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ORIGINAL PAPER

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Low-level laser therapy on the rat's gastrocnemius morphometry submitted to a rheumatoid arthritis model

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ABSTRACT

Introduction. Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune inflammatory disease of unknown origin, mainly affecting synovial joints and related structures, including the adjacent musculature, generating great disability and reduction in quality of life.

Aim. This study was designed to investigate the effect of low-level laser therapy (LLLT) on gastrocnemius of Wistar rats subjected to an experimental model of RA.

Material and methods. Forty male Wistar rats were used, separated into: acute and chronic, being subdivided into Control Group (CG): without intervention, Lesion Group (LG): submitted to lesion, Laser Control Group (LCG): without lesion and with treatment, and Laser Lesion Group (LLG): submitted to lesion and LLLT. The treatment with LLLT occurred in four points of the right knee, wavelength of 660 nm, energy density of 5 J/cm^2, energy per point of 0.003 J. Morphometric analysis was performed using a 40x magnification photomicrograph and analyzed using the Image-Pro-Plus 6.0 program.

Results. As result of the acute group there was a difference only for muscle mass, being higher in CG. For the chronic group there was significant difference for cross-sectional area, larger and smaller diameter, again with the control group obtaining higher values than the others, for the number of nuclei LG was lower than CG and LCG, but LLG was not different from any of them.

Conclusion. It is concluded that treatment with LLLT was not very effective in reversing the harmful effects of RA on the gastrocnemius muscle.

Keywords. photobiomodulation therapy, rheumatic diseases, skeletal muscle

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune inflammatory disease of unknown origin, mainly affecting synovial joints and related structures, including the adjacent musculature, generating great disability and reduction in quality of life.[1-4] The prevalence of RA in the United States from 2004 to 2014 ranged from 0.41 to 0.54% of the population, affecting 1.28 to 1.36 million

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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people in 2014, at a rate ranging from 0.29-0.31% among men to 0.73-0.78 among women.[5]

There are several therapies for the RA, however, they have many side effects, so new therapies are developed to overcome these limitations, such as immunotherapy and gene therapy.[1] Since the main therapeutic objectives are: reduction of pain and inflammatory activity, prevention of tissue degradation, increase function and improve quality of life, non-pharmacological resources have their relevance, such as thermotherapy, electrotherapy, whole body vibration, orthoses and low-level laser therapy (LLLT).[6,7]

LLLT has been used in the treatment of RA and has its mechanism of action described through the absorption of red and infrared radiation by the chromophores, which can increase enzymatic activity, production of adenosine triphosphate (ATP), protein synthesis and cell proliferation, resulting in analgesic effects.[8] In addition, it can assist in the joint protection process, reducing pain and stiffness.[9] Another important action of LLLT is the inhibition of chemotactic factors and prostaglandin synthesis in the early stages of inflammation.[10]

The anti-inflammatory, analgesic and healing effects of radiation emitted by LLLT are dependent on the characteristics of the laser, including wavelength, the mode of application, dose, duration and location.[11]

Aim

Given the existence of research gaps that evaluate the repercussion of RA treatment with LLLT on possible histological changes observed in skeletal muscle tissue, the objective of the present study was to analyze the effects of treatment with LLLT on the gastrocnemius muscle of Wistar rats submitted to an experimental model of complete Freund adjuvant (CFA) induced RA.

Material and methods

This research is characterized as quantitative, experimental, nonblind and randomized. It was approved by the Ethics Committee on Animal Use of the Universidade Estadual do Oeste do Paraná (Unioeste). The group consisted of 40 male Wistar rats, kept in plastic polypropylene boxes, with access to water and feed at will, controlled temperature at 21±1°C, light/dark photoperiod of 12 hours.

The animals were randomly separated into: acute, with a 7-day period of inflammation, and chronic with 28 days of inflammation, totaling 20 animals each. Then, for each period, the animals were again separated into four groups (n=5), for each period: Control Group (CG) – animals that were not submitted to injury with CFA, nor treated with LLLT; Lesion Group (LG) – animals submitted to RA induction, without treatment; Laser Control Group (LCG) – animals that were not submitted to injury but were treated with LLLT; Laser Lesion Group (LLG) – animals injured and submitted to treatment with LLLT.

