ORIGINAL ARTICLE

Photobiomodulation increases mitochondrial citrate synthase activity in rats submitted to aerobic training

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Abstract

This study investigated the effects of photobiomodulation by low-laser laser therapy (LLLT) on the activities of citrate synthase (CS) and lactate dehydrogenase (LDH) and the anaerobic threshold (AT) in rats submitted to treadmill exercise. Fifty-four rats were allocated into four groups: rest control (RCG), rest laser (RLG), exercise control (ECG), and exercise laser (ELG). The infrared LLLT was applied daily on the quadriceps, gluteus maximum, soleus, and tibialis anterior muscles. Muscle samples (soleus, tibialis anterior, and cardiac muscles) were removed 48 h after the last exercise session for spectrophotometric analysis of the CS and LDH. The CS activity (μmol/protein) in ELG (16.02 and 0.49) was significantly greater ($P < 0.05$) than RCG (2.34 and 0.24), RLG (6.25 and 0.17), and ECG (6.76 and 0.26) in the cardiac and soleus muscles, respectively. The LDH activity (in 1 Mm/protein) in soleus muscle was smaller ($P < 0.05$) for ELG (0.33) compared to ECG (0.97), RLG (0.79), and RCG (1.07). For cardiac muscle, the LDH activity was smaller ($P < 0.05$) in ELG (1.38) compared to ECG (1.91) and RCG (2.55). The ECG and ELG showed increases in the maximum speed and a shift of the AT to higher effort levels after the training period, but no differences occurred between the exercised groups. In conclusion, the aerobic treadmill training combined with LLLT promotes an increase of oxidative capacity in this rat model, mainly in muscles with greater aerobic capacity.

Keywords Lactate dehydrogenase · Oxygen consumption · Exercise tolerance · Low-level laser therapy

Introduction

Photobiomodulation (PBM) is the use of light, usually through low-level laser therapy (LLLT) or light-emitting diode therapy (LEDT), to stimulate the healing and regeneration of damaged tissue [1]. Classically used to increase adenosine triphosphate (ATP) synthesis by cytochrome c oxidase activity in mitochondrial electron transport chain, recently PBM by red or near-infrared light has been applied before or after exercise to improve muscle performance by attenuation of exercise-induced muscle damage [2] and higher fatigue resistance [1].

Previous studies have suggested that on the irradiated cells, the first photochemical and photophysical events occur in the mitochondria, promoting respiratory modifications as a result of structural changes [3, 4] as well as metabolic modifications in these cytoplasmic organelles. This probably occurs, because changes on the membrane potential [5–7] and/or in the enzymatic activities [8–10] all contributing towards more available energy to perform cellular activities and may also to develop more effective aerobic metabolic pathways [4, 10].

Specifically, the enzyme citrate synthase (CS), which plays a central role in the mitochondrial oxidative capacity, may have its activity increased by PBM [11]. In addition, the enzyme lactate dehydrogenase (LDH) that has high activity

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when lactic anaerobic metabolism predominates may have its function altered by LLLT [12].

However, there is limited information about the bioenergetic modulation and about the muscular aerobic performance (AP) in clinical or experimental studies, mainly when aerobic exercises are combined with LLLT irradiation on skeletal muscles. The analyses of bioenergetics and AP in different skeletal muscles are extremely important, considering that these variables have obvious applications in therapeutic field, including those involved in sports.

Therefore, the aim of this study was to determine the adaptations of CS and LDH enzymes and the anaerobic threshold (AT) after treadmill exercise training in rats submitted to LLLT. It was postulated that the LLLT could stimulate aerobic metabolism through the increase of the Krebs' cycle enzymatic activity and glycolytic activity reduction, resulting in a better AP after training.

Methods

Animals

This study was conducted according to the Guide for Care and Use of Laboratory Animals and approved by the local Animal Ethics Committee. Sixty male Wistar rats weighting 112 ± 4.7 g and 30 days old were used. All rats had food and water "ad libitum." The temperature during experimental period was controlled (22–27 °C), as well as the photoperiod: 12/12 h light and darkness [13]. Only 54 rats remained in the experiment (one rat died and another five did not demonstrate an AT curve in one of the tests during serum lactate dosage).

