Effect of 808 nm Diode Laser on Swimming Behavior, Food Vacuole Formation and Endogenous ATP Production of *Paramecium primaurelia* (Protozoa)

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**ABSTRACT**

Photobiomodulation (PBM) has been used in clinical practice for more than 40 years. To clarify the mechanisms of action of PBM at cellular and organism levels, we investigated its effect on *Paramecium primaurelia* (Protozoa) irradiated by an 808 nm infrared diode laser with a flat-top handpiece (1 W in CW). Our results led to the conclusion that: (1) the 808 nm laser stimulates the *P. primaurelia* without a thermal effect, (2) the laser effect is demonstrated by an increase in swimming speed and in food vacuole formation, (3) the laser treatment affects endogenous adenosine triphosphate (ATP) production in a positive way, (4) the effects of irradiation dose suggest an optimum exposure time of 50 s (64 J cm⁻² of fluence) to stimulate the *Paramecium* cells; irradiation of 25 s shows no effect or only mild effects and irradiation up to 100 s does not increase the effect observed with 50 s of treatment, (5) the increment of endogenous ATP concentration highlights the positive photobiomodulating effect of the 808 nm laser and the optimal irradiation conditions by the flat-top handpiece.

**INTRODUCTION**

Photobiomodulation (PBM) (earlier terms: low-level laser therapy, LLLT, laser biostimulation) has been used in clinical practice for more than 40 years and its mechanisms of action at cellular and molecular levels have been studied for about 30 years (1). PBM is the term applied to the manipulation of cellular behavior using low-intensity light sources and works on the principle of inducing a biological response through energy transfer (2). Photonic energy delivered into the tissue modulates the biological processes within that tissue and within the biological system of which that tissue is a part (3).

Despite many reports of positive findings from experiments conducted in vitro in animal models and in randomized controlled clinical trials, PBM remains controversial. It is possible that this may be due to incomplete knowledge surrounding the biochemical mechanisms underlying the PBM positive effect; additionally, there is the complex influence of choice among a large number of operating parameters such as wavelength, fluence, power density, pulse structure and treatment timing, the homogeneity and the intrinsic disease state of animal models and operator’s sensitivity (4).

Photobiomodulation is known to act at cellular level. It is suggested that the mechanism of PBM at cellular level is based on the absorption of monochromatic visible and near-infrared radiation by component of mitochondria respiratory chain (5). The absorption of photons by molecules leads to electronically excited states, and consequently can lead to an acceleration of electron transfer reaction. More electron transport necessarily leads to the increased production of ATP. The light-induced increase in ATP synthesis and increased proton gradient lead to an increasing activity of the Na⁺/H⁺ and Ca²⁺/Na⁺ antiporters, and of all the ATP-driven carriers ions, such as Na⁺/K⁺ ATPase and Ca²⁺ pumps (see review, 6). Ca²⁺ regulates almost every process in the human body as well as in the *Paramecium* (Protozoa), where it is known to influence the membrane polarization (7). Moreover, we recently showed the positive effect of the 808 nm diode laser on the fission rate (8) and the oxygen consumption of *Paramecium cell* (9).

To clarify the mechanisms of action of the PBM at cellular and organism levels in our study, we investigated its effect on the eukaryotic unicellular organism *Paramecium primaurelia* (Protozoa) irradiated by the 808 nm diode laser with a flat-top handpiece. The use of a flat-top handpiece compared to a defocused conventional handpiece with a Gaussian profile, enables irradiation of a target surface with a homogenous energy density. This would make the application repeatable and not operator sensitive (8,10).

The protozoa were chosen because of their short cell cycles that allow the analysis of the effects of environmental perturbations on a conspicuous number of cells, genetically homogeneous populations (clones) and successive generations within short time-frame sequences. Furthermore, because of a common ancestor with the metazoan, the experimental response of the protozoa can be correlated with those of the more-developed organisms (11). It is important to emphasize how in protozoa, the identification of molecules responsible for neurotransmission in metazoan (12–17) as well as the genome sequencing of several protozoa (18,19), has demonstrated that they can represent excellent...
bioassay in field studies on human health (20) such as the effects of pesticides (11) and extremely low frequency electromagnetic field (21–24) as well as PBM (8,9,25).

