Collagen birefringence in skin repair in response to red polarized-laser therapy

Daniela de Fátima Teixeira da Silva
IPEN-CNEN/SP
Instituto de Pesquisas Energéticas e Nucleares-Comissão Nacional de Energia Nuclear/SP
Centro de Lasers e Aplicações
CEP 05508-000, São Paulo
São Paulo, Brazil
E-mail: dftsilva@ipen.br

Benedicto de Campos Vidal
IB-UNICAMP
Instituto de Biologia
Universidade Estadual de Campinas
CEP 13084-971, Campinas
São Paulo, Brazil

Denise Maria Zezell
IPEN-CNEN/SP
Instituto de Pesquisas Energéticas e Nucleares-Comissão Nacional de Energia Nuclear/SP
Centro de Lasers e Aplicações
CEP 05508-000, São Paulo
São Paulo, Brazil

Telma Maria Tenório Zorn
ICB-USP
Instituto de Ciências Biomédicas
Universidade de São Paulo
CEP 05508-000, São Paulo
S ão Paulo, Brazil

Silvia Cristina Núñez
Martha Simões Ribeiro
IPEN-CNEN/SP
Instituto de Pesquisas Energéticas e Nucleares-Comissão Nacional de Energia Nuclear/SP
Centro de Lasers e Aplicações
CEP 05508-000, São Paulo
São Paulo, Brazil

Abstract. We use the optical path difference (OPD) technique to quantify the organization of collagen fibers during skin repair of full-thickness burns following low-intensity polarized laser therapy with two different polarization incidence vectors. Three burns are cryogenerated on the back of rats. Lesion $L_{\parallel}$ is irradiated using the electric field vector of the polarized laser radiation aligned in parallel with the rat's occipital-caudal direction. Lesion $L_{\perp}$ is irradiated using the electric field vector of the polarized laser radiation aligned perpendicularly to the aforementioned orientation. Lesion C is untreated. A healthy area labeled H is also evaluated. The tissue samples are collected and processed for polarized light microscopy. The overall finding is that the OPD for collagen fibers depends on the electric field vector of the incident polarized laser radiation. No significant differences in OPDs are observed between $L_{\parallel}$ and H in the center, sides, and edges of the lesion. Lesions irradiated using the electric field vector of the polarized laser radiation aligned in parallel with the rat's occipital-caudal direction show higher birefringence, indicating that collagen bundles in these lesions are more organized. © *2006 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.2187418]

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1 Introduction

The process of wound healing is dependent on the coordination of numerous cellular processes. It consists of an inflammatory phase, a proliferative phase, and a remodeling phase. The remodeling phase includes the reorientation and reorganization of the granulation tissue into a scar.[1]

The current interest in the effects of low-intensity laser therapy (LILT) on wound healing arose from the studies of Mester et al.[2] The advantageous use of the laser radiation is mostly observed in the treatment of persistent wounds. In particular, the irradiation of wounds with helium-neon laser (HeNe, radiation at 632.8 nm) has demonstrated an acceleration of healing in animals and humans.[3] The main reason for using the light sources radiating in the red and near-IR spectral region is that water and hemoglobin have a weak absorption in the range of 600 to 1300 nm. In addition, photons are preferably scattered in the forward direction, promoting a high transdermal penetration of light.[4]

Trends in LILT research have looked for grounding the effects of the laser radiation on wound healing *in vivo*. More particularly, an augmented knowledge of the physical characteristics of the radiation is desirable for the effective application of LILT. For example, in biological tissue, laser random speckles are less pronounced than the speckles caused by laser radiation with a preferential direction and orientation, thus, this phenomenon could enhance the effects of radiation

Address all correspondence to Daniela de Fátima Teixeira da Silva, Center for Lasers and Applications, IPEN, Av. Lineu Prestes, 2242, São Paulo 05508–000, Brazil. Tel: +55 11 3816 9313; Fax: +55 11 3816 9315; E-mail: dftsilva@ipen.br

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coherence when the tissue is irradiated.[5] Furthermore, a recent morphological semiquantitative study concerning LILT effects on wound healing showed that a specific direction of the polarized laser incidence could improve the healing process.[6]

The polarization state of light can be detected and quantified.[7–12] This fact is particularly significant when birefringent tissues are evaluated for diagnostic purposes, because birefringent tissues present remarkable modifications due to the transition from normal to a pathologic state.