Experimental procedures

The rats were randomly allocated into four groups and placed in boxes. Each box had five rats of the same group as follows: (1) rest control group—non-trained and non-irradiated with LLLT (RCG; $n = 10$); (2) rest laser group—non-trained but irradiated with LLLT during 30 days (RLG; $n = 9$); (3) exercise control group—trained and non-irradiated with LLLT (ECG; $n = 16$); and (4) exercise laser group—trained and irradiated with LLLT (ELG; $n = 19$). Only ECG and ELG groups were submitted to a daily aerobic treadmill training protocol performed from Sunday to Thursday over 5 weeks, and to an incremental, multistage exercise test, once a week, totalizing five measurements.

Instrumentation

The aerobic treadmill training and incremental effort tests (IET) were performed on an ergometric treadmill for small-sized animals. This equipment has seven individual lanes that allowed each rat to training in isolated mode and simultaneously. In addition, the ergometric treadmill allows manual adjustments in treadmill slope, ascending and descending up to 20%. In order to analyze blood lactate level and to determine AT, an electroenzymatic lactimer (YSI MODEL I500 Sport Lactate Analyzer, Yellow Springs, Ohio, USA) was used.

Aerobic treadmill conditioning and training

Only ECG and ELG groups were submitted to an initial period of adaptation on treadmill. The adaptation procedure consisted on a walk/run at lower speed (6 m/min) and 0% of treadmill inclination during 5 min, per 6 consecutive days, until the rats reach 10 min of exercise at 17.5 m/min on the sixth day (minimum speed recommended) [14].

The training program began with the introduction of daily speed increments (2 min during the training period with a variable speed progression) until predetermined goal have been reached on the last day of the training week. This gradation in training was aimed to reach an optimum adaptation of the rats in the aerobic training routine, hence favoring the predominance of aerobic metabolism [14]. The predominance of aerobic metabolism could be confirmed based on the AT values presented by the animals that was always superior to the weekly proposed work for training program. Each training session was carried out at darkness. The training room was also kept dark for possible better adaptation of the rats to the treadmill training [15], respecting the light/dark photoperiod [13].

After concluded the program of aerobic training on treadmill, all rats were sacrificed in order to analyze CS and LDH activities.

Incremental effort test and blood sample

An incremental and multistage effort test with blood samples was carried out to measure maximum running speed (MRS) and lactate to determine AT, respectively, for ELG and ECG. AT was visually identified by blood lactate concentration versus workload curve, considering the workload corresponding to the inflection point of blood lactate concentration within a range of 2–4 mM, according to previous studies [16, 17]. This analysis was performed by a blinded researcher for group allocation. These effort tests were performed once a week, every Friday (1 day after the last training of the week), totaling five evaluations. On Saturday, all the animals remained at rest and received no treatment.

The incremental and multistage effort test with blood sample had a discontinuous protocol, which was based on steps and increases in ergometric treadmill speed as following: (1) an initial baseline blood sample was taken before to start the incremental effort test; (2) all rats were submitted to sets of

physical effort for 3 min followed by 2 min of rest; (3) blood was collected directly from rat tail at the all rests by heparinized capillary and transferred to "eppendorf" tubes containing 50 µl of sodium fluoride for analysis of lactate. The blood collected was kept in cool ice during the whole procedure and afterwards was stored at − 20 °C until lactate analysis.

Treadmill speed during incremental effort test was increased at 4 m/min at each set of rat physical effort with constant inclination of 10% [18, 19]. This incremental effort test was performed until rat's exhaustion (inability to run the set speed).

Low-level laser therapy

The infrared irradiation was applied on the quadriceps femoris, tibialis anterior, soleus, and gluteus maximus muscles of both hind limbs of the RLG and ELG groups, totaling eight irradiation points daily in each rat. The points of laser irradiation were shaved, and LLLT probe was applied on the skin using contact technique with light pressure in order to prevent and/or minimize reflection and refraction. The irradiation points were chosen to obtain measurable responses on systemic proportions.

The LLLT parameters used in this study were the following: laser diode (Class IIIb-CEI-IEC 825-1; MM Optics, São Carlos, Brazil) with mean output power of 15 mW and wavelength of 780 nm (GaAlAs), beam area of 0.04 cm^2, energy per point of 0.15 J, total energy of 1.2 J (per session for animal), and fluency of 3.8 J/cm^2; irradiance of 37.5 mW/cm^2; time of irradiation per point equal 10 s; continuous mode; during 30 days of training, except Saturdays (resting days). The rat muscles were irradiated immediately after physical effort (days of training and effort tests) for its better efficiency under metabolic stress [20, 21]. The laser equipment was properly calibrated by the manufacturer according to its light specifications immediately prior to start the study.