In particular, the bacterivore ciliate *P. primaurelia* is an excellent model system for eukaryotic sensory transduction studies. The advantages for using this ciliate for cellular sensory transduction studies are that behavioral, electrophysiological, biochemical and genetic (both forward and reverse) approaches can all be used in this simple unicellular organism. Swimming behavior is used to estimate the physiological state of the *Paramecium* cell (26). The swimming of *Paramecium* is an extreme case where the cell’s body is covered with several thousands of cilia; their beating produces the swimming motion of the cell and its feeding, by driving bacteria into the oral groove. Ciliary-based motility is crucial to survival and reproduction of these organisms and is based on the ability to sense environmental stimuli and to respond through an appropriate attraction, or avoidance and escape. For this purpose, based on the coupling of sensing and motile functions of its cilia whose beating frequency is modulated by a membrane depolarization or by a hyperpolarization, *Paramecium* is able to respond to chemical, mechanical, physical, thermal or gravitational stimuli by adapting the frequency, coordination and direction of the ciliary beating (27).

On this basis, our study involved the assessment of changes on both swimming behavior and ability to eat and produce food vacuoles as well as the variation in endogenous adenostine triphosphate (ATP) concentration of *Paramecium primaurelia* irradiated by the 808 nm diode laser with a flat-top handpiece.

**MATERIALS AND METHODS**

*Paramecium primaurelia* and *Paramecium bursaria* are ciliates that are used as model organisms in both basic and applied research. Some of these applications are that behavioral, electrophysiological, biochemical and genetic (both forward and reverse) approaches can all be used in this simple unicellular organism. Swimming behavior is used to estimate the physiological state of the *Paramecium* cell (26). The swimming of *Paramecium* is an extreme case where the cell’s body is covered with several thousands of cilia; their beating produces the swimming motion of the cell and its feeding, by driving bacteria into the oral groove. Ciliary-based motility is crucial to survival and reproduction of these organisms and is based on the ability to sense environmental stimuli and to respond through an appropriate attraction, or avoidance and escape. For this purpose, based on the coupling of sensing and motile functions of its cilia whose beating frequency is modulated by a membrane depolarization or by a hyperpolarization, *Paramecium* is able to respond to chemical, mechanical, physical, thermal or gravitational stimuli by adapting the frequency, coordination and direction of the ciliary beating (27).

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25, 50, 75 or 100 s by the 808 nm infrared diode laser (Wiser; Doctor Smile) with a flat-top handpiece (AB2799; Doctor Smile), while the other Eppendorf tube was placed in contact with a petri dish filled with water at the temperature of 24°C for 25, 50, 75 or 100 s, nonirradiated and considered as control. The irradiated cells and the nonirradiated control cells were then processed for the endogenous ATP detection.

The other experiments were performed irradiating the Eppendorf tube placed in contact with a petri dish filled with water at the temperature of 14°C, for 50 s by the 808 nm infrared diode laser with a flat-top handpiece. After 0, 1, 2, 15 and 30 min from the irradiation, the cells were processed for the endogenous ATP detection. The second Eppendorf tube was placed in contact with a petri dish filled with water at the temperature of 24°C for 50 s, nonirradiated and considered as control. After 0, 1, 2, 15 and 30 min, the control cells were processed for the endogenous ATP detection too.

To evaluate the cellular ATP concentration, a highly sensitive luciferin/luciferase method was used. One hundred μL of 0.25 M perchloric acid was added to *Paramecium* cells to block the enzyme activities. The sample was sonicated and neutralized with 100 μL of 2M K₂CO₃, at pH 7. The sample was centrifuged and 100 μL of supernatant was used to assay the cellular ATP concentration, after the addition 100 μL of luciferin/luciferase solution (Roche, Basel, Switzerland), on a Luminometer (Triathlon, Bioscan, Washington, DC). ATP standard solutions (Roche) in the concentration range 10⁻¹⁰–10⁻⁷ M were used for calibration (30).

Statistical analysis. The statistical analysis was performed using a two-way ANOVA followed by the Student–Newman–Keuls multicomparison test to discriminate the statistically significant results.

RESULTS

Effect of 808 nm infrared diode laser on the temperature variations of the sterile lettuce medium

Figure 1 shows the data relevant to the effects of the irradiation by 808 nm infrared diode laser, on the temperature of *Paramecium*’s lettuce infusion medium. The irradiation affects the medium temperature when the experiments were performed placing the Eppendorf tube or the plastic semimicro cuvette on a petri dish filled with water at the temperature of 24, 22, 20 or 18°C. In fact, the temperature increased significantly (*P* < 0.05) by about 2.8, 2.5, 1.9, 1.4°C, respectively. When placed on the petri dish filled with water at the temperature of 16°C, there was not a significant (*P* > 0.05) thermal increase after 25 s of irradiation; the water temperature of 14°C allowed the temperature of the medium to be maintained, for exposure from 25 s up to 100 s, at values not significantly different (*P* > 0.05) from the temperature control. Thus, we chose this last experimental condition as optimal for our experiments to prevent thermal increases during exposition.

