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Communication

Effects of photobiomodulation on mitochondria of brain, muscle, and C6 astroglioma cells

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ABSTRACT

The purpose of this study was to investigate the effect of different doses of photobiomodulation (PBM) on mitochondrial respiratory complexes and oxidative cellular energy metabolic enzymes in the mitochondria of brain, muscle, and C6 glioma cells after different time intervals. C6 cells were irradiated with an AlGaInP laser at 10, 30, and 60 J/cm^2 for 20, 60, and 120 s, respectively. After irradiation, the cells were maintained in serum-free Dulbecco's Modified Eagle's medium for 24 h, and biochemical measurements were made subsequently. Mitochondrial suspensions from adult rat skeletal muscles/brains were irradiated with an AlGaInP laser at the abovementioned doses. In one group, the reaction was stopped 5 min after irradiation and in the other 60 min after irradiation.

Both the C6 cells that received the doses of 10 and 30 J/cm^2 showed increased complex I activity; the cells that were irradiated at 30 J/cm^2 showed increased hexokinase activity. Five minutes after the introduction of PBM of the muscle mitochondria (at 30 and 60 J/cm^2), the activity of complex I increased, while the activity of complex IV increased only at 60 J/cm^2. One hour after the laser session, complex II activity increased in the cells treated with 10 and 60 J/cm^2; however, complex IV activity showed an increase in all PBM groups. In brain mitochondria, 5 min after irradiation only the activity of complex IV increased in all PBM groups. One hour after the laser session, complex II activity increased at 60 J/cm^2, and complex IV activity increased for all PBM groups when compared to controls. PBM could increase the activity of respiratory chain complexes in an apparently dose- and time-dependent manner.

1. Introduction

Although photobiomodulation (PBM) is one of the most promising procedures for treating several diseases, the mechanism by which such a procedure works, as well as the light target(s) responsible for triggering the observed effects, remain uncertain [1].

Evidence has shown that laser light can stimulate several biological processes, such as cell growth and proliferation [2]. In particular, infrared laser irradiation can affect the bioenergetics in mammalian mitochondria, as well as their biogenesis [3]. Some enzymes, such as NADH dehydrogenase and cytochrome c oxidase (CCO), and substrates (adenine nucleotides) show a significant change in their biochemical properties following Irradiation [4].

Studies have observed that PBM could target the mitochondria for biostimulation [5]. Isolated irradiation of mitochondria induces changes in mitochondrial transcription and translation, increasing cascade reactions and several respiratory chain components (e.g., cytochromes, cytochrome oxidase, and flavin dehydrogenase) [6].

Abbreviations: ANOVA, analysis of variance; ATP, adenosine triphosphate; CCO, cytochrome c oxidase; CK, creatine kinase; DCIP, 2,6-dichlorophenolindophenol; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBM, photobiomodulation.

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PBM seems to have physiological effects, including increasing microcirculation [7], enhancing ATP synthesis, and stimulating the mitochondrial respiratory chain [8] and mitochondrial function [9], that may influence soft tissue metabolism in several pathologies. In addition, PBM seems to reduce the release of reactive oxygen species and increase the activity of creatine kinase (CK) and hexokinase, as well as antioxidant production [10].

Appropriate PBM dosing for inducing biostimulation is a difficult task, because it depends on several factors such as the wavelength, power output, continuous or pulsed emission, power density, irradiation time, dose in J/cm^2, total energy delivered, application technique, and intervals between sessions. A successful dose delivery into a biological system could alter cellular metabolism; however, ambiguity still exists in this regard [11].

Nowadays the PBM is being used a lot in muscular tissue to improve the regenerative capacity and performance gain. Furthermore, the cerebral tissue is gaining a lot of attention in use of this therapy for treatment of injuries such ischemia and neurodegenerative diseases. Due to this, this study aimed at investigating the effects of different PBM doses and time intervals on mitochondrial respiratory complexes and oxidative cellular energy metabolic enzymes (CK and hexokinase) of C6 astroglioma cells and muscle/brain mitochondria.

