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BRIEF REPORT

The effects of low-level light emitting diode on the repair process of Achilles tendon therapy in rats

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Abstract Thirty Wistar rats (350±20 g) were subjected to total Achilles tendon tenotomy of the right fore limb. They were submitted to a daily dose of 20 J/cm^2 light emitting diode (LED) (640±20 nm) therapy. The LED was applied punctually and transcutaneously to the lesioned region. The animals were separated into six groups, C1 and L1, C2 and L2, C3 and L3. The C groups were used for control and the L groups, treated for 7, 14 and 21 consecutive days, respectively. The animals were killed on the 7th, 14th and 21st days after surgery. After the animals had been killed, their tendons were extracted and dissected, fixed in formaldehyde at 10%, and sent for histological analysis by light microscopy in which the repair process was analysed. This study demonstrated that LED interfered in the repair process of the tendon tissue, reducing the number of fibroblasts in the initial periods and improving the quality of the repair in all periods studied.

Keywords Achilles tendon · Light emitting diode (LED) · Low-level laser therapy · Tissue repair

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Introduction

Many scientific works have been published about tendon lesions with the objective of optimizing tissue repair. According to Goffi [1], surgeons worry about tendon lesions in reconstructive surgeries, because, when there is a suture, there may be adherence which hinders its full recovery. Schimitt called attention to the use of physiotherapic resources in speeding the tissue repair process in 1993 [2] in a study that showed the effects of laser radiation on the regeneration of tendons in dogs. Nowadays, researchers are still looking for the best way to restore the functions and improve the repair in tendons. Most studies suggest that coherent light (low-power laser) can start the modulation of physiological processes.

The high cost of laser-emitting devices stimulates research on the effects of alternative light sources such as light emitting diode (LED). According to Solear et al. [3] and Clark and colleagues [4], irradiation with non-coherent light has a lower cost and can be as efficient as laser radiation. Vinck et al. [5] obtained satisfactory results in their study and suggest beneficial effects with LEDs on different kinds of skin lesions.

The stimulant effects produced by low-power laser on biological tissues were attributed to their coherence by Boulton and Marshall [6]. The light transmitted by an LED, unlike that from laser, is non-coherent. However, in more recent studies, Pontinen [7] and Whelan et al. [8] state that the light coherence is not responsible for the therapeutic effects of low-power laser, because this property is lost in the first layers of biological tissue. The growth of cellular activity, both in division and synthesis, has been related to the length and dosage and not specifically to the light source [9]. Nowadays, LEDs are being commercially introduced as alternatives to low-power laser therapies. It

is known that the LED activity has an influence on the regenerating process of wounds [5].

The aim of the study was to investigate the effect of LED (640±20 nm) therapy on tissue regeneration of the Achilles tendon of rats through the quantitative evaluation of the number of fibroblasts and the quality of tissue repair. This study would allow evaluation of the effect of LED therapy on the tissue repair process of the tendon, aiming at a low-cost alternative to low-level laser therapy (LLLT) in clinical treatments.

Materials and methods

Experimental groups

Thirty albino male Wistar rats (approximately 350±20 g, 3 months old) were used in this study. They were kept in the Research and Development Institute of the University of Vale do Paraíba in appropriate standard polyethylene cages, in random groups of five animals per cage. They underwent a period of adaptation for 5 days in a room with constant temperature and humidity (24°C e 60%), natural light, water and food ad libitum.

The 30 animals were randomly separated into six groups, C1, L1, C2, L2, C3 and L3. Table 1 shows the groups and the respective days on which they were killed.

Surgical procedure

A total tenotomy of the medium region of the right Achilles tendon, between the tendon insertion and the myotendon articulation, was made in all animals. For this procedure all animals were subcutaneously pre-treated with atropine (analgesic); the dosage was 0.04 ml/100 g body weight. After this, there was an interval of 15 min before the anaesthetic procedure [10]. The anaesthetic drug was given in a single intramuscular injection of cetamine chloridrate10%, 10 ml (Syntec, 0.1 ml/100 g body weight) and xylazine chloridrate 2%-10 ml (Syntec, 0.1 ml/100 g) [11], injected with a 1 ml insulin syringe for each animal. The skin of the right limb was shaved and scrubbed with a 2% iodine alcohol solution. The tendon was exposed through a 1 cm incision and transversally tenotomized in the medial region. The skin was closed with 6.0 polyester monofilament (Prolene®) and disinfected with 2% iodine alcohol [10, 11].