Rheumatoid arthritis was induced by two injections of CFA (Mycobacterium butyricum, 0.5 mg/ml; 50 µl). The first injection was administered at the base of the animal's tail, intradermally. For this, the area of administration was trichotomized for subsequent asepsis with iodinated alcohol (1%). For the injection a 1 mL syringe and a 13x4.5mm needles were used, inserted approximately 1cm into the base of the tail in a subcutaneous manner, this being the first inflammatory stimulus. After seven days, the second injection was administered intra-articularly to the right tibiofemoral joint of the animals. For this application, the anterior area of the knee was trichotomized and the animals were manually contained for asepsis with iodinated alcohol (1%) and injection (1mL syringe and 13x4.5mm needle). The animals belonging to CG and LCG underwent the same protocol but received an injection with saline solution (0.9% sodium chloride).

Treatment with LLLT (Ibramed®) was performed punctually at four points on the right knee: anterior to the patella, medial side at the tibiofemoral joint, lateral side at the tibiofibular joint, and posterior in the popliteal region. Following the parameters: wavelength of 660 nm, energy density of 5 J/cm², power of 30 mW, spot area: 0.06 cm², irradiated energy per point of 0.003 J, irradiation time per point of 10 s. The treatment was performed on interspersed days during one week in the acute group, totaling four days of treatment. For the chronic group, 8 applications were performed, totaling 14 days of treatment. The animals belonging to the CG and LG of both groups were submitted to pen contact, but without the emission of the beam.

At the end of the experiment, the animals of both the acute and chronic groups were euthanized with an overdose of the association of anesthetic (ketamine - 240 mg/Kg) and a muscle relaxant (xylazine - 45 mg/kg). The gastrocnemius muscle of the right pelvic limb of the animals was collected, weighed, measured and fixed in Methacarn (70% Methanol + 20% chloroform + 10% glacial acetic acid), for 24 hours and stored in 70% alcohol. Subsequently, the muscles were included in histological paraffin, cut transversely in 7 µm of thickness (microtome Olympus CUT 4055), and the slides stained in hematoxylin and eosin to perform morphometric analysis.

For morphometric analysis, photomicrographs with 40x magnification were performed under the Olympus® DP71 microscope (USA) and analyzed using the Image-Pro-Plus 6.0 program (USA), which is calibrated to measure the cross-sectional area (µm²), the largest and smallest diameter of muscle fibers (µm), number of nuclei, number of fibers and ratio of nuclei per muscle fiber (nuclei ÷ fiber number).

The data were analyzed with the SPSS 20.0 program and presented with means followed by their respective

95% confidence intervals. After confirming the normality of the data, to perform the comparison of the different groups, it was used Generalized Linear Models, followed by the Sidak post-test, according to the different variables evaluated. In the area variables, the largest and smallest diameter, the distribution used was Gamma, and for the others the Normal distribution was used. The accepted level of significance was 5%.

Results

As a result for the gastrocnemius muscle mass, a significant difference was observed between the control animals for animals that had acute RA induction [Wald $X^2(3)$= 50.691; p<0.01], in which CG and LCG presented higher mass when compared to LG (p<0.01) and LLG (p<0.01) (Table 1). On the other hand, the chronic treatment animals did not present significant differences in the weight of the gastrocnemius muscle [Wald $X^2(3)$= 4.584; p=0.205] (Table 1).

Table 1. Mean and their respective 95% confidence intervals of the gastrocnemius muscle mass and length of Wistar rats

	Groups	Acute	Chronic
Mass	CG	1.48 [1.3-1.5]^a	1.54 [1.4-1.6]^a
	LCG	1.43 [1.3-1.5]^a	1.57 [1.4-1.6]^a
	LG	1.05 [0.9-1.1]^b	1.44 [1.3-1.5]^a
	LLG	1.13 [1.0-1.2]^b	1.49 [1.4-1.5]^a
Length	CG	24.85 [22.9-26.7]^a	25.55 [24.4-26.6]^a
	LCG	22.88 [20.9-24.8]^a	25.657 [24.5-26.7]^a
	LG	21.29 [19.3-23.2]^a	25.55 [24.4-26.6]^a
	LLG	22.28 [20.3-24.2]^a	25.29 [24.1-26.4]^a

Legend: CG – Control Group; LCG – Laser Control Group; LG – Injury Group; LLG – Injury Group + Laser. Different letters mean statistically different values (p≤0.05)

Regarding muscle length, both for chronic and acute treatment, no significant differences were observed between the experimental groups [Wald $X^2(3)$= 6.983; p=0.072], [Wald $X^2(3)$= 0.215; p<0.975] (Table 1).