Tissue removal and enzyme activities

To minimize acute effects from the last exercise session and ensure that eventual enzymatic activity changes would be results from the effects of LLLT, all rats were sacrificed with anesthetic after 48 h of the last treadmill exercise session.

Heart, tibialis anterior, and soleus were removed by two specialists, and all tissue samples were sectioned in two equal parts (50% of total mass each) for CS and LDH analyses. All muscle tissue was rapidly frozen in liquid nitrogen and stored at − 80 °C until analysis.

All tissues removed were weighed and allocated together in accordance with its type. Next, all similar tissues were weighed and homogenized together (pool) with a specific cooled buffer for each enzymatic kinetics analysis, following a ratio of 1:4 (weight/volume):

1. LDH activity: 150 mM of IMIDAZOL (SIGMA), 1 mM of EDTA, 5 mM of DTT (Dithiothreitol – SIGMA) at 1% TRITON X-100, pH 7.4 at 25 °C.
2. CS activity: 20 mM of HEPES (SIGMA), 1 mM of EDTA (SIGMA) at 1% TRITON X-100 (SIGMA), pH 7.4 at 25 °C.

Homogenates were centrifuged for 15 min at 27.000g (4 °C). Next, the extracts containing the pool for each type of tissue were used in measurements of CS and lactate LDH activities. Both enzyme assays were performed in triplicate for each sample pool with volume less than 1.0 ml at 25 °C. All samples were analyzed on the same day in order to avoid variation between them. The analyses of CS and LDH activities were performed using technique previously described [22] for interpretation and relative measurements of the metabolism.

Metabolic differentiation

The metabolic characteristics were determined assuming that metabolic differentiation in striated muscle is connected with relative capacities of aerobic and anaerobic bioenergetic pathways [23]. Therefore, CS and LDH were chosen as markers of aerobic and anaerobic bioenergetic pathways, respectively. Thereby, muscles with high oxidative capacities will have low LDH/CS ratios, whereas muscles with low oxidative and high glycolytic capacities will have high LDH/CS ratios.

Statistical analyses

Data normality was verified by Shapiro-Wilk's test. All statistical analyzes of enzyme activities were tested by one-way ANOVA followed by Tukey HSD post-hoc test when significant interactions among groups were observed. MRS and AT were tested by two-way ANOVA. Tukey HSD post-hoc test was used when there was a significant interaction between groups and/or time effects. The significance was set at $P < 0.05$.

Results

Enzyme activities

The enzymatic analysis was performed only in tibialis anterior and soleus muscles, since these muscles present predominantly anaerobic and aerobic metabolic profiles, respectively [24]. Thus, tibialis anterior and soleus were considered key muscles with appropriate metabolic profiles to calculate the LDH/CS ratio. Quadriceps femoris and gluteus maximus muscles were not analyzed, since they have more mixed metabolic characteristics [24], thus hindering inferences based on the LDH/CS ratio. Finally, all results of enzyme activities are presented for
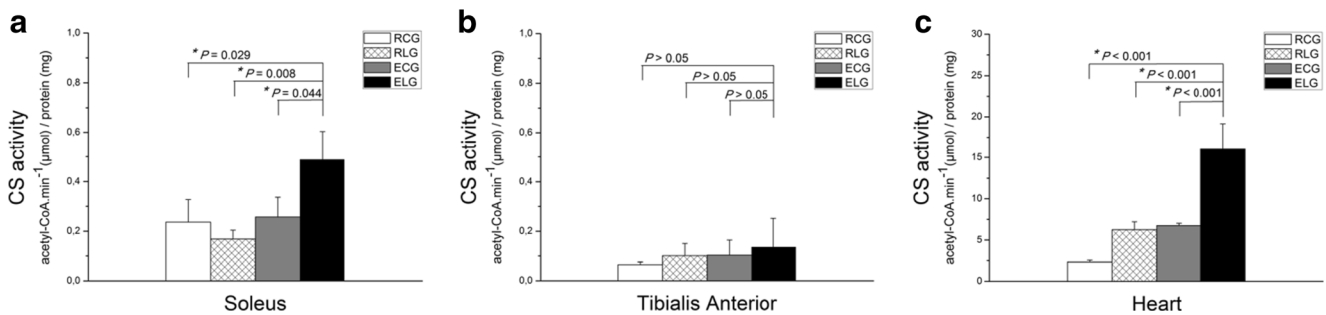
Fig. 1 Citrate synthase activity (CS) in extracts of soleus (**a**), tibialis anterior (**b**), and cardiac muscles (**c**) of the four groups studied. Values are means ± SEM. Units are micromoles of substrate per minute per milligram of protein. RCG (rest control group); RLG (rest laser group); ECG (exercise control group); RLG (exercise laser group). *$P < 0.05$

muscle (soleus, tibialis anterior, and heart) versus groups (RCG, RLG, ECG, and ELG).

