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ORIGINAL ARTICLE

The action of pre-exercise low-level laser therapy (LLLT) on the expression of IL-6 and TNF-α proteins and on the functional fitness of elderly rats subjected to aerobic training

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Abstract The aim of the present study was to determine whether low-level laser therapy (LLLT), when used in conjunction with aerobic training, interferes with the expression of inflammatory markers IL-6 and TNF-α, thereby influencing the performance of old rats participating in swimming. A total of 30 Wistar rats (*Rattus norvegicus albinus*) were used for this study: 24 aged rats, and 6 young rats. The older animals were randomly divided into four groups designated as follows: aged-control, aged-exercise, aged-LLLT, aged-LLLT/exercise group, and young-control animals. Aerobic capacity (VO_2max) was analyzed before and after training period. The aged-exercise and aged-LLLT/exercise groups were trained for 6 weeks. LLLT laser was applied before each training session with 808 nm and 4 J of energy to the indicated groups throughout training. The rats were euthanized, and muscle tissue and serum were collected for muscle cross-sectional area and IL-6 and TNF-α protein analysis. In VO2 showed statistical difference between young- and aged-control groups (used as baseline) ($p<0.05$). The same difference can be observed in the young control group compared with all intervention groups (exercise, LLLT and LLLT+exercise). In comparison with the aged-control group, a difference was observed only for comparison with the exercise group ($p<0.05$), and exercise associated with LLLT group ($p<0.001$). Levels of IL-6 and TNF-α for the aged-exercise and the aged-LLLT/exercise groups were significantly decreased compared to the aged-control group ($p<0.05$). Analysis of the transverse section of the gastrocnemius muscle showed a significant difference between the aged-exercise and aged-LLLT/exercise groups ($p<0.001$). These results suggest that laser therapy in conjunction with aerobic training may provide a therapeutic approach for reducing the inflammatory markers (IL-6 and TNF-α), however, LLLT without exercise was not able to improve physical performance of aged rats.

Keywords Low-level laser · Aerobic exercise · Inflammatory markers · VO_2max

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Introduction

Aging is characterized by adverse physiological and functional changes, including poor homeostatic balance and increasing

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incidence of pathology. In older individuals, these physiological changes often impair immune function, a condition referred to as immunosenescence. Moreover, aging is also linked to negative effects on the cardiovascular, respiratory and nervous systems [1], as well as impaired muscle strength and increased fatigability from only minor physical exertion. Overall, these changes due to aging have been shown to impair the quality of life and survival of older subjects [2, 3].

Researchers have suggested the idea of aging inflammation, a chronic condition in which low-grade inflammation is associated with the normal aging process and age-related diseases [4, 5]. A key role of inflammation has been clearly established in several large epidemiologic studies of older adults [6]. Thus, aging is associated with increased inflammatory activity in the blood, including increased levels of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, c IL-1ra, and neopterin [7]. In addition, increased inflammatory activity in older populations may reflect age-related pathological processes [8].

Based on the aforementioned findings, the use of pro-inflammatory status as a therapeutic target is proposed. Numerous studies have suggested that low to moderate exercise training may decrease the levels of TNF-α and C-reactive protein (CRP) in the elderly. In addition, similar types of exercise have recently been associated with lower plasma concentrations of IL-6 and CRP in various age groups, ranging from young adults to the elderly [9].

In addition to exercise training, low-level laser therapy (LLLT) has also been used to boost repair processes by reducing pro-inflammatory markers, including TNF-α and IL1-β, as well as increasing anti-inflammatory cytokines, such as IL-10 [10–13]. More recently, researchers have shown that in addition to the modulatory effects of LLLT on inflammatory processes, LLLT also results in the reduction of creatine kinase levels immediately after exercise [14, 15]. LLLT also acts on the markers of oxidative stress, such as protein carbonyls, superoxide dismutase, and TBARS [16], in addition to yielding clinical signs of improvement, delayed muscle fatigue, and improved physical performance [17–19].

Considering the anti-inflammatory properties of exercise training and LLLT, here, we wanted to determine whether LLLT, when associated with aerobic training, interferes with protein expression of the inflammatory markers IL-6 and TNF-α, thus influencing the performance of old rats subjected to exercise in the form of swimming.