In the animals of the acute treatment group, all the variables analyzed showed no difference between the groups: cross section [Wald $X^2(3)$=2, 061; p=0.560], larger diameter [Wald $X^2(3)$= 7.306; p=0.063] and smaller muscle fiber diameter [Wald $X^2(3)$= 1.629; p=0.653], as well as number of nuclei [Wald $X^2(3)$= 6.324; p=0.097], number of fibers [Wald $X^2(3)$= 7.067; p=0.070], and nucleus ratio per fiber [Wald $X^2(3)$= 4.022; p=0.259] (Table 2).

Regarding the morphometric analysis of the chronic RA animals, it was observed a significant reduction in the cross-sectional area of the gastrocnemius muscle [Wald $X^2(3)$= 50.74; p<0.01], where CG differed from LCG (p<0.01), LG (p<0.01) and LLG (p<0.01), presenting a larger area when compared to other groups. The same result was obtained for the largest [Wald $X^2(3)$= 61.445; p<0.01] and smallest muscle fiber diameter [Wald $X^2(3)$= 83.476; p<0.01] (Table 3).

In respect to the number of muscle fibers of these animals, there was no significant difference between the groups [Wald $X^2(3)$= 6.565; p=0.087]. For the number of nuclei [Wald $X^2(3)$= 12.863; p=0.05] LG presented a lower number of nuclei when compared to CG (p<0.05) and LCG (p<0.05), an effect that was reversed by the effect of LBP (LLG p=0.01). Regarding the core/fiber ratio there was no significant difference [Wald $X^2(3)$= 0.803; p=0.849] (Table 3).

Discussion

In the present study it was observed that the model used to trigger RA was able to produce acute changes in muscle mass, and chronically there were changes in diameters and cross-sectional area, in addition to a reduction in the number of myonuclei, and only in this variable did LLLT produce an effect.

The CFA induced RA model has been widely used, being able to efficiently mimic the symptoms of human RA, as well as the effects triggered by this type of inflammation. The changes generated by the RA start in synovia and progress to the joint and muscle structures, generating a systemic effect.[12–14]

Table 2. Mean and their respective 95% confidence intervals of the cross-sectional area, largest and smallest diameter, number of fibers and nuclei, besides the nucleus/fiber ratio of the muscle fibers of the gastrocnemius muscle of the acute group

	Cross-sectional area (μm²)	Largest diameter (μm)	Smallest diameter (μm)	Number of fibers	Number of nuclei	Nucleus/fiber ratio
CG	159.21 [128.4-08.9]^a	16.03 [14.5-19.1]^a	9.55 [8.11-11.9]^a	29.84 [18.3-37.1]^a	57.3 [36.6-64.8]^a	1.96 [1.7-2.2]^a
LCG	174.49 [139.4-161.4]^a	16.53 [16.0-17.2]^a	10.97 [10.5-11.5]^a	22.2 [19.7-26.5]^a	39.24 [33.9-49]^a	2.10 [1.5-2.1]^a
LG	154.94 [134.8-180.1]^a	15.45 [14.2-17.7]^a	9.51 [9.0-10.3]^a	27.26 [19-30.9]^a	59.78 [34.6-76.7]^a	2.18 [1.7-2.5]^a
LLG	169.74 [150.3-201.2]^a	16.43 [14.0-19.5]^a	9.87 [8.7-12.0]^a	24.94 [16.7-31]^a	36.1 [16-77.4]^a	2.14 [1.2-2.9]^a

Legend: CG – Control Group; LCG – Laser Control Group; LG – Injury Group; LLG – Injury Group+Laser.
Different letters mean statistically different values (p≤0.05)

Table 3. Mean and their respective 95% confidence intervals of the cross-sectional area, largest diameter, smallest diameter, in μ, number of fibers and nuclei, besides the nucleus/fiber ratio of the muscle fibers of the gastrocnemius muscle of the chronic group

