Influence of Low Level Laser Therapy on Wound Healing and Its Biological Action Upon Myofibroblasts

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Background and Objective: In re-evaluating the effects of laser therapy in wound healing, the role of extracellular matrix elements and myofibroblasts, was analyzed.
Study Design/Materials and Methods: Cutaneous wounds were inflicted on the back of 72 Wistar rats. Low level laser was locally applied with different energy densities. Lesions were analyzed after 24, 48, 72 hours and 5, 7, and 14 days. Tissues were studied by histology, immunohistochemistry, and electron microscopy.
Results: In treated animals, the extent of edema and the number of inflammatory cells were reduced ($P < 0.05$), but the amount of collagen and elastic fibers appeared slightly increased. Desmin/smooth muscle alpha-actin-phenotype myofibroblasts were statistically more prominent on the 3rd day after surgery ($P < 0.05$) in treated wounds than in controls. Treatment with a dosage of 4 J/cm^2 was superior to that with 8 J/cm^2.
Conclusions: Laser therapy reduced the inflammatory reaction, induced increased collagen deposition and a greater proliferation of myofibroblasts in experimental cutaneous wounds. Lasers Surg. Med. 32:239–244, 2003.

Key words: biostimulation; low-level laser; skin wounds

INTRODUCTION

Low level laser therapy has been found to accelerate wound healing [1] and reduce pain [2,3], possibly by stimulating oxidative phosphorylation [4,5] and reducing inflammatory responses, thus exhibiting several beneficial effects upon inflammation and healing.

Wound healing presents three phases which have been well described: an inflammatory phase, a proliferative phase, and a remodeling phase [6]. Most accounts of laser biostimulation suggest that its greatest effect occurs during the proliferative phase. It seems that cell metabolic processes are enhanced due to mitochondrial photoreception of monochromatic light. It has been suggested that the laser increases the respiratory metabolism of certain cells, thereby affecting their electrophysiological properties [7]. Aspects of wound healing that are reportedly affected in vitro are fibroblast proliferation, collagen synthesis, macrophage stimulation, and extracellular matrix production [8,9]. Other studies suggest that laser light causes rearrangements in the cell cyto-skeleton, thus inducing cell modulation [10,11]. For instance, stimulation of connective-tissue cells toward a myoid phenotype would result in the differentiation of myofibroblasts, the cell-type mainly responsible for the contraction force during wound healing [12–14]. Myofibroblasts share morphologic features in common with fibroblasts and smooth muscle cells [15]. These cells are observed in normal tissue, granulation tissue, and some pathological conditions [16,17]. They may exhibit different skeletal phenotypes, according to the filaments they present, such as desmin, vimentin, and alpha smooth muscle actin. The influence of low level laser therapy on the phenotypic modulation of myofibroblasts has been little explored.

Thus, the present study investigated the effects of low level laser therapy on the participation of myofibroblasts in the wound healing process. A model of standardized skin wound in rats was utilized. The several components of the repair process were examined by histological, ultrastructural, and immunohistochemical methods, to study the specific effects of two dosages of laser treatment.

MATERIALS AND METHODS

Animals

Seventy-two healthy male and female Wistar rats weighing 150–250 g were maintained in individual cages with free access to water and pellets containing a balanced commercial diet for rats. Rats were randomly divided into three groups of 24 animals each.

Surgical Procedure

Under aseptic conditions and light anesthesia with ketamine-xylazine, a single, standardized, 8-mm-diameter round skin wound was produced in an area of approximately 50 mm^2, by means of a punch-skin biopsy device at the right dorsal region of each animal.

Experimental Groups

The wounds of two groups of animals were treated with a gallium-aluminum-arsenide diode laser, at an

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energy-density of 4 J/cm^2 (Group 1) or 8 J/cm^2 (Group 2), while a third group consisted of untreated control animals (Group 3).

