Journal of Clinical Laser Medicine & Surgery
Volume 22, Number 1, 2004

Pp. 59–66

Effects of Low-Intensity Polarized Visible Laser Radiation on Skin Burns: A Light Microscopy Study

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ABSTRACT

***Objective*: This study was carried out to investigate the influence of low-intensity polarized visible laser radiation on the acceleration of skin wound healing. *Background Data*: Low-level laser therapy (LLLT) at adequate wavelength, intensity, and dose can accelerate tissue repair. However, there is still unclear information about light characteristics, such as coherence and polarization. Some studies indicate that linearly polarized light can survive through long propagation distance in biological tissue. *Materials and Methods*: Three burns about 6 mm in diameter were created on the back of rats with liquid N_2. Lesion "$L_{//}$" was irradiated by He-Ne laser (λ= 632.8 nm), D= 1.0 J/cm^2, with linear polarization parallel to the spinal column of the rat. Lesion "$L_{\wedge}$" was irradiated using the same laser and dose, but the light polarization was aligned perpendicularly to the relative orientation. Lesion "C" was not irradiated in order to be considered as control. The animals were sacrificed at day 3–17 after lesion creation. Samples were collected and prepared for histological analysis. *Results*: Histological analysis showed that the healing of irradiated wounds was faster than that of non-irradiated wounds. Moreover, it was observed that skin wound repair is dependent on polarization orientation with respect to a referential axis as the animal's spinal column. Consequently, "$L_{//}$" was completely healed after 17 days, whereas "$L_{\perp}$" showed a moderate degree of healing after the same period. *Conclusions*: These results indicate that the relative direction of the laser polarization plays an important role in the wound healing process when highly coherent He-Ne laser is used.**

INTRODUCTION

Low-level laser therapy (LLLT) is largely used in clinical practice in the treatment of rheumatoid arthritis, pain management, dental diseases, herpes treatment, healing of trophic ulcers, and healing of indolent wounds and burns.[1–3] The physical model and the biological reasons for the effectiveness of laser treatment have not yet been clarified. Although several studies have been published, the results are frequently conflicting, and only a few have presented some scientific argument.[4–8] Moreover, there is little information about the dependence of the irradiation dose, wavelength, laser regime, and intensity on the beneficial effects of LLLT, and few double-blinded studies in clinical trials.

In previous studies using burns created by the application of liquid nitrogen on the skin of rats, we noticed that the use of polarized laser light can enhance the stimulation of healing, if compared to the lesions irradiated with non-polarized laser light.[9] Those experiments were performed using a low-intensity He-Ne laser beam, linearly polarized. However, the relative direction of the polarization was arbitrarily set. The theoretical approach then consisted of a simple model that connected the effect of the polarized light with the microscopically rough tissue using Maxwell's equations for optical properties of surfaces.[10]

In the present study, we evaluated the role of the relative orientation of the laser polarization in LLLT stimulation of skin wounds artificially produced on the back of adult rats. A highly

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coherent low-power laser source was used: He-Ne gas laser at $\lambda = 632.8$ nm. We used the spinal column of the rats as a preferential axis for the rats' back skin optical properties, and we aligned the linear laser polarization parallel, and then perpendicular to this axis. The wounds firstly were monitored by histological sections of skin, which allowed the morphological evaluation of the effects of the visible laser therapy on the organization of the epithelial and connective tissue and its cells during the process of skin repair.

MATERIALS AND METHODS

The source of laser light was a non-polarized He-Ne 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 a 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 in order to obtain a linearly polarized beam. The polarizer was held on a precision disk, which managed to rotate it 90° and thus change the direction of the incident polarization.

Twenty male adult Lewis rats weighing 300 g each were used. The animals were anesthetized with Avertin (0.025 ml/g body mass) and had their backs shaved. Due to the individual variability on the duration and quality of regeneration, each experimental animal acted as its own control. Hence, irradiated and non-irradiated (control) burns were produced in the same animal. Three round burns measuring about 6 mm in diameter were created at the end of the spinal column of each animal using a cylindrical brass rod cooled at 77 K[9] and randomly assigned to different treatments. The brass rod was kept for 5 sec on each animal, twice a day with an interval of 5 min for 3 days. After the last application, lesion "$L_{//}$" was irradiated using laser polarization aligned in parallel with the rat's spinal direction, lesion "$L_{\perp}$" was irradiated using laser polarization aligned perpendicularly to the relative orientation, and lesion "C" was not irradiated (control). The total dose was 1 J/cm^2 per irradiation, corresponding to an exposure of approximately 3 min.

