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Comparative Study Using 685-nm and 830-nm Lasers in the Tissue Repair of Tenotomized Tendons in the Mouse

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ABSTRACT

Objective: **The objective of this study was to evaluate the effects of 685- and 830-nm laser irradiations, at different fluences on the healing process of Achilles tendon *(Tendon calcaneo)* of mice after tenotomy.** ***Background Data:*** **Some authors have shown that low-level laser therapy (LLLT) is able to accelerate the healing process of tendinuos tissue after an injury, increasing fibroblast cell proliferation and collagen synthesis. However, the mechanism by which LLLT acts on healing process is not fully understood.** ***Methods:*** **Forty-eight male mice were divided into six experimental groups: group A, tenomized animals, treated with 685 nm laser, at the dosage of 3 J/cm^2; group B, tenomized animals, treated with 685-nm laser, at the dosage of 10 J/cm^2; group C, tenomized animals, treated with 830-nm laser, at dosage of 3 J/cm^2; group D, tenomized animals, treated with 830-nm laser, at the dosage of 10 J/cm^2; group E, injured control (placebo treatment); and group F, non-injured standard control. Animals were killed on day 13 post-tenotomy, and their tendons were surgically removed for a quantitative analysis using polarization microscopy, with the purpose of measuring collagen fibers organization trough the birefringence (optical retardation [OR]).** ***Results:*** **All treated groups showed higher values of OR when compared to injured control group. The best organization and aggregation of the collagen bundles were shown by the animals of group A (685 nm, 3 J/cm^2), followed by the animals of group C and B, and finally, the animals of group D.** ***Conclusion:*** **All wavelengths and fluences used in this study were efficient at accelerating the healing process of Achilles tendon post-tenotomy, particularly after the 685-nm laser irradiation, at 3 J/cm^2. It suggests the existence of wavelength tissue specificity and dose dependency. Further studies are required to investigate the physiological mechanisms responsible for the effects of laser on tendinuos repair.**

INTRODUCTION

INNOVATIVE CLINICAL APPROACHES to repairing damaged tissues are being developed, including low-level laser therapy (LLLT).[1] A series of studies have demonstrated that LLLT is effective at reducing post-injury inflammatory processes and accelerating soft tissue healing.[1] Moreover, it is suggested that LLLT, at the cellular level, produces increased ATP synthesis, increased mitochondrial respiration, and increased production of molecular oxygen, thus stimulating DNA synthesis and cell proliferation.[1–3]

Some authors state that LLLT can accelerate the healing process of tendinuos tissue after injury. LLLT seems to create new blood vessels, to increase collagen fiber deposition, and to promote higher fibroblast cell proliferation in the site of the lesion.[2–8] Enwemeka and Reedfont[2] demonstrated that the 632.8-nm laser (He-Ne) produced a higher deposition of collagen, increasing the tensile strength of completely severed and surgically repaired rat tendons. These results corroborate those of Salate et al.,[7] Wanderer et al.,[9] Pereira et al.,[10] and Almeida-Lopes et al.,[11] who also found increases in vascularization,

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higher deposition of collagen, and an acceleration of the healing process of an injured tendon.

Collagen is the main structural protein of the body, and it is the major component of the extracellular matrix of the tendon. The biologic and mechanophysical properties of collagen fibers are, to a great extent, determined by the aggregation and molecular structure of their components.[12,13] Variation in the order of organization states has been detected and measured in collagen bundles during the processes of repair through their anisotropic properties (birefringence).[12,13] The total birefringence is determined by the addition of both birefringences, and this measurement remains the best way to study molecular order and the degree of ordered aggregation in collagen bundles.[12] Many studies have used polarized light microscopy to qualitatively and quantitatively examine the organization, aggregation state and molecular order of collagen fibers through the measurements of optical retardation (OR; in nm) due to birefringence.[13]

Although studies have demonstrated the stimulatory effects of LLLT in cell proliferation and acceleration of the healing process, the mechanisms by which LLLT acts on tissues are not completely understood. Moreover, a wide range of wavelengths and fluences have been used in the different works, making it difficult to compare studies.

The aim of this study was to compare the effects of 685-nm and 830-nm lasers, at different fluences, on healing processes after tenotomy.