Materials and methods

Experimental animals

A total of 30 Wistar rats (*R. norvegicus albinus*) were used in this study, with the animal groups consisting of 24 aged animals (24-month old) with a mean body weight of 517.7± 27.54 g and 6 young animals (12-weeks old) with a mean body weight of 266, 19:30 0±g. The animals were from the Animal Facility of the Federal University of São Paulo (UNIFESP), where they were housed and kept under conditions with controlled light and temperature, and with water and food "ad libitum". All the experimental procedures were carried out in accordance to the standards established by the Brazilian College for Animal Experimentation (COBEA). The animals were handled in compliance with the national guidelines for the humane treatment of laboratory animals. All the experimental procedures were approved by the Research Ethics Committee of the UNIFESP.

Experimental groups

It was used a total of 30 animals, 6 young and 24 aged rats. The 24 aged animals were randomly divided into four groups, with 6 animals per group as follows: aged-control group, with no LLLT irradiation and no exercise training; aged-LLLT group (GLI), treated with LLLT irradiation and no exercise training; aged-exercise group, that performed exercise training without LLLT; aged-LLLT/exercise group (GLTI), treated with irradiation and subjected to exercise training. The six young animals were allocated to young-control group, with no LLLT irradiation and no exercise training. Thus, we totaled a sample of 30 animals (24 animals aged 6 and younger animals).

Functional fitness assessment (maximal oxygen uptake, VO_2max)

The test protocol to assess functional fitness was performed using a motorized treadmill coupled with a gas analyzer (Panlab, Harvard Bioscience Company, MA, USA) in which VO_2 and VCO_2 were continuously recorded. Three days prior to testing, the rats were introduced to running on the treadmill for 15 min (5-min stages) as follows: first day (25 cm/s, 35 min/s, 35 cm/s); second day (25 cm/s, 45 min/s, 55 cm/s); and third day (25 cm/s, 55 min/s, 65 cm/s). To evaluate VO_2max, each rat had to undergo a 2-min warm-up period at 25 cm/s, and the treadmill speed was increased by 9 cm/s every 2 min to reach physical exhaustion. A VO_2 steady state obtained by a progressive increase in running speed and a respiratory exchange ratio of ≥1.05 were taken into account to determine the VO_2max. Assessment of VO_2max was performed before and after the exercise training. VO_2max measured for aged-control group (without LLLT and without exercise training) was used as baseline.

Exercise training

The exercise training consisted of swimming in a pool built using a fiberglass water box with a diameter of 130 cm and a height of 80 cm. The water was heated by a thermostat

associated with a gas heating system to temperatures ranging from 32 to 34 °C. The training protocol was based on the study of Takeda et al. (1988) [20] and was divided into two phases:

1. Adaptation: On the first day of exercise, the animals swam for 15 min and for times increased by 15 min on each subsequent day until the sixth day when the animals were able to swim for 90 min.
2. Main training: The training time was maintained at 90 min, 6 times a week for 6 weeks.

Low-level laser application

The DMC Laser Photon Laser III ® (DMC—Sao Carlos, SP, Brazil) system was used for irradiation with conditions as shown in Table 1. The laser was applied transcutaneously at a single point for 40 s in the central area of the gastrocnemius muscle according Sussai et al. (2010) [14]. The application was done before every training session for both in the specified aged group that received exercise training and the group that received LLLT only.

Euthanasia

At the end of the 6-week training period, animals from each experimental group were identified, weighed, and then euthanized by decapitation according to the protocol detailed in the Report of the AVMA Panel on Euthanasia (2001).

The gastrocnemius muscle was collected immediately after euthanasia. The incised areas were surgically removed with a 1-cm margin of skin surrounding the lesion to the depth of the fascia. The muscle (two from each animal) was divided into two parts, with one piece intended for histological analysis, and the other piece frozen in liquid nitrogen and stored at −80 °C for subsequent ELISA analysis of protein expression.

Histological procedures

Collection of muscle tissues

The gastrocnemius muscles of the right hind paw were collected and stored in 10 % buffered formalin for histological processing. Hematoxylin and eosin (H&E) staining was performed based on a routine protocol. Slides were imaged, and the morphology of the striated skeletal muscle fibers was analyzed (Eclipse E-200; Nikon, Tokyo, Japan).

The whole muscle cross-sectional area (CSA) at the mid-belly was measured by tracing an outline of each muscle using Image Pro Plus. To measure individual muscle fiber CSA, independent of fiber type, four images (×10) from the H&E sections of the gastrocnemius muscles were captured for each age group. A grid with 25 random dots was placed over the images, and the CSA of the fibers marked with the dots was measured. A total of 600 fibers were measured from each muscle at each age group ([4 images per muscle]×[25 fibers per image]×[6 animals per age group]=600 fibers).

