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Immunohistochemical Assessment of Myofibroblasts and Lymphoid Cells During Wound Healing in Rats Subjected to Laser Photobiomodulation at 660 nm

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Abstract

Objective: The goal of this study was to assess the biomodulatory effect of low-level laser therapy (LLLT) on myofibroblasts and T and B cells during wound healing. ***Background Data:*** Photobiomodulation using LLLT has been extensively applied to improve wound healing. ***Materials and Methods:*** Standardized artificial surgical wounds were made on the backs of 24 male rats. Half of them underwent LLLT (20 J/cm^2) at 660 nm delivered for 7 d. At 8 and 14 d post-surgery the healing wounds were removed and immunohistochemical analysis of myofibroblasts, T cells, and B cells was carried out. The mean of each cell subset was calculated and compared to one another using two-way analysis of variance (ANOVA) and Tukey's test. ***Results:*** The average number of myofibroblasts was statistically significantly higher in the irradiated group than in the non-irradiated group on the eighth ($p = 0.001$) but not the 14th ($p = 0.555$) day. B and T cells were significantly more conspicuous in the irradiated group on both the eighth ($p = 0.004$ and 0.02, respectively) and 14th days ($p = 0.04$ and 0.03, respectively). ***Conclusions:*** Our results suggest that LLLT facilitates myofibroblastic differentiation during the early stages of the cicatricial repair process. Furthermore, LLLT also appears to modulate the inflammatory response by downregulating lymphocytic proliferation during the wound healing process.

Introduction

Low-level laser therapy (LLLT) in the far red to near-infrared range is known to modulate various biological processes, a phenomenon known as photobiomodulation.[1] Thus, a wide variety of biological effects, such as trophic regenerative,[2] anti-inflammatory,[3] and analgesic effects[4] induced by photobiomodulation at low fluences have been reported, most likely as a result of photo-induced electronically excited states of different molecules that accelerate electron transfer in the respiratory chain and consequently increase mitochondrial ATP synthesis. This mechanism is thought to promote increases in cellular metabolism in several tissue types and to improve a wide variety of physiologic and pathophysiologic processes, such as wound healing.[5] The wound-healing process represents a defensive response of the human body to a variety of injuries, including penetrating trauma, burn trauma, and blunt trauma. All of these insults set into motion an orderly sequence of events that are involved in a highly controlled repair process, characterized by the movement of specialized cells to the wound site, which are responsible for providing key functions and signals needed for the influx of connective tissue cells and angiogenesis.[6] The healing response begins the moment the tissue is injured. As the blood components flood the site of injury, inflammatory cells such as neutrophils at initial stages, and lymphocytes and plasma cells at later stages, spread out within the tissue in order to perform the critical task of phagocytosis to remove foreign materials, bacteria, and damaged tissue. Once the wound site is cleaned out, fibroblasts migrate into the area and deposit new extracellular matrix. The new collagen matrix then becomes cross-linked and organized during the final remodeling phase of the healing process. In order for this efficient and highly controlled repair process to take place, there are numerous cell-signaling events that are required.[7] Data obtained from many

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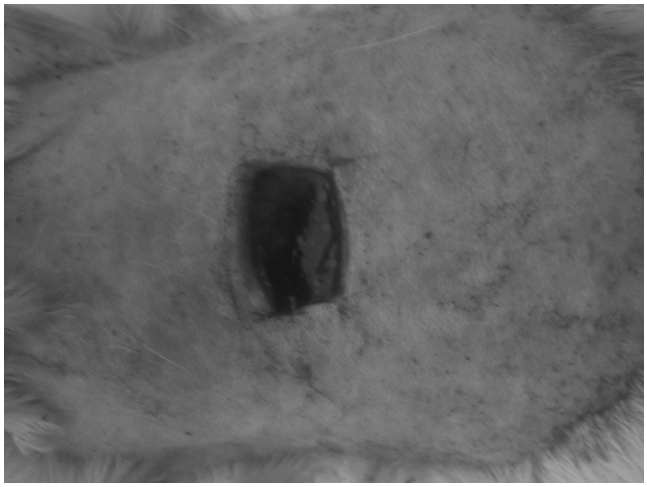