METHODS

Forty-eight male mice (*Rattus norvegicus albinus,* Wistar lineage), aged 12–13 weeks (225 ± 25 g) were used in this study. The animals were housed in cages made of polypropylene, in a 12-h light/dark environment and provided with water and food *ad libitum*. Animals were randomly divided into six groups: group A (n = 8), animals irradiated with the 685-nm laser, at the fluence of 3 J/cm^2; group B (n = 8), animals irradiated with the 685-nm laser, at the fluence of 10 J/cm^2; group C (n = 8), animals irradiated with the 830-nm laser, at the fluence of 3 J/cm^2; group D (n = 8), animals irradiated with the 830-nm laser, at the fluence of 10 J/cm^2; group E (n = 8), animals injured, without treatment; and group F (n = 8), non-injured standard control.

Tenotomy

Forty animals were submitted to tenotomy of the calcaneal tendon. Each animal was previously weighed and anesthetized by intraperitoneal injection of ketamine and xylazine 2%, at the dose of 95 and 12 mg/kg, respectively. The superficial skin around the right Achilles tendon was incised medially and the tendon was released from surrounding soft connective tissue via this incision. The tendon was cut sharply and transversely midway between its calcaneal insertion and the musculotendineous junction. The severed ends of the tendon were not sutured. Afterward, the skin incision was closed. All surgical procedures were performed under aseptic conditions.[12] The study was conducted in compliance with the American and Brazilian Laws on animal experimentation (NIH, Rockville, MD).[12]

The animals of injured groups did not receive any kind of immobilization and the animals of the standard control were not submitted to any procedure.

Irradiation procedures

The irradiation started 24 h after the tenotomy. A total of 12 sessions were performed, during 12 consecutive days. A 685-nm InGaAlP and a 830-nm GaAlAs were used (15 mW, CW, 5.4 J/cm^2, spot of 0.0028 cm^2, Teralaser; DMC® São Carlos, SP, Brazil). Treatments were made through the contact technique, at one point, on the injured area. All the procedures were carried out in a stipulated period of the day. During the treatments, irradiated animals were sedated (Xylazine 2%, at the dose of 12 mg/kg) and they were kept in a special container from which their hind limbs extended.[11]

The rats were killed on day 13 after tenotomy by an overdose of general anesthetic. Their right tendons were surgically removed by dissection from the musculo-tendinous junction to the calcaneal attachment. Immediately, tendons were washed in physiological solution, where they were prepared for further procedures used in the data collection.

Preparation of samples and data collection

For a quantitative evaluation of tendinous repair, the removed tendons were submitted to fixation in 10% formaline for 24 h and subsequently were prepared for inclusion in paraffin blocks before mounting on glass slides to be analyzed through polarized light microscopy.[12,13] The purpose of this procedure was to analyze the organization, aggregation state and molecular order of the collagen fibers in the tendons through the measurement of birefringence. The tendons were cut longitudinally in semi-seriated sections through a rotative microtome, with thickness standardized at 7 μm. The slices were disposed in a glass laminae with no stain.

Birefringence measurements

Optical retardation (OR) due to birefringence was measured using the Senarmont's compensator λ/4. Forty measurements were performed from each subgroup studied, using monochromatic light and the interference filter Schott λ = 546 nm. The resulting measurements in degrees were transformed to nm by multiplying the degrees by 3.03. Total birefringence of the collagen fibers was measured after imbibing distilled water.[12,13]

To carry out the measurements along the axis of the tendon, the longitudinal axis of collagen fibers were orientated at 45° from the polarizing light direction of propagation. In this position, the collagen fibers introduced the highest OR. The measures were made in different points of central areas of tendons that correspond to the lesion area.[12,13]

Statistical analysis

The results are given as means and standard deviations (SD). The data were compared by analysis of variance (ANOVA). When the analysis indicated the presence of a significant difference, the means were compared with the Kruskal-Wallis test. Values were analyzed using the statistical package MINITAB. Differences were accepted at a level of 5% ($p \leq 0.05$) as significant and a level of 1% ($p \leq 0.01$), as highly significant.

