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ORIGINAL ARTICLE

Effect of pre-irradiation with different doses, wavelengths, and application intervals of low-level laser therapy on cytochrome c oxidase activity in intact skeletal muscle of rats

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Abstract Modulation of cytochrome c oxidase activity has been pointed as a possible key mechanism for low-level laser therapy (LLLT) in unhealthy biological tissues. But recent studies by our research group with LLLT in healthy muscles before exercise found delayed skeletal muscle fatigue development and improved biochemical status in muscle tissue. Therefore, the aim of this study was to evaluate effects of different LLLT doses and wavelengths in cytochrome c oxidase activity in intact skeletal muscle. In this animal experiment, we irradiated the tibialis anterior muscle of rats with three different LLLT doses (1, 3, and 10 J) and wavelengths (660, 830, and 905 nm) with 50 mW power output. After irradiation, the analyses of cytochrome c oxidase expression by immunohistochemistry were analyzed at 5, 10, 30 min and at 1, 2, 12, and 24 h. Our results show that LLLT increased ($p<0.05$) cytochrome c oxidase expression mainly with the following wavelengths and doses: 660 nm with 1 J, 830 nm with 3 J, and 905 nm with 1 J at all time points. We conclude that LLLT can increase cytochrome c oxidase activity in intact skeletal muscle and that it contributes to our understanding of how LLLT can enhance performance and protect skeletal muscles against fatigue development and tissue damage. Our findings also lead us to think that the combined use of different wavelengths at the same time can enhance LLLT effects in skeletal muscle performance and other conditions, and it can represent a therapeutic advantage in clinical settings.

Keywords Phototherapy · Mitochondrial activity · Muscular metabolism

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Introduction

LASER means light amplification by stimulated emission of radiation. Laser was developed in 1960s and is light with special proprieties including monochromaticity and low divergence. Low-level laser therapy (LLLT) is the application of light for therapeutic purposes usually using a class 3B laser device with a mean output range of 10–500 mW. There is strong evidence that LLLT promotes tissue regeneration,

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reduces inflammation, and relieves pain [1–3]. The light used in LLLT is typically of narrow spectral width and in the red or near infrared (NIR) spectrum (600–1,000 nm), with a power density (irradiance) up to 5 W/cm^2 [4].

In musculoskeletal pain conditions, the first placebo-controlled clinical LLLT trial was published in 1980, and it was found that LLLT improved erythema, pain, and grip strength in patients with rheumatoid arthritis of the hands [5]. Since then there has been a steady growth of the evidence of efficacy for the management of various conditions including osteoarthritis [6], tendinopathies [1, 7], wounds [8, 9], low back pain [10], neck pain [3, 11], peripheral nerve injuries [12], and stroke [13]. A major advantage of LLLT versus pharmacological management is that LLLT has fewer side effects.

Over the years, it was observed that LLLT has a biphasic dose-response pattern. This means that intermediate doses situated inside a "therapeutic window" have stimulatory effects in biological tissue, while doses below this window have no effects, while higher doses may have also induce inhibitory or even destructive effects [3, 4, 14]. However, the "therapeutic window" changes depending on the nature of the pathology and the affected cells and tissue types. In clinical practice, the optimal power density, energy dose, and irradiation time are parameters extremely important to give adequate treatment with positive results, while inadequate parameters will, at best, give zero results.

Skeletal muscle fatigue and recovery are a novel area of research in low-level laser therapy (LLLT). Recent studies of our research group with LLLT and light emitting diode therapy (LEDT) have shown positive results delaying skeletal muscle fatigue in both animals and humans [14–18] and improving the status of biochemical markers related to skeletal muscle recovery [14, 17–20] when LLLT was applied before exercise.

Over the years, the modulation of cytochrome c oxidase (complex IV of mitochondrial respiratory chain) activity has been identified as a key mechanism of interaction between laser radiation and different biological tissues [4]. More recently, Hayworth et al. [21] demonstrated that the activity of cytochrome c oxidase is enhanced by phototherapy in skeletal muscle fibers of rats. It is important to highlight that in this study, intact muscles were irradiated; this means that muscles were uninjured as in previous studies. In this study, the activity of cytochrome c oxidase was assessed 24 h after the application of phototherapy. These results demonstrated that the metabolism of intact muscles (healthy) may be enhanced by low-level laser therapy (LLLT), offering one possible explanation for why LLLT works. However, the results of Hayworth et al. [21] leaves an open question as to whether the peak increase in cytochrome c oxidase activity really occurs exactly 24 h after phototherapy.