CS (soleus and heart): there was a significant main effect interaction ($P < 0.05$) among all groups. Post-hoc has shown that CS activity of ELG was higher ($P < 0.05$) than ECG, RLG, and RCG in both muscles (Fig. 1a, c).

CS (tibialis anterior): there was no significant main effect interaction ($P > 0.05$) among all groups (Fig. 1b).

LDH (soleus and heart): there was a significant main effect interaction ($P < 0.001$) among all groups in both muscles. For soleus, post-hoc has shown that LDH activity of ELG was lower ($P < 0.001$) than ECG, RLG, and RCG (Fig. 2a). For heart muscle, post-hoc have shown that LDH activity of ELG was lower ($P < 0.05$) than ECG and RCG, but did not present significant difference ($P = 0.531$) in relation to RLG (Fig. 2c).

LDH (tibialis anterior): there was a significant main effect interaction ($P < 0.001$) among all groups. Post-hoc has shown that LDH activity of ELG was higher ($P < 0.01$) than ECG and RLG and lower ($P < 0.001$) than RCG (Fig. 2b).

LDH/CS (soleus and heart): there was a significant main effect interaction ($P < 0.05$) among all groups. For soleus, post-hoc has shown that LDH/CS activity of ELG was lower ($P < 0.05$) than RLG and RCG, but did not present significant ($P = 0.057$) difference in relation to ECG (Fig. 3a). For heart muscle, post-hoc has shown that LDH/CS activity of ELG was lower than ($P < 0.001$) ECG, RLG, and RCG (Fig. 3c).

LDH/CS (tibialis anterior): there was a significant main effect interaction ($P = 0.040$) among all groups. Post-hoc did not show any significant differences among groups (Fig. 3b).

Maximum running speed

There was a significant time effects and group versus time effects ($P < 0.001$) for MRS of ELG and ECG. Post-hoc has shown that MRS of ELG at all five IET was increased. MRS of the fifth incremental test (MRS5) was higher ($P < 0.002$) than MRS 1, MRS 2, and MRS 3. The velocity was increased from 31 ± 3.39 m/min to 52 ± 3.15 m/min for ELG. Post-hoc for ECG has shown that MRS at all five IET was also increased (from 25 ± 3.97 m/min to 41 ± 10.05 m/min). However, MRS of the fifth incremental test (MRS5) did not present any statistical significance ($P > 0.05$) compared to MRS1, MRS2, MRS3, and MRS4 (Fig. 4a).

Anaerobic threshold at MRS

There were significant time effects and group versus time effects ($P \leq 0.001$) for AT of ELG and ECG. Post-hoc for ELG has shown that AT at MRS of all five IET were increased. AT at MRS of the fifth incremental test (MRS 5) was higher ($P < 0.05$) than AT1, AT2, AT3, and AT4. The AT was increased from 25 ± 3.97 m/min to 41 ± 10.05 m/min for
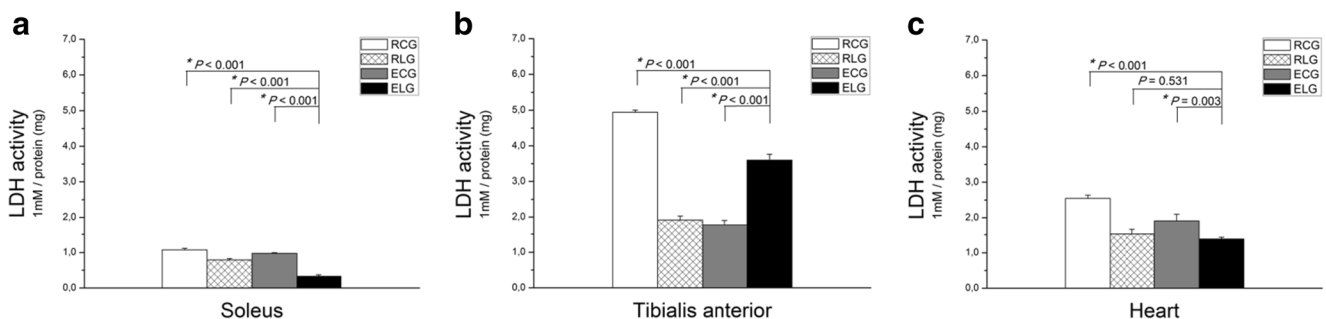