RESULTS

Figure 1 represents the mean values of OR of colagenous fibers of calcaneuos tendon found in the experimental groups. The standard control group (group F) showed higher values of OR of collagen fibers than the other groups (OR mean of 72.19 ± 5.54; $p \leq 0.01$). We also demonstrated that both laser wavelengths and both dosages used produced a statistically significant increase of the OR of the collagen fibers in the injured tendons compared to injured control (OR mean of 12.11 ± 1.75; $p \leq 0.01$), suggesting that the laser irradiation was able to accelerate tendon healing. However, the better tissue response was observed after the irradiation with the 685-nm laser, at the dosage of 3 J/ cm^2 (Group A, OR mean of 37.67 ± 6.13). On the other hand, the animals irradiated with the 830-nm laser, at the dosage of 10 J/ cm^2 (group D, OR mean of 24.43 ± 6.66) presented the weaker response to laser irradiation. Moreover, there were no statistical differences between groups B (OR mean of 28.32 ± 5.36) and C (OR mean of 32.32 ± 7.83).

Tables 1 and 2 present p values and percentage increase of OR values for the treated groups compared to the OR for the injured control group and comparison of the results obtained in the different treated groups. As mentioned above, all treated groups demonstrated a statistically significant difference compared to the injured control group.

As highlighted in Figure 1 and Table 2, the best tissue response was obtained after the 685-nm laser irradiation, at the dosage of 3 J/cm^2 ($p \leq 0.05$ compared to groups B and C and $p \leq 0.01$ compared to group D). These animals showed an OR value of the collagens fibers 33%, 16%, and 54% higher than groups B, C, and D, respectively. Group B and group C did not show any difference between them ($p \geq 0.05$), and they demonstrated a percentage of 15% and 32% higher in comparison to group D, respectively (Table 2).

DISCUSSION

Our results have demonstrated that laser irradiation, at the wavelengths and dosages used in the present work, produced an acceleration of the healing process of the tenotomized tendons, particularly after 685-nm laser irradiation, at the fluence of 3/cm^2 (group A). The second best response was observed in the animals of group C (830-nm laser, 3 J/cm^2) and group B (685-nm laser, 10 J/cm^2), followed by the animals of group D (830-nm laser, 10 J/cm^2).

To analyze the level of collagen fiber organization at the site of injury, we found the OR value due to birefringence for all animals of each group using polarized light microscopy. The intensity of brightness in the birefringence image (represented by the OR value) reveals the level of collagen fibers aggregation and molecular order.[12] During the regeneration process of an injured tendon, there is a tendency toward increased values of OR, which represents an improvement of the collagen organization. Our findings demonstrated the lowest OR values for the injured control group, suggesting that these animals showed a more disorganized molecular structure of the collagen fibers in the lesion area. Probably, the laser was able to produce an increase in collagen synthesis and a better aggregation and alignment of collagen fibers in the irradiated animals during the tissue-repair process, which was represented by the highest OR value presented by the treated groups (Fig. 1). These findings