With this perspective in mind, the present study aims to evaluate effects of different LLLT doses and wavelengths in cytochrome c oxidase activity in intact skeletal muscle as well as to determine time/effect interval profile for cytochrome c oxidase activity in healthy skeletal muscle.

Materials and methods

Materials

The experiments were carried out with male Wistar rats weighing 200-230 g with food and water ad libitum. Central animal house of Nove de Julho University provided animals. All rats were randomly divided into groups of six animals. The policies and procedures of the animal laboratory are in accordance to Brazilian laws and with those detailed by the US Department of Health and Human Services. The experimental protocol was submitted and approved by the Animal Research and Care Committee of the Nove de Julho University.

Experimental groups

Animals were randomly divided in experimental groups of six animals each as follows:

- Control group—animals that did not undergo any type of procedure;
- LLLT 660 nm—animals that were irradiated with 660-nm LLLT;
- LLLT 830-nm group—animals that were irradiated with 830-nm LLLT;
- LLLT 905-nm group—animals that were irradiated with 905-nm LLLT.

Animals of LLLT-irradiated groups were further randomly redistributed according laser doses given as follows: 1, 3, or 10 J (six animals each).

Experimental procedures

Laser irradiation Diode lasers with mean output power of 50 mW; spot size of 0.028 cm^2; continuous mode; and wavelengths of 660 nm (red), 830 nm (infrared), or 905 nm (infrared) were used. The optical power was calibrated using a Newport multifunction optical meter model 1835C. The stability of laser during the laser irradiation was measured collecting light with a partial reflect (4 %). In order to irradiate animals, the laser spot was placed in direct contact with the skin with slight pressure at the central part of the tibialis anterior muscle area. Irradiation lasted 20, 60, and 200 s, respectively, with a fixed power density of 1.78 W/cm^2. The total delivered energy for irradiated groups were 1, 3, and 10 J, respectively, [22].

Removal of muscle tissue for analysis After LLLT irradiation with different doses (1, 3, or 10 J) and different wavelengths (660, 830, or 905 nm) (exception for the control group), animals were stored at the central animal house of Nove de Julho University until experimental time needed to remove the tibialis anterior muscle. Animals were anesthetized with pentobarbitone sodium (40 mg/kg), and the tibialis anterior muscle was removed surgically and processed for further analysis exactly in experimental times of 5, 10, and 30 min and 1, 2, 12, or 24 h after LLLT.

Immunohistochemistry analysis

For this analysis, paraffin was removed with xylene from serial sections of 5 μm. After this procedure, the sections were rehydrated in graded ethanol and pre-treated with pepsin 0.4 % for 20 min for antigen retrieval. Subsequently, the material was pre-incubated with 0.5 % hydrogen peroxide in phosphate-buffered saline (PBS) solution for 5 min to inactivate the endogenous peroxidase. The specimens were incubated with anti-cytochrome c oxidase polyclonal primary antibody (Santa Cruz Biotechnology, USA) at a concentration of 1:20. Incubation was performed overnight at room temperature (24 ° C) and followed by two washes in PBS for 10 min. Afterwards, the sections were incubated with biotin-conjugated secondary antibody anti-goat IgG (Santa Cruz Biotechnology, USA) at a concentration of 1:200 in PBS for 1 h. The sections were washed two times with PBS followed by the application of avidin biotin complex conjugated to peroxidase (Vector Laboratories, USA) for 30 min. The visualization of the bound complexes was realized by the application of a 0.05 % solution of 3-3′-diaminobenzidine solution and counterstained with Harris Hematoxylin. Finally, for the control analyses of the antibodies, the serial sections were treated with goat IgG (Santa Cruz Biotechnology, USA) at a concentration of 1:200 instead of the primary antibody. Furthermore, internal positive controls were performed with each staining bath. The percentage of colored area per muscle fiber and the percentage of fibers with positive coloration for cytochrome c oxidase (more than 50 % of muscle fiber area colored) were then determined by an observer who was blinded to group allocation.

Statistical analysis

Another blinded observer first plotted obtained data for the analysis of normal distribution, and statistical analysis was then performed with parametric tests since data were normally distributed. Data were tested statistically by an ANOVA test with Dunnet post-test. The statistical level of significance was set at $p<0.05$.

Results

Figure 1 illustrates the percentage of colored area for cytochrome c oxidase per muscle fiber through immunohistochemistry analysis for the control group, 660-nm LLLT with a 1-J dose at 2 h, 830-nm LLLT with a 3-J dose at 10 min, and 905-nm LLLT with a dose of 1 J at 2 h.