Laser Treatment

A VR-KC-610 laser device containing a continuous-wave GaAlAs diode emitting light with a wavelength of 670 nm, and an output of 9 mW (Dentoflex, Brazil). Power output was calibrated by a Lasermate 10 power meter (Coherent, USA). In order to determinate the irradiance time of cutaneous applications, the spot size was measured to calculate the density of energy based upon power output, which was of 28.27×10^{-2} cm^2, during either 31 or 62 seconds). The laser probe was locally applied in contact with the wound. Only a single session of treatment was applied, immediately following surgery.

Morphological Studies

On day 1, 2, 3, 5, 7, and 14 following surgery and laser treatment, the wounds were grossly inspected and measured with a flexible ruler and two in each group were randomly chosen and totally removed to be submitted to the following procedures.

Histology

Fragments of the skin, including the margin of the wound and subcutaneous tissue were fixed in 10% phosphate buffered formalin and embedded in paraffin. Five-micrometer thick sections were stained with hematoxylin and eosin, sirius red for collagen and orcein for elastic fibers.

Immunohistochemistry

Paraffin-embedded 3 μm-thick sections were mounted on aminopropyl-triethoxy-silane coated slides and submitted to staining with either monoclonal anti-α-smooth muscle actin (1:800) or anti-desmin (1:100) (DAKO, Cardinteria, CA, USA). The antigenic retrieval was obtained by previous microwave treatment. All procedures were according to the manufacturers' instructions. Briefly, all incubations were performed at room temperature as follows: (a) primary antibodies were incubated from 30 minutes to 24 hours according to the manufacturers' instructions; (b) link antibody was incubated for 20 minutes; (c) streptavidin-conjugated enzyme was incubated for 20 minutes, (d) peroxidase substrate was incubated for 5 min. After immunostaining, slides were counterstained with Gil's hematoxylin and covered with Canada balsam. Sections from control animals were processed identically to sections from treated animals. Contribution of nonspecific staining of primary antibody was evaluated by substitution of this antibody with normal rat serum or phosphate buffer solution (PBS).

Electron Microscopy

Tiny fragments of tissue were fixed for 1 hour at room temperature in 2% glutharaldehyde in 0.1 M cacodylate buffer, post-fixed in 2% OsO_4, dehydrated in graded acetone, and embedded in Epon 812. Semi-thin sections were cut and stained with methylene-blue. Representative areas were selected for ultra-thin sections. These were collected on copper grids, double-stained with uranyl acetate and lead citrate, and examined with a Zeiss EM-109 electron microscope at 50 Kv.

Semi-Quantitative Analysis

Changes affecting the number of polymorphonuclear inflammatory cells, the degree of edema, collagen and elastic fibers deposition, and the number of cells marked for desmin, vimentin and alpha actin smooth muscle were semi-quantitatively evaluated in coded slides and registered as absent (0), mild (+), moderate (++), and marked (+++).

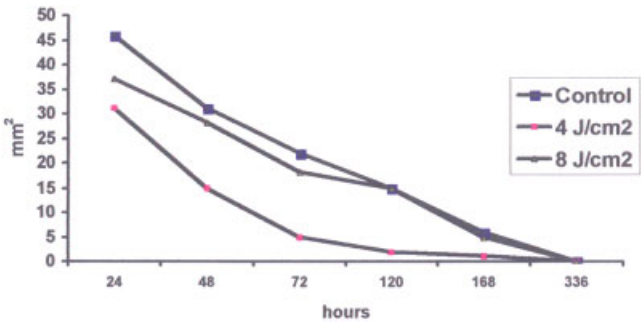
Statistical Analysis

The differences between the control group and the experimental groups were analyzed by using the nonparametric Kruskal Wallis test or the parametric one-way ANOVA test for the histological data. Significance was accepted at $P < 0.05$.

RESULTS

Statistically significant differences in the areas of laser-treated and untreated cutaneous ulcers were observed as early as 24 hours after surgery (Table 1). After 72 hours, the low level laser-treated ulcers exhibited the greatest difference in diameter when compared to untreated ulcers. This tendency for treated ulcers to be smaller than untreated ulcers was observed until the 7th day. By the 14th day, cutaneous wounds in all animals were completely healed (Graphic 1). The group treated with a dosage of 4 J/cm^2 showed a greater reduction of the diameter of the wounded area at every timepoint than the group treated with a dosage of 8 J/cm^2.