The collagen content of the skin is particularly important, since combined with elastin in the dermis it provides the extracellular matrix on which the impermeable epidermal layer is grown. The loss of the collagen structure and integrity is frequently associated with abnormalities of the skin, including skin cancer and thermal injury. Thus, birefringence measurements may be seen as a valuable diagnostic indicator of the skin condition.[13]

Polarized light microscopy is an example of a tool to analyze birefringent material such as collagen, providing information about the orientation and distribution of the collagen.[14] On the illumination of the sample on the polarized microscopy the ratio between incident/emergent light is changed by the retardation or by the optical path difference (OPD) of the object.[15] When light contacts a birefringent material, the light wave is decomposed into two perpendicularly polarized beams; one beam is polarized along the slowest direction and another along the fastest direction. Polarized light along any random orientation will become elliptically polarized due to the phase difference (δ). If the incoming polarization is in the same direction as either the slowest or the fastest direction, the light will remain linearly polarized when exiting the sample, giving a brighter image usually called birefringence brightness. δ is proportional to the thickness of the sample (L) and the difference in refractive indices (Δn) and it is inversely proportional to the wavelength of the light (λ): $\delta = (2\pi/\lambda)L\Delta n$, where $L\Delta n$ is the optical retardation or OPD.

Although the OPD technique constitutes a powerful instrument to perform quantitative analysis during the healing process, to the best of our knowledge collagen birefringence measurements following LILT have never been reported. In this paper, we quantify the organization of collagen fibers in burned skin during the healing process following LILT applied in two different directions of incidence of the electric field vector of the polarized laser radiation through the OPD technique.

2 Materials and Methods

Twenty male adult Wistar rats weighing 300 g each were studied. The animals were anesthetized with Avertin (0.025 mL/g body mass) and had their backs shaved. Due to the individual variability in the duration and quality of regeneration, each experimental animal acted as its own control. Thus, irradiated and nonirradiated (control) deep third-degree burns were created in the same rat. Three round burns measuring about 6 mm in diameter were cryogenerated at the end of the spinal column of each animal using a cylindrical brass rod cooled to 77 K. The brass rod was kept in contact with the skin of the animal in the areas that received the injury. The contact was made in two sequences of 5 s each with an interval of 5 min. This procedure was repeated for 3 days. After

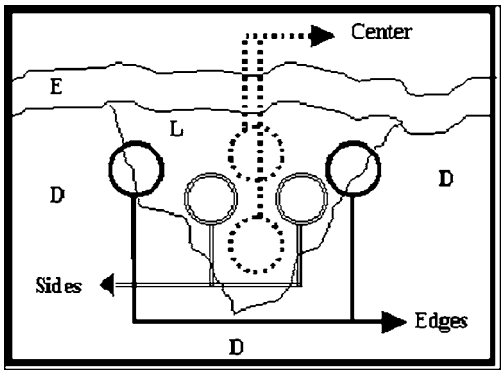

Fig. 1 Areas analyzed with polarization microscope: center, sides, and edges of lesion; E (epidermis), D (dermis), and L (lesion).

the last application, lesion $L_{\parallel}$ was irradiated using the electric field vector of the incident polarized laser radiation aligned in parallel with the rat's occipital-caudal direction, lesion $L_{\perp}$ was irradiated using the electric field vector of the incident polarized laser radiation aligned perpendicularly to the aforementioned orientation, and lesion C was not irradiated (control). The dose was 1 J/cm^2 per irradiation, corresponding to an exposition of approximately 3 min. The laser dose was chosen according to previous studies, which are of relevance for procedures on soft tissues.[6,16] Four animals were irradiated and sacrificed at days 3, 7, 10, and 14 after the burn creation. The last animals were killed at day 17 postwounding (p.w.). During the experiment, the rats were singly caged in a 12-h light/dark schedule at 22 °C, with access to food and water *ad libitum*. National and international principles of laboratory animal care were followed.

The light source was a HeNe laser at $\lambda = 632.8$ nm, with 10 mW of output power (Uniphase, USA) mounted in a convenient setup. A lens system and an optical filter were used to ensure an uniform exposure at the wound position, obtaining an expanded beam with 6 mW at 1 cm^2. A Glan-Thompson prism was inserted in the beam path to obtain a plane-polarized beam. The polarizer was held on a precision disk, which enabled us to rotate it 90 deg, and thus, change the direction of the incident polarization. The output power of the laser was confirmed prior to irradiations, using a power meter (LaserCheck®—Coherent, USA).

After sacrifice, the areas of the skin under study were collected and fixed routinely by immersion in Bouin's solution at 4 °C for 24 h and thereafter dehydrated and embedded in paraffin to provide 5-μm transversal sections of the skin. After cutting, the samples were deparaffined. A healthy skin area labeled "*H*" was also processed and analyzed.