The percentage of colored area per muscle fiber observed through immunohistochemistry analysis in the control group (non-irradiated animals) was 12.64 % (SEM 0.65). Regarding LLLT irradiation with 660 nm, best results were observed with 1 J that showed a significant increase ($p<0.05$) in the percentage of colored area at 5 min (19.86 %, SEM 0.37), 30 min (16.73 %, SEM 1.26), 1 h (16.43 %, SEM 0.75), 2 h (23.74 %, SEM 0.48), and 24 h (23.74 %, SEM 0.48) after LLLT irradiation. LLLT with 830 nm showed best results with a 3-J dose that significantly increased ($p<0.05$) the percentage of colored area at 5 min (21.83 %, SEM 0.32), 10 min (24.9264 %, SEM 1.46), and 24 h (24.83 %, SEM 0.30) after LLLT irradiation. Finally, 905-nm LLLT showed best results with a 1-J dose that significantly increased ($p<0.05$) the percentage of colored area at 5 min (21.52 %, SEM 0.78), 1 h (16.58 %, SEM 1.70), 2 h (29.16 %, SEM 0.13), 12 h (20.09 %, SEM 0.20), and 24 h (17.81 %, SEM 0.23) after LLLT irradiation. Results are summarized in Fig. 2.

The percentage of fibers with positive coloration for cytochrome c oxidase (muscle fibers with more than 50 % of area colored) observed through immunohistochemistry analysis in the control group (non-irradiated animals) was 14.28 % (SEM 0.44). Regarding LLLT irradiation with 660 nm, best results were observed with 1 J that showed a significant increase ($p<0.05$) in the percentage of muscle fibers with more than 50 % of area colored for cytochrome c oxidase at 5 min (21.09 %, SEM 4.159) and 2 h (32.74 %, SEM 0.5845) after LLLT irradiation. LLLT with 830 nm showed best results with a 3-J dose that significantly increased ($p<0.05$) the percentage of muscle fibers with more than 50 % of area colored for cytochrome c oxidase at 5 min (18.76 %, SEM 0.09), 2 h (20.88 %, SEM 1.86), and 24 h (22.20 %, SEM 0.83) after LLLT. Finally, 905-nm LLLT showed best results with a 10-J dose that significantly increased ($p<0.05$) the percentage of muscle fibers with more than 50 % of area colored for cytochrome c oxidase at 1 h (19.55 %, SEM 0.42) and 2 h (20.13 %, SEM 3.17) after LLLT. Results are summarized in Fig. 3.

Discussion

This is the first time that several doses and several wavelengths have been tested in the same experiment to investigate the modulation of cytochrome c oxidase in skeletal muscle tissue. We choose to use the same three doses (1, 3, and 10 J)