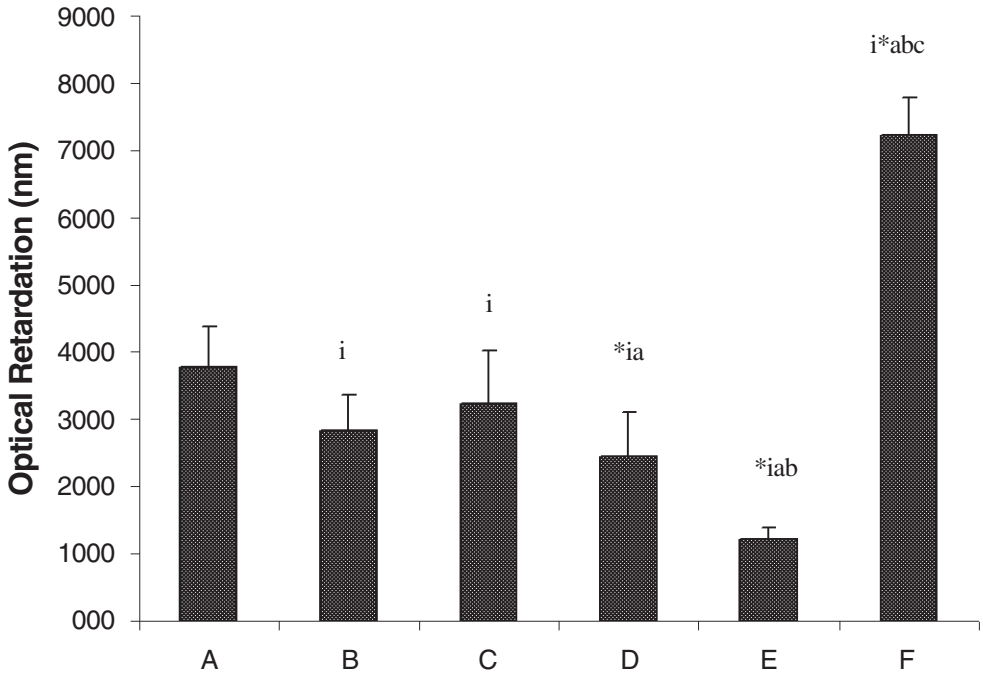

FIG. 1. Means and SD of optical retardation values of collagen fibers of calcaneal tendons of mice. i vs group A: (685 nm, 3 J/cm^2); *vs group B: (685 nm, 10 J/cm^2); a vs Group C: (830 nm, 3 J/cm^2); b vs Group D: (830 nm, 10 J/cm^2); c vs Group E: injuried control; d vs Group F: Standard control.

TABLE 1. *P* VALUES AND MEAN PERCENTAGE CHANGES IN THE OR OF THE COLLAGEN FIBERS AFTER EXPOSURE TO LASER IRRADIATION AT DIFFERENT WAVELENGTHS AND FLUENCES COMPARED TO THE INJURED CONTROL GROUP

	Group E		
Group	*p*	*Multiple comparisons*	*Percentage (%)*
Group A	0.000001	**	208%
Group B	0.000007	**	113.8%
Group C	0.000003	**	166.8%
Group D	0.000005	**	101.2%

*Significant ($p \leq 0.05$).
**Highly significant ($p \leq 0.01$).
Group A, 685 nm, 3 J/cm^2; group B, 685 nm, 10 J/cm^2; group C, 830 nm, 3 J/cm^2; Group D, 830 nm, 10 J/cm^2; group E, injured control.
NS, not significant.

agreed with the results obtained by several authors, who also observed that LLLT produced a stimulatory effect on tendon healing process.[1–9]

Our results suggest that laser irradiation (particularly using the 685-nm laser, at the dosage of 3 J/cm^2) produced an increase of cell proliferation through changes in mitochondrial physiology, subsequently affecting RNA synthesis, which, in turn, alters the expression of various cell regulatory proteins.[14–17] The effects of LLLT may also be due to stimulating the release of fibroblastic growth factors and increased fibroblast cell proliferation, which contributed to the higher deposition of collagen and reorganization of these fibers at the injured area.[14,18] It seems that fibroblast growth factor (FGF) promotes proliferation and differentiation of healing cells. FGFs can be considered natural mediators of the tissue repair process since they stimulate the growth fibroblasts and endothelial cells, and enhance mitogenic activities of vascular cells.[18]

It can also be suggested that the LLLT was able to promote neovascularization, re-establishing circulation at the site of injury, thus limiting isquemic necrosis and accelerating tissue repair.[7,9,13,17,19]

TABLE 2. *P* VALUES AND MEAN PERCENTAGE CHANGES IN THE OR OF THE COLLAGEN FIBERS AMONG THE DIFFERENT GROUPS

Group	*p*	*Multiple comparisons*	*Percentage (%)*
Group A vs. group B	0.0017	*	33%
Group A vs. group C	0.0291	*	16%
Group A vs. group D	0.0000	**	54%
Group B vs. group C	0.2816	NS	14%
Group B vs. group D	0.033	*	15%
Group C vs. group D	0.0093	**	32%

*Significant ($p \leq 0.05$).
**Highly significant ($p \leq 0.01$).
Group A, 685 nm, 3 J/cm^2; group B, 685 nm, 10 J/cm^2; group C, 830 nm, 3 J/cm^2; group D, 830 nm, 10 J/cm^2.
NS, not significant.