FIG. 1. Photograph showing the 1-cm^2 square-shaped wound made on the back of each rat.

biological assays appear to indicate a possible role of laser photobiomodulation in stimulating cellular proliferation, collagen synthesis, and release of growth factor from stromal cells.[8] Paradoxically, several studies with negative results with regard to the activity of laser irradiation in the wound-healing process have also been reported.[9] It has been suggested that wavelength and dose are relevant factors to be considered to assure successful laser irradiation–induced modulation of wound healing. In addition, optical characteristics inherent to a given tissue type are particularly relevant to evaluating the extent of the interaction between laser irradiation and cells.[10] Therefore, positive and negative results of photobiomodulation as applied to wound healing appear to depend significantly on the protocol of irradiation used in the various experiments reported in the literature.[7] There are only a few reports that examine the possible role of laser photobiomodulation on different phenotypes of stromal and inflammatory cells during wound healing. Thus the goal of this study was to analyze the effect of a specific protocol of low-energy laser irradiation on myofibroblasts, B cells, and T cells during two different phases of wound healing in rodents.

Materials and Methods

Animals

Before the start of the experiments, this study was approved by our animal care and use committee. The animals used in this study were adult male *Rattus norvegicus albinus*, Wistar lineage, weighing 250–300 g each. The rats were housed in clear plastic cages with solid floors and loose hardwood chip bedding, and supplied with food and water ad libitum in a temperature- and humidity-controlled environment.

Surgical procedure and experimental groups

Twenty-four rats were anesthetized with IP ketamine-xylazine (100 mg/kg and 5 mg/kg, respectively) and a 1-cm^2 surgical standardized wound was made on the back of each animal (Fig. 1). The animals were handled in accordance with aseptic principles to avoid any possibility of exogenous bacterial contamination. Subsequently the rats were separated into four groups of six animals each, which were randomly assigned to one of four treatment groups: group 1 (G1), an untreated group sacrificed 8 d post-surgery; group 2 (G2), a photoirradiated group sacrificed 8 d post-surgery; group 3 (G3), an untreated group sacrificed 14 d post-surgery; and group 4 (G4), a photoirradiated group sacrificed 14 d post-surgery. The sacrifice of the animals was carried out by IM administration of 0.8 mL/kg of zolazepam/tiletamine, 0.43 mL/kg thiopental, and 5 mL/kg potassium chloride. After death, the area containing the wounded region on the back of each animal was surgically removed and the specimens were formalin-fixed and paraffin-embedded according to routine laboratory techniques.

Photoirradiation protocol

The animals in G2 and G4 were treated with Twin Laser Ga-Al-As array emitting at 660 nm (MMOptics, São Paulo, Brazil). The treatment consisted of daily transcutaneous irradiation at a power intensity of 40 mW, spot size of 0.04 cm^2, and energy density of 5 J/cm^2 for 120 sec, daily for 7 d. The laser array was positioned directly over the animal at a vertical distance of 0.3 cm from the edge of the wound and irradiation was performed at four different points equidistant from one another, delivering a total dose of 20 J/cm^2 (480 sec in all). These laser parameters were previously demonstrated to be beneficial to wound healing.[11] The control groups (G1 and G3) were submitted to the same stress by manipulation and sham irradiation.

Immunohistochemical analysis

Cytoplasmatic immunostaining of myofibroblasts, B cells, and T cells was performed by using murine monoclonal antibodies against the antigens. Serial 4-μm sections were obtained from the samples previously fixed in buffered 10% formalin and paraffin embedded. Immunohistochemistry was performed using the streptavidin-biotin-peroxidase