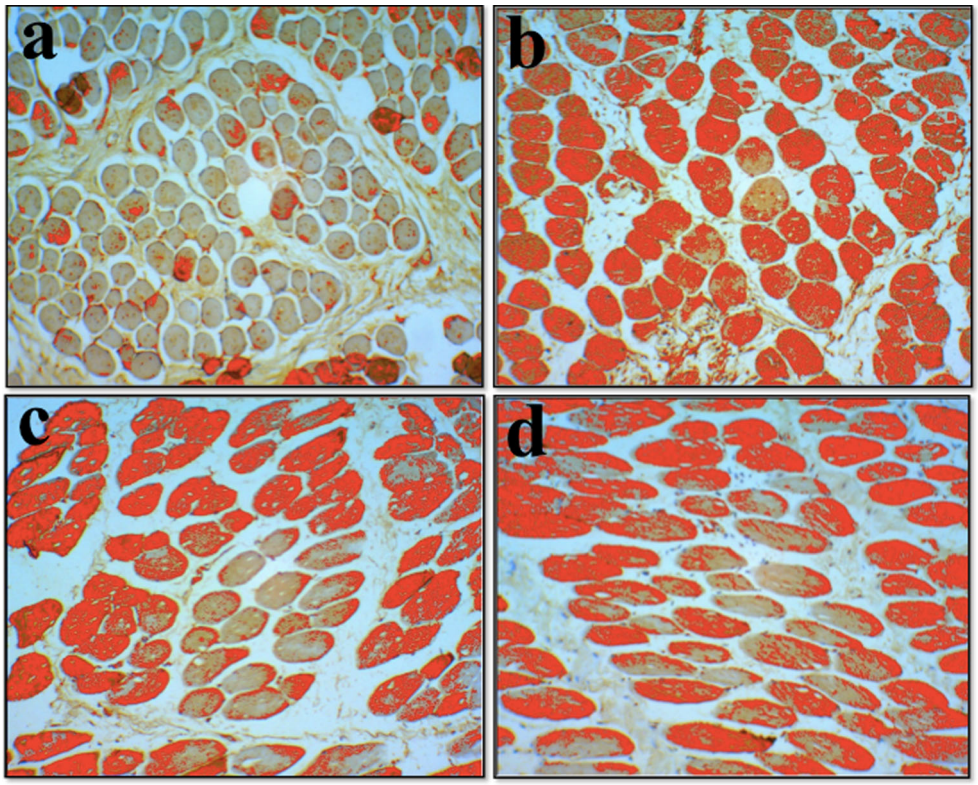

Fig. 1 Representative images of the percentage of colored area for cytochrome c oxidase per muscle fiber by immunohistochemistry of the tibialis anterior muscle. **a** Control group. **b** 660-nm LLLT with 1 J at 2 h. **c** 830-nm LLLT with 3 J at 10 min. **d** 905-nm LLLT with 1 J at 2 h. Magnification of ×40

for each to allow us to perform a direct comparison between wavelengths. And for each wavelength and dose, we made a direct comparison to the non-irradiated control group.

Regarding the assessment of the percentage of colored area per muscle fiber, we observed that LLLT with 660-nm wavelength and a dose of 1 J, 830-nm wavelength and 3 J, and 905-nm wavelength and 1 J showed the best results for increasing cytochrome c oxidase expression. This increase was significant for 660 nm/1 J and 905 nm/1 J at almost all time points investigated and for 830 nm/3 J at shorter time points (5 and 10 min) and also at 24 h.

The percentages of fibers with positive coloration for cytochrome c oxidase (muscle fibers with more than 50 % of area colored) results were not very consistent, and best data were observed with 660-nm wavelength and dose of 1 J at 5 min and 2 h; 830-nm wavelength and 3 J at 5 min, 2, and 24 h; and 905-nm wavelength and 10 J at l and 2 h. This issue deserves further investigation in future studies.

When we put together our results, we can observe that LLLT can stimulate cytochrome c oxidase from 5 min to 24 h after irradiation, but it is dependent of the dose and wavelength used. As mentioned before, recently, Hayworth et al. [21] demonstrated that the activity of cytochrome c oxidase is enhanced by phototherapy at 24 h after irradiation. However, authors did not evaluate cytochrome c oxidase at shorter times. Our data are in line with Hayworth et al. [21] since the best doses for all wavelengths significantly enhanced the percentage of colored area per muscle fiber at 24 h.

Our results open new perspectives for the use of laser phototherapy and respond to the question of what the optimal time is between irradiation and the beginning of exercise activity. Actually, this is dependent of the parameters employed, and it can be from 5 min to 24 h before exercise. Our findings are limited to pre-irradiation of LLLT and also for cytochrome c oxidase analyses. Further studies must include pre-, co-, and post-irradiation of LLLT as well as analyses of reactive oxygen species (ROS), reactive nitrogen species (RNS), glycogen phosphorylase, phosphofructokinase, and lactate dehydrogenase in order to fully elucidate mechanisms through LLLT acts in skeletal muscle metabolism.

It is known that light from lasers and LEDs interact with cellular mitochondria and promotes structural changes (appearance of giant mitochondria) and increases ATP synthesis in metabolic processes [23–25]. This interaction leads to enhancement in the synthesis of DNA and RNA of the cellular cycle of regulatory proteins [26]. When photons of light energize the metal sites in the mitochondrial complex IV (cytochrome c oxidase), it leads these complexes to alter both the conformation of the enzyme and redox reaction. This increases the electron transfer in the respiratory chain and/or pumping of protons across the inner mitochondrial membrane. The increase in the transfer of protons and electrons accelerates the oxidative metabolism leading to an increase in ATP, which in turn promotes an increase in cellular metabolism [27]. Since one of mechanisms behind