Effect of 808 nm infrared diode laser on swimming behavior

The *Paramecium* cells irradiated by 808 nm infrared diode laser did not show alterations in their swimming such as may be seen in one or more of the following: continuous ciliary reversal yielding relatively long periods of fast backward swimming mediated by full ciliary reversal, periodic ciliary reversal characterized by brief and repeated episodes of backward swimming or partial ciliary reversal giving rise to a spiral-like movement and resulting from the reversal of only part of the somatic cilia of the cells. Furthermore, during the irradiation after 25 s from the beginning of the laser treatment, the cells displayed a swimming speed not significantly different (Fig. 2, *P* > 0.05) to the control.

However, as shown in Fig. 2 after 50 s from the beginning of the irradiation, the swimming speed increased significantly (*P* < 0.05) and was kept after 75 and 100 s from the beginning of the irradiation (*P* > 0.05). At the completion of 50 s of laser irradiation, the cells kept their increased swimming speed for about 15 min. As shown in Fig. 3, the cells irradiated had a significantly (*P* < 0.05) increased swimming speed at the end of irradiation (0 min), which was kept after 1 min and increased again after 2 min (*P* < 0.05). After 15 min, the swimming speed decreased but was significantly (*P* < 0.05) higher compared to...
15 min after the 50 s irradiation, the number of food vacuoles exposure (data not shown). However, as show in Fig. 4, 1, 2, did not create food vacuoles during the 25, 50, 75 or 100 s of Paramecium The formation Effect of 808 nm infrared diode laser on food vacuole formation

The Paramecium cells irradiated by 808 nm infrared diode laser did not create food vacuoles during the 25, 50, 75 or 100 s of exposure (data not shown). However, as show in Fig. 4, 1, 2, 15 min after the 50 s irradiation, the number of food vacuoles was significantly higher than the control ($P < 0.05$). Thirty minutes after the laser treatment, the number of food vacuoles decreased, reaching a value similar to that of the control ($P > 0.05$). Similar results were observed in the samples irradiated for 75 and 100 s, which had no significant difference ($P > 0.05$) in the number of food vacuoles with respect to the sample irradiated for 50 s (data not shown). Lastly, the sample irradiated for 25 s had no significant difference ($P > 0.05$) with respect to the control (data not shown).

Effect of 808 nm infrared diode laser on endogenous ATP production

Figure 5 shows the variation in the level of endogenous ATP in P. primaurelia during 100 s of irradiation with the 808 nm infrared diode laser. The ATP increased significantly ($P < 0.05$) during the first 25 s of irradiation, reaching the highest level after 50 s ($P < 0.05$). Seventy-five seconds and 100 s of irradiation increased significantly the ATP level with respect to the control ($P < 0.05$) but not when compared with the level at 50 s ($P > 0.05$).

Figure 6 shows the variation in the level of endogenous ATP in P. primaurelia after 0, 1, 2, 15 and 30 min from the irradiation with the 808 nm infrared diode laser. At the end of irradiation (0 min), the ATP level was significantly ($P < 0.05$) higher than the control and increased again in the first minute (1 min) reaching the highest level ($P < 0.05$). Two minutes (2 min) after the irradiation, the ATP level was significantly lesser than at 1 min but higher than the control ($P < 0.05$). The ATP level decreased again and at 15 min reached the value of the control ($P > 0.05$). However 30 min after the irradiation, the ATP level increased significantly compared to the control ($P < 0.05$) reaching a value similar to at 2 min ($P > 0.05$).

DISCUSSION

Paramecium propels itself by whiplash movements of its cilia, which are arranged in tightly spaced rows around the outside of their body. It can rapidly modulate both the beating frequency and the direction of the ciliary power stroke to swim
transiently backward or forward at various speeds (31). As such, the locomotion of Paramecium depends on the ciliary movements which are controlled by the electrical changes in the cell membrane (7).