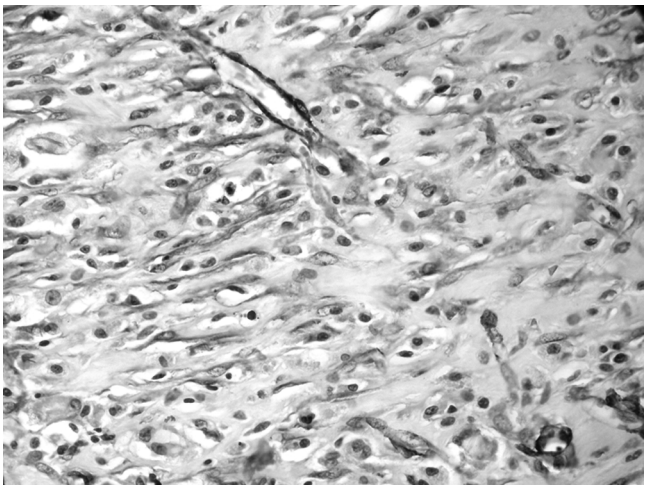

FIG. 2. Myofibroblasts showing immunopositivity to α-SMA can be seen here within the cicatricial area of a specimen 8 d post-surgery (SABC, 100×).

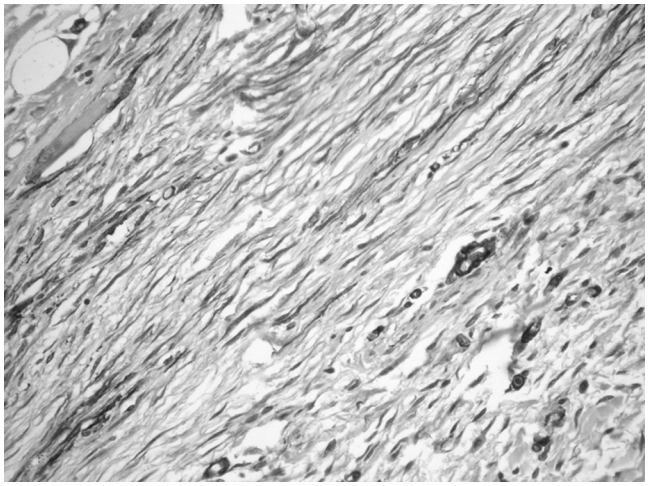

FIG. 3. Photograph showing that myofibroblast immunostaining was less conspicuous on the 14th day, and the cells were arranged predominantly in a parallel pattern following the deposition of collagen fibers (SABC, 100×).

complex (SABC) method (Ultra Vision Large Volume Detection System antipolyvalent, HRP; Lab Vision, Fremont, CA, USA), with monoclonal antibodies against α-smooth muscle actin (α-SMA) (clone 1A4, 1:200, 18 h; Dako, Glostrup, Denmark), CD3 (UCTH 1, 1:100, 18 h; Dako), and CD20 (L26, 1:100, 18 h; Dako, Glostrup) present in myofibroblasts, T cells, and B cells, respectively. Heat-induced antigen retrieval using Dako Antigen Retrieval Solution was previously performed in a water bath for 20 min. Histological sections of uterine leiomyoma and reactional lymph nodes were used as positive controls for α-SMA and B/T cells, respectively. Negative controls were assessed using normal serum as the primary antibody.

Quantitative analysis of myofibroblasts and lymphoid cells

Counting of immunostained cells was done with an image analysis system (Imagelab). All the images were sent to the PC using an analog video camera (PAL system), after being converted to the RGB (red-green-blue) system necessary for digitizing and processing the sections. Ten histological fields (200× magnification) of the wounded area of each rat were selected. The images were recorded and automatically processed to find the cell density (CD) in each reference area (RA) (CD = number of cells/RA). As long as the antigen α-SMA is expressed by smooth muscle cells of the arteriolar muscular walls, mature well-formed blood-vessel-rich fields formed. Statistical analysis was performed using Student's *t*-test to compare CDs of the myofibroblasts, B cells, and T cells between the experimental and control groups at 8 and 14 d post-surgery. Data were expressed as mean ± SD.

Results

Myofibroblasts

Eight days after surgery, myofibroblasts were seen copiously spreading out within the abundant granulation tissue that occupied nearly all of the cicatricial area of the specimens (Fig. 2). Even though no specific pattern of cellular arrangement could be identified, this cell subset seemed to concentrate particularly in the margins and bottom of the wounds. At the 14th day, however, myofibroblast immunostaining was less conspicuous and these cells were arranged predominantly in a parallel pattern following the deposition of collagen fibers (Fig. 3). In addition, quantitative analysis of myofibroblasts, as shown in Table 1, demonstrated that these cells were significantly more abundant in irradiated than in non-irradiated wounds at 8 ($p = 0.00$) but not at 14 d ($p = 0.55$). Moreover, G2 was statistically significantly different from G3 and G4 ($p = 0.00$), whereas G1, G3, and G4 showed similar values.