Table 1 Summary of the laser parameters

Wave length (nm)	Output power (mW)	Power density (W/cm^2)	Laser beam (cm^2)	Energy density (J/cm^2)	Energy per point (J)	Irradiation time per point (s)
808	100	1.071	0.028	144	4	40

Evaluation of IL-6 and TNF-α protein expression

IL-6 and TNF-α protein expression was quantified using an ELISA kit, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Briefly, 96-well plates were coated with 100 μL of the monoclonal antibodies specific for the cytokines, anti-IL-6 and TNF-α, that were diluted in sodium carbonate buffer (0.1 M, pH 9.6), and the plates were incubated at 4 °C for 18 h. To block the plates, they were washed four times with phosphate-buffered saline containing 0.05 % Tween 20 (PBST), then filled with blocking solution containing 3 % gelatin in PBST (Sigma, St. Louis, MO, USA) (300 μL/well) and incubated at 37 °C for 3 h before being subjected to a new washing cycle. Next, 100 μL of the appropriately diluted samples or recombinant cytokine standard were added to the plates, and they were incubated at 4 °C for 18 h. After washing, 100 μL of the biotinylated antibodies specific for the detection of each cytokine were added to the wells, and they were incubated at room temperature (22 °C) for 1 h. After the plates were washed, 100 μL of streptavidin-peroxidase was added to the wells, and the plates were incubated at room temperature (22 °C) for 1 h, followed by additional washes. The reaction was visualized by adding 3,3′,5,5′ tetramethylbenzidine solution (100 μL/well), followed by the addition of 2 N sulfuric acid (50 μL/well) to stop the reaction. The plates were read using a Spectrum Max Plus 384 spectrophotometer (Sunnyvale, CA, USA) at a wavelength of 450 nm with correction at 570 nm. The concentrations of the cytokines in the samples were calculated from the standard curves obtained from the recombinant cytokines [11].

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). The post-training VO_2max values were compared using one-way ANOVA with a Tukey post hoc test. Data from the ELISA and histological analysis were also compared using one-way

ANOVA with a Tukey post hoc test. Data are expressed as mean±standard deviation of the mean, and differences with $p<0.05$ were considered significant.

Results

Functional fitness

Assessment of cardiopulmonary function was conducted using the standard approach before and after swim training. All values of VO2 obtained after the period of training of groups suffering intervention, statistically were crossed with control groups of young and aged basal values of VO2. In statistical analysis, groups were compared using one-way ANOVA with a Tukey post hoc test. In the comparison between the young-control group (62.7±4.1 ml/kg/min) and aged-control group (42.1±4.9 ml/kg/min) for values of VO2max baseline statistically showed significant difference ($p<00:05$) when the young-control group (baseline) compared to the aged-was LLLT/exercise group (53.8±5.3 ml/kg/min), the statistical difference ($p<12:05$) was observed. In addition, when the aged-control group (baseline) was compared to the aged-LLLT/exercise group, the statistically significant difference was also observed ($p<0:01$). A statistical difference was observed between the young-control group (baseline) and the aged-LLLT group (42.5±4.4 ml/kg/min) ($p<0:05$), and when the aged-control group (baseline) was compared to the aged-LLLT group, the statistical difference was observed ($p>0:05$). Comparison of the young-control group (baseline) to the aged-exercise group (54.4±8.0 ml/kg/min) showed a statistical difference ($p<0:05$), and when the aged-control group (baseline) was compared with the aged-exercise group, a significant difference ($p<0:05$) (Fig. 1) was also observed.

IL-6 and TNF-α protein expression

The analysis of the protein expression results for the cytokine IL-6 showed a statistically significant difference ($p<0.001$) between the sgroup (795±64.73). When the young-control group was compared to the aged-exercise group (539.1±40.38), a statistical difference ($p<0.001$) was observed. In addition, when the aged-control group was compared to the aged-exercise group, a statistically significant difference was also observed ($p<0.001$). A statistical difference was observed between the young-control group and the aged-LLLT group (539.1±40.38) ($p<0.001$), and when the aged-control group was compared to the Aged–LLLT group, a statistical difference was also observed ($p<0.05$). Comparison of the young-control group to the aged–LLLT/exercise group (310.0±88.9) showed that the combination of these therapies resulted in a lower level of protein expression that was very close to the young-control, and that there was no statistical difference. The intersection between the aged-control group and the aged-LLLT/exercise group showed a statistical difference ($p<0.05$). A statistical difference was also observed between the aged-exercise group and the aged-LLLT/exercise group ($p<0.01$). Finally, a significant difference was observed between the two groups irradiated with the laser, the aged-LLLT group and the aged-LLLT/exercise group ($p<0.001$) (Fig. 2).