Four animals of each group were irradiated and sacrificed at days 3, 7, 10 and 14 after the wound creation. The last animals were sacrificed at day 17 post-wounding (p.w.) according to the schedule shown in Table 1. During the experiment, the rats were singly housed in solid-bottomed cages in a 12-h light/12-h darkness schedule at 22ºC, with unlimited access to food and water. National and international principles of laboratory animal care were followed.

After sacrifice, the areas of the skin containing the wounds were collected and fixed by immersion in Bouin's liquid at 4ºC for 24 h. The tissues were dehydrated and thereafter embedded in Paraplast (Oxford, USA). The specimens were embedded so as to provide transversal sections of the skin. The 5-µm sections were stained with hematoxylin and eosin. They were then observed and photographed with a Nikon Labophot AFX-II light microscope.

The histological analysis of the specimens was done in a blind test; that is, prior to the examination, the observers were not informed about the experimental conditions of the samples. At the same time, analysis was independently carried out by two histologists.

At least four slides containing three sections each from each animal were analyzed. A semiquantitative evaluation was performed using the following morphological parameters: continuity of the surface epithelium, polimorphonuclear infiltration, number and morphological features of the blood vessels (neovascularization), and number and morphological features of the fibroblasts.

The epidermal thickness was measured in distinct locations in a selected area of healthy and repaired tissue 17 days p.w. The average and standard deviations were computed and the significance of the differences between lesions "$L_{//}$" and "$L_{\perp}$" was analyzed by using the ANOVA test. Significance was accepted at $p < 0.05$.

RESULTS

Morphological analysis of the wounds showed that the structural characteristics of the repaired skin is dependent on

TABLE 1. EXPERIMENTAL SCHEDULE: DIFFERENCES AND CHARACTERISTICS OF GROUPS 1–5

	Day 1	*Day 2*	*Day 3*	*Day 7*	*Day 10*	*Day 14*	*Day 17*
Group 1	Lesion	Lesion	Lesion + 1st irradiation + sacrifice				
Group 2	Lesion	Lesion	Lesion + 1st irradiation	2nd irradiation + sacrifice			
Group 3	Lesion	Lesion	Lesion + 1st irradiation	2nd irradiation	3rd irradiation + sacrifice		
Group 4	Lesion	Lesion	Lesion + 1st irradiation	2nd irradiation	3rd irradiation	4th irradiation + sacrifice	
Group 5	Lesion	Lesion	Lesion + 1st irradiation	2nd irradiation	3rd irradiation	4th irradiation	Sacrifice

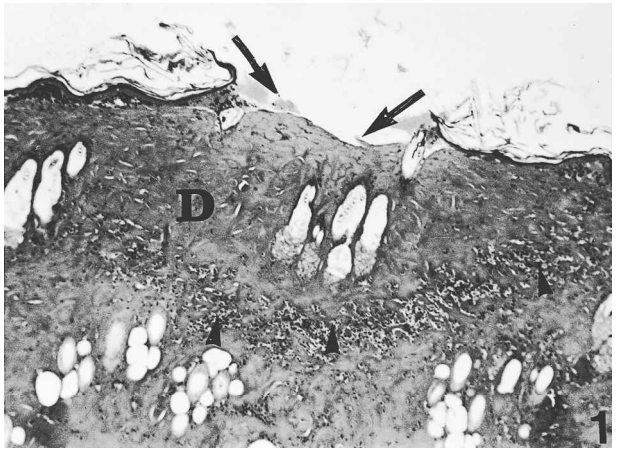


FIG. 1. Lesion "$L_{//}$" showing an injured area 7 days post-wounding. Note that the injured area is devoid of epidermis (arrows). Necrotic areas (arrowheads) can be seen in the subjacent dermis (D). HE, ×200.

the relative orientation of laser polarization. It was also observed that, during the first 7 days p.w., no significant differences were observed when irradiated and control lesions were compared. After 14 days, however, morphological differences were observed among the groups.

On days 3 and 7 p.w. the injured area was devoid of a healthy epidermis in both irradiated and control lesions (Fig. 1). A variety of inflammatory cells and cell debris was present in the dermis subjacent to the damaged epidermis. Neutrophils were the predominating cell type in the superficial dermis, whereas monocytes, macrophages, and giant cells were present in the deep dermis (Fig. 2). Vascularization was also affected, and many small blood vessels were dilated, (most of them filled with blood cells).