Table 1 Number of animals, groups, treatments and days on which they were killed

Group (n=5)	Therapy	Day killed
C1	Control	7th day after surgery
L1	LED	
C2	Control	14th day after surgery
L2	LED	
C3	Control	21th day after surgery
L3	LED	

After surgery the animals received a single intramuscular injection of broad-spectrum antibiotics (Fort Dodge®, 0.02 ml/100 g body weight).

LED therapy

The equipment used in the study was an LED (640± 20 nm), Red Star LED® model (100 mW) with a 0.5 cm^2 area in direct contact with the right limb of the animal, on the lesion area. The application time was 120 s, with a final dose of 20 J/cm^2 at one point on the injured area. Before the beginning of the experiments, the LED equipment was checked with a power checker (13PEM001/J, Mellers Griot, Netherlands).

The therapeutic procedure was begun 1 h after the surgery and was repeated every 24 h. All animals were treated the same way. For the procedure, the animals were positioned on a table in ventral decubitus, and manually immobilized. The LED was used on their hind limbs, directly on the injury, at a 90° angle. The LED pen was protected by plastic film after each application.

Experiment

The animals were killed on the 7th, 14th and 21st days after surgery in groups C1 and L1, C2 and L2, C3 and L3, respectively. Before the rats were killed, the same sedation procedure was used as in the surgery, after which an intracardiac injection of sodium thiopental (Cristalia) (1 ml/100 g body weight) was given.

The tendons were removed by dissection, from the calcaneal insertion to the myotendon articulation. They were fixed in 10% formaldehyde and sent for histological processing.

Histological technique

After fixation, the tendons were dehydrated and embedded in paraffin, followed by microtomy in a semi-automatic revolving microtome to produce sections 5 μm thick, eight sections per animal. Four sections were stained with haematoxylin and eosin (HE) and four with Masson trichrome.

Morphometry

A Nikon® optical binocular microscope, model YS100, was used for the morphometry, with a Zeiss® ocular with

millimetre reticulated integrator. The analysis was done in 12 microscopic fields, equivalent to a 150 μm^2 area, with histological sections dyed with HE and Masson trichrome. The area chosen for analysis in all samples was the most proximal to the edges of the tenotomized regenerating tissue. The numbers of fibroblasts and gradation of the repair were evaluated in the tissue of this area.

The tissue regeneration in the tenotomized area was graded as follows:

- Group $^{+}$. Absence of regeneration. Evidence of hypercellularity associated with the presence of delicate and rare collagen fibrils with no specific orientation in relation to the edges of the tenotomized area. Abundant amorphous fundamental substance. Diffuse infiltrate of chronic inflammatory and phagocytic cells.
- Group $^{++}$, Initial regeneration. Evidence of hypercellularity associated with the presence of delicate and thick collagen fibres, already presenting indications of orientation (angulation of up to 25°) in relation to the to the edges of the tenotomized area. Decrease in the presence amorphous fundamental substance. Chronic inflammatory and phagocytic cells.
- Group $^{+++}$. Intermediate regeneration. Cellularity near normal, associated with mature collagen fibres, positioned in orientated clusters in relation to the edges of the tenotomized area (25° to 10° angle). Occasional presence of inflammatory cells and absence of phagocytic cells.
- Group^{++++}. Complete regeneration. Normal cellularity associated with clusters of mature collagen of fibres in parallel orientation (180°) in relation to the edges of the tenotomized. Absence of inflammatory cells and absence of phagocytic cells.

