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Effect of 670-nm Laser Therapy and Dexamethasone on Tissue Repair: A Histological and Ultrastructural Study

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Abstract

Objective: In this study we investigated the role of extracellular matrix elements and cells during the wound healing phases following the use of low-level laser therapy (LLLT) and anti-inflammatory drugs.
Background Data: There are few scientific studies that characterize the possible interactions of LLLT and anti-inflammatory medications.
Materials and Methods: Thirty-two rats submitted to a wound inflicted with a 6-mm-diameter punch. The animals were divided into four groups: sham treated, those treated with the GaAlAs laser (4 J/cm^2, 9 mW, λ = 670 nm, spot size 28.27×10^{-2} cm^2), those treated with dexamethasone (2 mg/kg), and those treated with both LLLT and dexamethasone. After 3 and 5 d, the cutaneous wounds were assessed by histopathology using polarized light and ultrastructural assessment using transmission electron microscopy. Changes seen in polymorphonuclear inflammatory cells, edema, mononuclear cells, and collagen fiber deposition were semi-quantitatively evaluated.
Results: The laser-treated group demonstrated increased collagen content and better arrangement of the extracellular matrix ($p < 0.05$). Fibroblasts in these tissues were increased in number and were more synthetically active. In the dexamethasone group, the collagen was shown to be non-homogenous and disorganized, with a scarcity of fibroblasts. In the group treated with both types of therapy, fibroblasts were more common and they exhibited vigorous rough endoplasmic reticulum, but they had less collagen production compared to those seen in the laser group.
Conclusion: LLLT alone accelerates post-surgical tissue repair and reduces edema and the polymorphonuclear infiltrate even in the presence of dexamethasone.

Introduction

Repair is comprised of different stages following tissue injury. Acute inflammation is the first stage of this process, wherein pro-inflammatory molecules participate and are important for inducing the cascade of later events such as cell migration and extracellular matrix synthesis.[1] The wound microenvironment is made up of granulation tissue comprised of a dense population of macrophages, fibroblasts, and new blood vessels arranged in a loose extracellular matrix of proteins and polysaccharides. This provisional matrix may be modified by various factors, both inhibitory and stimulatory, that alter the course of inflammation and eventually lead to fibrogenesis. Dexamethasone, an anti-inflammatory steroid commonly prescribed after oral surgical procedures to alleviate symptoms of acute inflammation such as pain and edema, has a suppressive effect on cellular migration and alters the activity of growth factors like TGF-β1.[2] It is also observed to result in decreased collagen synthesis and inhibition of the tissue repair process.[3] On the other hand, photoradiation is capable of stimulating the cellular constituents of different tissues, including cicatricial tissue, through a biostimulatory effect. In addition, its contribution is particularly evident with regard to modulation of collagen[4] and elastic fiber expression.[5] There are few scientific studies that analyze the possible interactions between low-level laser therapy (LLLT) and anti-inflammatory medications.[6] Thus a better understanding of LLLT and dexamethasone and their interactive effects on healing outcomes becomes possible by focusing on the morphologic aspects of wound healing. The goal of this study was to use histopathology and polarized light microscopy, as well as

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Table 1. Criteria Used for Light Microscopic Analysis

Criterion	*Score*		
Acute inflammation	Slight Presence of <25% of neutrophils present in the field	Moderate Presence of 25–50% of neutrophils in the field	Intense Presence of >50% of neutrophils in the field
Edema	Slight Presence of <25% of edema in the field	Moderate Presence of 25–50% of edema in the field	Intense Presence of >50% of edema in the field
Mononuclear cells	Slight Presence of <25% of mononuclear cells in the field	Moderate Presence of 25–50% of mononuclear cells in the field	Intense Presence of >50% of mononuclear cells in the field
Amount of collagen fibers	Slight Sirius red staining is less intense than that seen on healthy adjacent tissue	Moderate Sirius red staining is similar to that seen on healthy adjacent tissue	Intense Sirius red staining is more intense than that on healthy adjacent tissue

ultrastructural analysis using transmission electron microscopy, to evaluate the qualitative and quantitative characteristics of the initial phases of the healing process.

