Polarized Light (400–2000 nm) and Non-ablative Laser (685 nm): A Description of the Wound Healing Process Using Immunohistochemical Analysis

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ABSTRACT

Objective: This study aimed to describe, through morphologic and cytochemical analysis, the healing process of wounds submitted (or not) to laser therapy (λ685 nm) or polarized light (λ400–2000 nm). *Background Data:* There are many reports on different effects of several types of phototherapies on the treatment of distinct conditions, amongst them, on wound healing. Laser therapy and the use of polarized light are still controversial despite successive reports on their positive effects on several biological processes. *Methods:* Thirty male Wistar rats, approximately 4 months old, were used, and standardized excisional wounds were created on their dorsum. The wounds were irradiated in four equidistant points with laser light or illuminated with polarized light, both with doses of 20 or 40 J/cm2. Group 1 acted as untreated controls. Animals were irradiated every 48 h during 7 days, starting immediately after surgery, and were humanely killed on the 8th post-operative day. Specimens were taken and routinely processed and stained with H&E, and for descriptive analysis of myofibroblasts and collagen fibers, the specimens were imunnomarked by smooth muscle α-actin and picrosirius stain. *Results:* Control specimens showed the presence of ulceration, hyperemia, discrete edema, intense, and diffuse inflammation, collagen deposition was irregular, and myofibroblasts were seen parallel to the wound margins. Wounds treated by laser therapy with a dose of 20 J/cm2 showed mild hyperemia, inflammation varied from moderate to intense, the number of fibroblasts was large, and the distribution of collagen fibers was more regular. Increasing the dose to 40 J/cm2 evidenced exuberant neovascularization, severe hyperemia, moderate to severe inflammation, large collagen deposition, and fewer myofibroblasts. On subjects illuminated with polarized light with a dose of 20 J/cm2, mild to moderate hyperemia was detectable, and collagen matrix was expressive and unevenly distributed; a larger number of myofibroblasts was present and no re-epithelialization was seen. Increasing the dose resulted in mild to moderate hyperemia, no re-epithelialization was seen, edema was discrete, and inflammation was moderate. *Conclusion:* The use of 685-nm laser light or polarized light with a dose of 20 J/cm2 resulted in increased collagen deposition and better organization on healing wounds, and the number of myofibroblast was increased when polarized light is used.

INTRODUCTION

LASER THERAPY results in non-thermal effects in tissues, so its biological effects cannot be attributed to heating.[1] The magnitude of the effects depends on the physiologic status of the cells or the clinical stage of the condition before irradiation, and this may explain why positive biomodulation may not always be detectable.[2]

Controversies regarding doses continue, though previous reports have pointed out that low doses result in positive effects on living organisms.[3,4] On the other hand, higher doses have not been shown to have positive effects on stimulating living tissues.[3,5–7] Wavelength is also important, as visible laser light seems to be more effective in inducing cell proliferation than infrared light.[8] Laser light is monochromatic, possesses high intensity, and is coherent—unlike visible light. The disadvantage of laser light is the possibility of increasing proliferation of malignant cells.[3,9–11]

It has been shown that cellular proliferation is more intense 24 h after irradiation and decreases, in an energy-dependent way, up to 72 h.[12] If the same output power is used but time is increased, the amount of energy within the tissue will increase in the same ratio, so a larger volume of tissue will receive doses within the therapeutic window.[13] Coherent light, in a correct protocol, accelerates the healing process; but incoherent light does not.[14,15] Although coherence is reduced when laser light penetrates tissue, it is still coherent enough to form laser polarized speckles through interference.[13]

Alternative light sources have been used to improve wound healing, and previous reports suggested that polarization is the characteristic of laser light responsible for the biomodulation; because of that, other polarized light sources may also biomodulate biological systems.[6,16]

The present study used morphologic, cytochemical, and imunohistochemical analysis to describe and compare the healing process of wounds submitted (or not) to laser therapy (λ685 nm) or polarized light of λ400–2000 nm (Bioptron[a]) with doses of 20 or 40 J/cm2.

MATERIALS AND METHODS

Thirty young adult male Wistar rats were maintained on environmental conditions of temperature and brightness at the Animal Experimentation Laboratory of the School of Dentistry of the Federal University of Bahia. They were fed standard laboratory pelted diet, had drinking water *ad libidum,* and were maintained on individual cages during the whole period of the study.