These gross changes were positively correlated with the microscopic findings. During the first days, signs of acute inflammation were less intense and subsided earlier in the treated animals. The degree of edema, vascular congestion and the exudation of neutrophilic polymorphonuclear leukocytes were particularly affected (Tables 2 and 3). The semi-quantitative estimation of collagen and elastic tissues deposition did not differ for the three groups during the 24–48 hour period (Table 4). After 72 hours the laser-treated groups showed prominent granulation tissue. The vascular proliferation was most apparent when immuno-



Graphic. 1. Area reduction (square millimeters) between the three groups of each experimental timepoint.

TABLE 1. Average of Skin Wound Areas (mm^2) in Laser Treated Animals, at Two Dosages, and Nontreated Controls at 24, 48, and 72 Hours and 5, 7, and 14 Days

Period of sacrifice	Group	Mean ± SD	*P*
24 hours	Control	46.2 ± 2.7[a]	0.01*
	4 J/cm^2 laser	31.16 ± 3.2[b]	
	8 J/cm^2 laser	37.39 ± 9.7[a,b]	
48 hours	Control	31.16 ± 8.7	0.051
	4 J/cm^2 laser	15.85 ± 4.0	
	8 J/cm^2 laser	28.17 ± 9.7	
72 hours	Control	22.27 ± 6.3[a]	0.001*
	4 J/cm^2 laser	5.39 ± 2.0[b]	
	8 J/cm^2 laser	18.59 ± 3.9[a,b]	
5 days	Control	15.80 ± 7.8[a]	0.01*
	4 J/cm^2 laser	2.54 ± 0.9[b]	
	8 J/cm^2 laser	15.30 ± 4.8[a,b]	
7 days	Control	6.03 ± 2.1[a]	0.01*
	4 J/cm^2 laser	1.17 ± 0.3[b]	
	8 J/cm^2 laser	5.83 ± 2.6[a,b]	
14 days	Control	0.00	
	4 J/cm^2 laser	0.00	
	8 J/cm^2 laser	0.00	

*The mean difference is significant at the 0.05 level.
[a,b]Means followed by the same letter does not differ significantly.

histochemically sections marked for smooth muscle actin were examined (Fig. 1). Fibroblast proliferation and collagen deposition accompanied vascular proliferation. These changes progressively increased toward the 5th day for all the groups. Actin and desmin-positive cells became prominent among these fusiform cells (Fig. 2). They appeared distributed predominantly at the periphery of capillaries and venules. The group that received 4 J/cm^2 laser treatment exhibited significantly more

TABLE 2. Distribution of Polymorphonuclear Infiltrate in the Control and Laser Treated Groups at 24, 48, and 72 Hours and 5, 7, and 14 Days

Period of sacrifice	Group	Mean ± SD	*P*
24 hours	Control	3.00 ± 0.0	
	4 J/cm^2 laser	2.00 ± 0.0	
	8 J/cm^2 laser	2.00 ± 0.0	
48 hours	Control	2.50 ± 0.5[a]	0.025*
	4 J/cm^2 laser	1.25 ± 0.5[b]	
	8 J/cm^2 laser	1.50 ± 0.5[a,b]	
72 hours	Control	2.25 ± 0.5[a]	0.004*
	4 J/cm^2 laser	1.00 ± 0.0[b]	
	8 J/cm^2 laser	1.25 ± 0.5[a,b]	
5 days	Control	1.00 ± 0.0	1.00
	4 J/cm^2 laser	0.25 ± 0.5	
	8 J/cm^2 laser	0.50 ± 0.5	
7 days	Control	0.50 ± 0.5	0.10
	4 J/cm^2 laser	0.00	
	8 J/cm^2 laser	0.00	
14 days	Control	0.00	
	4 J/cm^2 laser	0.00	
	8 J/cm^2 laser	0.00	

*The mean difference is significant at the 0.05 level.
[a,b]Means followed by the same letter does not differ significantly.