Statistical analysis

We evaluated the data for coefficient variance and sample distribution to determine the statistical test, considering a statistical significance level of 5%($P<0.05$) [12]. The numbers of fibroblasts in the treated and untreated groups were subjected to analysis of variance (ANOVA), with the Bonferroni post-test. The level of significance was 5% ($P<0.05$). The program used was GraphPad Prism®, version 2.0

Results

Qualitative analyses of tissue repair

In the histopathological analyses of group C1 the most observed gradation of remodelled collagen fibres was in the absence of the regeneration phase and, in rare cases, in the initial regeneration phase. The C1 group presented hypercellularity associated with the presence of delicate and rare collagen fibrils with no specific orientation in relation to the edge of the wound. There was evidence of an amorphous fundamental substance and a diffuse infiltrate of chronic and phagocitic inflammatory cells (Fig. 1a).

Group L1 presented a remodelled gradation of the collagen fibres, especially in the initial remodelling. Evidence of hypercellularity was observed, in association with delicate and sparse collagen fibres, but with signs of orientation in relation to the edge of the wound. The presence of an amorphous fundamental substance and a diffuse infiltrate of chronic and phagocitic inflammatory cells diminished (Fig. 1b).

In group C2 the gradation of collagen fibre remodelling was mostly observed in the initial remodelling phase. Evidence of hypercellularity was observed, in association with delicate and rare collagen fibres, with signs of orientation in relation to the edge of the wound (Fig. 2a).

The frequently observed gradation of remodelled collagen fibres in group L2 was in the intermediate remodelling phase. The group presented near normal cellularity, with

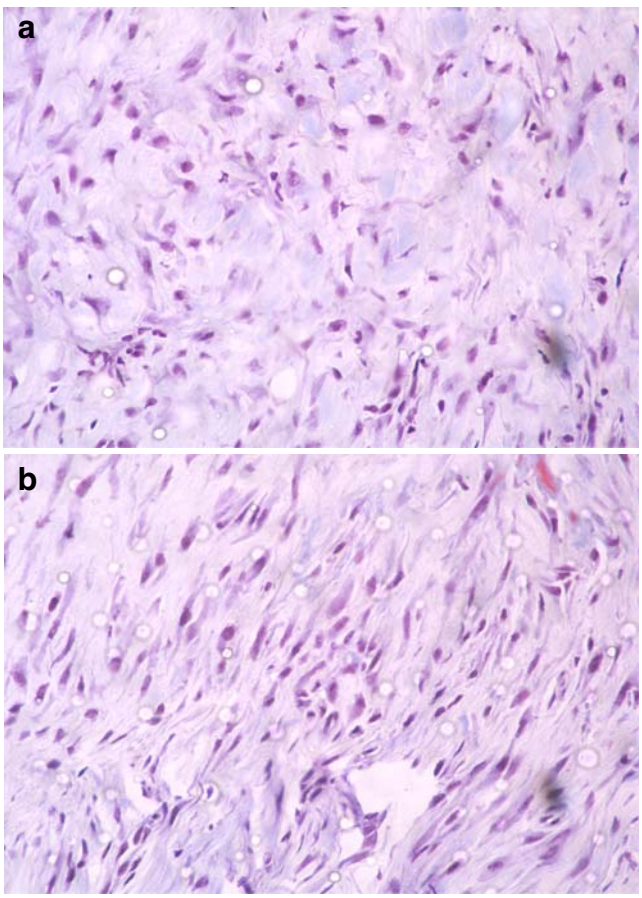

Fig. 1 Microscopy of calcaneal tendon of rats killed on the 7th day after surgery. **a** Group C1; **b** group L1. Masson trichrome, ×400