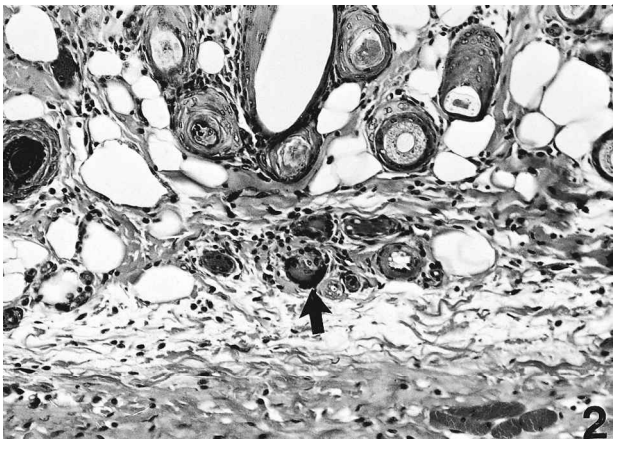


FIG. 2. Higher magnification of the deep dermis from lesion "$L_{//}$" on the 7th day post-wounding showing a mononuclear infiltration and giants cells (arrow). HE, ×400.

FIG. 3. Lesion "$L_{//}$" showing an injured area 10 days post-wounding. The arrow indicates the direction of the reepithelization process. An inflammatory infiltration can be seen on the left bottom (*). Note that the superficial skin still contains cell debris dermis (d). HE, ×200.

At 10 days p.w., a large area of the damaged epidermis was still devoid of epithelial layer, although signals of epidermal wound repair were clearly observed in both irradiated and control lesions (Fig. 3). In both sides of the wound, the epithelial layer had migrated over a very loose connective tissue of the subjacent dermis (Fig. 4). This new loose connective tissue was easily distinguished from the old one because it contained a few and thin collagen fibers and many large fibroblasts with nuclei that were euchromatic and bigger. Although reepithelization of the skin occurred in both non-irradiated and irradiated lesions, it appeared to be more advanced in irradiated lesions.

At 14 days after injury, detailed analysis showed some morphological differences between irradiated and non-irradiated lesions. The most obvious difference was the more advanced epithelization of the skin in irradiated wounds when compared to the controls (Figs. 5 and 6). Another morphological difference between the groups was related to the number of the migratory infiltrated cells, which was much higher in the dermis of the control lesions than in the dermis of the irradiated animals. Accumulation of cell debris was also frequently ob-

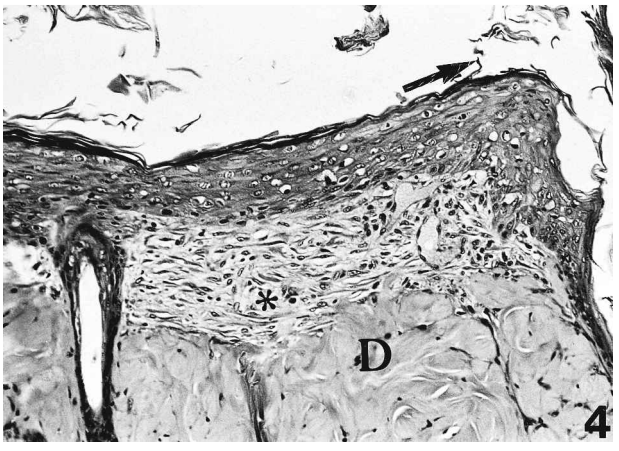


FIG. 4. Higher magnification of epidermis from lesion "$L_{//}$" on the 10th day post-wounding. In the subjacent dermis (D), the fibroblasts have a large cytoplasm indicative of cellular activation (*). The arrow indicates the direction of the reepithelization process. HE, ×400.