Three slides containing two sections from each group "*C*," "$L_{\parallel}$," "$L_{\perp}$," and "*H*" were analyzed. Within each section were carried out six measurements, called center 1, center 2, side 1, side 2, and edge 1, edge 2 (Fig. 1). The same measures were performed in the healthy skin sections.

To evaluate the total birefringence (intrinsic and textural) of the samples, sections were mounted with distilled water, which was placed between the slides. This procedure was performed to reduce the refractive index mismatch, which leads to considerable alteration of OPD between slides. The mea-

surements were made with a Zeiss polarized light microscope equipped with a 10× objective and a mica λ/4 compensator. The same compensator was used to monitor the stability of the system during the experiments: the compensator, without the fibers, was used to obtain standard measurements after each register to guarantee and to test the system stability. Light of wavelength equal to 550 nm was obtained with an interference filter and was used in all of the analyses.

There is a phase difference between the intensity of the incident light and the intensity of the emergent light, which is altered by the retardation of the object and by the polarization system. The angle δ obtained through the measurements was converted to the OPD (in nanometers) of the birefringent material and was calculated using the Brace-Kohler method as follows:[17]

$$\mathrm{OPD} = -47.20 \sin(2\theta),$$

where −47.20 is the retardation of the compensator, derived from the ratio λ/11.65, where λ is the incident wavelength, in this particular case 550 nm; and 11.65 is the constant of the compensator resolution; θ is 45 deg-δ, where 45 deg is the position of the long axis of the collagen bundles relative to the electric vector of the plane of the polarization system.

The mean and the standard deviations of the OPD values were computed. Statistical analyses were accomplished using Student's t test, to compare different values obtained among groups C, $L_{\parallel}$, $L_{\perp}$, and H. Significance was accepted at $p < 0.01$.

3 Results

During the first 14 days p.w., there were no statistical significant differences in OPD values among C, $L_{\parallel}$, and $L_{\perp}$ in each area analyzed. Significant differences were observed between H and the other specimens in this period.

On the 17th day p.w., the overall finding was that the OPD depends on the electric field vector of the incident polarized laser radiation and on the area analyzed within each lesion. Note that the OPD showed a minimum value in the center in comparison to the sides and the edges of the lesion. The OPD values were lower in the center than at the sides and edges, and they progressively increased approaching the lesion edges.

Analyzing the center of the lesion, group C presented an OPD mean value of 9.78±1.79 nm; $L_{\parallel}$, 12.51±1.97 nm, $L_{\perp}$, 8.59±1.02 nm; and H presented 13.16±1.65 nm (Fig. 2). No statistically significant differences were observed between C and $L_{\perp}$, and $L_{\parallel}$ and H ($p > 0.01$). There was significant statistical difference between $L_{\parallel}$ and $L_{\perp}$, $L_{\parallel}$ and C, C and H, and $L_{\perp}$ and H ($p < 0.01$).

The OPDs measured in the lesion sides for C was 10.91±2.81 nm; $L_{\parallel}$, 14.59±2.73 nm; $L_{\perp}$, 13.61±2.29 nm; and H, 15.50±3.03 nm (Fig. 2). Significant differences were observed between C and $L_{\parallel}$ and between C and H; however, no statistically significant differences were detected between C and $L_{\perp}$, $L_{\parallel}$ and $L_{\perp}$, $L_{\perp}$ and H, and $L_{\parallel}$ and H.

At the lesion edges, the groups C, $L_{\parallel}$, $L_{\perp}$, and H exhibited OPDs of 13.93±3.50, 15.18±2.44, 16.90±2.15, and 15.16±2.89 nm, respectively (Fig. 2). No significant differences among the samples were observed. Noteworthy was that $L_{\parallel}$ and H exhibited a similar OPD mean value at the lesion center, sides, and edges.