The 808 nm infrared diode laser used in our work affects the swimming behavior of P. primaurelia, increasing the swimming speed during the 50, 75 and 100 s of irradiation, without inducing changes in the swimming direction such as: continuous ciliary reversal, periodic ciliary reversal or partial ciliary reversal. The effect reaches a maximum after 2 min from the irradiation, and then returns to the value of the control after 30 min. It has been reported that membrane hyperpolarization is correlated with the augmented ciliary beating and accelerated forward swimming (32), whereas depolarization occurs with the ciliary reversal and backward swimming (33). Relying on the work of Nakaoka et al. (34), our results suggest that the laser irradiation induces in P. primaurelia an increase in Ca²⁺ concentration of above 10⁻⁷ M and below 10⁻⁶ M. In fact, Nakaoka et al. (34) reported that the intracellular concentration of Ca²⁺ is kept below 10⁻⁷ M in the resting state (in which the swimming velocity and beating frequency are low and cell shape is normal), must rise above 10⁻⁷ M to increase the beating frequency and stay above 10⁻⁶ M to increase the beating frequency and induce ciliary reversal.

The cilia are also used by Paramecium to gather food and their effectiveness is linked to beating frequency. The Paramecium uses its cilia to sweep prey organisms, along with some water through the oral groove and into the mouth opening. The food passes through the cell mouth into the gullet. When enough food has accumulated at the gullet base, it forms a food vacuole in the cytoplasm (35).

The Paramecium cells irradiated by the 808 nm infrared diode laser do not make food vacuoles during the 25, 50, 75 or 100 s of irradiation. Conversely, 1, 2 and 15 min after the laser treatment the number of food vacuoles increases. The increase in the food vacuole formation can be correlated with the increased beating frequency of the cilia, but the beating frequency does not explain the absence of food vacuole formation during the irradiation with the laser. It is possible to assume two effects of the laser treatment on the Paramecium cell. The first one is an effect on the concentration of Ca²⁺ in the Paramecium, as reported by Friedmann and Lubart (36,37) in mammalian cells, which is visible with the increased swimming speed, supported by an increment of cilia beating; the second is a transient effect on the membrane fluidity of Paramecium, as described in other cell models (38) which blocks the formation of food vacuoles. These hypotheses are supported by the results of Otto et al. (39), which show that the Ca²⁺ permeability of the ciliary membrane is a membrane property which is not directly affected by the fluidity of its lipid environment.

In Paramecium cilia as well as in the cilia of metazoan, the dynein ATPase is responsible for the movements of cilia driven by microtubule sliding. For active microtubule sliding, ATP must be readily available and diffuses into the cilia from the cytoplasm and forms a concentration gradient; therefore, the distal regions of the cilia are starved of ATP (40). It has been calculated that Paramecium expends more than half of its energy in propelling itself through the water (41).

The Paramecium cells, irradiated by the 808 nm infrared diode laser increase incrementally the endogenous ATP concentration during the 25, 50, 75 and 100 s of irradiation and the increased ATP synthesis is kept for 15 min above the 50 s of irradiation. Fifteen min from the laser treatment, the endogenous ATP concentration returns to the baseline level, to increase again after 30 min. It is known that cytochrome C oxidase is the primary photoacceptor for the PBM in mammalian cells (42) as well as ATP synthesis being stimulated by the infrared diode laser (43). Consequently, it is possible to assume that the increase in the endogenous ATP level is not only a consequence of variation in Ca²⁺ concentration, inducing the accelerated swimming, but also a promoting effect of the laser on ATP synthesis, thus explaining the increase in ATP after 30 min from the irradiation when the speed of swimming and, consequently, the consumption of ATP progressively decrease.

CONCLUSION

Our results lead to the conclusion that:

1. The 808 nm infrared diode laser with a flat-top handpiece stimulates the P. primaurelia without a thermal effect.
2. The laser effect is demonstrated by an increase in swimming speed and in food vacuole.
3. The laser treatment affects the endogenous ATP production in a positive way.
4. Exposure to laser irradiation of 50 s (64 J cm⁻²) optimizes the stimulation of Paramecium cells; irradiation of 25 s shows no effect or only mild effects and irradiations up to 100 s do not increase the effect observed during the 50 s of treatment sequence.
5. The increment of endogenous ATP concentration highlights the positive PBM effect of the 808 nm infrared diode laser and the optimal treatment conditions used. In fact, as suggested by Friedmann and Lubart (37), the irradiation has not induced high calcium levels, which interfere with the respiration process of the respiratory chain by using part of the NADH, enhancing Ca²⁺ uptake by mitochondria and slowing down ATP production.
REFERENCES