2. Material and methods

2.1. Cell culture

The C6 astroglioma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and was cultured as previously described [12].

2.2. Preparation of tissue mitochondria for measuring the activity of respiratory chain complexes

Mitochondrial suspensions from adult rat skeletal muscle/brain were prepared [13]. Briefly, tissues were homogenized in 10% (v/v) of 4.4 mM potassium phosphate buffer, at pH 7.4, containing 0.3 M sucrose, 5 mM MOPS, 1 mM EGTA, and 0.1% bovine serum albumin. Homogenates were centrifuged at 3000 g for 10 min at 4 °C. The pellets were discarded, and the supernatants were centrifuged at 17,000 × g for 10 min at 4 °C. The pellet was dissolved in the same buffer. For the tissue preparations, an Eppendorf 5415 R centrifuge (Eppendorf, Hamburg, Germany) was used.

2.3. Laser irradiation of C6 glioma cells

A low-energy AlGaInP laser (Ibramed Equipamentos Médicos Ltda, Amparo, Brasil), with a 660 nm continuous wave and 0.6 mm beam diameter was used. Laser irradiation was at fluences of 10 J/cm^2 for 20 s (total energy of 0.6 J), 30 J/cm^2 for 60 s (total energy of 1,8 J), and 60 J/cm^2 for 120 s (total energy of 3.6 J), i.e., doses considered as low, medium, and high-intensity, respectively [14,15]. All the parameters are described in the manual of the apparatus wich was calibrated following the manufacturer's instructions. The AlGaInP laser was applied to the C6 cells (10 × 10^3 cells/well in 96-well plates) at a perpendicular angle, a distance of 2 mm. The cells was irradiated inside a laminar flow in a dark room without radiation. Control cells were not irradiated. In order to minimize light scatter, an empty column of wells separated the irradiated cell-containing wells. After irradiation, cells were maintained in serum-free DMEM for 24 h. Afterwards, biochemical measurements were made.

2.4. Application of laser to mitochondrial suspensions from adult rat skeletal muscles/brains

One-hundred and ninety-five microliters of mitochondrial suspensions (protein concentration of 20 mg/mL) and 5 µL of substrate (10 mM pyruvate and 5 mM malate) were added to 96-well plates and incubated for 5 min at 37 °C. The plates were placed in laminar flows and irradiated using the AlGaInP laser at doses of 10, 30, and 60 J/cm^2 for 20, 60, and 120 s, respectively. Each sample received a single irradiation treatment with the laser perpendicular to the well at a 2-mm distance. The reaction was stopped 5 min after irradiation in one group and 60 min after in the other group. Control mitochondria were not irradiated. For minimizing contact with light, the well columns of the irradiated groups were far apart from the controls.

2.5. Mitochondrial function measurement

Mitochondrial function of C6 astroglioma cell was assessed by following the MTT (3-[4,5 *dimethylthiazol*-2-*y*1]−2,5-diphenyltetrazolic bromide) reduction. Active mitochondrial dehydrogenases cleavage and reduce the soluble yellow MTT dye into the insoluble purple formazan [16]. At the end of the incubation period, MTT test were performed. The formazan formation was spectrophotometrically assayed at 570 nm and 630 nm, and the net $\Delta A_{(570-630)}$ was taken as an index of mitochondrial function. Results are indicated as percentage of controls, to which 100% activity was attributed.

CK activity was measured in a 60 mM Tris–HCl buffer, pH 7.5, containing 7 mM phosphocreatine, 9 mM $MgSO_4$, and approximately 1 µg protein in a final volume of 0.13 mL. After 20 min pre incubation at 37 °C, the reaction was started by the addition of 0.42 µmol ADP (2.8 mM final concentration). The reaction was stopped after the incubation for 15 min by the addition of 1 µmol p-hydroxymercuribenzoic acid (6.25 mM final concentration). The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure the spontaneous hydrolysis of phosphocreatine. The creatine formed was estimated according by colorimetric measurement [17] The color was developed by the addition of 0.1 mL 2% α-naphtol and 0.1 mL 0.05% diacetyl in a final volume of 1 mL and read after 20 min at 540 nm. Results were expressed as nmol creatine formed/min/mg protein.