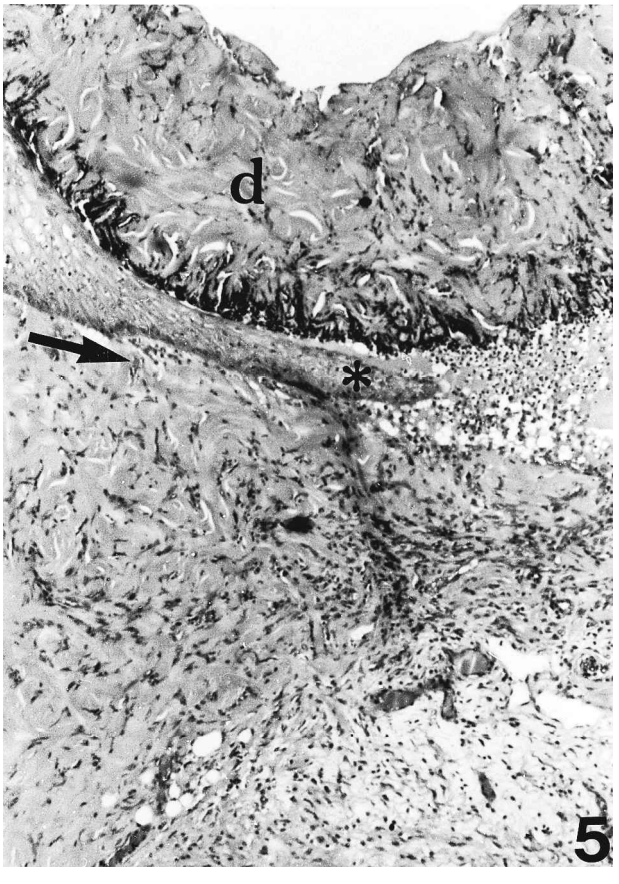


FIG. 5. Lesion "C" (control) showing an injured area 14 days post-wounding. On this day, the epidermis still does not line the complete wounded area (*). Cell debris (d) are deposited on the skin surface. The arrow indicates the direction of the reepithelization process. HE, ×200.

served in the superficial dermis of non-irradiated lesions, particularly in the regions subjacent to the non-epithelalized areas of the skin (Fig. 5).

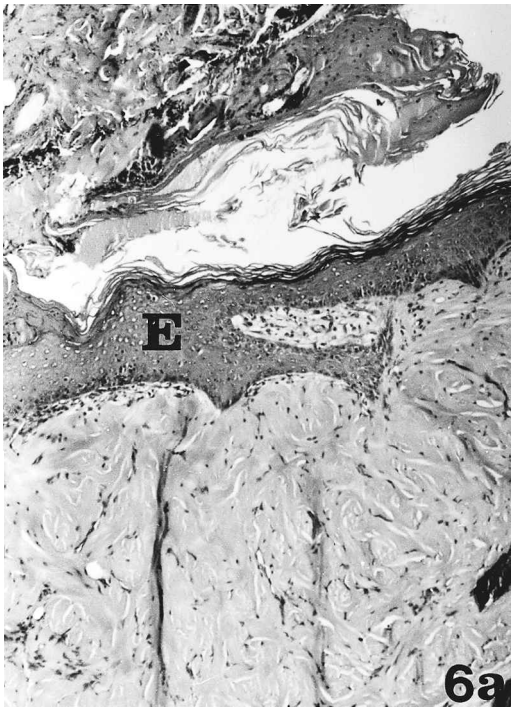
At 17 days after the creation of the burns, part of the injured skin of the control lesions was still devoid of epidermis and many inflammatory cells were still present in the connective tissue of the dermis subjacent to the lesion (Fig. 7). At the same period, however, only rare leukocytes were observed in the dermis of irradiated lesions, which were completely recovered by a well-organized epidermis (Figs. 8 and 9). Something that drew our attention was the fact that, in irradiated lesions, the new epidermis was significantly thicker than that of the healthy skin, and it was formed by several layers of epithelial cells. At this time, the damaged subjacent dermis was replaced by a new loose connective tissue, which was easily distinguished from the old one by the large number of large fibroblasts. The extracellular matrix of the regenerating dermis was also distinguished from the surrounding original dermis by the presence of a few thin collagen fibers immersed in abundant non-fibrilar matrix (Figs. 8 and 9). No appendages of the skin or sebaceous glands were observed in the regions of repaired dermis. A rich network of small blood vessels was present in these areas, and most of them were seen in close proximity to the epithelial layer (Figs. 8 and 9). In this new connective tissue, the fibroblasts were enlarged and exhibited a large basophilic cytoplasm, distinguished among the few acidofilic collagen fibers. The nuclei of the fibroblasts were large and contained loose chromatin and conspicuous nucleoli. In both sides of the wound, the non-injured connective tissue contained predominantly fibrocytes, which had small and dense nuclei and small cytoplasmic areas.

On day 17 p.w., some morphological differences were observed between lesion "$L_{//}$" and lesion "$L_{\perp}$". Compared to lesion "$L_{\perp}$," a large area of the original dermis had been replaced by a reparative connective tissue in lesion "$L_{//}$". Although no quantitative evaluation was performed, it was possible to observe that, in lesion "$L_{//}$," both cellular and extracellular components appeared to be more organized than in lesion "$L_{\perp}$." The blood supply in lesion "$L_{//}$" was also more exuberant than in lesion "$L_{\perp}$" and was represented mostly by venules and capillaries (Figs. 8 and 9). Another important difference between the lesions was the maintenance of inflammatory cells in lesion "$L_{\perp}$," particularly neutrophils. Furthermore, in lesion "$L_{//}$," the new epidermal thickness was statistically significant when compared to the epidermal thickness of lesion "$L_{\perp}$" (Fig. 10). Also, both of them were significantly thicker than the epidermis of healthy skin.