TABLE 3. Distribution of Edema in the Control and Laser Treated Groups at 24, 48, and 72 Hours and 5, 7, and 14 Days

Period of sacrifice	Group	Mean ± SD	*P*
24 hours	Control	3.00 ± 0.0[a]	0.02*
	4 J/cm^2 laser	2.00 ± 0.8[b]	
	8 J/cm^2 laser	2.00 ± 0.0[a,b]	
48 hours	Control	2.25 ± 0.5	0.06
	4 J/cm^2 laser	1.25 ± 0.5	
	8 J/cm^2 laser	1.50 ± 0.5	
72 hours	Control	1.25 ± 0.9	0.47
	4 J/cm^2 laser	0.50 ± 0.5	
	8 J/cm^2 laser	0.75 ± 0.9	
5 days	Control	0.50 ± 0.5	0.10
	4 J/cm^2 laser	0.00	
	8 J/cm^2 laser	0.00	
7 days	Control	0.00	
	4 J/cm^2 laser	0.00	
	8 J/cm^2 laser	0.00	
14 days	Control	0.00	
	4 J/cm^2 laser	0.00	
	8 J/cm^2 laser	0.00	

*The mean difference is significant at the 0.05 level.
[a,b]Means followed by the same letter does not differ significantly.

TABLE 4. Distribution of Collagen Fibers in the Control and Laser Treated Groups at 24, 48, and 72 Hours and 5, 7, and 14 Days

Period of sacrifice	Group	Mean ± SD	*P**
24 hours	Control	1.00 ± 0.0	
	4 J/cm^2 laser	1.00 ± 0.0	
	8 J/cm^2 laser	1.00 ± 0.0	
48 hours	Control	1.25 ± 0.5	0.74
	4 J/cm^2 laser	1.50 ± 0.5	
	8 J/cm^2 laser	1.25 ± 0.5	
72 hours	Control	1.50 ± 0.5	0.76
	4 J/cm^2 laser	1.75 ± 0.5	
	8 J/cm^2 laser	1.50 ± 0.5	
5 days	Control	1.50 ± 0.5	0.10
	4 J/cm^2 laser	2.00 ± 0.5	
	8 J/cm^2 laser	2.00 ± 0.0	
7 days	Control	2.25 ± 0.5	0.51
	4 J/cm^2 laser	2.75 ± 0.5	
	8 J/cm^2 laser	2.25 ± 0.9	
14 days	Control	3.00 ± 0.0	
	4 J/cm^2 laser	3.00 ± 0.0	
	8 J/cm^2 laser	3.00 ± 0.0	

*The mean difference is significant at the 0.05 level.

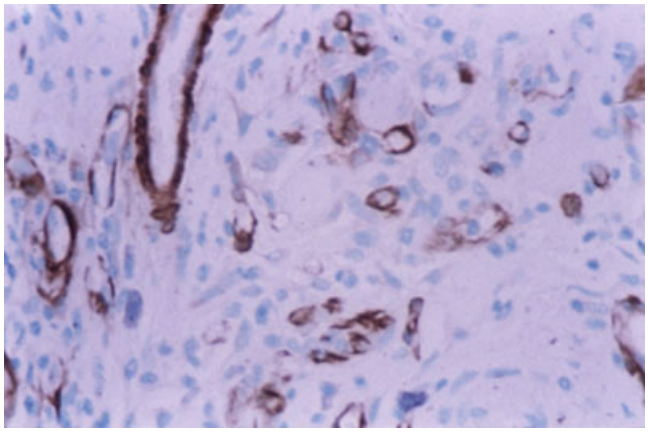


Fig. 1. Actin positive cells are seen around blood vessels. The animal was treated with 4 J/cm^2 and sacrificed 72 hours after surgery. Alpha smooth muscle actin antibody. Streptavidin-byotin. 400×. [Figure can be viewed in color online via www.interscience.wiley.com].

actin/desmin-marked cells in correlation with its more marked vascular proliferation (Tables 5 and 6). The period from the 7th to the 14th day showed a progressive remodeling of the lesion toward an almost normal skin. Collagen fibers appeared more regularly organized (in densely-packed parallel bands) in laser-treated groups than in nontreated controls. Elastic fibers were scarce and scattered in all experimental groups, even at the latest observational points (data not showed).