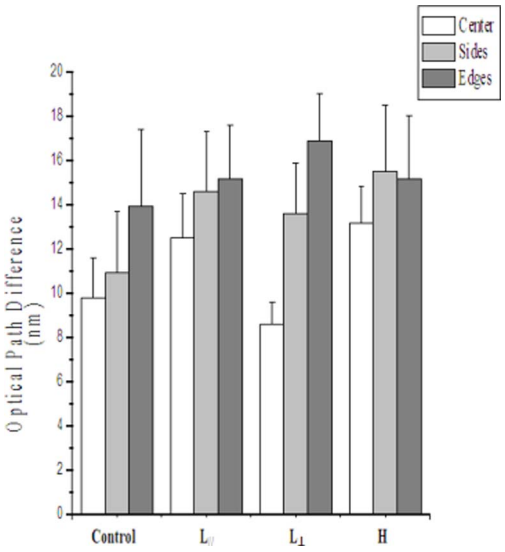

Fig. 2 For OPDs measured at the lesion center, no significant differences were observed between C and $L_{\perp}$ and $L_{\parallel}$ and H ($p > 0.01$); there was significant difference statistically between $L_{\perp}$ and $L_{\parallel}$, $L_{\parallel}$ and C, C and H, and $L_{\perp}$ and H ($p < 0.01$). For OPDs measured at the lesion sides, significant differences were observed between C and $L_{\parallel}$ and between C and H ($p < 0.01$); no statistically significant differences between C and $L_{\perp}$, $L_{\parallel}$ and $L_{\perp}$, $L_{\perp}$ and H, and $L_{\parallel}$ and H were observed ($p > 0.01$). For OPDs measured at the lesion edges, there were no significant differences among the samples ($p > 0.01$).

4 Discussion

In this study, we observed the ability of the components of the skin to alter the polarization state of light. This finding can be attributed to the birefringent nature of collagen fibers, which along with elastin fibers, provides the dermal layer with mechanical strength and organization.

There are two types of birefringence, intrinsic birefringence and textural birefringence. The first type is determined by the orientation, oscillation, and the strengths of all electronic transitions of the molecules that constitute the filaments. The second is dependent on partial volume (concentration), aggregational state, and orientation of the bundle components.[18] Birefringence measurement remains a powerful technique to study molecular order and the degree of ordered aggregation (packing conditions) in collagen bundles. Changes in either the collagen fibrils or in the extrafibrillar matrix can both, therefore, alter birefringence. A direct illustration of this is provided in situations where the normal structure has been disrupted. For example, the thermal denaturation temperature of collagen range from 70 to 100 °C, and in this range, the individual fibrils that makeup the fibers unravel and there is a reduction in the birefringence.[19]

Our results agree with those of Tang et al.,[20] who demonstrated that thermally denatured collagen does not exhibit birefringence since the collagen fibers might become merged into an amorphous random state. In fact, birefringence loss is due to the induced necrosis and to the collagen denaturation

that corresponds with the separation of the α-chain from the triple helix. In addition, the importance of the action of the metalloproteinase matrix in the course of inflammation must be taken in account regarding the collagen fibers breaking down and birefringence loss.[21]

During the first 14 days p.w. no significant differences were observed between irradiated and control lesions, but both exhibited a lower OPD value than H for each area analyzed. It is well known that a high fibroblast density, thin randomly oriented collagen fibers, and a rich capillary bed characterize early granulation tissue development, which contributes to a low of OPD value. These characteristics explain our results in the first 14 days.

On the 17th day, no significant differences were observed between H and $L_{\parallel}$, which presented the highest birefringence in the center of the lesion. Also, no significant differences were observed between C and $L_{\perp}$, which presented the lowest birefringence in the mentioned area. These results suggest that lesions $L_{\parallel}$ are in a more advanced repair stage because the birefringence in this group was similar to that from healthy skin.

The birefringence measured outside of the burn center tended to increase with the distance and reached the normal level at the edges of the lesion. This finding is probably caused by increased healthy tissue content, decreased repaired tissue content, and better fibril organization, which is consistent with a secondary intention repair and in conformity with the results obtained through birefringence measurements in the cornea.[19]

Also on the 17th day, the groups C and $L_{\perp}$ showed no significant differences when analyzed at the lesion center, sides, and edges. This finding suggests that the electric field vector of the polarized laser radiation that was perpendicularly aligned to rat's occipital-caudal direction led to analogous effects to untreated wounds. Consequently, the birefringence is low. This fact is according to Berry et al.,[22] who showed that the wound granulation tissue of untreated rats has minimal birefringence at the third week of wound healing.

To summarize, the birefringence analysis showed that the orientation of the collagen bundles represented by OPDs seems to be dependent on the relative orientation between the electric field vector of the polarized laser radiation and a reference direction on the tissue (rat's occipital-caudal direction). The electric field vector of the polarized laser radiation has a competent effect on the collagen organization in the dermis as observed by the fact that both $L_{\parallel}$ and H exhibited higher birefringence 17 days p.w., while C and $L_{\perp}$ exhibited smaller birefringence during the same period.

Some possible factors that may contribute to this variation of OPD during wound healing include the fact that the collagen fibers are randomly distributed in the dermis at the beginning of the healing process, and the thickness of the collagen fibers varies: fibers are thinner in the beginning of the healing process (newly synthesized collagen or younger collagen fibrils) and thicker in the final process.