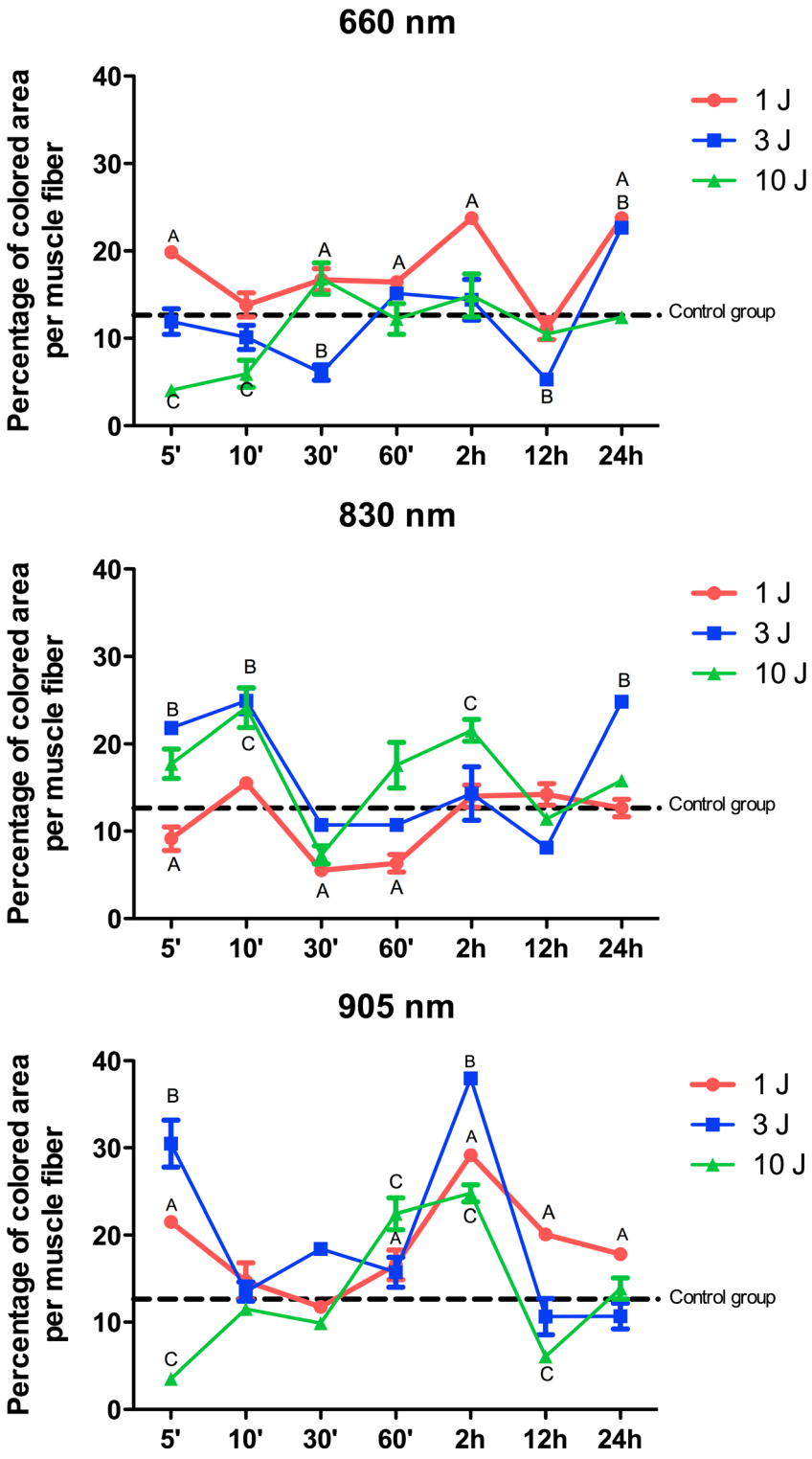

Fig. 2 Analysis of the percentage of colored area for cytochrome c oxidase per muscle fiber by immunohistochemistry of the tibialis anterior muscle. "*A*" indicates a significant difference ($p<0.05$) between 1 J and control group, "*B*" indicates a significant difference between 3 J and control group, and "*C*" indicates a significant difference between 10 J and control group

skeletal muscle fatigue is the decrease of ATP inside muscles, the results of our study can partially explain how skeletal muscle performance is enhanced by phototherapy. Our findings also challenge the paradigm that only tissues under stress (such as inflammation) are suitable to be treated with phototherapy [28].