Under intraperitoneal general anesthesia (Ketamin® and Midazolam ®, 1:1, 0.15 mL/100 g), one standardized excisional surgical wound was created on the dorsum of each animal with a scalpel. The animals were divided into five groups (*n* = 6) as follows: group 1, untreated control; group 2, laser therapy (λ685 nm, ~2 mm, 40 mW, 20 J/cm2); group 3, laser therapy (λ685 nm, ~2 mm, 40 mW, 40 J/cm2); group 4, polarized Light (Bioptron®, λ400–2000 nm, ~2 cm, 40 mW, 20 J/cm2); and group 5, polarized light (Bioptron®, λ400–2000 nm, ~2 cm, 40 mW, 40 J/cm2). The subjects of groups 2, 3, 4, and 5 were transcutaneously irradiated or illuminated immediately and at every 48 h during 7 consecutive days according to the instructions of the manufacturers.

At the end of the experimental period, the animals were humanely killed by an overdose of general anesthetics and specimens were taken for light microscopy. The specimens were routinely processed and stained with H&E

and Picrosírius stains and immunomarked with α–smooth muscle actin (αSMA); they were then semi-qualitatively or quantitatively analyzed under light microscopy by an experienced oral pathologist.

For the semi-qualitative analysis, two scores were used: (1) mild, moderate or severe; or (2) mild, moderate or intense. The quantitative analysis was based on the counts of the number of immunomarked cells in an area delimited by a grid. The parameters used as markers of the healing process were re-epithelialization, interstitial edema, hyperemia, collagen deposition, inflammatory reaction, and myofibroblast counts.

RESULTS

Controls

Light microscopy showed the presence of ulceration, underlined by granulation tissue and large amounts of vessels sprouts, hyperemia, and discrete edema. Intense and diffuse lymphoplasmocitary inflammatory infiltrate could also be seen at the end of the experimental period. Deposition of collagen was irregular and was restricted to deeper portions of the wound site. At the surface, the collagen deposition was not organized and was mostly observed close to perivascular region (Fig. 1). Immunomarking by α-SMA showed the presence of fibroblast-like cells, here referred as myofibroblasts, whom were paralleled distributed on the wound margins (Fig. 2).

Laser therapy

On wounds treated with 20 J/cm2, despite granulation tissue being richly vascularized, hyperemia was less intense than that observed on control wounds. The inflammatory infiltrate was mostly mononuclear and varied in intensity from moderate to intense (Fig. 3). Epithelial cell were seen migrating on most specimens, and on two of them, the wound was completely reepithelized. The number of fibroblasts was higher than the observed on control specimens and the distribution of collagen fibers was more regular. The density of these fibers was higher deeply on the wounded site (Fig. 4). The underlying connective tissue was more compact than on control samples and adypocites were seen on the wound surface of one specimen.

Immunomarking showed fewer myofibroblasts when compared to controls. Increasing the dose to 40 J/cm2 resulted in exuberant neovascularization and severe hyperemia similar to control specimens. Superficial edema was also observed. There was evidence of re-epithelialization on two subjects. Moderate to severe diffuse mononuclear inflammatory infiltrate was also seen and was more intense than the observed at lower dose (Fig. 5). Increased collagen deposition was observed when compared to controls especially at the wound surface. However, the fibers were smaller; fewer in number; and less organized than the observed with lower dose (Fig. 6). The number of myofibroblasts as immunomarked by α-SMA was smaller than the observed on the controls.

Polarized light

When 20 J/cm2 was used, granulation tissue was present and a mild to moderate hyperemia was detectable, inflammatory cells could be seen at the margins (Fig. 7) and surface necrosis was seen on two specimens. Collagen deposition was expressive and showed different patterns and were not regularly distributed.

Thick bundles of collagen fibers further evidenced the healing. Larger number of myofibroblasts was present (Fig. 8). No re-epithelialization was seen. Increasing

the dose resulted on a richly vascularized granulation tissue with a mild to moderate hyperemia. No re-epithelialization was detectable and discrete edema was seen. A moderate mild mononuclear inflammatory infiltrate was seen as well as localized areas of dense connective tissue. Picrosirius stain showed expressive deposition of parallel collagen fibers (Fig. 9). α-SMA imunnomarking showed a number of cells higher than the observed on control and laser-treated wounds, but smaller than the observed when 20 J/cm/2 was used (Fig. 10).
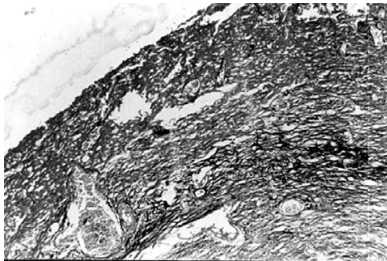