Materials and Methods

All animal experiments were carried out in compliance with the laws and guidelines for experimental use and care of animals in accordance with the Committee for Ethics in Animal Experimentation, Bahia Foundation for Science Development, Salvador, Bahia, Brazil.

Animals

Thirty-two male Wistar rats weighing 200–250 g were randomly divided into four groups of eight rats each. Each treatment group consisted of four animals per time point (3 and 5 d post-wounding).

Surgical procedures

The animals were anesthetized with tiletamine chloride and zolazepam chloride at a dose of 50 mg/kg of body weight. Under aseptic conditions, a 6-mm-diameter punch was used to inflict a wound in the dorsal skin of each rat.

Experimental groups

Group I: Sham. This group was submitted to wounding and laser treatment with the laser apparatus unplugged. We also preinjected 0.9% saline as a vehicle for the dexamethasome (0.13 mL/250 g).

Group II: Treated with LLLT. Four 1 J/cm^2 laser doses were applied to the edges of the wounds, so that a total of 4 J/cm^2 was administered, soon after the surgical wounding. The time used for each dose was 31 sec times 1 J/cm^2 for a total of 124 sec and 4 J/cm^2. To estimate the area of the light beam on the wounded site, we measured the area of the spot size (28.27×10^{-2} cm^2). The appliance used complied with the specifications for a GaAlAs diode semiconductor laser, in continuous emission mode (9 mW power, $\lambda = 670$ nm, 0.031 W/cm^2) (Laser VR-KC-610; Dentoflex, São Paulo, Brazil).

Group III: Treated with intramuscular dexamethasone. A single dose of dexamethasone (Decadron®, Aché, Guarulhos, São Paulo, Brazil) was given to each animal (2 mg/kg [0.5 mg/250 g]) 1 h before the surgical wound was inflicted.

Group IV: Treated with both dexamethasone and LLLT. These animals were preinjected with dexamethasone 1 h prior to being wounded, and then were treated with LLLT as described above.

Histology

The animals were sacrificed by an overdose of anesthetic. We removed half of the wounded cutaneous tissue and fixed them sections in 10% buffered formalin solution for a minimum of 18 h. Then the tissues were routinely processed for hematoxylin and eosin and picrosirius red-F3B staining for histopathology. The picrosirius-stained sections were examined under polarized light microscopy and assessed specifically for collagen content and organization. Changes affecting the number of polymorphonuclear inflammatory cells, the degree of edema, and mononuclear inflammatory cell and collagen fiber deposition were semi-quantitatively evaluated by blinded evaluation of coded slides with the fol-

Table 2. Distribution of Polymorphonuclear Infiltrate in Healing Tissues

Time of death	*Group*	*Mean ± SD*	p[a]
3 d	Sham	3.00 ± 0.0[b]	0.001[a]
	Laser	0.25 ± 0.5[b]	
	Dexamethasone	1.25 ± 0.5	
	Laser + dexamethasone	1.00 ± 0.8	
5 d	Sham	1.25 ± 0.5[c]	0.033[a]
	Laser	0.25 ± 0.5	
	Dexamethasone	0[c]	
	Laser + dexamethasone	0.50 ± 0.5	

[a]The mean difference was significant at $p < 0.05$.
[b]Significant differences between the sham and laser groups.
[c]Significant differences between the sham and dexamethasone groups.

lowing criteria: absent (0), slight (+), moderate (++), and intense (+++) (Table 1).

Transmission electronic microscopy

For the ultrastructural analysis, the other half of the wound tissue was fixed in 2.5% glutaraldehyde and 0.1 M cacodylate buffer for 1 h at 4°C. Post-fixation was performed with a 1% osmium tetroxide solution and 0.15 M cacodylate for 1 h at 4°C. The fragments were dehydrated in successive dilutions of acetone and embedded in PolyBed 812 resin (Polysciences, Inc., Warrington, PA, USA). These blocks were then sectioned using a Reichert-Supernova ultramicrotome (Ceica, Austria). After careful analysis, selected sections were submitted to ultra-fine cuts and stained with uranyl acetate and then lead citrate for analysis by transmission electron microscopy (Zeiss EM-109; Zeiss, Germany) at 80 kV. Analyses of the results obtained from this assessment were descriptively expressed. The ultrastructural study of fibroblasts dispersed in extracellular matrix revealed a characteristic profile, as they exhibited fusiform morphology, and the outline of the nuclear membrane was similar to that of the cytoplasmic one. They all exhibited rich rough endoplasmic reticulum, Golgi complexes, and mitochondria. Small fatty vacuoles were occasionally seen, and their activity was indicated by the hyperplastic and dilated endoplasmic reticulum and abundant nuclear euchromatin.