The activity of mt-HK was determined by a coupled assay according to Camacho-Pereira et al. [18]. Briefly, mt-HK activity was determined by NADH formation following the A_{340} at 28 °C. The assay medium contained: 20 mM Tris–HCl pH 7.4, 5 mM glucose, 6 mM $MgCl_2$, 1 mM β-NAD$^+$, 1 unit/mL G6PDH, 2 mM phosphoenolpyruvate, 0.1% (v/v) Triton X-100, and 10 units/mL pyruvate kinase. The reaction was started by adding 1 mM ATP. Results were expressed as nmol/min/mg protein.

2.6. Mitochondrial respiratory chain enzyme activity

The mitochondrial suspensions and C6 cells were centrifuged at 800 *g* for 10 min and the supernatants were used for determining the activity of mitochondrial respiratory chain enzymes (complexes I, II, and IV). On the day of the assays, samples were frozen and thawed thrice in hypotonic assay buffer to fully expose the enzymes to the substrates and achieve maximal activity.

NADH (nicotinamide adenine dinucleotide) dehydrogenase (complex I) was evaluated as described previously [19], i.e., by the rate of NADH-dependent ferricyanide reduction at 420 nm. The activity of complex I was measured before the addition of rotenone (20 g/mL) and the absorbance was monitored for a further 5 min. The activity of complex I was determined as the sensitivity to rotenone and was expressed as nmol/min/mg protein. Activity of succinate (DCIP oxidoreductase – complex II) was determined according to Fischer et al. [20], by following the decrease in absorbance due to the reduction of 2,6-DCIP) at 600 nm with 700 nm