Figure 11 summarizes the main histological results obtained from the analysis of laser treated and untreated burns.

DISCUSSION

The present morphological analysis showed that the improvement of skin repair as well as the structural characteristic of the healing represented by the amount and quality of both reparative connective tissue and inflammatory cells appear to be dependent on the relative orientation between the electrical field polarization and the reference direction (spinal column).



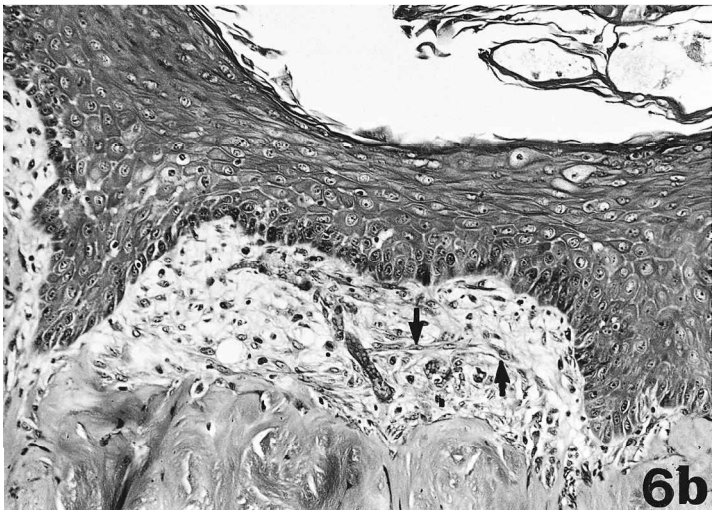


FIG. 6. Lesion "$L_{//}$" showing an injured area on the 14th day of the experiment. **(a)** The dermis is completely repaired by a new epidermis (E). HE, ×160. **(b)** In the superficial dermis, there are fibroblasts with loose chromatin and large cytoplasm (arrows). HE, ×400.

Laser radiation has a competent effect on the epithelial cell proliferation and migration, as both irradiated groups ("$L_{//}$" and "$L_{\perp}$") exhibited a completed and renewed epithelial layer at 17 days p.w., while the control group had not completed the reepithelization process during the same period. This result indicates that laser radiation is able to accelerate epidermis formation. The significant increase of the thickness of the epithelial layer, the great amount of blood vessels, and the higher organization of collagen fibrils observed in lesion "$L_{//}$" strongly suggest that the orientation of the laser polarization may enhance improvement of the wound healing process.

The biostimulatory effects of LLLT have been reported since the initial years of laser development. Many studies focused on the effects of laser photostimulation on a variety of pathologi-

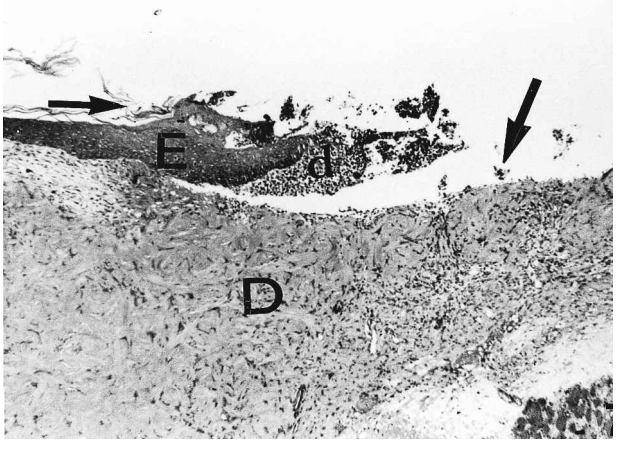


FIG. 7. Non-irradiated skin (control lesion) showing an injured area 17 days post-wounding. A large area of the dermis is still devoid of epidermal layer (large arrow). Cell debris are observed near the edge of the injured epidermis (d). E, epidermis; D, dermis. The arrow indicates the direction of the reepithelization process. HE, ×100.

