No effect of low-level lasers on in vitro myoblast culture

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Effects of phototherapy using low-level lasers depend on irradiation parameters and the type of laser used. The aim of the present study was to evaluate the effect of phototherapy on the proliferation of cultured C2C12 myoblasts under different nutritional conditions using low-level GaAlAs and InGaAlP lasers with different parameters and incubation periods. C2C12 cells cultured in regular and nutrient-deficient medium were irradiated with low-level GaAlAs (780 nm) and InGaAlP (660 nm) lasers with energy densities of 3.8, 6.3 and 10 J/cm², and 3.8, 10 and 17.5 J/cm², respectively. Cell proliferation was assessed 48 and 72 h after irradiation by MTT assay. There were no significant differences in cell proliferation between laser-treated myoblasts and control cultures for any of the parameters and incubation periods. Further studies are necessary to determine the correct laser parameters for optimizing the biostimulation of myoblasts.

Keywords: C2C12 cells, Cell proliferation, Low-level lasers, Myoblasts

Maintenance and regeneration of skeletal muscles depend mainly on resident stem cells known as satellite cells. Upon injury, the adult muscle tissue demonstrates a remarkable regeneration capacity supported by satellite cells that are stimulated to proliferate and fuse with surrounding myoblasts or preexisting myofibers in order to regenerate their functionality.

A growing body of evidence suggests that low-level laser therapy (LLLT) promotes skeletal muscle regeneration by reducing the duration of acute inflammation and accelerating tissue repair. The biological mechanisms behind the beneficial results of LLLT are becoming clearer due to a considerable scientific research, including the use of cell cultures, animal models and clinical studies.

Majority of in vitro investigations on muscle regeneration have used primary cultures of satellite cells and the C2C12 murine line to experimentally delineate the multi-step process of muscle cell proliferation and differentiation. The C2C12 line is a myoblast cell line isolated from satellite cells of adult mice and exhibits most of the characteristics of normal myoblast cells. The use of cell lines as models for the analysis of cell proliferation eliminates the possibility of the influence of laser irradiation on the production of growth factors by non-myogenic cells contained in primary cultures, such as fibroblasts and macrophages.

Laser irradiation seems to induce the synthesis of cell cycle regulatory proteins due to the activation of early cell cycle regulatory genes, mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) cascades. The effects of low-level laser radiation on biological tissue regeneration depend on the exposure time, dosage, wavelength and frequency of irradiations. A recent study found that LLLT with the GaAlAs (Gallium-Aluminum-Arsenide) laser (810 nm) at 0.33 to 8.22 J/cm² had a photobiomodulation effect on C2C12 myotubes, as manifested by the improvement of mitochondrial function, while doses of 11.22 to 14.16 J/cm² were beyond the point at which photomodulation is beneficial and such doses could actually worsen cellular function. In the present study effect of different LLLT parameters on C2C12 myoblast proliferation has been investigated using a muscle injury model.

Materials and Methods

Cell Culture—C2C12 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Cultilab, Campinas, SP, Brazil), supplemented with 10% fetal bovine serum (FBS, Cultilab) and 1%
antibiotic–antimycotic solution (Cultilab) at 37°C in a humidified atmosphere of 5% CO₂. The cells were maintained at subconfluent densities and passaged every two to three days.

**Experiments**—Prior to the experiments, all cell cultures were examined under a light microscope and the viability of the cultures was confirmed by the trypan blue exclusion test. In order to induce cell stress, half of the cultures were grown in DMEM supplemented with only 5% FBS for 24 h prior to the assay. This *in vitro* situation produces stresses that are similar to *in vivo* stress conditions, reducing rates of cell growth and allowing the observation of the possible effects of phototherapy on cell growth.\(^{15,19}\)

**Laser irradiation**—Cells were irradiated with GaAlAs (780 nm) and InGaAlP (Aluminium gallium indium phosphide, 660 nm) (MMOptics Ltd., São Carlos, SP, Brazil). The parameter settings are listed in Table 1 and exposure time was set at 10 sec. The power output of the lasers was measured with a LaserCheck power meter (Coherent, Santa Clara, CA, USA). In order to standardize the results, the distance between the laser probe and cell cultures was kept constant. The bottom of the test tubes containing the cell cultures was irradiated with laser, as described elsewhere.\(^{20}\) Thus, the laser beam did not pass through the culture medium, but rather was applied directly to the cell pellet. The control cell pellets were subjected to the same experimental conditions as the irradiated cells, except for the irradiation. Irradiation was performed in the dark in order to avoid the influence of other light sources.