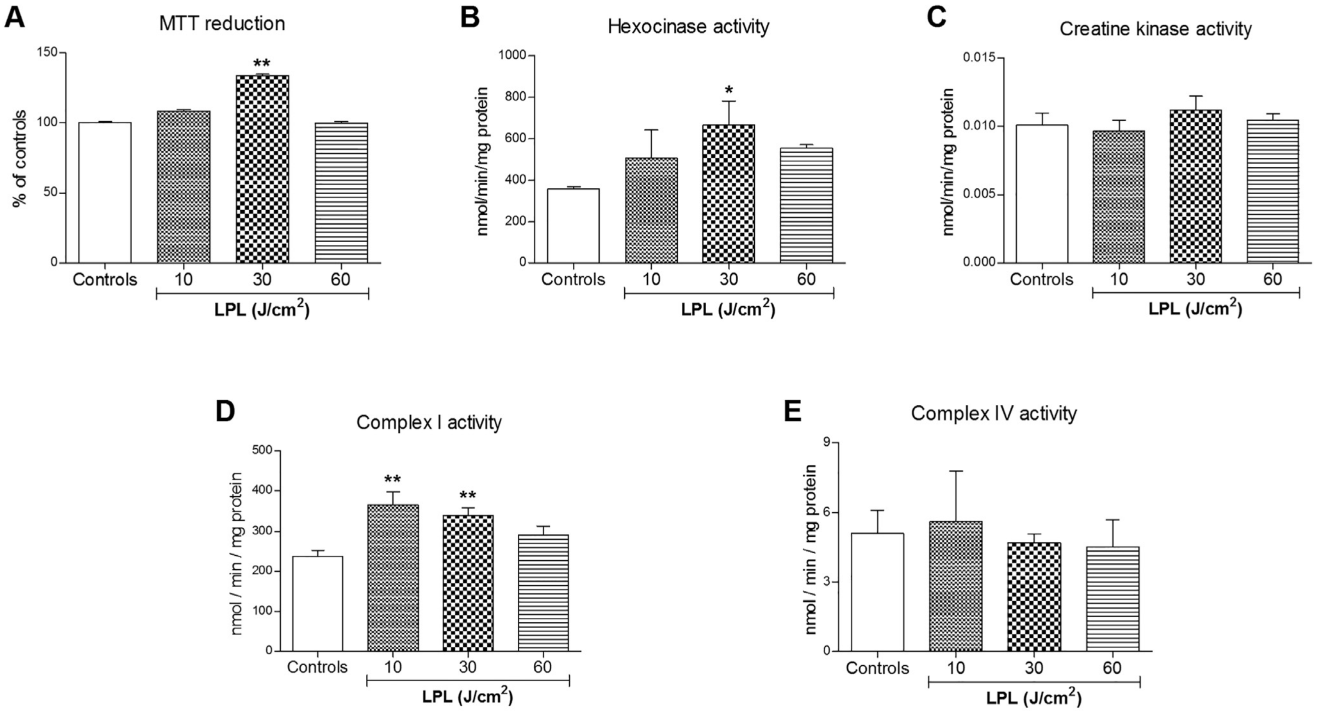

Fig. 1. *In vitro* effect of low-power laser (LPL) irradiation on energy metabolism in C6 astroglioma cells. The reduction of MTT (A) and the activity of hexokinase (B), creatine kinase (C), complex I (D), and complex IV (E) were assessed 24 h after exposing C6 cells to increasing fluences (10, 30, and 60 J/cm^2) of the AlGaInP laser. Bars represent mean values ± standard deviations from four to six independent experiments. $^{*}p < 0.05$ compared to non-irradiated cells (one-way ANOVA followed by post-hoc Tukey's test). $^{**}p < 0.01$ compared to non-irradiated cells (one-way ANOVA followed by *post-hoc* Tukey's test).

as reference wavelength ($\varepsilon = 19.1$ mM^{-1} cm^{-1}). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 µM DCIP was preincubated with 40–80 µg homogenate protein at 30 °C for 20 min. Subsequently, 4 mM sodium azide and 7 µM rotenone were added and the reaction was initiated by addition of 40 µM DCIP and was monitored for 5 min. Complex IV activity [21] was measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome c at 550 nm with 580 nm as reference wavelength ($\varepsilon = 19.1$ mM^{-1} cm^{-1}). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-D-maltoside, 2–4 µg homogenate protein and the reaction was initiated with addition of 0.7 µg reduced cytochrome c. The activity of complex IV was measured at 25 °C for 10 min.

2.7. Protein determination

The protein content in the samples was determined by the Lowry method using bovine serum albumin as the standard [22].

2.8. Statistical analysis

Data were analyzed using one-way ANOVA followed by Tukey's test when p-values were significant ($p < 0.05$ and 0.01). All analyses were performed using the Statistical Package for the Social Science software (SPSS, v 17.0; IBM Corp, Armonk, New York).

3. Results

Mitochondrial electron transport activity is associated with the activity of different complexes in the electron transport chain. For assessing the exact complex that may respond to PBM, the activity of mitochondrial complexes I and IV in C6 cells was determined (Fig. 1). Groups irradiated with doses of 10 and 30 J/cm^2 saw an in increase in complex I activity compared to the control group. MTT reduction and hexokinase activity increased significantly at the dose of 30 J/cm^2 when compared to the control.

Similarly, PBM at different time intervals affected the activity of mitochondrial complexes from the muscle/brain (Fig. 2 and 3). In the muscles, the activity of complex I at 30 and 60 J/cm^2 and of complex IV at 60 J/cm^2 increased 5 min after PBM compared to the control. One hour after muscle irradiation with 10 or 60 J/cm^2, the activity of complex II increased compared to the control. On the other hand, the activity of complex IV in all PBM groups (10, 30, and 60 J/cm^2) showed a significant increase compared to the controls.

In brain mitochondria, only the activity of complex IV increased in all groups 5 min after PBM when compared to the control. After a 1 h interval, complex II activity increased in the group irradiated with 60 J/cm^2, and complex IV activity increased in all PBM groups (10, 30, and 60 J/cm^2) when compared to the control.