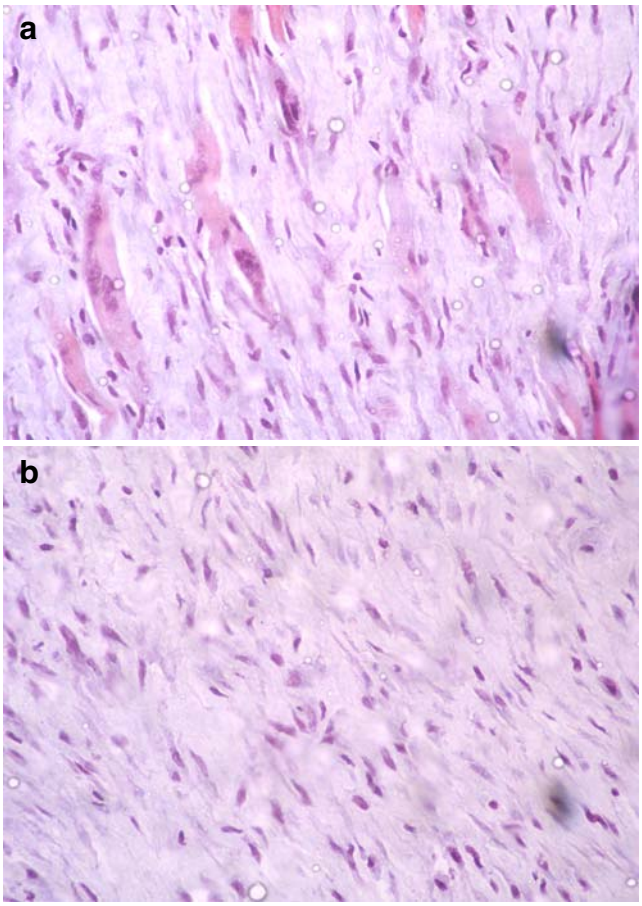

Fig. 2 Microscopy of calcaneal tendon of rats killed on the 14th day after surgery. **a** Group C2; **b** group L2. Masson trichrome, ×400

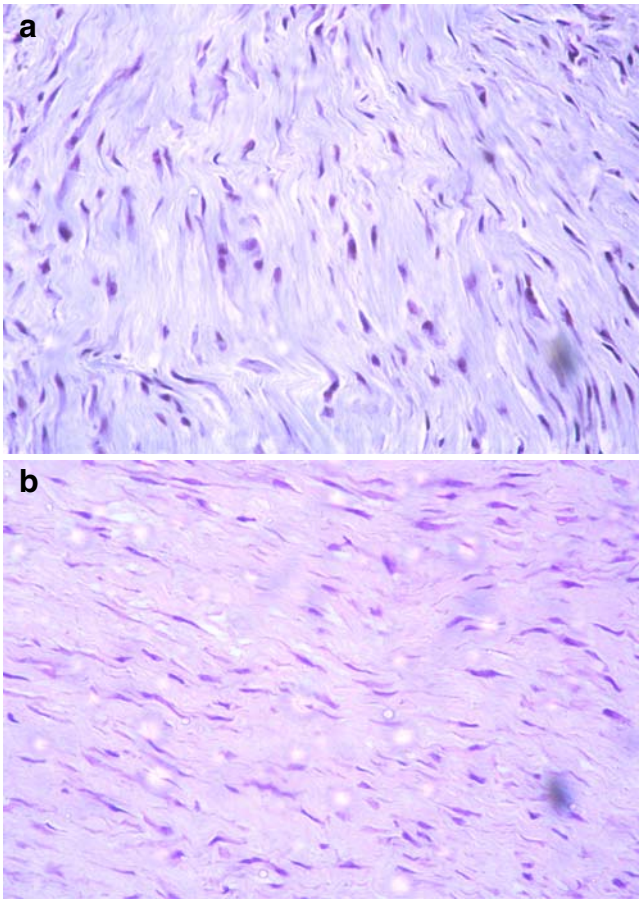

Fig. 3 Microscopy of treated calcaneal tendon of rats killed on the 21st day after surgery. **a** Group C3; **b** group L3. Masson trichrome, ×400

mature collagen fibres disposed in clusters aligned with the edge of the wound. Occasional chronic inflammatory cells were observed (Fig. 2b).

Group C3 presented a remodelation gradation of collagen fibers especially in the intermediate remodelation phase and in rare cases in the complete remodelation phase. Cellularity near normal was observed with the presence of clusters of mature collagen fibers aligned with the edge of lesion area (Fig. 3a).

In group L3 the gradation of the collagen fibres presented a complete remodelling phase. Evidence of normal cellularity was observed, in association with clusters of mature collagen fibres aligned with the edge of lesion area (Fig. 3b).