Evaluation of protein expression for the cytokine TNF-α showed a statistically significant ($p<0.001$) difference between the young-control group (462.4±116.3) and the aged-control group (776.5±113). When the young-control was compared to the aged-exercise group (574.0±76.96), no

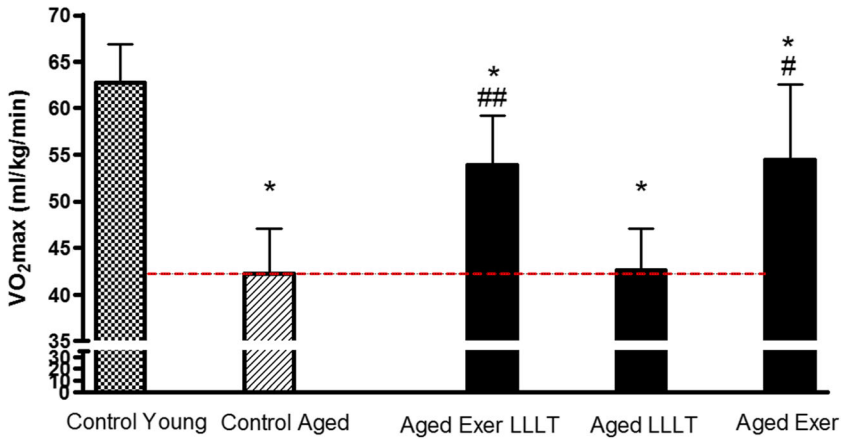

Fig. 1 Comparison of the mean and standard deviation of the maximum oxygen consumption (VO_2max) of young-control rats and aged rats before and after laser therapy or LLLT and swim training. *$p<0.05$ and **$p<0.001$, using Tukey's test with comparisons against the young-control group; $^{\#}p<0.05$ and $^{\#\#}p<0.001$ using Tukey's test with comparisons against the aged-control group; $^{\lambda}p<0.05$ using Tukey's test comparing the group aged-LLLT/exercise with the aged-LLLT and $p<0.05$ using Tukey's test comparing the group aged-LLLT with the aged-exercise

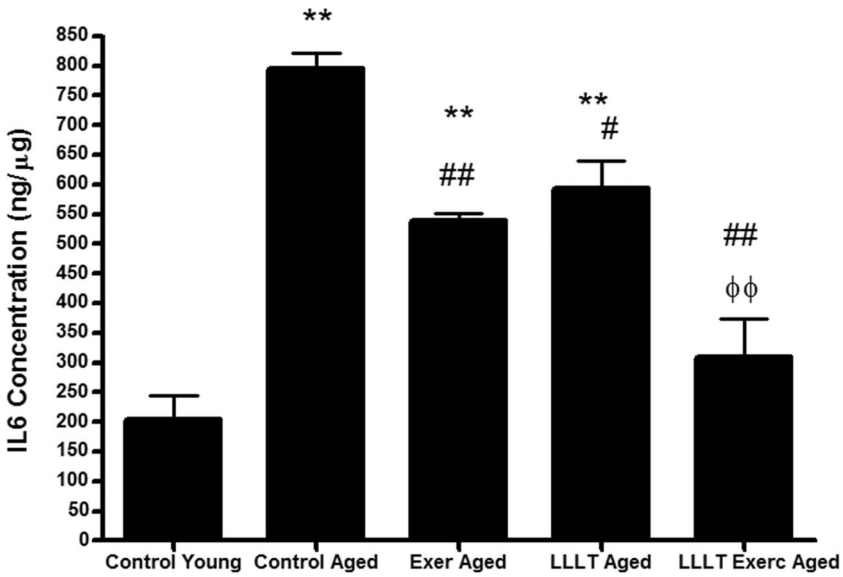

Fig. 2 Comparison of the mean and standard deviation of the IL-6 concentrations obtained using the ELISA kit. Figure represents the concentrations in serum after 6 weeks of aerobic training through swimming, where $*p<0.05$ and $**p<0.001$, using Tukey's test with comparisons against the young-control group; $^{\#}p<0.05$ and $^{\#\#}p<0.001$ using Tukey's test with comparisons against the aged-control group; and $p<0.05$ using Tukey's test comparing the young rat group administered LLLT with the aged-LLLT group