The propagation of polarized light through a biological tissue is an intricate process because it leads to a change in the polarization status of photons owing to tissue birefringence and tissue scattering.[12] Since biological tissues are not homogeneous samples, parameters such as the size, shape, and density of the scatters in the medium as well as the polarization state of the incident light are relevant factors.[23]

It is well known that light polarization remains unchanged through a thin layer of cells; however, in highly scattering mediums, such as living tissue, the light is depolarized after a penetration of a millimeter or so. However, the epidermis and the initial papillary dermis apparently allow the penetration of linearly polarized light with modest depolarization.[7] The light can travel a distance of 1.2 mm in normal human skin without the complete loss of the linear polarization.[23] In fact, Sankaran et al. demonstrated the different patterns of depolarization for linear and circular incident polarization for different tissues. Their results indicate that for dense tissues, linearly polarized light is better preserved than circularly polarized light.[11]

Note that in this study linear polarized light was used to irradiate the tissue during the first 14 days of healing process. The extracellular matrix (ECM) is the scaffold of the skin that supports cells in either unwounded or wounded states. The ECM is dynamic during healing process; it is constantly undergoing remodeling.[24] Thus, one can expect that light propagation into a live tissue during the healing process will change due to the dynamic changes inside the tissue. After the initial inflammatory phase, the early wound matrix is gradually replaced by granulation tissue. Indeed, the transmittance of an He–Ne laser ($\lambda = 632.8$ nm) and a semiconductor laser ($\lambda = 675$ nm) in granular tissue is about 2.5 times higher than in normal skin.[25]

In addition to transmittance, the degree of linear polarization was investigated in healthy and burned skin.[26] The results indicated that linearly polarized light could survive in the superficial layers of skin; furthermore, the preservation was even higher in burned skin. Moreover, a more recent work investigated the depolarization properties of gamma-irradiated pig skin at different wavelengths. The authors observed that for the red wavelength, an increase of gamma irradiation dose, which results in skin erythema, decreased the depolarization. As a result, healthy skin promotes more depolarization than a 20-Gy-irradiated skin sample.[27]

A dense population of blood vessels, macrophages, and fibroblasts embedded within a loose provisional matrix of fibronectin, hyaluronic acid, and collagen characterize the granulation tissue that covers the wound at the beginning of the healing process. The high cellular content and the increased cellular activity of the granulation tissue should contribute to the optical changes during healing process, since Mourant et al. showed that changes in light scattering from both isolated cellular nuclei and cells can be correlated with the DNA content.[28]

Our findings suggest that in the center of the burn on the 17th p.w., differences were observed between $L_{\parallel}$ and $L_{\perp}$, indicating that the irradiation $L_{\parallel}$ might have anticipated the remodeling phase of the healing process. In fact, our results, as well as those reported previously, demonstrated the importance of the direction of incidence of the electric field vector of the polarized laser radiation, since on day 17 p.w. it was reported[6] that in $L_{\parallel}$, both cellular and extracellular components, as collagen fibrils, appeared to be more organized and thick than in $L_{\perp}$. Linearly polarized light presented better therapeutic results; in addition, a preferential direction of the

light incidence was also considered a relevant parameter.

According to Nickell et al., scattering in the skin is anisotropic, with a factor of up to 2 between orthogonal directions.[29] The observed scattering anisotropy can be described by assuming a preferential orientation of the collagen fibers in the dermis. As well as the Nickell et al. results, in our study, the awareness of the anisotropy in burned skin could help in the interpretation of the results. If the occipital-caudal direction of incidence is running parallel with the tension lines, which comprise an anatomical equivalent consisting of a preferential parallel orientation and a straightening of collagen bundles,[30] it is possible to hypothesize that the polarization memory is better preserved in this direction than perpendicularly with the tension lines, and thus, it could assist in cutaneous wound healing acceleration.

Although some reports have suggested that treatment with polarized light may accelerate wound healing,[5,31–33] literature is still scarce concerning the influence of laser-induced biomodulation on skin repair regarding polarization. For example, it would be interesting to compare the directions of minimum light scattering to those of the skin tension lines to discover the component that makes the difference between the studied incidence directions.

5 Conclusion

Our results demonstrated that the electric field vector of the polarized laser radiation affects the collagen organization in the dermis. Lesions irradiated using the parallel polarization aligned with the rat's occipital-caudal direction showed higher birefringence, indicating that collagen bundles in these lesions are more organized than lesions irradiated using the perpendicular relative direction.

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