Ultrastructurally, the most significant changes appeared in sections from the 72-hour period. Proliferated fibroblasts showed rich coarse endoplasmic reticulum forming distended cisternae filled with an amorphous secretory content, prominent Golgi apparatus and increased number of mitochondria. Some of these cells exhibited large fat droplets in the cytoplasm. Myofibroblasts, isolated or in clusters, were frequently found, also exhibiting a rich secretory endoplasmic reticulum and variable amount of cytoplasmic myofibrils and submembrane stress fibers (Fig. 3).

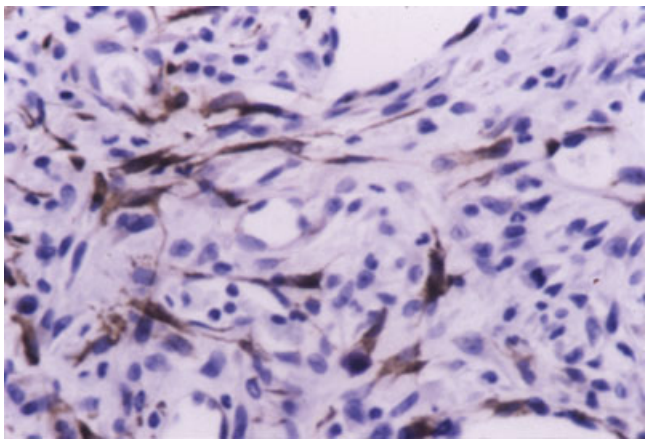


Fig. 2. Desmin-positive cells within granulation tissue in an animal that received 4 J/cm^2 and was sacrificed on the 5th day after the surgical procedure. Desmin antibody. Streptavidin-byotin. 400×. [Figure can be viewed in color online via www.interscience.wiley.com].

TABLE 5. Distribution of Positive Desmin Cells in the Control and Laser Treated Groups at 24, 48, and 72 Hours and 5, 7, and 14 Days

Period of sacrifice	Group	Mean ± SD	*P*
24 hours	Control	0.00 ± 0.0	
	4 J/cm^2 laser	0.00 ± 0.0	
	8 J/cm^2 laser	0.00 ± 0.0	
48 hours	Control	0.00 ± 0.0	
	4 J/cm^2 laser	0.00 ± 0.0	
	8 J/cm^2 laser	0.00 ± 0.0	
72 hours	Control	0.50 ± 0.5[a]	0.004*
	4 J/cm^2 laser	2.00 ± 0.0[b]	
	8 J/cm^2 laser	1.50 ± 0.5[a,b]	
5 days	Control	2.00 ± 0.8	0.10
	4 J/cm^2 laser	3.00 ± 0.0	
	8 J/cm^2 laser	2.50 ± 0.5	
7 days	Control	2.50 ± 0.5	0.32
	4 J/cm^2 laser	3.00 ± 0.0	
	8 J/cm^2 laser	2.75 ± 0.5	
14 days	Control	1.50 ± 0.5	0.74
	4 J/cm^2 laser	1.75 ± 0.5	
	8 J/cm^2 laser	1.75 ± 0.5	

*The mean difference is significant at the 0.05 level.
[a,b]Means followed by the same letter does not have significative difference between each other.