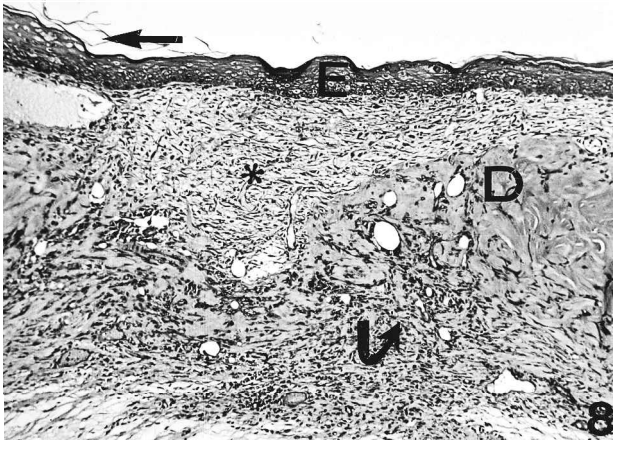


FIG. 8. Irradiated skin (lesion "$L_{//}$") from day 17 post-wounding. Note that the wounded area is completely lined by a new epidermis (E). The connective tissue of the superficial dermis is formed by a loose connective containing active fibroblasts (*). In the deep dermis, it is possible to observe inflammatory cells (curved arrow). The arrow indicates the direction of the reepithelization process. D, dermis. HE, ×200.

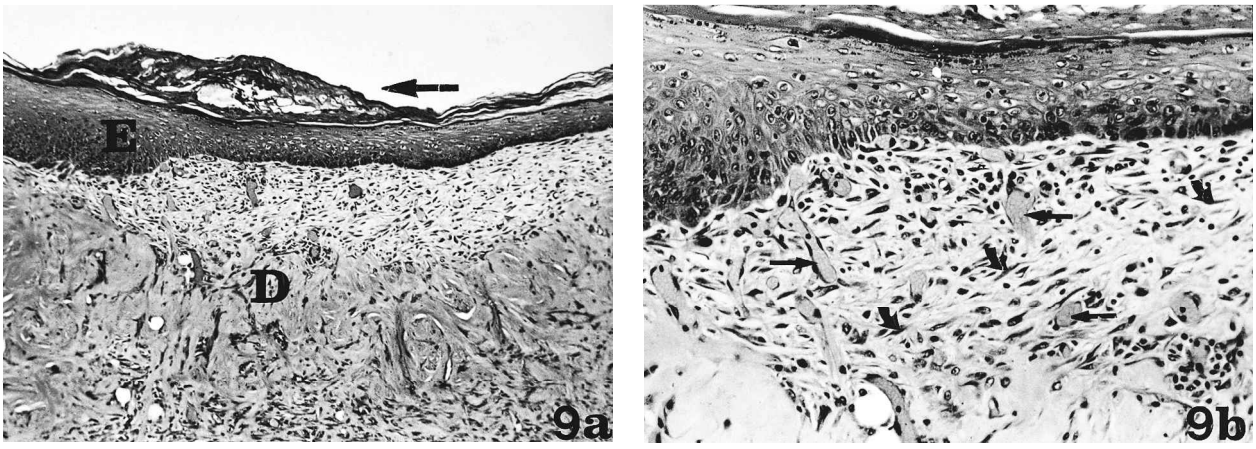


FIG. 9. Irradiated skin (lesion "$L_{//}$") on the 17th day post-wounding. **(a)** The dermis is also lined by a new epidermis (E). HE, ×200. **(b)** The connective tissue of the superficial dermis is formed by a loose connective tissue rich in active fibroblasts (curve arrows) and numerous blood vessels (arrows). D, dermis. HE, ×400.

cal conditions, including wounds[11,12]. It has been shown that laser photostimulation can lead to the enhancement of the collagen production in the regeneration of the Achilles tendon of rabbits[13]. Increase of collagen type I and type III procollagen mRNA in skin wounds treated with He-Ne laser has also been demonstrated by Saperia et al.[14] These results demonstrated that therapy with He-Ne laser might exert effects on the transcriptional level of the collagen gene expression.

Although there is rich literature about laser biostimulation, the information is quite diverse and conflicting, especially because of the differences in experimental procedures used by different laboratories. For example, *in vivo* morphological studies are not usually performed.

According to Karu,[4] if the exposure is by visible wavelength, the primary photoacceptors will be the components of the respiratory chain in the mitochondria through photochemical events. However, Smith proposed that infrared radiation starts the cascade of metabolic events by photophysical effects on the membranes.[8] Kamikawa and Ohnishi investigated the fluctuation of proton signals by laser irradiation using ^{1}H-NMR experiments, and suggested that the photophysical action of laser on the hydrogen bond is one of the essential mechanisms of LLLT effects.[7] Despite these important studies showing the primary photoacceptors in the cells, the physical model and the biological reasons for the effectiveness of the laser treatment as clinically observed in several countries have not yet been clarified. There is still divergent information about the dependence of the effect on the irradiation dose, wavelength, regime, intensity, and light characteristics such as coherence and polarization.