Lymphoid cells

Positive immunostaining of both T (Fig. 4) and B cells (Fig. 5) was seen in the healing areas of the wounds. As shown in Table 2, the T cells were less conspicuous in the irradiated than in the control groups, and this difference was statistically significant at the 8th ($p = 0.02$) and 14th days ($p = 0.03$). Similar findings were found for B cells (Table 3) at 8 ($p = 0.00$) and 14 d ($p = 0.04$) post-surgery. Nevertheless, the numbers of T cells in both the control and experimental groups were statistically similar at 8 ($p = 0.99$) and 14 d ($p = 0.92$) post-surgery. The same behavior was seen with regard to B cells ($p = 0.22$ at 8 d, and $p = 0.83$ at 14 d). It was apparent that a many more B cells were seen at both 8 and 14 d than T cells. Furthermore, independently of the lymphoid phenotype, inflammatory cells appeared to concentrate at the top of the wound at the 8th day, but they were scattered diffusely in the connective tissue, infiltrating the newly-formed bundles of collagen fibers (Fig. 6).

Discussion

Photobiomodulation has been widely applied in the treatment of soft-tissue injuries, particularly due to the biostimulatory properties of laser energy, which accelerates wound healing[12] in spite of the mixed results reported in the literature. Therefore, factors such as wavelength and dose are thought to be extremely important parameters to effectively speed wound healing.[10]

It has been suggested that wavelengths in the 600- to 700-nm range have been chosen for treating superficial tissues, whereas wavelengths between 780 and 950 nm have been used for deeper tissues.[13] Furthermore, choosing the correct dosage of light (energy density) for a specific condition has proved to be particularly difficult.[5]

When the proper protocols of photobiomodulation are employed, the beneficial effects of LLLT on wound heal-

TABLE 1. QUANTITATIVE ANALYSIS OF MYOFIBROBLASTS IN THE STUDY GROUPS SACRIFICED AT THE 8TH AND 14TH DAYS

Groups	*n*	*Mean (±) SD*	*Variance*	
G1	6	10.42 ± 5.64	13.652	a
G2	6	20.46 ± 3.69	13.853	b
G3	6	12.4 ± 0.97	0.06	a
G4	6	15.28 ± 2.44	0.957	a

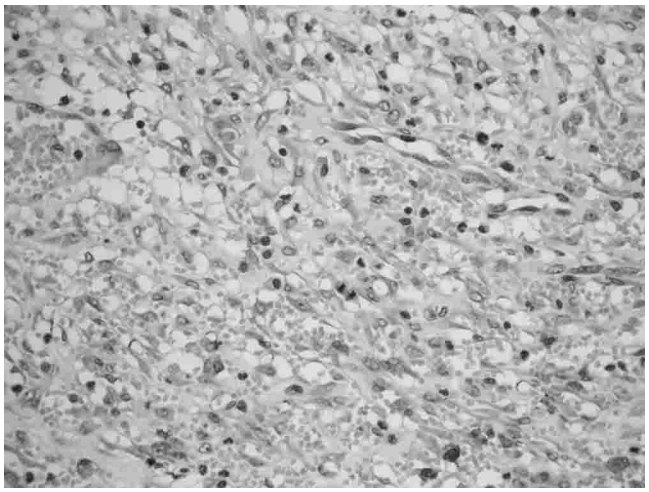

FIG. 4. T cells can be seen here spreading within the healing area and showing extensive immunopositivity for anti-CD 3 (SABC, 100×).

ing can be seen due to its stimulation of biological mechanisms that trigger several phases of cicatricial repair of soft-tissue injuries, including the induction of cytokines and expression of growth factors by keratinocytes and stromal cells.[5]