**Experimental groups**—Control group (no laser irradiation); groups treated with a 780-nm laser operating at 3.8, 10 and 17.5 J/cm\(^2\); and groups treated with a 660-nm laser operating at 3.8, 6.3 and 10 J/cm\(^2\). As there are no previously defined irradiation parameters in the literature for experiments involving low-level lasers and C2C12 myoblasts, the experimental groups were irradiated with the two diode lasers (660 nm, 780 nm) at the same energy densities of 3.8 and 10 J/cm\(^2\) for the comparison of results between groups, as well as with the 660-nm laser at an intermediate energy density of 6.3 J/cm\(^2\), and the 780-nm laser at a higher energy density of 17.5 J/cm\(^2\) for the comparison of results within groups.

**Effect of laser irradiation on cell proliferation**—After irradiation, control (non-irradiated) and treated (irradiated) cell pellets were resuspended in fresh DMEM (containing 10% or 5% FBS) and plated (1\times10^4 cells/well for 48 h and 1\times10^5 cells/well for 72 h) in 96-well plates. For cell viability analysis, the cultures were incubated in a humidified atmosphere of 5% CO₂ for 48 and 72 h. Analysis of mitochondrial activity was then performed to assess cell proliferation.

**Analysis of mitochondrial activity**—The mitochondrial function of C2C12 myoblasts was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA). After laser irradiation and incubation for 48 and 72 h, MTT was added to the cell cultures to a final concentration of 0.5 mg/ml and the cells were incubated in an atmosphere of 5% CO₂ at 37°C for 3 h. Then, 100 µl of isopropanol was added to each well to dissolve the formazan crystals. Absorbance was measured at 620 nm using a microplate reader (Anthos2020, Anthos Labtec Instruments, Wals, Austria).

**Statistical analysis**—Absorbance data corresponding to cell viability were obtained in quadruplicate and are presented as mean value ± standard deviation (SD). Comparisons between groups were made using one-way analysis of variance (ANOVA) and the Dunnett test was used to determine significant differences between the laser irradiated groups and control (non-irradiated) group. \(P<0.05\) were considered statistically significant. Data were analyzed using the GraphPad Prism 4.0 statistical software (GraphPad Software, San Diego, CA, USA).

All experiments were performed in quadruplicate in three assays, although only a single representative experiment is shown in each figure.

**Results**

**Effect of GaAlAs (780nm) Laser Irradiation on Cell Proliferation at 3.8, 10 and 17.5 J/cm\(^2\) in regular nutritional condition and injury model**—There were no significant differences in cell proliferation, as

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Output power (mW)</th>
<th>Beam spot area (cm(^2))</th>
<th>Power density (mW/cm(^2))</th>
<th>Energy density (J/cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>780</td>
<td>15</td>
<td>0.04</td>
<td>37.5</td>
<td>3.8</td>
</tr>
<tr>
<td>780</td>
<td>40</td>
<td>0.04</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>780</td>
<td>70</td>
<td>0.04</td>
<td>175</td>
<td>17.5</td>
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<tr>
<td>660</td>
<td>15</td>
<td>0.04</td>
<td>37.5</td>
<td>3.8</td>
</tr>
<tr>
<td>660</td>
<td>25</td>
<td>0.04</td>
<td>62.5</td>
<td>6.3</td>
</tr>
<tr>
<td>660</td>
<td>40</td>
<td>0.04</td>
<td>100</td>
<td>10</td>
</tr>
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evaluated by the MTT assay, between InGaAlP laser-treated myoblasts and control cultures after 48 and 72 h of incubation. However, the proliferation of cells cultured in the medium supplemented with 5% FBS (injury condition) was significantly lower than that of cells cultured in the regular medium containing 10% FBS. The comparison of the proliferation of C2C12 cells irradiated with a 780-nm laser at energy densities of 3.8, 10 and 17.5 J/cm² with that of non-irradiated control cells cultured in normal medium (10% FBS) and in nutrient-deficient medium (5% FBS) is shown in Figs 1 and 2.

Effect of InGaAlP (660 nm) Laser Irradiation on Cell Proliferation at 3.8, 6.3 and 10 J/cm² in regular nutritional condition and injury model—Likewise, in the groups treated with the GaAlAs 660 nm laser, there were no significant differences in cell proliferation, as evaluated by MTT assay, between laser-treated myoblasts and control cultures after 48 and 72 h of incubation. However, the viability of cells cultured in the medium supplemented with 5% FBS (injury condition) was significantly lower than that of cells cultured in the normal medium containing 10% FBS. The comparison of the viability of C2C12 cells irradiated with a 660-nm laser at 3.8, 6.3 and 10 J/cm² energy densities with that of non-irradiated control cells cultured in normal medium (10% FBS) and in nutrient-deficient medium (5% FBS) is shown in Figs. 3 and 4.