4. Discussion

PBM is the use of light, usually through low-level laser or light-emitting diode treatment, for stimulating the healing and regeneration of damaged tissue [1]. Studies have suggested that, in irradiated cells, the first photochemical and photophysical events occur in the mitochondria and promote respiratory modifications as a result of structural changes [3,4], as well as metabolic modifications in these organelles. Thus, it appears that PBM can alter the activity of respiratory chain complexes, CK, and hexokinase in C6 cells and mitochondria isolated from muscles/brain.

This study examined the changes in mitochondrial activity after irradiation with a 670-nm, low-energy AlGaInP laser. When the low and medium doses were used (10 and 30 J/cm^2), the activity of complex I increased, and only the dose of 30 J/cm^2 increased hexokinase activity and MTT reduction in C6 cells, while a high dose did not affect any of the analyzed mitochondrial parameters.

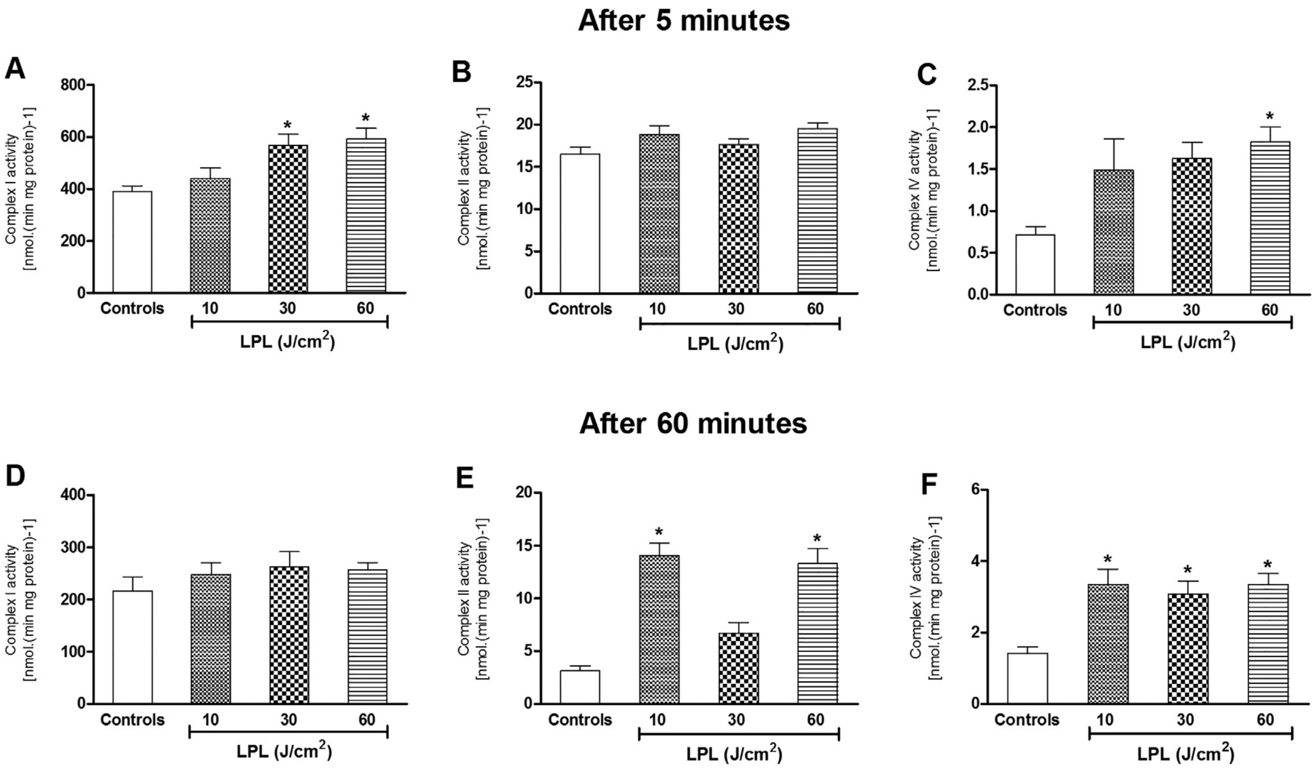

Fig. 2. Effect of low-power laser (LPL) irradiation on the activity of mitochondrial respiratory chain complexes I, II, and IV in skeletal muscles 5 and 60 min after irradiation with increasing fluences (10, 30 and 60 J/cm^2) of the AlGaInP laser. Bars represent mean values ± standard deviations from four to six independent experiments. $*p < 0.05$ compared to non-irradiated cells (one-way ANOVA followed by *post-hoc* Tukey's test).