Fig. 2 Lactate dehydrogenase activity (LDH) in extracts of soleus (**a**), tibialis anterior (**b**), and cardiac muscles (**c**) of the four groups studied. Values are means ± SEM. Units are micromoles of substrate per minute per milligram of protein. RCG (rest control group); RLG (rest laser group); ECG (exercise control group); RLG (exercise laser group). *$P < 0.05$
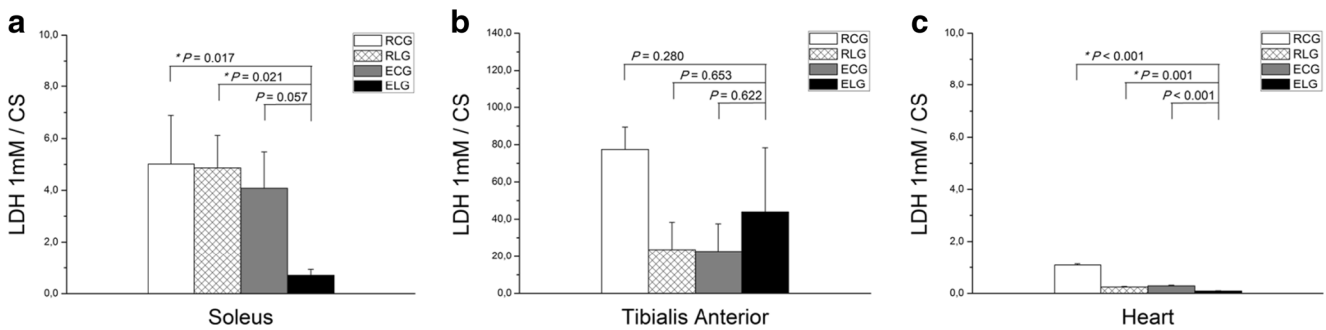
Fig. 3 LDH/CS ratios in extracts of soleus (**a**), tibialis anterior (**b**), and cardiac muscles (**c**) of the four groups studied. Values are means ± SEM. Units are micromoles of substrate per minute per milligram of protein.

LDH (Lactate dehydrogenase); CS (Citrate synthase); RCG (rest control group); RLG (rest laser group); ECG (exercise control group); RLG (exercise laser group). *$P < 0.05$

ELG. Post-hoc for ECG has shown that AT at MRS of all five IET was increased too (from 28 ± 3.04 m/min to 31 ± 7.32 m/min). But AT at MRS of the fifth incremental test (MRS5) did not present any statistical significance ($P > 0.05$) compared to AT1, AT2, AT3, and AT4 (Fig. 4b).

Discussion

The present study investigated the physiological adaptations related to CS and LDH activities, and the AT in rats submitted to an aerobic training program on the treadmill combined with LLLT. Our main finding was a significant increase in CS activity in the soleus and cardiac muscles in the ELG group compared to RCG, RLG, and ECG. A decrease in LDH activity and a higher running speed over the weeks in the ELG group was also observed.

The effects of isolated aerobic exercise on CS activity have been demonstrated in the literature. In previous study, a 10-day protocol of exercise on treadmill increased CS levels in the plantar muscle of rats [25]. Additionally, the levels of CS

activity were higher in the soleus muscle of rodents submitted to 8 weeks of running training that in untrained rats [26]. However, trials about the effects of PBM on CS activity in the context of exercise are scarce.