Wound healing can be divided into three phases, namely inflammation, proliferation, and tissue remodeling. Soon after tissue injury, inflammatory cells migrate to the injury site as a result of local vascular and biochemical changes, and this characterizes the inflammatory phase. Neutrophils and macrophages migrate into the wound to prevent the invasion and proliferation of microorganisms. These polymorphonuclear cells are then gradually replaced by lymphocyte and plasma cells as the healing process continues. The proliferation phase starts with the migration of fibroblasts into the wound to produce granulation tissue components, such as fibronectin, collagen, and hyaluronic acid.[14] Some fibroblasts differentiate into myofibroblasts, which are responsible for tissue contraction as well as the production of extracellular matrix components.[15] Soon after re-epithelialization, which occurs by proliferation and migration of epithelial cells from the wound edges, wound contraction stops, and myofibroblasts start to disappear, probably through apoptosis.[16] Finally, during the remodeling phase, the number of blood vessels declines, and apoptosis of fibroblasts results in scar tissue with a low cell density.[14]

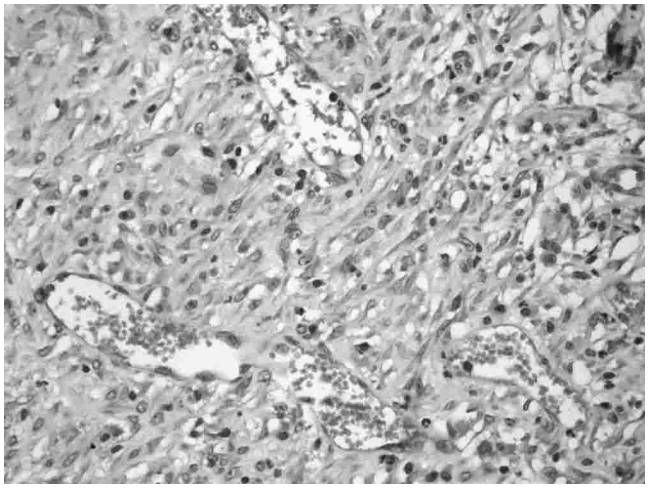

FIG. 5. B cells can be seen here, as identified by anti-CD 20 immunostaining (SABC, 100×).

TABLE 2. QUANTITATIVE ANALYSIS OF CD3-POSITIVE T CELLS IN THE STUDY GROUPS SACRIFICED AT THE 8TH AND 14TH DAYS

Groups	*n*	*Mean (±) SD*	*Variance*	
G1	6	10.7 ± 1.5	2.857	a
G2	6	6.66 ± 2.5	2.458	b
G3	6	11.0 ± 1.1	1.107	a
G4	6	7.5 ± 1.9	1.347	b

Previous studies have demonstrated that LLLT is able to upregulate the release of cytokines responsible for fibroblast proliferation and collagen synthesis, such as FGF-β and TGF, respectively.[8,9,17] Furthermore, some reports have pointed out that LLLT also enables fibroblasts to undergo transformation into myofibroblasts.[18]

Myofibroblasts are a cell type with a contractile phenotype characterized by a cytoskeleton rich in α-SMA. Additionally, their contractile apparatus contains bundles of actin microfilaments and associated contractile proteins, such as non-muscle myosin and desmin. This contractile apparatus is thought to be the major force-generating element involved in wound contraction, so this cell subset is thought to play an important role in the wound healing process by reducing the cicatricial area of the scar.[20] These factors indicate that myofibroblastic differentiation may be a crucial event leading to adequate healing of larger wounds, which have more extensive loss of cells and tissue (wound healing by secondary intent).

The LLLT protocol used in this study promoted a significant increase in numbers of myofibroblasts 8 d post-surgery, suggesting the ability to stimulate myofibroblastic differentiation. Similar immunohistochemical findings were previously reported by Medrado et al.[18] Furthermore, this phenomenon is likely responsible for the substantial reduction seen in the cicatricial area of post-irradiation wounds, when similar wavelengths and energy densities are used.[21] Recently, it was reported that there was ultrastructural evi-

TABLE 3. QUANTITATIVE ANALYSIS OF CD20-POSITIVE B CELLS IN THE STUDY GROUPS SACRIFICE AT THE 8TH AND 14TH DAYS

Groups	*n*	*Mean (±) SD*	*Variance*	
G1	6	39.5 ± 4.6	214.587	a
G2	6	18.1 ± 2.7	7.775	b,c
G3	6	30.0 ± 15.5	43.988	a,b
G4	6	14.9 ± 1.6	4.147	c