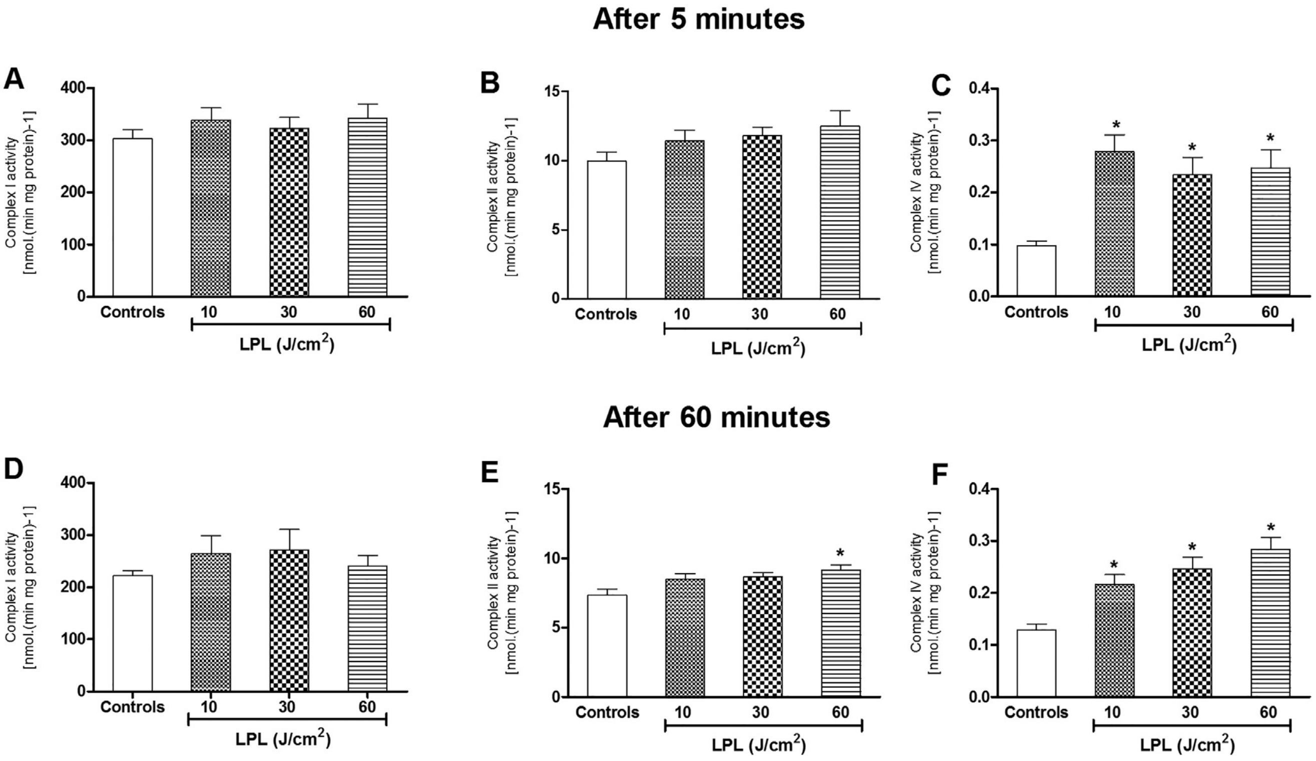

Fig. 3. Effect of low-power laser (LPL) irradiation on the activity of mitochondrial respiratory chain complexes I, II, and IV in the brain 5 and 60 min after irradiation with increasing fluences (10, 30, and 60 J/cm2) of the AlGaInP laser. Bars represent mean values ± standard deviations from four to six independent experiments. $*p < 0.05$ compared to non-irradiated cells (one-way ANOVA followed by *post-hoc* Tukey's test).