Statistical analysis

A non-parametric version of the exact Kruskal-Wallis method for the determination of differences between groups was used, together with the Dunn's multiple comparison post-test for pair analysis. Results were considered significant when reached $p \leq 0.05$.

Results

Group I

In the histological sections of the sham group stained with hematoxylin and eosin and examined 3 d post-surgery, edema was evident, and a moderate inflammatory infiltrate of predominantly polymorphonuclear cells was observed (Tables 2 and 4). A mild lymphocytic infiltrate was also observed (Table 3). In some areas, the sections examined under polarized light showed a predominance of greenish-yellow fibers with irregular birefringence. In the sections examined on post-surgery day 5, granulation tissue with intense angiogenesis and an increased mononuclear (round) cellular infiltrate with many macrophages and lymphocytes were observed (Table 3). Under polarized light, these tissues demonstrated collagen fibers with a predominance of greenish-yellow fibers and few thick yellowish-orange fibers arranged in various directions.

Table 3. Distribution of Mononuclear (Round) Cell Infiltrate in Healing Tissues

Time of death	*Group*	*Mean ± SD*	p[a]
3 d	Sham	1.00 ± 0.0	1.000
	Laser	1.00 ± 0.0	
	Dexamethasone	1.00 ± 0.0	
	Laser + dexamethasone	0.75 ± 0.5	
5 d	Sham	1.75 ± 0.5	0.029[a]
	Laser	1.00 ± 0.0	
	Dexamethasone	1.00 ± 0.0	
	Laser + dexamethasone	1.00 ± 0.0	

[a]The mean difference was significant at $p < 0.05$.

Table 4. Distribution of Edema in Healing Tissues

Time of death	*Group*	*Mean ± SD*	p[a]
3 d	Sham	2.00 ± 0.0	0.009[a]
	Laser	1.00 ± 0.0	
	Dexamethasone	1.00 ± 0.0	
	Laser + dexamethasone	1.75 ± 0.9	
5 d	Sham	1.25 ± 0.5	0.629
	Laser	0.75 ± 0.5	
	Dexamethasone	0.75 ± 0.5	
	Laser + dexamethasone	1.00 ± 0.0	

[a]The mean difference was significant at $p < 0.05$.

The ultrastructural analysis of the samples taken on day 3 post-surgery exhibited fibroblasts with increased cytoplasm and elongated, irregular nuclei with loose chromatin. In the samples taken on day 5 post-surgery, cisterns of the rough endoplasmic reticulum with dilated and electrodense lumens were observed. The extracellular matrix demonstrated dispersed and loosely arranged collagen fibers. Edema was also observed, which formed large pale spaces (Fig. 1).

Group II

On day 3 post-surgery, the group treated with LLLT demonstrated a statistically significantly decreased exudative process than the sham group, with decreased edema and

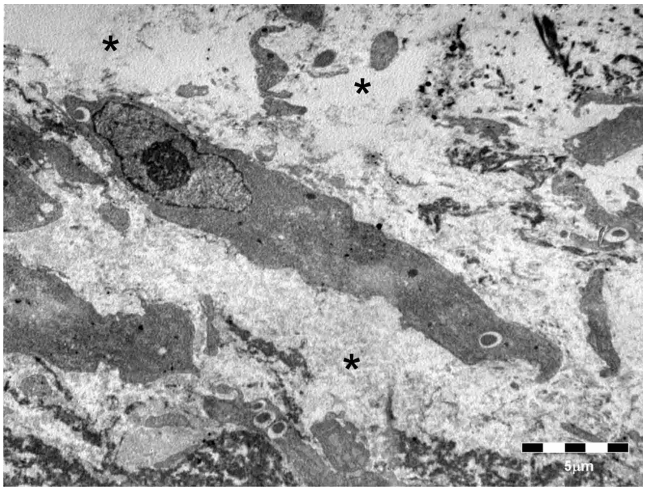

FIG. 1. Electron micrograph of a sample from the sham group taken on day 5 post-surgery showing dissociated fibroblasts in a slightly electro-dense matrix. Edema can be seen as pale spaces (asterisks) (3000×).