We propose the existence of a wavelength dependency response of the tendon to laser irradiation. We compared the effectiveness of two distinct wavelengths and the best response was observed after the irradiation with 685-nm laser. A series of authors have found different results in cell metabolism after the irradiation with different wavelengths. Almeida-Lopes et al.[11] found a significant increase in fibroblast cell proliferation after 780-nm laser irradiation and a decrease of cell proliferation after 692-nm laser irradiation. Moore et al.[20] observed increased fibroblast proliferation after 665-nm and 675-nm laser irradiation, whereas the 810-nm laser irradiation produced an inhibitory effect. Thus, it is clear the importance of identifying the most appropriate wavelength to stimulate a specific tissue.

Moreover, we found that the lower fluence used (3 J/cm^2) was more likely to produce a more pronounced effect of tendon healing. The existence of a curve dose-response of the tissues to laser irradiation has been postulated.[16,19–24] Many authors have stated that a dose of 1–5 J/cm^2 increases the tensile force of tenotomized tendons, accelerating collagen synthesis and facilitating the formation of membrane-bound intracytoplasmic collagen fibrils in tendon fibroblasts and myofibroblasts.[6,8] For example, Bayat et al.[25] and Parizotto and Baranauskas[3] state that the 632-nm laser, at the dosages between 0.5 to 5.0 J/cm^2 can stimulate the healing process of the conjunctive tissue in studies *in vivo*, whereas it was found that dosages higher than 10 J/cm^2 produced a decrease in fibroblast cell proliferation.[11]

Although the effects of LLLT have been highlighted in many works, the mechanisms by which LLLT acts on tissues are not totally understood and the use of the laser as a treatment is still controversial.[11,15,18–20] Therefore, before LLLT can be used as a therapeutic modality, it is necessary to investigate the effects of different wavelengths and fluences on tissues, to determine its safety and efficacy.

CONCLUSION

The results of the present study show a beneficial effect of LLLT on the repair process of the Achilles tendon in mice. Moreover, it was observed that the tissue response to laser irradiation has a dose and a wavelength dependence. It was found that the 685-nm laser, at the dose of 3 J/cm^2, appeared to be most effective at accelerating tendinous repair, although all treated groups showed a positive response to laser irradiation compared to the injured control animals. Further investigations are required to study the interaction of laser and tissues, and to study the effects of different parameters of laser on the healing process of the tendon, which may contribute to a better understanding of the efficacy of LLLT.

REFERENCES

1. Basford, J.R. (1995). Low-intensity laser therapy: still not an established clinical tool. Lasers Surg. Med. 16, 331–342.
2. Enwemeka, C.S., and Reddy, K. (2000). The biological effects of laser therapy and other physical modalities on connective tissue repair processes. Laser Ther. 12, 22–30.
3. Parizotto, N.A., and Baranauskas, V. (1998). Hidrogen bonding of collagen molecule stimulated by He-Ne laser in the regenerating of