statistical difference was observed. However, when the comparison was made between the aged-control group and the aged-exercise group, a statistically significant difference ($p<0.01$) was observed. Comparison between the young-control group and the aged-LLLT group (646.7±54.98) showed a statistical difference ($p<0.05$); however, there was no statistical difference between the aged-control group and the aged-LLLT group. When comparing the results for the young-control group to the aged–LLLT/exercise group (506.1±106.3), we observed that the combination of these therapies resulted in a lower level of protein expression that was very close to the young-control, and no statistical difference was observed for the intersection between the aged-control group and the aged-LLLT/exercise group. The comparison of the two groups irradiated with the laser, the aged-LLLT group and the aged-LLLT/Exercise group, showed a statistically significant difference ($p<0.05$) (Fig. 3).

Muscle fiber morphology

The CSA was determined using planimetry (Fig. 4), and as expected, the young animals (66.23±26.6 area/mm^2) had a larger CSA than the aged-control group (31.64±5.8 area/mm^2) ($p<0.001$). On the other hand, comparison of the young-control group to the aged-exercise group, with (64.29±8.8 area/mm^2) and without LLLT (58.62±6.0 area/mm^2), did not show a statistical difference. These findings indicate that exercise training was effective in reducing common muscular atrophy during aging, although the values for the group subjected to swim training in conjunction with the LLLT, were higher than the average values.

Comparison of the aged-control group to the aged-exercise group and the aged-LLLT/exercise group showed a statistical difference ($p<0.001$), indicating that there was an increase in the transverse section of the muscle compared to the control group that did not undergo any intervention. When the aged-control group was compared to the aged-LLLT group (35.24±7.8 area/mm^2), there was no statistically significant difference, demonstrating that application of LLLT alone was not able to promote changes in muscle morphology. We also observed that there was no statistical difference between the aged-exercised groups with and without LLLT irradiation (Fig. 4).

Discussion

In the current study, we hypothesized that LLLT in conjunction with aerobic training may be an effective approach to reduce pro-inflammatory markers, IL-6 and TNF-α, in skeletal muscle. Studies have demonstrated this problem, and in addition, there is data that LLLT plus swim training could improve the functional performance of old rats. LLLT plus swimming could improve the functional performance in the old rats. Thus, we investigated the VO_2 max before and after 6 weeks of swimming.

Circulatory levels of inflammatory markers, including IL-1b, IL-6, CRP, and TNF-α are known to increase during aging and are thus frequently measured in research and diagnostic applications.

Previous studies have shown that aerobic exercise decreases the production of pro-inflammatory, atherogenic cytokines, IL-1b, TNF-α, interferon gamma (INF-c), and CRPs, as well as increases the anti-inflammatory levels of atheroprotective cytokines, such as IL-10. In contrast, other studies have reported that exercise training has no such effects. Moreover, exercise training results in an up-regulation of antioxidant molecules in several bodily tissues, presumably due to