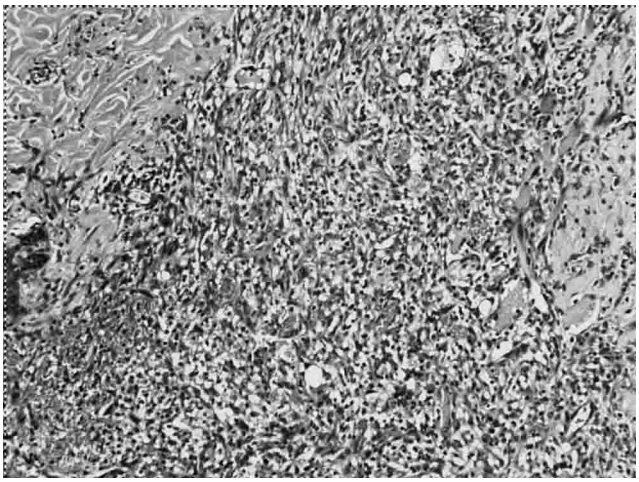

FIG. 2. Distribution of fusiform cells and vessel proliferation can be seen in this sample taken from the laser group 5 d post-surgery. Mononucleated cells can also be seen (hematoxylin and eosin, 400×).

numbers of polymorphonuclear cells (Tables 2, 3, and 4; $p < 0.5$). The tissue sections exhibited qualitative and quantitative alterations as evidenced by higher levels of collagen synthesis that was better organized and had thicker fibers that stained intensely. These differences were statistically significant in relation to the animals treated with dexamethasone (Table 5; $p < 0.5$). On the day 5 post-surgery, angiogenesis was more evident with an increase in fusiform cells, probably endothelial cells, that were linearly distributed in some areas (Fig. 2). There was clear deposition of thicker collagen in parallel bundles, forming more compact and organized areas with characteristic birefringence.

In the ultrastructural analysis of the tissues in this group, the fibroblasts were more numerous and had a better-developed synthetic system (Golgi complexes and rough endoplasmic reticulum) compared to the sham group. The most relevant aspect in this group was the organization of the extracellular matrix. A denser matrix deposition was noted, made up of orderly collagen bundles that appeared to become progressively more organized over the two time points studied. Some of these fibers thickened adjacent to the fibroblasts and formed wave-like patterns (Fig. 3).

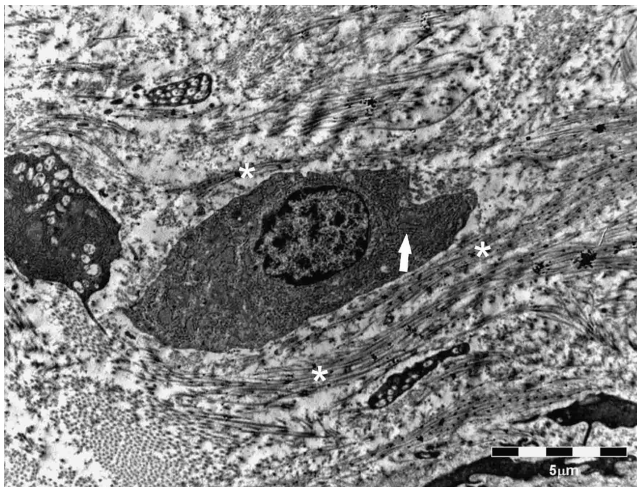

FIG. 3. A fibroblast from the laser group taken 5 d post-surgery, with well-developed endoplasmic reticulum (arrow), surrounded by organized collagen fibers in tight bundles (asterisk) (4400×).