Conclusion

LLLT leads to a dose- and wavelength-dependent increase in cytochrome c oxidase expression in intact skeletal muscle tissue, which means that muscular metabolism can be enhanced by phototherapy. These results are helpful in

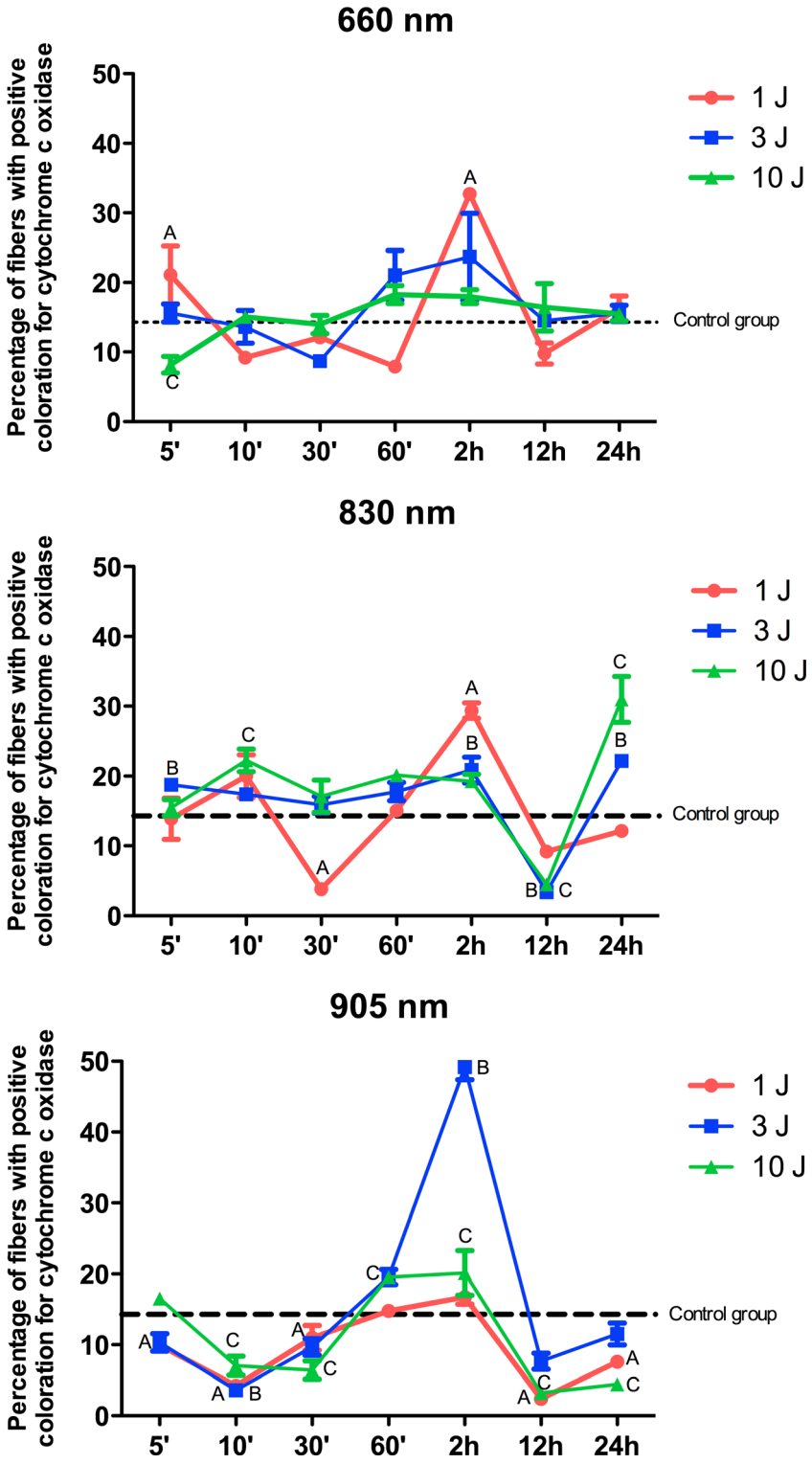

Fig. 3 Analysis of the percentage of fibers with positive coloration for cytochrome c oxidase (muscle fibers with more than 50 % of area colored) by immunohistochemistry of the tibialis anterior muscle. "*A*" indicates a significant difference ($p<0.05$) between 1 J and control group, "*B*" indicates a significant difference between 3 J and control group, and "*C*" indicates a significant difference between 10 J and control group

explaining how skeletal muscle performance is enhanced by pre-irradiation with phototherapy and in opening a new avenue in use of phototherapy, mainly in the prevention of progression in disorders and diseases. Our findings also lead us to think that the combined use of different wavelengths at the same time can enhance LLLT effects in skeletal muscle performance and other conditions, and it can represent a therapeutic advantage in clinical settings.

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Conflict 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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