FIG. 1. Photomicrography of a control specimen. Delicate collagen fibers are seen deeper on the wounded site. (Picrosírius: original magnification, X100.)
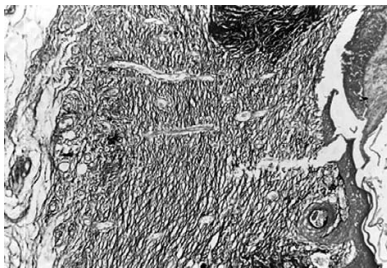
FIG. 4. Photomicrography of specimen irradiated with laser light at a dose of 20 J/cm2. Delicate and parallel collagen fibers are observed. (Picrosírius; original magnification, X100.)
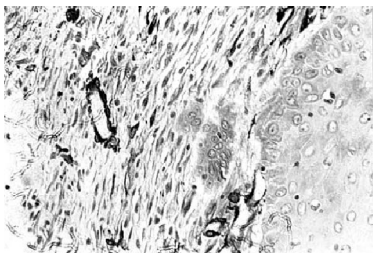
FIG. 2. Photomicrography of a control specimen. Immunoreactivity to α-SMA on myofibroblasts that are seen parallel to the wound surface. (Streptoavidin Biotin; original magnification, X400.)
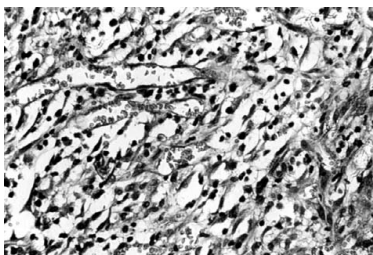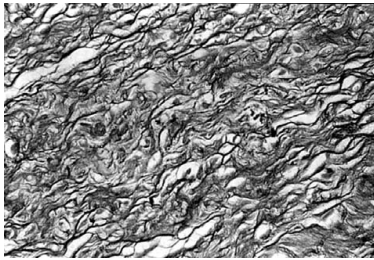

FIG. 5. Photomicrography of specimen irradiated with laser light at a dose of 40 J/cm2. Severe hyperemia, superficial edema, and a moderate to severe diffuse mononuclear inflammatory infiltrate. (H&E; original magnification, X400.)
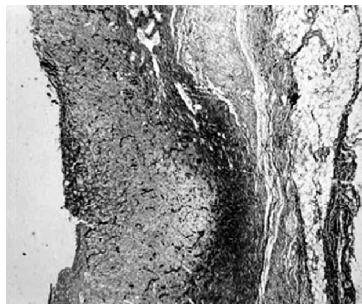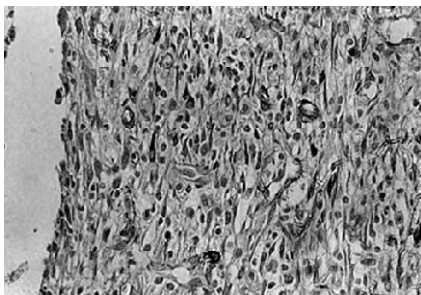
FIG. 3. Photomicrography of specimen irradiated with laser light at a dose of 20 J/cm2. Moderate inflammatory infiltrate, mostly mononuclear. (H&E; original magnification, X400.)

FIG. 6. Photomicrography of specimen irradiated with laser~light at a dose of 40 J/cm2. Higher collagen deposition and a few less organized fibers. (Picrosírius; original magnification, X400.)

FIG. 7. Photomicrography of specimen illuminated with polarized light at a dose of 20 J/cm2. Ulcer and deeper distribution of collagen fibers is observed. (H&E; original magnification, X400.)

FIG. 8. Photomicrography of specimen illuminated with polarized light at a dose of 20 J/cm2. Myofibroblasts immunomarked by α-SMA are sparsely distributed on the wounded site. (Streptoavidin Biotin; original magnification, X400.)
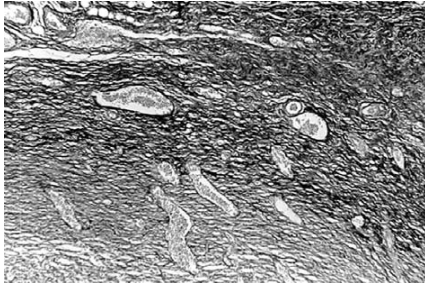
FIG. 9. Photomicrography of specimen illuminated with polarized light at a dose of 40 J/cm2. Collagen matrix formation is distributed in a parallel manner. (Picrosírius; original magnification, X100.)
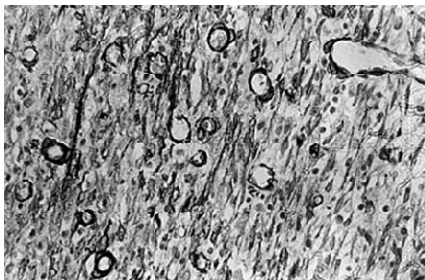

FIG. 10. Photomicrography of specimen illuminated with polarized light at a dose of 40 J/cm2. Small blood vessels and endothelial cells displaying immunoreactivity to α-SMA and the presence of immunomarked myofibroblasts. (Streptoavidin Biotin; original magnification, X400).