	Cross-sectional area (μm²)	Largest diameter (μm)	Smallest diameter (μm)	Number of fibers	Number of nuclei	Nucleus/fiber ratio
CG	120.69 [97.3-149.6]^{a}	14.77 [13.4-16.2]^{a}	9.46 [8.7-10.1]^{a}	35.30 [26.3-44.2]^{a}	94.60 [77.4-115.6]^{a}	2.88 [2.5-3.2]^{a}
LCG	55.21 [44.5-68.4]^{b}	9.93 [9-10.8]^{b}	6.21 [5.5-6.8]^{b}	32 [23-40.9]^{a}	94.50 [77.3-115.4]^{a}	3.08 [2.6-3.5]^{a}
LG	55.65 [44.8-69.0]^{b}	9.99 [9.1-10.9]^{b}	6.14 [5.4-6.8]^{b}	23.80[14.8-32.7]^{a}	67.10 [54.9-82]^{b}	2.91 [2.5-3.3]^{a}
LLG	42.47 [34.2-52.6]^{b}	9.22 [8.4-10.1]^{b}	5.51 [4.8-6.1]^{b}	39.80 [30.8-48.7]^{a}	110.9 [90.7-135.5]^{a}	2.85 [2.4-3.2]^{a}

Legend: CG – Control Group; LCG – Laser Control Group; LG – Injury Group; LLG – Injury Group + Laser. Different letters mean statistically different values ($p \leq 0.05$)

In clinical findings of RA, muscle atrophy is observed related to the affected region, with several factors that may be related to its appearance, including sedentariness, advanced age, inflammatory, infectious, autoimmune diseases and malnutrition. Such conditions also modify the capacity of the muscle to regenerate.[15]

In the present study, a significant loss of muscle mass was observed in animals submitted to the lesion already in its acute form, emphasizing that joint inflammation induced by CFA produces a pro-atrophic deleterious effect. Corroborating these findings, Silva et al., demonstrated a reduction of muscle mass in 20% of patients with RA as a result of an intense inflammatory process, which can lead to muscle fatigue, weakness and functional deficit, in addition to changes in the quality of life of patients with this type of arthritis.[16] Similar findings were reported by Ancuta et al., showing physiological changes in the deltoid muscle, including muscle fiber atrophy, increase in the number of mitochondria and nuclei, in addition to the presence of inflammatory cells invading the muscle fibers.[17] This was different from the present study in which the lesion group presented a lower number of nuclei and was reverted by LLLT.

In the present study, the experimental model did not affect muscle changes in the other variables in animals induced to acute RA. The harmful effects of RA are linked to its progressive character, which is caused by chronic inflammation in tissues.[18] Muscular involvement appears in the form of myalgia, weakness and muscular atrophy, which end up producing sarcopenia, being an element of rheumatoid cachexia, a frequent event in the RA.[16,19] This research corroborates this fact, since the morphological findings for the chronic lesion group, since a decrease of the cross-sectional area was noted, and consequently of its larger and smaller diameter.

Regarding the number of muscle fibers between the groups in this study, there was no significant difference, contradicting studies that present the decrease in the number of fibers caused by the loss of motor units due to muscle atrophies caused by rheumatoid sarcopenia, in addition to direct degenerative changes of muscle fibers.[18] This fact explains not only the direct alteration in muscle fibers, but also the loss of muscle mass observed in the acute phase, in the chronic phase the loss of mass was not observed, however, it may have been masked by a fat infiltration in muscle tissue, which was not evaluated, which is one of the limitations of the present study.[20]

In conditions of skeletal muscle loss, intracellular signaling cascades cause cell death, decrease of satellite cells and protein deterioration, thus generating a decrease of nuclei in the muscle when affected by the RA, explaining the result found in the decrease in the number of nuclei in the group affected by the RA compared to the other groups, which was reverted by laser radiation, given its protective action on muscle tissue.[21,22]

However, in the present study, LLLT was not competent to revert muscle changes from the joint inflammatory process in the other variables analyzed. This fact may be related to insufficient irradiation, due to the small energy delivered[23] or even the time of exposure to radiation.[24] Thus, new studies with dosimetry variations are suggested, seeking an effective design to act directly on inflammatory cells, producing minimal side effects and contributing to the quality of life of patients with this chronic rheumatic disease.

Conclusion

Thus, it is concluded that the RA model, especially in the long term, resulted in deleterious effects on muscle morphometry, and changes were observed in the measurement of the cross section, greater diameter and smaller diameter of the muscle fiber. Also, LLLT was effective in reversing only the change in the amount of myonuclei.

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