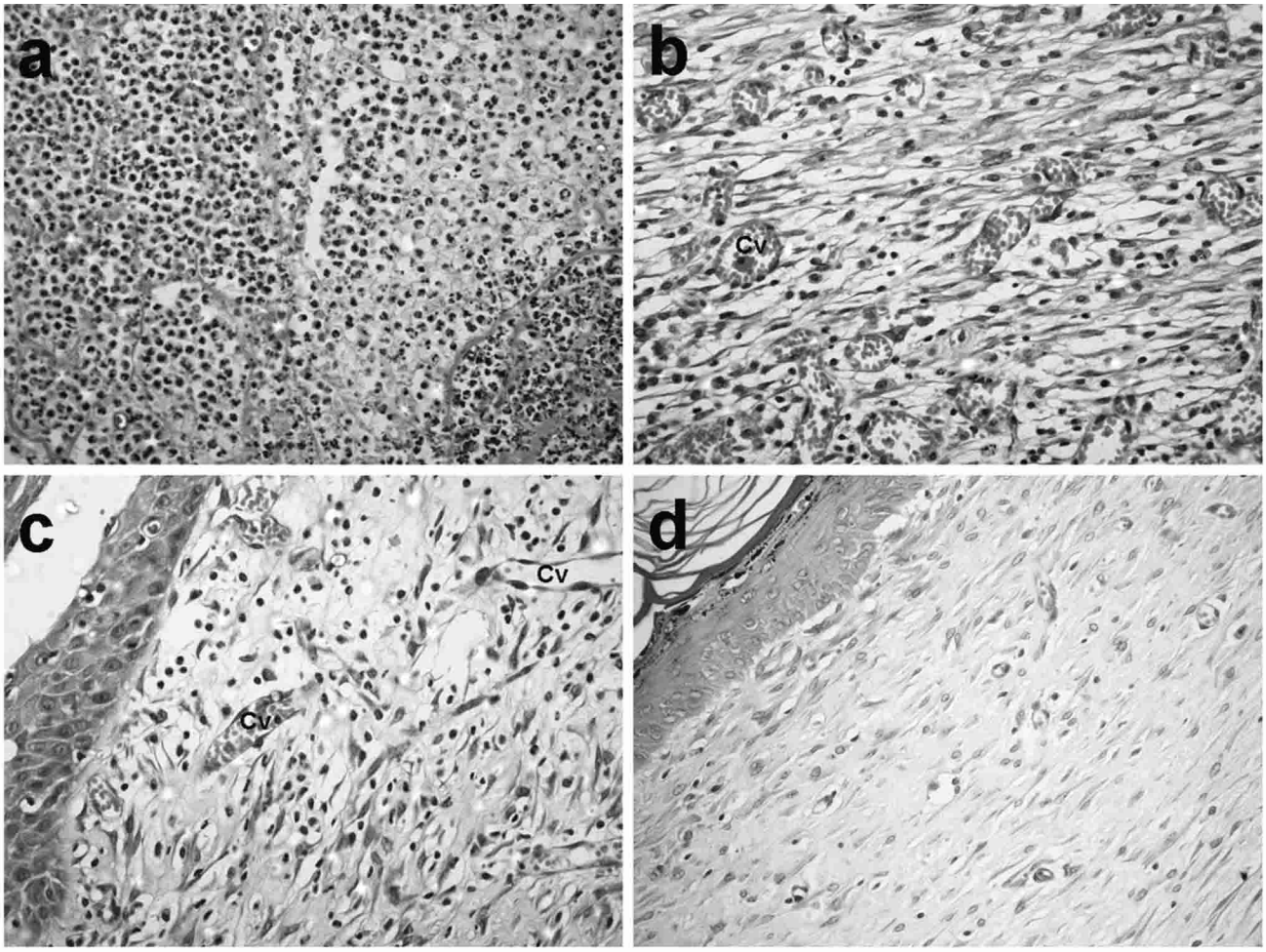

FIG. 6. An intense neutrophil-rich infiltrate can be seen in G1 (**a**), whereas a granulation reaction, with conspicuous formation of capillary vessels (CV) can be seen in G2 (**b**). In G3, the cicatricial process is advanced, but there is still residual leukocytic infiltration and a rich capillary network (CV) (**c**), whereas the collagen tissue is already well established in G4 (**d**) (hematoxylin and eosin, 100×).