Figure 4 shows the comparison of results of the gradation of tendon tissue remodelling between the control and LED groups in the three different phases observed.

Quantitative analysis

ANOVA was performed between groups, and all groups (control and treated) presented significant statistical differences in relation to the numbers of fibroblasts with the passage of time: C1 and C2 ($P<0.01$), C1 and C3 ($P<0.001$), L1 and L3 ($P<0.05$). In inter-group evaluations a significant statistical difference was observed between groups C1 and L1 ($P<0.01$). In Table 2 the numbers of fibroblasts in the control and LED animals at the 7th, 14th

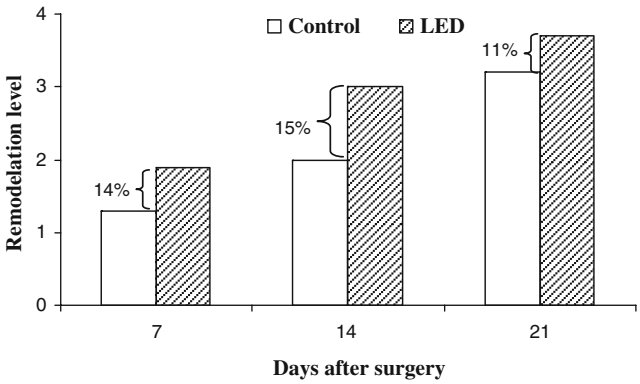

Fig. 4 Comparison of the intensity of remodelling between the control and LED groups in the three different phases observed. The numbers 1, 2, 3 and 4 represent tissue regeneration grades +, ++, +++ and ++++, respectively. The difference between the control and LED is shown in percentages

Table 2 Means, standard deviations, and ANOVA results of the numbers of fibroblasts found on the 7th, 14th and 21st days in the control and LED groups

Number of fibroblasts		
Day	Control	LED
7th day	201.5±6.3 *	167.6±5.3***
14th day	166.5±11.5	160.1±1.4
21 st day	161.6±1.2 **	138.8±1.4 **

$P<0.05$=* (7th day vs 14th day), ** (7th day vs 21 st day), *** (control vs LED)

and 21st days are shown. The lowest number of fibroblasts was found on the 21st day, with the lowest mean value in the LED group (L3). On the 14th day intermediate values were observed; the lowest mean value was in the LED group (L2). The group killed on the 7th day presented the highest number of fibroblasts; the lowest mean value was in the LED group (L1).

Discussion

This study was the result of the limited research into the use of LEDs in tendon healing and the need for alternative therapies to help in the process. It considered the success of photobiomodulation in tendon wounds with light sources, coherent or not, demonstrated by previous studies [10]. However, this is a controversial area, because of conflicting results, justifying the need to identify the best parameters to be used in clinical practice [13–17].

Studies have demonstrated the effects of LED irradiation similar to the ones obtained with laser on cells [5]. However, the differences between the in vitro and in vivo systems must be heeded, for the presence of the circulatory and lymphatic systems, among other aspects, can lead to modifications in the light–cell interaction. For this reason, we carried out this study in vivo, using young male rats to avoid interferences such as menopause and age [18]. The calcanear tendon, known as the Achilles tendon, was selected for its easy access, because it is near the skin and is large, which makes it easy to undergo surgical techniques [10, 11]. The irradiation followed the recommended procedure. It was punctual, with a beam covering the whole injured area. The pen was positioned at 90° to the tendon's longitudinal axis, in contact with the animal's skin (transcutaneously). According to these authors, these conditions allow the deposited energy to penetrate the tissue with less loss by specular reflection [11, 19].

The tendon tissue has poor regenerative capacity, because of its scarce blood circulation, oxygenation and nutrition, which are very important in tissue repair, for the adequate evolution of regeneration [20, 21]. Thus, the use of non-coherent light as an auxiliary in tendon repair was a relevant factor in this study because this kind of light source is similar to coherent light sources such as the laser, but at a lower cost [3, 4, 7, 9, 22].