Group III

The samples taken on day 3 post-surgery in this group demonstrated decreased acute inflammation compared to sham-treated animals (Tables 2 and 4). The thin collagen fibers had a greenish-yellow color under polarized light (Table 5). On day 5 post-surgery, the proliferative phenomena were less exuberant, and were characterized primarily by granulation tissue. There seemed to be a paucity of polymorphonuclear leukocytes (Table 2). This group also demonstrated decreased synthesis of the collagen matrix (Table 5). Ultrastructural analysis demonstrated a scarcity of fibroblasts in the healing tissue. They exhibited fewer organelles, mainly mitochondria and rough endoplasmic reticulum. The extracellular matrix showed a scarcity of dense and organized collagen (Fig. 4).

Group IV

On day 3 post-surgery, this group demonstrated vigorous angiogenesis with congested vessels. These tissues also showed an exudate consisting of polymorphonuclear leukocytes and a small contingent of mononuclear cells, particularly macrophages and lymphocytes (Tables 2, 3, and 4). The extracellular matrix showed collagen fibers of varying thicknesses with yellow-orange fibers and more evident organization than that seen in the sham-treated and dexamethasone-only groups. On day 5 post-surgery, there was more mature granulation tissue compared to the dexamethasone-only group (Fig. 5). Ultrastructural analysis demonstrated more numerous fibroblasts in comparison to the dexamethasone-only group, and they exhibited slightly more developed rough endoplasmic reticulum. Deposition of a denser collagen matrix was seen adjacent to the fibroblasts. Although they were still scarce compared to the laser group,

TABLE 5. DISTRIBUTION OF THE COLLAGEN IN HEALING TISSUES

Time of death	*Group*	*Mean ± SD*	p[a]
3 d	Sham	1.25 ± 0.5	0.03[a]
	Laser	2.00 ± 0.0[b]	
	Dexamethasone	1.00 ± 0.0[b]	
	Laser + dexamethasone	1.75 ± 0.5	
5 d	Sham	1.25 ± 0.5	0.026[a]
	Laser	2.25 ± 0.5	
	Dexamethasone	1.25 ± 0.5	
	Laser + dexamethasone	2.00 ± 0.0	

[a]The mean difference was significant at $p < 0.05$.

[b]Significant differences between the dexamethasone and laser groups.

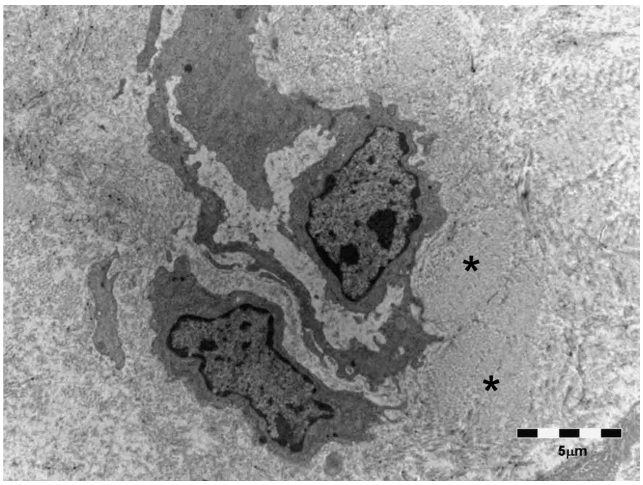

FIG. 4. A few fibroblasts exhibiting elongated cytoplasm, dispersed in an extracellular matrix with a scarcity of organized collagen (asterisks) can be seen in this sample taken on day 5 post-surgery from the dexamethasone-only group (4400×).