Although non-polarized and non-coherent light have been indicated as responsible for many biological effects *in vitro*,[15,16] some reports support the hypothesis that the biostimulative effects *in vivo* using coherent light are more efficient than those obtained with non-coherent light.[17,18] The effects of polarization studies *in vivo* are still scarce. Some studies have shown the therapeutic effect of polarized light on cells and on biological processes *in vitro*,[19,20] and it is well known that light polarization remains unchanged through a thin layer of cells. If we illuminate a highly scattering medium such as living tissue,

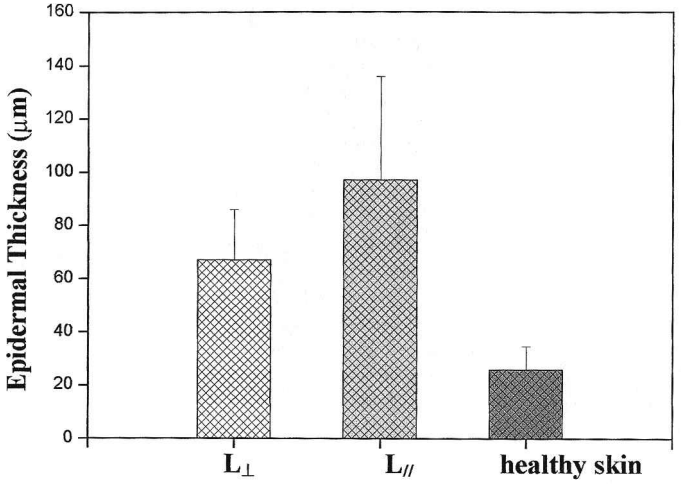


FIG. 10. Epidermal thickness for healthy and healed skin at 17 days post-wounding. Mean values ± standard deviation.