TABLE 6. Distribution of Positive Actin Cells in the Control and Laser Treated Groups at 24, 48, and 72 Hours and 5, 7, and 14 Days

Period of sacrifice	Group	Mean ± SD	*P*
24 hours	Control	0.00 ± 0.0	
	4 J/cm^2 laser	0.00 ± 0.0	
	8 J/cm^2 laser	0.00 ± 0.0	
48 hours	Control	0.00 ± 0.0	
	4 J/cm^2 laser	0.00 ± 0.0	
	8 J/cm^2 laser	0.00 ± 0.0	
72 hours	Control	0.50 ± 0.5[a]	0.004*
	4 J/cm^2 laser	2.00 ± 0.0[b]	
	8 J/cm^2 laser	1.50 ± 0.5[a,b]	
5 days	Control	2.00 ± 0.8	0.10
	4 J/cm^2 laser	3.00 ± 0.0	
	8 J/cm^2 laser	2.50 ± 0.5	
7 days	Control	2.50 ± 0.5	0.32
	4 J/cm^2 laser	3.00 ± 0.0	
	8 J/cm^2 laser	2.75 ± 0.5	
14 days	Control	1.50 ± 0.5	0.74
	4 J/cm^2 laser	1.75 ± 0.5	
	8 J/cm^2 laser	1.75 ± 0.5	

*The mean difference is significant at the 0.05 level.
[a,b]Means followed by the same letter does not have significative difference between each other.

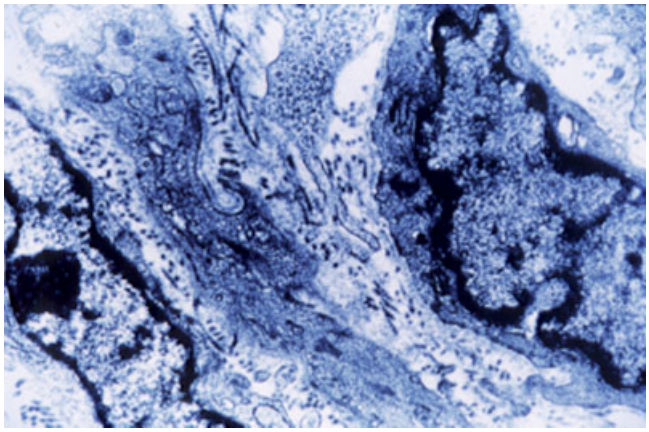


Fig. 3. Myofibroblasts, exhibiting submembrane bodies (head arrows), indented nuclei and myofilaments (narrow arrows) and a perimembrane material like basal membrane. The animal received 4 J/cm^2 of energy density and was sacrificed 5 days after surgery. Electron microscopy. 7000×. [Figure can be viewed in color online via www.interscience.wiley.com].

By the 7th day, some fibroblasts and myofibroblasts appeared quiescent, without signs of synthetic activity, but containing internalized fragments of collagen fibers in cytoplasmic membrane-bound vesicles. At the same time, a few inflammatory cells showed signs of apoptosis in the form of a condensed nucleus and a shrunken cytoplasm containing mummified organelles (Fig. 4).

DISCUSSION

Although the overall cicatrization time of the standardized skin wound did not differ in laser-treated animals compared to untreated controls, several gross and histological differences between the two groups were evident. Present results confirm findings of Mester et al. [18] and Braverman et al. [19] that laser light accelerates some phases of wound healing. Sequential semiquantitative histological examination revealed that laser treatment abbreviated the exudation phase of the wound and stimulated the reparative process. Low level laser therapy exhibited the greatest wound area reduction, especially observed at 24 and 72 hours after treatment, a finding well-correlated with a prominent presence of myofibroblasts. Mester et al., [20] used photos to document the wound's contraction. In the present study, such contraction was demonstrated by direct measurements of the wound diameters.