dence of increased myofibroblastic differentiation in He-Ne laser-irradiated lesions than in non-irradiated ones.[19] These data point out at the significant role played by different types of laser arrays on the dynamics of laser-induced myofibroblastic differentiation. Since no study confirming the photobiomodulatory effects of the different types of laser arrays has to date been reported, further investigations are necessary to verify not only what type is most effective, but also if there is any difference in their mechanisms of cellular photostimulation.

We also verified that the irradiated group had a peak of myofibroblastic differentiation at the 8th day of the experiment, and it then decreased by the 14th day. Surprisingly, the opposite phenomenon happened in the control group, even though this difference was not statistically significant. These data seem to suggest that LLLT may cause fibroblastic transformation into myofibroblasts at the early stages of the healing process, and accelerate wound contraction. Thus by the 14th day, the number of myofibroblasts decreased, most likely due to apoptosis, and scar tissue formed. On the other hand, in non-irradiated wounds (control group), the healing process appeared to be considerably slower, so that by the 14th day the process of myofibroblastic apoptosis had not yet taken place.

It is well established that fibroblasts can differentiate into mature myofibroblasts in response to specific factors, such as TGF-β^1 and fibronectin.[22] Thus despite the fact that our research showed that LLLT promoted an increase in the number of myofibroblasts during wound healing, further studies are necessary to clarify if this cell phenotype transformation is a direct effect of the irradiation itself, or a result of the laser-stimulated release of differentiating factors by other cells involved in the healing process.

The inflammatory reaction represents the earliest event to take place after tissue injury, and its main functions are to eliminate microorganisms and clean the wound. Therefore inflammation is required to facilitate wound healing, although the long-term persistence of inflammatory cells at an injured site is thought to be one of the most important factors that delays healing.[14] The initial phase of the inflammatory response is acute, and it is characterized by progressive accumulation of neutrophils at the injured area. During this phase, intense phagocytosis of microorganisms is carried out, and simultaneously significant amounts of

lysosomal enzymes are released, inducing secondary damage to the tissues.[15] Subsequently, neutrophils replace mononuclear cells (lymphocytes and plasma cells), and the process of collagen synthesis begins.[14] In this study, it was observed that the average numbers of both T and B cells was significantly higher in the irradiated group on the 8th day than in controls. Nevertheless, at the 14th day the average numbers of these lymphocyte subsets was shown to be statistically similar in both the irradiated and control groups. These data may indicate that photobiomodulation is able to induce a significant reduction in T- and B-cell populations during the early stages of the proliferation phase of the healing process. Photobiomodulation was found to inhibit synthesis and release of prostaglandin, an important chemical mediator of the inflammatory response required for proliferation and maturation of B and T cells.[23] Therefore, the reduction of both lymphocyte subsets in the early stages of the proliferation phase of the inflammatory response may be a result of reductions in prostaglandin synthesis induced by laser energy.

On the contrary,[24] it was demonstrated that photobiomodulation led to proliferation of peripheral blood lymphocytes in experiments *in vitro*. It must be taken into account that the laser irradiation was performed directly on lymphoid cells in culture, and not on injured tissues that were undergoing wound healing, indicating that laser energy causes lymphocyte proliferation *in vitro,* but not *in vivo*. This apparent paradox can be explained by the fact that clonal expansion and cell maturation of lymphocyte subsets during wound healing may require the participation of other co-stimulatory biochemical agents, such as prostaglandin, in addition to intracellular laser-induced proliferative stimuli. This theory is supported by the fact that the average numbers of T and B cells were similar in both the control and irradiated groups by the 14th day, and the release of biochemicals such as prostaglandin is significantly reduced at this stage healing. However, other studies are needed to verify this theory.

Conclusion

Our results strongly indicate that this protocol of photobiomodulation with the 660-nm laser is able to improve wound healing. Despite the findings detailed here, further studies are needed to clarify the effects of laser energy on the different cell subsets participating in wound healing.

Disclosure Statement

No conflicting financial interests exist.

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