It is plausible to hypothesize that the association of aerobic exercise and LLLT may potentiate cellular bioenergetic activity by increasing CS activity, a key enzyme of Kreb's cycle that is intimately linked with mitochondrial membrane potential and with cytochrome c oxidase that in turn are modulated by PBM [7, 21, 26]. In this context, the literature has pointed a therapeutic optical window (600–900 nm) to be used in photobiomodulation [27] based on light absorption by chromophores such as hemoglobin, melanin and cytochome c oxidase, and the light penetration through the tissue. Thus, the wavelength 780 nm was chosen due to a previous critical review pointed that wavelengths ranging from 760 to 895 nm can be absorbed by cytochrome c oxidase, mainly by its binuclear copper centers (Cu_A, Cu_B), and then promote stimulation of mitochondrial function [28]. Moreover, infrared wavelengths as 780 nm has better tissue penetration compared
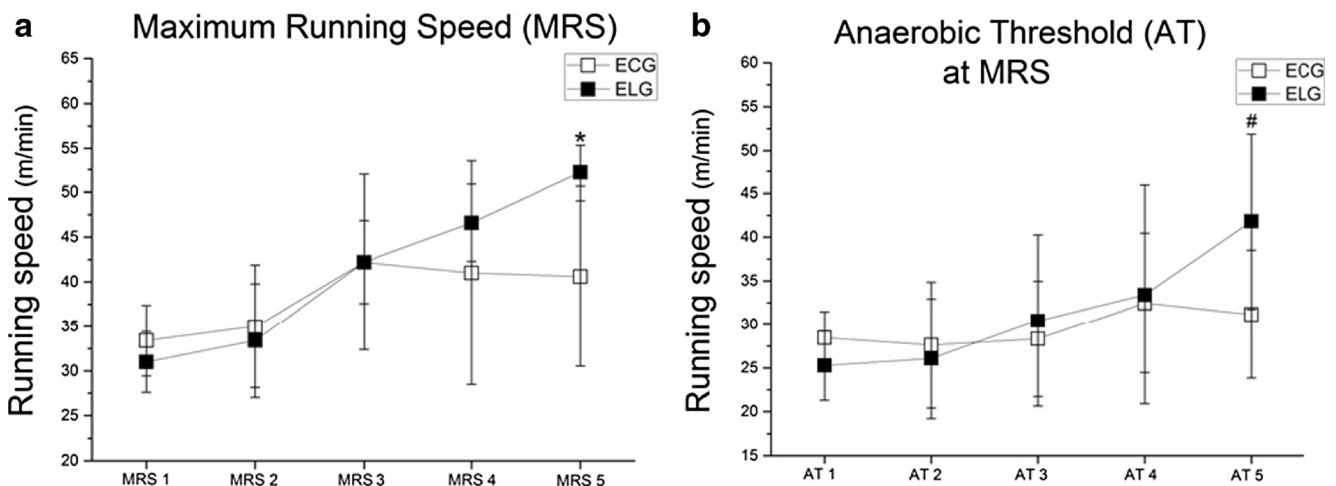

Fig. 4 Running speed at anaerobic threshold (AT) over five evaluations of the incremental effort test in ECG and ELG. Values are means ± SEM. ECG (exercise control group); ELG (exercise laser group). *intragroup

difference ($P < 0.05$) at MRS5 compared to MRS1, MRS2, and MRS3 in ELG; #intragroup difference ($P < 0.05$) at AT5 compared to AT1, AT2, AT3, and AT4 in ELG

to short wavelengths such as blue (~ 480 nm) or green (~ 530 nm), and also is less absorbed by tissue chromophores as hemoglobin and melanin [27].

Corroborating this assumption, Aquino et al. [11] showed that rats submitted to a daily LLLT (830 nm) over the gastrocnemius muscles after swimming exercises presented higher CS activity than rats submitted to the same exercise without LLLT. It is important to note that the response in CS activity after a period of aerobic training with LLLT irradiation seems to be dependent on the characteristic of the evaluated muscle. Our data point to a higher CS activity in the soleus and cardiac muscles (predominantly aerobic), which was not observed in the tibialis anterior (predominantly anaerobic).

As an adaptive consequence to the stimuli (exercise and/or LLLT) lower LDH levels would be expected in the groups submitted to LLLT, compared with their respective controls. According to this statement, it was demonstrated that association of LLLT and exercise promoted a lower elevation in LDH levels compared to the use of exercise alone and isolated LLLT irradiation in rats [29]. However, this result was not observed in tiabilis anterior muscle. Conversely, a key point should be raised and discussed deeply regarding LDH enzyme. According to previous study [29], LDH enzyme presents two different isozymes: LDH pyruvate reductase (predominantly in glicolitic muscles) and LDH pyruvate oxidase (predominantly in oxidative tissue). Therefore, it is possible that LDH pyruvate oxidase could be upregulated to increase its activity due to the exercise stimuli plus LLLT, justifying the results of Fig. 2b; or LDH pyruvate reductase increased its activity, refuting the previous hypothesis. We suggest more studies to investigate LDH isozymes as previous study [29], since this analysis was a limitation of the present study.