DISCUSSION

Light is isotropically scattered in all directions and, because of this, the effects of the light is independent of its the direction due to the isotropic diffusion, which creates spherical equal intensity zones.17 Polarization propagation in biological tissues is a complicated process that is fundamental to tissue optics. Several parameters, such as size, shape, refractive index, concentration of the scattering particles, and incident polarization state play important role in the scattering of light.

Usually when the light passes through biological tissues it follows a tortuous path and scatters many times. As a result of these diffuse multiple scattering, the direction, polarization and coherence are randomized. Mammalian tissues are a highly scattering medium for light with wavelengths between λ600–1300 nm.18

The polarization of the light causes brighter random intensity gradients that can enhance the manifestation of the effect of light coherence when the tissue is illuminated. So, in scattering tissues the coherence length plays the main role, in as much as this parameter determines the depth of the tissue where the coherent properties of the light can be potentially manifested on dependence of the attenuation that is the spatial coherence. This explains why coherence is not important for biological responses on cell monolayers, thin layers and cell suspensions and on tissue surface.19–21 But the effect of the coherence can be observed deeper in the tissues, so the absorption of low-intensity light by biological tissues is purely of non-coherent nature. 19–21 The effects of light therapy is related to both electric and magnetic polarization and not to wavelength.22 It is well known that light polarization remains unchanged trough a thin layer of cell but the polarization is lost after the penetration of a millimeter

or so. The optical penetration in skin is affected by the strong scattering produced mainly by collagen fibers.[23] Linear polarization can be preserved without the complete loss of polarization up to 1.2 mm in human normal skin.[24] The skin is a multi-layered tissue in which the stratum corneum (0.01–0.02 mm thick) shows low light absorption, and the transmitted energy is relatively uniform in the visible region of the spectrum). Approximately 5–7% of the light incident is reflected back to the environment because of the folds in the stratum corneum and the remaining is transmitted to the next layer, the epidermis, this phenomenon is know as surface scattering. The epidermis is about 0.027–0.15 mm thick and also propagates and absorbs light mainly due to the presence of the melanin. The scattering on this layer is mainly due to the presence of particles that are approximately the same size of the wavelength of light (mie scattering). Because of this, the forward scattering is wavelength dependent being broader towards shorter wavelengths.[25] The dermis, 0.6–3 mm thick, also propagates and absorbs light. The scattering on this layer occurs mainly due to the presence of thick collagen fibers and it is know as mie scattering. On the other hand, thinner collagen fibers and other microstructures are responsible for Rayleight scattering.[25] The light gets scattered several times on this layer before being either absorbed or propagated to the next layer. The hypodermis is an adipose tissue characterized by negligible absorption of light in the visible region of the spectrum. The presence of the adipose deposits causes the back reflection of the incident visible light to the upper layers.[25]

The incoherent light emitting polarized light is able to induce biostimulative effects in living cells similar to low-level lasers. As the Bioptron[a] lamp combines visible light at λ480–700 nm and infrared light at λ700–2000 nm, it is a low power light source such as low-level laser, but it is polychromatic and incoherent. Several of the mechanisms are responsible for photobioestimulating effects of both parts of the electromagnetic spectrum present in this polychromatic light source. These lead to the same final photoresponse, but start the cascade of metabolic events at different cellular levels.

One of the main effects of the absorption of visible light is the stimulation of the mitochondria, which results in increased cell energy and activation of nucleic acid synthesis, essential for wound repair. On the other hand, the use of infrared light also results in the same, being the process initiated by a response at the membrane level. Biomodulation is influenced by common characteristic of polarization of both types of light.[13]

A linearly polarized light has a particular effect on the bilipid layer of the cellular membrane as the polarized ends of lipids tend to rotate towards the electrical source changing its structure. Transference of energy from the lipids to proteins and consequent reorganization of the cellular membrane occurs due to a closer contact. These aspects interfere with all membrane-regulated processes.[16,26]