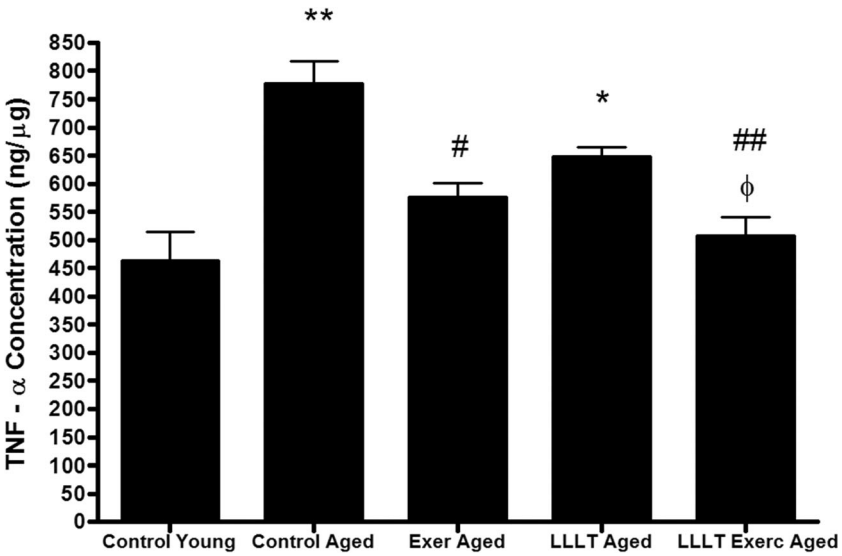

Fig. 3 Comparison of the mean and standard deviation of the concentrations of TNF-α obtained using the ELISA kit. Panel A represents concentrations in serum after 6 weeks of aerobic training through swimming, where $*p<0.05$ and $**p<0.001$, using Tukey's test with comparisons against the young-control group; $^{\#}p<0.05$ and $^{\#\#}p<0.001$ using Tukey's test with comparisons against the aged-control group; and $p<0.05$ using Tukey's test comparing the young rat group administered LLLT with the Aged-LLLT group

the increased levels of oxidative stress that occur during exercise [20].

Studies have indicated great anti-inflammatory potential of LLLT [11, 12], and we believe that the association of LLLT with aerobic exercise could be of great value to the physical performance of aged rats. Red or near-infrared photons are absorbed in cytochrome c oxidase (unit IV of the mitochondrial respiratory chain), thereby increasing mitochondrial respiration and ATP production and initiating signaling pathways mediated by reactive oxygen species, nitric oxide, and cyclic AMP. A large number of studies have examined the anti-inflammatory role of LLLT. These studies have reported reduction in inflammatory cells (neutrophils, macrophages, lymphocytes, and mast cells) and infiltration of multiple pathologies, as well as have shown to reduce several inflammatory cytokines, such as TNF-α and ILs.

In our study, we demonstrated that LLLT associated with exercise was able to intensely reduce pro-inflammatory TNF-α cytokine level compared to another experimental group. The statistical analysis showed no difference between the group subjected to LLLT and exercise and the young-control group, although the exercised animals showed decreased TNF-α.

Our results for the evaluation of levels of the IL-6 cytokine point to decreased expression of IL-6 at the end of the 6-week training period, in which case, LLLT associated with the exercise group also showed lower levels when compared to the other group. These findings, in which aerobic training presents

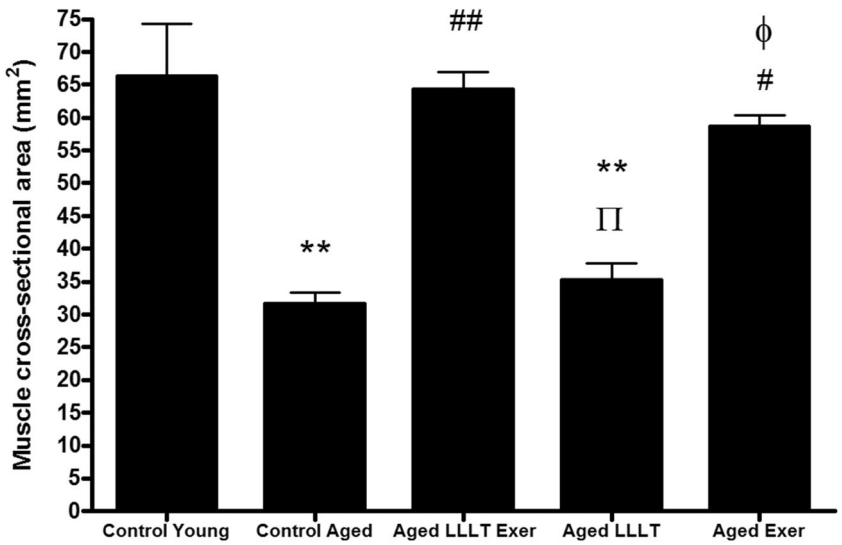

Fig. 4 Comparisons of the mean and standard deviation of the cross-sectional area (CSA) after 6 weeks of aerobic exercise training in the young-control and aged-control rats and groups undergoing training associated with LLLT laser or just LLLT. $*s<0.05$ and $**p<0.001$, using Tukey's test with comparisons against the young-control group; $\#p<0.05$ and $\#\#p<0.001$ using Tukey's test with comparisons against the aged-control group; and $p<0.05$ using Tukey's test comparing the young rat group LLLT with the aged-LLLT group

mitigation to levels of IL-6, goes against the results presented by Thompson [26], which performed a clinical study with 41 sedentary men aged 45–64 years. Volunteers performed a progressive exercise protocol with lengthening of 24 weeks. Authors observed that IL-6 levels only starts to decrease by the 12th week of training, and when training program was finished, IL-6 levels returned to baseline values.