Tissue remodelling demonstrated that there was superior healing in the groups treated with LED, resulting in a more mature tissue than in the other groups in this experiment. Our results agreed with those obtained by Faria [10] in a similar study with LEDs (4 J/cm^2).

The average obtained in the tissue remodelling analysis in the group treated with the LED therapy, on the 7th day after surgery, was very similar to the that obtained in the control group on the 14th day after surgery. On the other hand, the group treated with the LED therapy, on the 14th day after surgery presented an average similar to that of the control group on the 21st day after surgery. In the groups on the 21st day after surgery the average of the LED group almost reached the maximum remodelling previously established, with a higher quality than the average of the control group. The results suggested that the remodelling quality of the groups treated with LEDs might have been achieved more quickly than in the groups that did not undergo the treatment, confirming previous studies that stated that LLLT would optimize tissue healing in tendons [10, 11, 15, 23, 24].

An acceleration in the formation of fibrils, collagen fibres, simulating regeneration of the muscle tissue and a great decrease in the inflammatory response in tenotomized tendons treated with LLLT [25, 26] improves the healing quality of the tissue.

The repair process in tendon tissue happens in three overlapping phases: inflammatory, proliferative and remodelling [1, 20, 27, 28]. Our study demonstrated the presence of a larger number of fibroblasts in both groups killed on the 7th day after surgery (control and treated). It is known that until the 7th day an injury is in the inflammatory phase in which extrinsic regeneration happens and there is a proliferation of blood capillaries and fibroblasts that migrate to the injured area [20, 27, 29]. In the proliferation and remodelling phases intrinsic regeneration occurs in which there is fibroblast synthesis to produce collagen [1, 28, 30], which could explain the gradually diminishing number of fibroblasts in the groups killed on the 14th and 21st days after surgery.

Some specific studies have found that LLLT can stimulate or inhibit the various regenerating phases according to the dosage. The objective of our study was also to see whether this affirmation is also applicable to LED therapy. This could not be confirmed, because our results demonstrated that the LED therapy produced an acceleration of the healing process after total tenotomy. This was the expected effect of the electromagnetic radiation in the

red region, diffused inside the injured tissues [22]. Among the groups studied the biggest difference found in relation to the number of fibroblasts appeared when we compared group C1 with group L1, where the rats had been killed on the 7th day after surgery. The group stimulated with LED showed significant diminishing in the number of fibroblasts, which disagrees with some authors who described an increase as an effect of LLLT [5, 10, 31]. Important factors are mentioned as possible causes for this increase in the number of fibroblasts in the initial tissue repair phase, among which the dosage stands out. Probably, the results obtained were related to the high dosage used in our study (20 J/cm^2), pointing to new research in this direction.

Houreld and Abrahamse [32] observed the stimulatory effect of LLLT on cells that had received a 5 J/cm^2 dosage; meanwhile, cells that had received a 16 J/cm^2 dosage presented an inhibitory effect on the proliferation and fibroblast activity. Likewise, the results found in our work showed a significant increase in the numbers of fibroblasts after the rats had been irradiated with a 20 J/cm^2 dosage. This significant difference in the numbers of fibroblasts was only present in the control groups and the group treated in the initial phase of regeneration (C1 and L1), which might suggest little influence of the therapy in later phases. This would eliminate the need for LED treatment after 7 days. This proliferative aspect might be considered negative if the remodelling had not been superior in the irradiated groups in relation to the control.

It was observed that the effect of the LED therapy in the regenerating process of rats' Achilles tendons optimized the remodelling quality of the tendon tissue in all groups treated, presenting a great difference in all groups treated. There was a great difference in relation to the control groups and a significant difference between the days on which the rats had been killed, with a gradual decrease in the numbers of fibroblasts in both groups and between the groups killed on the 7th day after surgery.

To summarize, with the use of LED therapy in the parameters tested, the quality of the regenerated tissue was superior to that in the control group in all experimental phases, with significantly lower cellularity on the 7 days for the irradiated group. This information points to an increase in activity in collagen synthesis, probably related to the reduction of the inflammatory process in the first moments of regeneration. This would attest to the superiority of the quality of remodelling in the treated groups.

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