Complex I is the limiting step in oxidative phosphorylation [23], and its modifications or stimulations may directly affect the overall state of cellular energy. This may be related to the presence of photoreceptors, mainly in complexes I and IV of the respiratory chain, as previously proposed by Karu in HeLa cells [24].

The photoactivation of mitochondrial components sensitive to visible laser light can cause a short activation of the respiratory chain, NADH oxidation/reduction, and CCO, leading to beneficial cellular changes in the redox state of both the cytoplasm and the mitochondria, which may include increased activity of enzymes such as hexokinase and of the Krebs cycle and possibly increased ATP production [3].

Hexokinase is ubiquitously present in tissues, particularly in brain cells, where the regulation of glucose metabolism and its activity are mainly associated with the interaction of mitochondrial membranes. However, it seems that PBM alters the mitochondrial membrane potential, which might increase the proximity of hexokinase to the outer mitochondrial membrane to facilitate ATP transfer from the mitochondria to the cytosol [25].

In order to explain the effect of PBM on the activity of muscle and brain mitochondria, we interrupted the reactions 5 min and 1 h after irradiation. Triggering stimulation or inhibition depends on irradiation time, wavelength, and the focus of irradiation [26]. We observed fluctuation in mitochondrial activity at different fluences (10, 30, 60 J/cm^2). For example, 5 min after irradiation of muscle mitochondria with 30 or 60 J/cm^2, the activity of complex I increased, while the activity of complex IV increased only after irradiation with 60 J/cm^2 On the other hand, the activity of complex II increased 1 h after irradiation of muscle mitochondria with 10 or 60 J/cm^2, while the activity of complex IV increased 1 h after irradiation with 10, 30, or 60 J/cm^2. These results may be because exposure to higher irradiation after the short interval (5 min) caused increased electron transport in complexes I and IV, while, on the other hand, the long interval (1 h) allowed the low dose of 10 J/cm^2 to increase electron transfer from complexes II and IV in the muscle mitochondria.

In the brain mitochondria, 5 min after laser treatment, the activity of complex IV increased at all doses. Finally, 1 h after irradiation the activity of complex IV increased at all doses, while the activity of complex II increased only at 60 J/cm^2. The results taken together showed a fluctuation in activity of the respiratory chain complexes in relation to the doses used, but we can observe that the complex IV was more stimulated by PBM in the mitochondria of the brain.

According to Pastore et al. [27], purified CCO could be activated *in vitro* using a red laser at 670 nm. Several studies have reported that action spectra, i.e., relative efficiency of different wavelengths for mediating the aspects of the PBM process, correspond to the absorption spectrum of CCO [28,29]. Perhaps nitric oxide, which is known to inhibit CCO by non-covalently binding between heme-a3 and CuB, can be photodissociated by photon absorption (red or near-infrared light) [30]. One theory for explaining why PBM seems to have a greater effect in diseased or damaged cells and tissues, and not dramatically affecting healthy cells, is that unhealthy or hypoxic cells are more likely to have inhibitory concentrations of NO [31].

As the main PBM chromophores are located inside the mitochondria, it follows that cells with multiple mitochondria and a high metabolic activity are particularly responsive to light. This consideration applies to muscle cells (skeletal/cardiac) and neurons (especially those in the central nervous system). These cells are not commonly exposed to light during normal living activity, while cells of the skin have, due to the constant exposure to light evolved not to have multiple mitochondria [3].

This study suggests that PBM could increase the activity of mitochondria, possibly due to the absorption of laser light by the metal centers in the respiratory chain complexes, which may lead to the stimulation of the cytoplasm and mitochondrial enzymes.

Conflicts of interest

The authors declare no conflicts of interest. The authors alone are responsible about article content and writing.

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Ethical approval

All experimental procedures were in accordance with the Brazilian Guidelines for the Care and Use of Animals for Scientific and Didactic Purposes (DOU 27/5/13, MCTI, p. 7) and approved by the local ethical committee).

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