue that had been treated with varying doses of laser. Using previously validated and reliable measurement tools we observed that laser treatment, at an appropriate dose, could produce significant changes in tissue histology. This may

suggest that the changes in wound closure rates that were observed in photographic images may have resulted from underlying histological changes produced within the local wound environment. Further examination of the various component of the previously wounded tissue suggest that this was not related to an obvious change observed in the epidermal layer but rather was associated with elevated levels of collagen deposited both within the dermis and the bone regions of the wounded limbs. Accumulation of collagen within the wounded sections of limbs treated with 0.23, 2.75, and 3.66 J/cm² of laser occurred sooner (by Day 3) and remained higher at the end of the experiment (at seven days postwounding) than that observed in sham treated control limbs. In our study, lower doses of laser 0.23 J/cm², 2.75 J/cm², and 3.66 J/cm² were associated with higher amounts of collagen deposition within the wounded region, whereas higher doses of laser (4.58 J/cm²) did not stimulate collagen deposition. In general, the dose dependent pattern of collagen deposition related to that observed with wound closure rate, with better wound closure rates observed in limbs where there was greater collagen deposition in the underlying dermis and bone. A positive relationship between wound collagen and closure rate may be explained by enhanced epidermal migration over a wound bed that has an extracellular matrix composition that is high in collagen content⁽³⁵⁾.

Surprisingly, collagen deposition was not elevated in limbs treated with laser at a dose of 1.37 J/cm², whereas this dose was found to result in the greatest number of wounds that were completely closed (100%) by the end of the 7-day treatment period. The lower collagen/protein ratios were consistently detected in both Day 3 and Day 7 limbs, and in the all measurement taken in both the dermis and bones regions of the tissue sections

of wounded limbs with 1.37 J/cm² laser. It is possible that the faster wound closure rates that occurred in this laser treatment resulted in a diminution, or turning off, of the signal to wound fibroblasts to synthesize and secrete collagen. The corollary is certainly true clinically, i.e., wounds that take longer to heal are associated with greater collagenous scar production.

In conclusion, these results suggest that laser energy delivered at an appropriate energy density is capable of directly acting within the local wound environment to alter the activities of cellular and tissue components and result in augmented collagen deposition and accelerated wound closure. However, if laser energy is delivered at excessive levels, it can detrimentally affect cell function and impair wound healing.

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