The protocol used on the present investigation is based upon previous study carried out by our group which showed promising results.[27] The use of different doses was due to the fact that up to now no definite treatment parameter for laser therapy has been defined and there is still need for further investigations. [28–31]We decided to use 20 and 40 J/cm2 due to the experience of our team as well as previous reports that suggested that most biological response to laser therapy is found when doses of 1–50 J/cm2 are used.[32]

The timing of sessions followed the average suggested by previous reports.[33,34] The timing for sacrifice was based on previous study that reported

increased number of myofibroblasts between the 8th and 15th post-operative day.35 The presence of myofibroblast as early as the 4th post-operative day suggests early use of therapies. 36 A study with CO2 laser and scalpel wounds also found higher number of these cells at the 8th post-surgical day, similar to the present findings.37

The results of the present study regarding the collagen fibers evidenced a positive biomodulatory effect for both therapies as irradiated or illuminated subjects showed a more expressive expression of these structures. Increased amount of collagen fibers was reported previously following several laser therapy protocols.30,38,39 Similarly, studies carried out using polarized light sources also reported positive biomodulatory effects on wound healing.22,40,41 Polarized light may reproduce nearly 80% of the effects of the laser, but non-polarized light may not.42 Polarization has also been suggested as an important factor on tissue responses.26,43 Despite earlier reports suggesting that laser light may inhibit or have no effect on wound healing, meticulous analysis of evidence and problems on the parameters used30,44–48 or consequence of the systemic effect of the laser therapy remain.49,50

It is important to consider that the use of coherent and incoherent light results in different effects on living tissues and most studies suggested that the use of coherent light achieves better results.13 Considering that semiconductor lasers are coherent and mostly partially polarized, when the laser beam reaches the surface it becomes further polarized due to the formation of laser speckles. The degree of polarization decreases in a direct relationship of the depth of penetration within the tissues.13 The depth of the penetration of the laser light seems to be independent of the power and that biological effects depends on the absolute value and absolute penetration being these factors decisive to the intensity of the effect on the electrical field across the cellular membrane deep into the tissue. The effects of the laser therapy may be observed at least 4 cm deeper. Broadband non-coherent polarized light, such as the used on this study, results in biostimulative effects on more superficial lesions.13

The results of several studies demonstrated that polarized light have a significantly beneficial effect on wound healing due to a faster epithelialization, less exudation, improved quality of early scar tissue formation, quicker wound closure and increased tensile strength. Polarized light was also found to trigger human cellular and humoral defenses and increase the release of growth factors, cytokines, and increase collagen synthesis. The use of polarized light may also affect the local peripheral vasodilatation, which may enhance the blood flow of the skin and the delivery of oxygen to the wounded area, facilitating the transport of the nutrients to the site.40,51–57

The use of α-SMA as marker for myofibroblasts was due to the fact that it is specific for the contractile form of this type of cell.37,58,59 Those undergoing laser therapy showed a more uneven distribution and a smaller number than controls. The result of present investigation is aligned with previous reports, which also found fewer myofibroblasts on irradiated subjects.60,61

The use of polarized light also resulted in increased numbers of myofibroblast on illuminated subjects when compared to controls for both doses (20 or 40 J/cm2). Previous studies on wound healing stated that smaller numbers of myofibroblasts are related to smaller wound contraction during healing;37,62 this is despite the results found in this study, which showed that the use of polarized light resulted in large number of myofibroblasts and consequently it

would result on larger wound contraction, which is not desirable in most cases. However, wound contraction may favor wound healing in cases in which this phenomenon would accelerate the closure of the wound such as on extensive burns or ulcerations.

This study demonstrated that both treatments affected the number of myofibroblasts on opposite ways: Lasertherapy reduced and polarized light increased the number of cells. The reason for this is not completely clear but some characteristics such as coherence and monochromaticity, which differentiate both lights, may be responsible for the result. Dose was also shown to have influence on the outcome of the treatment as on both cases, the use of 20 J/cm2 resulted on better effects on the healing process resulting on better organization and distribution of collagen fibers and influencing the numbers of myofibroblast, as other study.[31] The use of λ904-nm laser light and different doses resulted increased collagen production, faster wound closure and less wound contraction on irradiated subjects and concluded that increasing doses could improve healing up to a threshold where inhibitory effects can be noticed.[30]

The reason why better results are observed when lower doses are used may be explained by a previous study using λ632.8 nm and doses of 10, 20, 30, and 40 J/cm2 and found positive biomodulatory effects for all doses was detectable and was progressive to a point in which it started to decrease.[28]

The present study indicates that the use of λ685-nm laser light or polarized light with a dose of 20 J/cm2 resulted in increased collagen deposition and better organization on healing wounds, and that the number of myofibroblasts is increased when polarized light is used.

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