tendon. Presented at the 2nd World Association of Laser Therapy (WALT) Meeting, Kansas City, MO.
4. Reddy, G.K., Stehno-Bittel, L., and Enwemeka, C.S. (1998). Laser photostimulation of collagen production in healing rabbit Achilles tendons. Lasers Surg. Med. 22, 281–287.
5. Reddy, G.K., Gum, S., Stehno-Bittel, L., et al. (1997). Biochemistry and biomechanics of healing tendon: Part II. Effects of combined laser therapy and electrical stimulation. Med. Sci. Sports Exerc. 97, 794–800.
6. Enwemeka, C.S., Conhen-Kornberg, E., Duswalt, E.P., et al. (1994). Biomechanical effects of three different periods of GaAs laser photostimulation on tenotomized tendons. Laser Ther. 6, 181–188.
7. Salate, A.C.B., Barbosa, G., Gaspar, P., et al. (2005). Effect of In-Ga-Al-P diode laser irradiation on angiogenesis in partial ruptures of Achilles tendon in rats. Photomed. Laser Surg. 23, 470–475.
8. Enwemeka, C.S. (1992). Ultrastructural morphometry of membrane-bound intracytoplasmic collagen fibrils in tendon fibroblasts exposed to HeNe laser beam. Tissue Cell. 24, 511–523.
9. Wanderer, C., Buchi, D.F., Tassini, C.M., et al. (1994). Braz. J. Med. Bio. Res. 27, 2241–2251.
10. Pereira, A.N., Eduardo, C.P., Matson, E., et al. (2002). Effect of low-power laser irradiation on cell growth and procollagen synthesis of cultured fibroblasts. Lasers Surg. Med. 31, 263–267.
11. Almeida-Lopes, L., Rigau, J., Zangaro, R., et al. (2001). Comparison of the low level laser therapy effects on cultured human gingival fibroblasts proliferation using different irradiance and same fluence. Lasers Surg. Med. 29, 179–184.
12. Cunha, A., Vidal, B.C., and Parizotto, N. (2001). The Effect of Therapeutic Ultrasound on Repair of the Achilles' Tendon (*Tendo calcaneus*) of the Rat. Ultrasound Med. Bio. 27, 1691–1696.
13. Vidal, B.C. (1986). Evaluation of the carbohydrate role in the molecular order of collagen bundles: microphotometric measurements of textural birefringence. Cell. Mol. Bio. 32, 527–535.
14. Enwemeka, C.S., Rodriguez, O., and Walsh, N. (1990). Morphometrics of collagen fibril populations in HeNe laser photostimulated tendons. Jour. Clin. Laser Med. Sur. 8, 47–62.
15. Passarella, S., Casamaxima, E., Molinari, E., et al. (1984). Increase of proton electrochemical potential and ATP synthesis in rat liver mitochondria irradiated *in vitro* by helium neon laser. FEBS Letter 175, 95–99.
16. Karu, T.I., Pyatibrat, L., and Kalendo, G. (1995). Irradiation with He-Ne laser increases ATP level in cells cultivated *in vitro.* Jour. Photochem. Photobiol. 27: 219–223
17. Karu, T. (2000). Mechanisms of low-power laser light action on cellular level. Proc. SPIE 4159, 1–17.
18. Wilden, L., and Karthein, R. (1998). Import of radiation phenomena of electrons and therapeutic low-level laser in regard to the mitochondrial energy transfer. J. Clin. Laser Med. Sur. 16, 150–165.
19. Webb, C., Dyson, M., and Lewis, W.H.P. (1998). Stimulatory effect of 660-nm low-level laser energy on hypertrofic scar-derived fibroblasts: possible mechanisms for increase in cell counts. Lasers Surg. Med. 22, 294–301.
20. Moore, P., Ridgway, T.D., Higbee, R.G., et al. (2005). Effect of wavelength on low-intensity laser irradiation-stimulated cell proliferation *in vitro*. Lasers Surg. Med. 1, 8–12.
21. Pinheiro, A.L.B., Limeira Junior, F., and Gerbi, M. (2001). Biomodulatory effects of LLLT on bone regeneration. Laser Ther. 13, 73–79.
22. Kujawa, J., Zavodnik, L., Zavodnik, I., et al. (2004). Effect of low-intensity (3.75–25 J/cm^2) near-infrared (810 nm) laser radiation on red blood cell ATPase activities and membrane structure. J. Clin. Laser Med. Surg. 22, 111–117.
23. Longo, L., and Mester, A. (1998). Present and future of laser cicatrization. In: Proceeding 2nd Congress World Association for Laser Therapy. Kansas City, Missouri, USA, September 2–5:10–11.
24. Kipshidze, N., Nikolaychik, V., and Keelan, M., et al. (2001). Low-power helium-neon laser irradiation enhances production of vascular endothelial growth factor and promotes growth of endothelial cells *in vitro.* Lasers Surg. Med. 28, 355–364.
25. Demir, H., Menku, P., Kirnap, M., et al. (2004). Comparison of the Effects of Laser, Ultrasound and Combined Laser*p*Ultrasound Treatments in Experimental Tendon Healing, Lasers Surg. Med. 35, 84–89.
26. Bayat, M., Delbari, A., Almaseyeh, M., et al. (2005). Low-Level Laser Therapy Improves Early Healing of Medial Collateral Ligament Injuries in Rats Photomed. Laser Surg. 23, 556–560.

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