Taking the CS, LDH activities and the ratios LDH/CS as metabolic markers, our data support the hypothesis that the metabolic properties of the rats submitted to LLLT irradiation differ from the non-irradiated rats, mainly when compared to the standard-control group (RCG).

Our findings may also suggest higher activity promoted by LLLT on muscle tissues metabolically stressed by the exercise. Such inferences have also been proposed by other researchers [20, 21], who suggested that the magnitude of PBM effects depend upon on the previous metabolic conditions of the tissue. In this way, the response tends to be optimum when the cellular redox potential is changed in damaged muscle cells, such as after an exhaustive exercise. For example, Ferraresi et al. [21] demonstrated that LEDT after strength training in rats was better to improve muscle performance than pre-exercise LEDT.

The increased CS activity observed in cardiac muscle of the ELG group compared with its ECG suggests a systemic effect of the LLLT, since the heart was not irradiated. Such finding was corroborated by Vieira et al. [29], who showed a reduction in LDH activity in the cardiac muscle, despite the LLLT being applied over the lower limb muscles. This possible

remote effect of PBM was recently shown by a significantly lower heart rate during the first 5 min of running exercise in maximal aerobic speed in humans submitted to LEDT compared to the placebo group [30].

These results could be explained due to the tissue submitted to LLLT produce substances (nitric oxidizes, interleukin-2, interleukin-6, interleukin-8 or TNF-α) which pass through the vascular system, resulting in distance responses [31–33]. Furthermore, PBM may have increased oxidative capacity and improved ATP synthesis and blood flow [33], improving the adaptations (lower heart rate) to the exercise.

The increases in the oxidative enzyme activity and decreases in the glycolytic in the ELG group suggest higher aerobic capacities in this group, mainly the cardiac and soleus muscles. This observation is important, since a high oxidative capacity would favor greater uses of fat metabolism [11]. This in turn can contribute to lower lactate production during periods of high contractile activity. Collectively, these adaptations may delay muscular fatigue when performing long and intensive contractile activities [1].

Thus, we also suggested that aerobic exercise associated with LLLT would generate higher levels of muscular performance compared with groups carrying out only exercise treatments. To test this hypothesis, the MRS and the AT of the rats in ECG and ELG were analyzed. The increases in the MRS and the shifts of AT to higher effort levels were found in this study after 5 weeks of training. These adaptations may be translated into increases in oxidative capacity, due to the superior mitochondrial content in the in skeletal (soleus) and cardiac muscles which are represented by higher CS activity and, consequently, higher O_2 availability for ATP synthesis. The significant changes observed in the enzymatic activity for the ELG and ECG, however, were not accompanied by a better performance. This find can be explained, in part, by the short training period (5 weeks) performed in this study, since any changes in aerobic capacity may be protocol-dependent or light dose-dependent.

In this context, regarding light dosimetry, earlier studies that applied LLLT only once over skeletal muscles in animal models used low energies (Joules - J) and doses (J/cm^2), such as Lopes-Martins et al. [34] who applied 0.04, 0.08, and 0.2 J corresponding to 0.5, 1, and 2.5 J/cm^2, respectively. These authors found a PBM dose-response in favor of 0.5 J/cm^2 (0.04 J) and 1 J/cm^2 (0.08 J) to prevent muscle fatigue. Thus, the present study assumed the assumption that is not necessary huge light doses or energies to stimulate skeletal muscles in animal models and then applied 0.15 J (3.8 J/cm^2) per point of irradiation, totaling 1.2 J (8 points of irradiation per animal).

Conclusions

Aerobic treadmill training combined with LLLT promotes physiological adaptations towards an increase of oxidative

capacity in tissues which have predominantly aerobic metabolism, such as in soleus and cardiac muscles.

Compliance with ethical standards

Conflict of interest   The authors declare that they have no conflict of interest.

Ethical approval   The experimental procedures with rats were conducted according to the Guide for Care and Use of Laboratory Animals and approved by the local Animal Ethics Committee.

Informed consent   It does not apply, since the study was developed with Wistar rats.

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