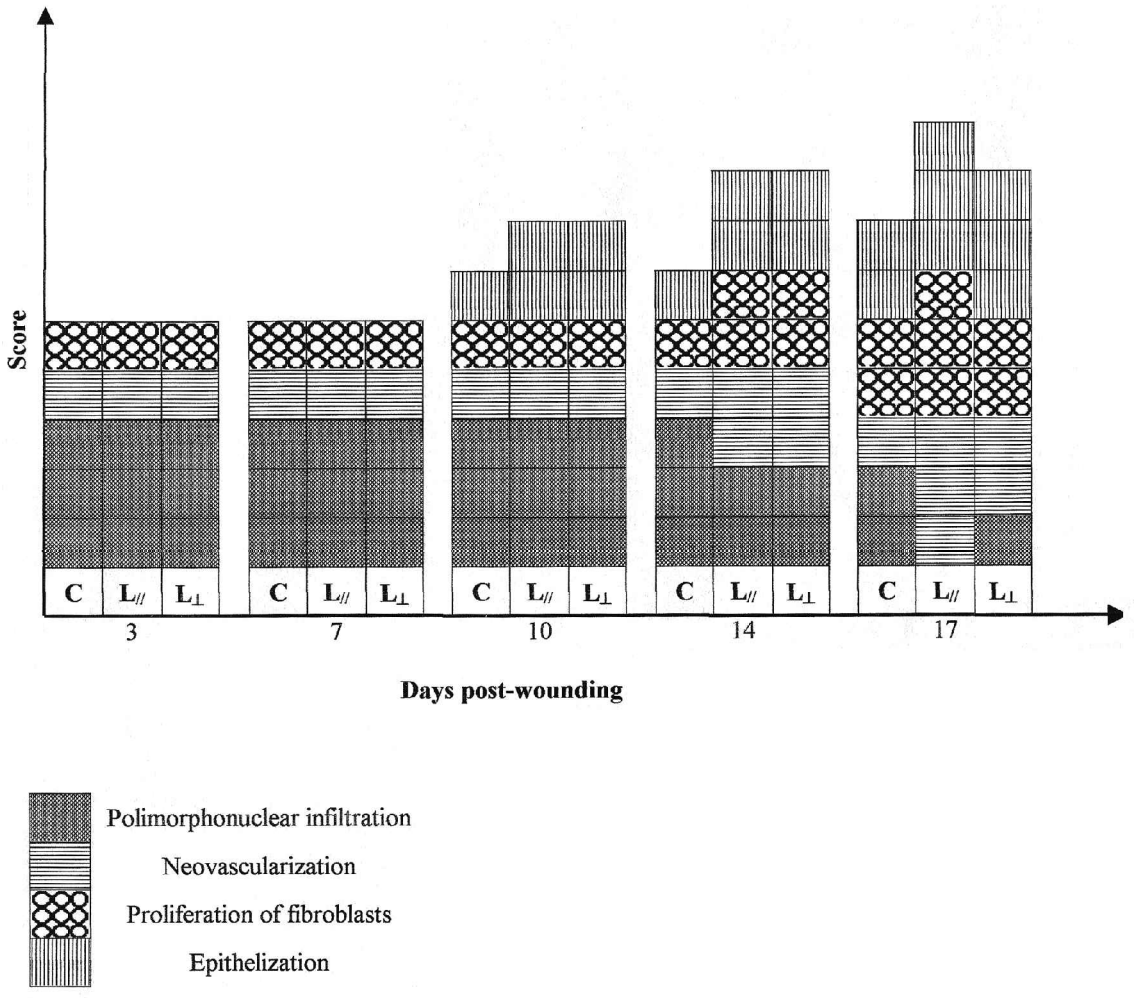


FIG. 11. Representation of the wound healing of the skin of the rat on different days post-wounding. Four animals were examined on each day. The results reflect the overview of each group of animals. Semiquantitative results were organized according to the following scores: Absent, no box; slight, 1 box; moderate, 2 boxes, intense, 3 boxes.

the polarization will be lost after penetration of a millimeter or so. The optical penetration in skin is affected by the strong scattering produced mainly by the collagen fibers.[21] However, Sankaran et al. concluded that linearly polarized light preserves its propagation properties through longer penetration depth in biological tissues.[22] In fact, linear polarization can be preserved over 2.5 transport paths in the red and near infrared wavelength ranges. Therefore, light can travel a distance of 1.2 mm in human normal skin without the complete loss of its linear polarization.[23]

It is suggested by Tunér and Hode that one could use polarized light to treat open wounds and improve healing, because the light would directly encounter the cells in the wound where there is no overlying skin to eliminate the polarization.[24] Actually, transmittance in granular tissue was about 2.5 times higher than that in normal skin according to Kolárová et al.[25]

Histological analysis in this study showed that, on day 3 p.w., the burned areas were still devoid of epidermis. In this case, laser radiation hit subjacent dermis where inflammatory cells, cell debris, dilated blood vessels, as well as normal cells of connective tissue were present. Therefore, light interacts with this granulation tissue in the process of re-formation. It was reported by Van Breugel and Bär that the absorption spectrum of fibroblast monolayers showed several absorption peaks, with one being at $\lambda = 630$ nm.[26] Therefore, we share the opinion that He-Ne wavelength starts the cascade of metabolic events after being absorbed by the endogenous porphyrins in the mitochondria.[27] It is known that the body's porphyrins possess absorption dipoles and both absorb and emit linearly polarized light.[24] Porphyrins also exhibit an absorption band at $\lambda = 630$ nm.[28]

Our results also suggest that the direction of the polarization of radiation may modulate biological responses during the skin wound repair if exposure is by He-Ne laser light. The present results indicate that the laser radiation, besides the stimulatory effect on the fibroblast, appeared to inhibit the inflammatory process, especially when the He-Ne laser was used with the polarization aligned with the animal spinal column. The inhibition of the inflammatory process may be important for the improvement of wound healing.[29]

Recently, Nickell et al. found a directional dependence on the propagation of visible and near infrared light through human skin *in vivo* using spatially resolved steady-state diffuse

reflectometry.[30] The skin's reduced scattering coefficient varied by up to a factor of two between different directions of propagation at the same position. This anisotropy is believed to be caused by the preferential orientation of the collagen fibers in the dermis; that is, the light is less scattered in the direction of the fibers under the surface of the skin. Consequently, a plausible explanation for our results is that, if the polarization is aligned with the preferential orientation of the collagen fibers in the dermis, scattering could be reduced and the polarization could be preserved, contributing to the acceleration and improvement of cutaneous wound healing. The complete mechanisms that support this observation are still unclear, and further investigation is necessary to obtain a more complete explanation.

CONCLUSION

In this study, we performed a morphological evaluation of the role of laser polarization orientation on the skin wound healing process using a He-Ne laser. Our results demonstrated that the direction of light polarization affects the healing process, particularly the formation of new connective tissue of the dermis. It was considered an important factor in the healing acceleration of inflammatory lesions.

ACKNOWLEDGMENTS

We thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support (grant no. 98–06784–1).

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