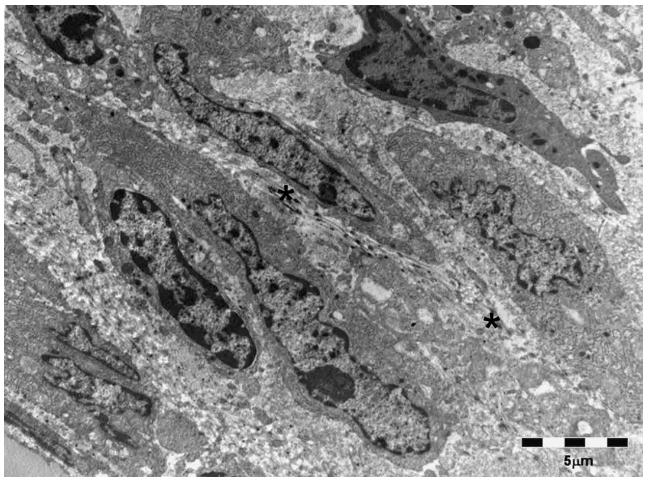

FIG. 6. Fibroblasts enveloped by a well developed and organized collagenous matrix (asterisks) can be seen in this sample from the laser-plus-dexamethasone group taken 5 d post-surgery (4400×).

the collagen fibers were more densely packed and well organized (Fig. 6).

Discussion

There are few reports in the literature on the ultrastructural appearance of cutaneous wounds treated with LLLT. One study demonstrated that in the initial phases of healing, especially on the third day following injury, animals treated with GaAlAs LLLT at an energy density of 4 J/cm^2 demonstrated a larger number of fibroblasts with rough endoplasmic reticulum, Golgi complexes, and numerous mitochondria, compared to the control sham group.[7] Our results confirm the findings of those authors. The electron micrographs taken on the third and fifth days post-surgery revealed that following LLLT, the fibroblasts were numerous with well developed synthetic organelles (Golgi complexes and rough endoplasmic reticulum). The fibroblasts were distributed among organized, parallel collagen bundles, implying a biostimulatory effect of LLLT on protein synthesis. Another important finding was the increased organization of the extracellular matrix in this group. It is known that tissue cells, especially fibroblasts, adjacent to extracellular matrix fibers regulate the direction of collagen molecules after secretion, leading to the formation of fibrils in close association with the cytoplasmic membrane.[8] As the spatial organization of collagen fibrils, at least partially, reflects the interaction with other extracellular matrix molecules, fibroblasts under the action of light stimulation may influence this organization even more, by secreting other matrix macromolecules along with its fibrillar collagens. In the irradiated animals, this improved matrix organization was also evident in the polarized light micrographs. Birefringence revealed supramolecular organization, and the laser group exhibited significant birefringence due to the presence of organized collagen fibers when examined by polarized light. In addition to collagen, birefringence was also seen in the actin filaments (F-actin), which are intermediate filaments, and myosin.[9,10]

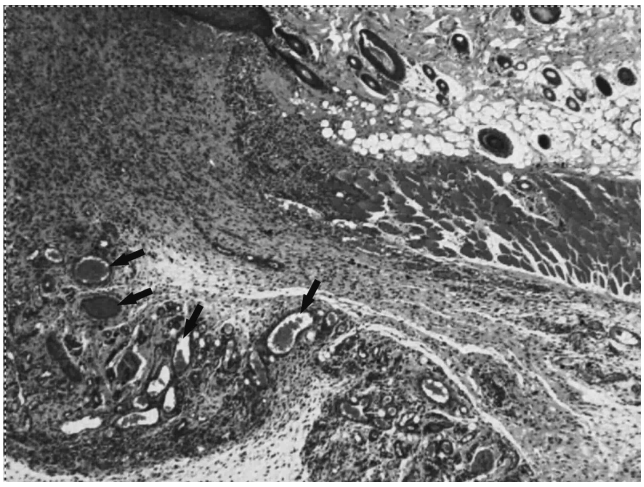

FIG. 5. The presence of inflammatory cells and intense angiogenesis (arrows) can be seen here in a sample from the laser-plus-dexamethasone group taken 5 d post-surgery (hematoxylin and eosin, 100×).