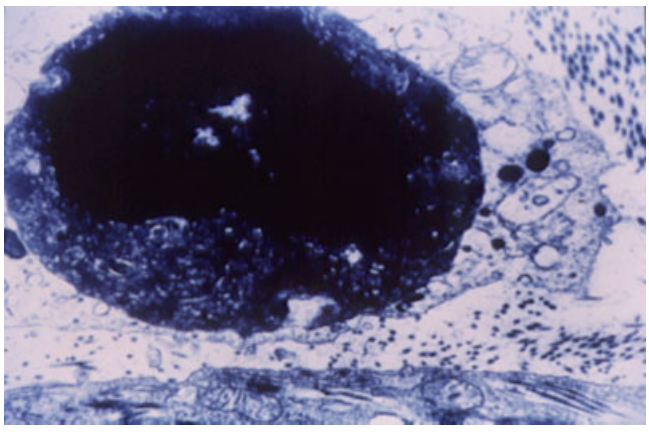


Fig. 4. Cell demonstrating condensation of nuclear cromatin and compaction of organelles. In the cytoplasm of the nearby cell, collagen internalization can be observed (arrows). Control animal sacrificed 7 days after surgery. Electron microscopy, 7000×. [Figure can be viewed in color online via www.interscience.wiley.com].

Besides confirming the beneficial effects of laser treatment upon wound healing, present investigation correlated clinical and pathological findings. Few previous studies have analyzed histological variables in relation to their clinical relevance. As reported by others [21,22], the reduction of the extent of edema was an important early effect of laser therapy. Although Røynesdal et al. [23] did not observe the prevention of early edema, it was a common event during this present study. The cellular infiltrate also differed among treated and control ulcers. Segmented leukocytes underwent earlier replacement by mononuclear cells in the laser-treated group than in the controls (data not showed). According to Calvin [24], inflammatory cells play an important role during wound healing, contributing to the release of lysosomal enzymes and oxygen products as well as facilitating the clearance of debris.

Another impressive difference between treated ulcers and controls was the presence of many more fusiform cells expressing desmin and alpha smooth muscle actin in the treated group 72 hours after surgery. This timepoint corresponds to the time the diameter of the cutaneous wound revealed its greatest reduction in treated compared to control ulcers, which indicated a direct effect of laser treatment. Also, an enhanced proliferation of fibroblasts and myofibroblasts coincided with an increased, albeit slight, collagen concentration observed in laser-treated animals. Laser influence on fibroblast proliferation has been observed in in vitro studies [25]. It is interesting that this finding was first observed in vitro by Skinner et al. [8] and was now confirmed in vivo. In humans, Porreau-Schneider et al. [11] demonstrated that myofibroblasts appeared in an intra-oral area within 48 hours of laser irradiation, while the control site did not exhibit these cells after the same time.

Sappino et al. [14] documented that vimentin, desmin and alpha smooth muscle actin are the main filaments expressed by myofibroblasts during wound healing. They suggested that fibroblast cells modulate toward a phenotype which is characterized by ultrastructural (stress fibers) and biochemical (alpha smooth muscle actin) features, typical of smooth muscle cells. The ultrastructural findings demonstrated myofibroblasts and fibroblasts with variable signs of synthesis activity, probably both cell types contributing to deposition of extracellular

matrix at the wound site. But, although the presence of desmin-actin-positive cells was more apparent at the 72-hour period, increase in collagen deposition appeared later on, suggesting that wound contraction comes before new matrix formation.

In relation to energy density, it was observed that the group which received a dose of 4 J/cm^2 always presented the greater reduction in the diameter of treated ulcers compared to the 8 J/cm^2 group. But while some authors agree that lower energy densities are more prone to enhance biostimulation [26], there are studies with negative results with low level laser treatment, as Basford discussed in a review article [27]. Several well-controlled experimental studies indicated a beneficial effect upon the wound by laser biostimulation [28]. Despite similarities of dose and a convergence in laser choice, significant methodological differences persist regarding experimental protocols. Among these differences are, for example, pulse rate, the irradiance, spot size, and applicator placement [29–31]. Even the anesthetics used may influence the action of laser light treatment [32].

An apparent paradox was noted at the end of the present investigation. Laser treatment induced a series of morphological changes, presumably favorable to the resolution of wound healing, but did not shorten cicatrization time. However, these effects may be important. By diminishing exudation of the early phase of wound healing, the general effects due to cytokine release, local pain, and tumefaction are probably ameliorated. Enhancement of wound contraction facilitates a more effective cicatrization, although not necessarily a more rapid one.

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