In group comparison of the aged rats irradiated with the laser and that were subjected to aerobic training showed it demonstrated a significant improvement in aerobic capacity; however, we did not obtain statistical difference between the two group's intervention which leads us to affirm that LLLT was able to produce direct benefits in aerobic capacity.

On the other hand, previous studies in our group showed that LLLT can attenuate serum creatine kinase in rats submitted to high-intensity exercise. Furthermore, LLLT can decrease [2, 21, 22] pro-inflammatory cytokine expression in mice subjected to cutaneous injuries [21] and damage to the tendons [12] or joints [11]. Other experimental [22, 23] and clinical studies [15–18] have also considered the role of LLLT in physical performance, thus improving aerobic capacity, resistance to fatigue, and decreasing oxidative stress [13]. Some of these studies have also reported that LLLT could interfere with the redox state to decrease reactive oxygen species (ROS), favoring physical performance [14, 15, 22, 24].

Patrocinio et al. (2013) [22] conducted an experimental study using thirty rats randomly divided into three groups: control group, trained group, and trained+laser-irradiated group. A resistance training program of a climbing exercise with weights attached to the tail of the animal was performed three times per week for 5 weeks. Furthermore, laser irradiation was performed in the middle region of the tibialis anterior (TA) muscle of both legs; authors observed that LLLT decreased resting lactate concentration and improved muscle fiber morphology, which may contribute to improvement of muscle performance in the exercised rats compared with the unirradiated animals.

A study [25] reported that phototherapy (LLLT and light-emitting diode therapy (LEDT)) has been used to combat ROS and reactive nitrogen species (RNS) delivered during physical exercise to improve the reduction of mitochondrial function, which contributes to the reduction of muscle fatigue to increase muscle performance. On the other hand, researchers have reported that other mechanisms may be involved in reducing muscle fatigue and increased performance, including (i) improvement in the sensitivity of myofibrils and Ca^{2+} channels to the Ca^{2+} ion; (ii) increase in Ca^{2+} uptake from the sarcoplasm to the sarcoplasmic reticulum via Ca^{2+} pump (ATP-dependent); (iii) improvement in the formation of cross-bridges and production of contractile forces; (iv) increase in the activity of the Na^{+}/K^{+} ATPase pump to reduce the excess of extracellular K^{+} and depolarization of the muscle fibers to continue the exercise; and (v) lower muscle damage and leakage of muscle contents into the blood, such as muscle CK and myoglobin.

Leal-Junior et al. [18] argued that the observed effects from phototherapy in muscle performance some times seems small, however, it is interesting to note that the positive effects of phototherapy seem to be even more consistent for surrogate outcome measures like the biochemical markers. This increases the credibility of the positive outcomes seen for muscle performance measures, because it strengthens the notion that there are underlying photochemical processes responsible for the observed effects. In addition, the no-significant results can be explained by inadequate irradiation in regards of either from too low doses or too small areas covered by irradiation.

In the present study, as evidenced, no significant improvement in aerobic function in the group submitted to LLLT; however, just parameters of the dose of LLLT and the application form may have influenced our results.

Although VO_2max is the gold standard to evaluate aerobic functional fitness, it is still possible that the test conducted on the treadmill may have affected the measurements. Thus, considering the specific training mode, a more appropriate assessment of VO_2max has been demonstrated using swimming tests. Unfortunately, there is no reliable approach to analyze VO_2max during swimming tests, because the pool water makes it difficult for incremental loading, which can result in unreliable measurements.

It is well accepted that the imbalance of pro- and anti-inflammatory cytokines is responsible for changes in the body composition of older adults [26]. We have shown that a swimming training program reduced pro-inflammatory cytokines in the skeletal muscle of older rats. Our finding that LLLT may potentiate the effects of exercise was quite intriguing. Overall, these findings may explain the maintenance of CSA in our case, which can mitigate sarcopenia during aging.

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Conflicts of interest Professor Ernesto Cesar Pinto Leal-Junior receives research support from Multi Radiance Medical (Solon, OH, USA), a laser device manufacturer. The remaining authors declare that they have no conflict of interests.

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