It is important to keep in mind the great variability of the parameters of LLLT used, which led to conflicting results. In one study with a diode GaAs laser and irradiation parameters of 3 J/cm^2 and 4 J/cm^2, an increase in the number of fibroblasts was observed on the third and sixth days, while pro-collagen synthesis was observed to be similar in the control and irradiated groups.[11] Another study demonstrated less collagen synthesis during healing after a myocardial infarction in animals treated with a 780-nm GaAlAs laser.[12] By using a 780-nm laser in a radiation-impaired wound healing model in murine skin, another study found no beneficial effects on the rate of wound closure.[13]

It is known that steroid anti-inflammatory drugs are, in a dose-dependent way, capable of inhibiting factors affecting

inflammation and tissue repair in different experimental models.[14–17] In addition to these actions, steroid anti-inflammatory drugs inhibit leukocyte migration and suppress the synthesis of interleukin (IL)-1α and IL-1β, as well as TNF-α.[13] These factors are chemotactic for mononuclear cells, which in turn participate in fibrogenesis.[14] Another study showed lower levels of hydroxyproline in rat colon anastomoses following 10 d of cortisone administration.[15] Rabbits that suffered tendon injuries and were treated with betamethasone demonstrated fewer fibroblasts and decreased type III collagen synthesis in the injured area.[18]

In the present study, the extracellular matrix of the dexamethasone group exhibited scarce collagen with a non-homogenous, disorganized appearance at the two time points we analyzed. Ultrastructural analysis demonstrated delayed extracellular matrix remodeling with the presence of immature collagen among sparse fibroblasts with poorly developed cellular synthetic organelles. Organized collagen was rarely seen under polarized light examination. In contrast to our observations, another group of researchers did not see any significant differences between a dexamethasone-treated (6 mg/kg in a single dose) group and controls at 7 d post-injury.[3]

Based upon our results, three questions arise. First, is the dexamethasone inhibiting the action of the LLLT? In our experimental model, when dexamethasone was injected prior to photoradiation, the effects of LLLT on wound polymorphonuclear infiltrate and collagen synthesis was reduced compared to LLLT alone. These findings were verified by the ultrastructural assessment of the subcellular synthetic machinery and collagen in these tissues. Recent studies have demonstrated that after the first 4 h following injury, down-regulation of cortisol receptors is demonstrated by endocrine cortisol gland excision,[19] and use of the steroid receptor antagonist mifepistrone[20] results in an anti-inflammatory effect. This suggests that the anti-inflammatory effects of photoradiation may occur through an endocrine-axis pathway, similar to that of glucocorticoid action.[20] Second, does the laser reduce the negative effects of dexamethasone treatment on tissue repair? A histopathological study demonstrated that GaAlAs LLLT in dexamethasone-treated wounds optimized healing outcomes by counterbalancing the anti-inflammatory-inhibiting effects of the drug.[21] It is well known that cellular responses to light stimuli are intensified under stressful conditions.[22] In injured tissue, we believe that LLLT may have a favorable effect in this situation. Although dexamethasone creates unsuitable conditions for tissue repair of the injured area, such as decreased protein synthesis, we have observed that LLLT is capable of accelerating cellular metabolism through its local effects, as seen by the increased number of fibroblasts, vasodilation, and synthesis of extracellular matrix elements, particularly collagen. Karu[23] suggests that these effects are due to an increase in mitochondrial synthesis.

Third, is LLLT able to improve tissue repair compared to sham treatment? Our results confirm previous data in the literature indicating that LLLT improves wound healing, especially via an improved anti-inflammatory response, throughout all phases of wound healing. Our findings suggest that LLLT is capable of modulating the extracellular matrix constituents qualitatively, particularly collagen, with improved results compared to those of sham treatment. Thicker collagen fibers that are more tightly bundled may contribute to the formation of tissue that is more similar to the original uninjured tissue.

Conclusion

The clinical use of dexamethasone should be limited in post-surgical conditions in which tissue repair is vital. Although dexamethasone reduces edema and polymorphonuclear infiltrate in the early inflammatory phase, the repaired tissue is no better than that seen without such treatment. LLLT alone accelerates the post-surgical tissue repair process by reducing edema and polymorphonuclear infiltrate to levels similar to those seen with dexamethasone in the inflammatory phase, but LLLT also increases collagen fiber production, and displays a better organized tissue matrix by day 5 post-surgery. If the use of dexamethasone cannot be avoided, simultaneous use of LLLT may reduce some of its deleterious long-term effects on healing, but not to the same levels seen with LLLT alone.

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