![Fig. 1](image1.png)

Fig. 1—Proliferation of C2C12 cells irradiated with a 780-nm laser at energy densities of 3.8, 10 and 17.5 J/cm² with that of non-irradiated control cells cultured in normal medium (10% FBS) and in nutrient-deficient medium (5% FBS) in 48 h.

![Fig. 2](image2.png)

Fig. 2—Proliferation of C2C12 cells irradiated with a 780-nm laser at energy densities of 3.8, 10 and 17.5 J/cm² with that of non-irradiated control cells cultured in normal medium (10% FBS) and in nutrient-deficient medium (5% FBS) in 72 h.

![Fig. 3](image3.png)

Fig. 3—Proliferation of C2C12 cells irradiated with a 660-nm laser at 3.8, 6.3 and 10 J/cm² energy densities with that of non-irradiated control cells cultured in normal medium (10% FBS) and in nutrient-deficient medium (5% FBS) in 48 h.

![Fig. 4](image4.png)

Fig. 4—Proliferation of C2C12 cells irradiated with a 660-nm laser at 3.8, 6.3 and 10 J/cm² energy densities with that of non-irradiated control cells cultured in normal medium (10% FBS) and in nutrient-deficient medium (5% FBS) in 72 h.
Discussion

Results revealed that C2C12 cell growth after LLLT stimulus was similar to the control group after 48 and 72 h of incubation period under both nutritional conditions and for all parameters tested. In fact, the question is no longer whether LLLT has biological effects, but rather how radiation from therapeutic lasers and light-emitting diodes works at the cellular and organism levels and what the optimal light parameters are for different uses of these light sources at various wavelengths and for different kinds of cells and tissues.

Epithelial cell cultures (Vero cell line) grown under regular (10% FBS) nutritional conditions as well as with nutritional deficit (2% FBS) irradiated with low-level laser from one to three times with a GaAlAs laser (780 nm) and InGaAlP (660 nm), 40 and 70 mW, respectively, at 3 or 5 J/cm² revealed that non-irradiated cell cultures grown in the regular nutritional medium produced greater cell growth than all cultures grown under nutritional deficit, whether irradiated or not. This is similar to the results of the present study. Furthermore, cell cultures irradiated once and twice with 780-nm (GaAlAs) and 660-nm (InGaAlP) diode lasers (40 and 70 mW, at 3 and 5 J/cm²) were similar to those of non-irradiated control cultures grown in the nutrient-deficient medium.

The same group of researchers found that growth rates of fibroblasts irradiated twice with a GaAlAs diode laser (2 J/cm²) were significantly greater than those for the control group and that osteoblast-like cultures irradiated with InGaAlP diode laser (10 mW, 3 J/cm²) had a higher number of cells than non-irradiated cultures. Moore et al. showed that fibroblasts proliferated with 665 and 675 nm light treatment, whereas 810 nm light was inhibitory to these cells. Even when we compare the results from the same kind of cells, if the dosage is too low, minimal or no effects are invoked; on the other hand, if the dosage is too high, no effects or even deleterious effects may result.

LLLT seems to act mainly on cells with committed functions and the intracellular responses of stem cells to laser phototherapy is not known. Results of the study by Xu et al. where LLLT irradiation (810 nm diode laser) at doses of 0.33 to 8.22 J/cm² had a photobiomodulation effect on C2C12 myotubes are different from the present study where LLLT irradiation induced C2C12 myoblast differentiation and obtained myotubes. In the present study the C2C12 line was used, which is a subclone of the C2 myoblast line isolated from satellite cells of adult mice kind of stem cell.

The muscle injury model used in the present study was based on the fact that, immediately after muscle injury (e.g., partial excision), the traumatized area suffers ischemic injury and a lack of nutrients and oxygen, and that cells cultured in low-serum and serum-free media mimic the initial post-traumatic phase. Using a culture medium supplemented with FBS (5%), we found that the viability of C2C12 cells was maintained, but growth rates were significantly lower than those of cells cultured in a normal medium supplemented with FBS (10%). Thus, this serum concentration was appropriate for the proposed experiment, i.e., nutrient-deficient conditions should not stop cell growth completely, but only reduce the cell growth rate; therefore, the effect of low-level laser irradiation on the growth rates of cells cultured under these conditions can be measured.

It was also observed that cell growth continued after LLLT stimulus, but was similar to the control group. This finding allows the conclusion that these cells are not in late differentiated stages because the number of myoblasts increased after 72 h when compared with the 48 h incubation period and mature skeletal muscle is a stable tissue characterized by multinucleated postmitotic muscle fibers. In a previous study, our group found that there were no significant differences in cell viability between laser-treated myoblasts and control cultures for the same parameters after 24 h of cell culture.

In conclusion, the data of present study showed that LLLT employed under the parameters described does not alter C2C12 cell growth under either regular or nutrient-deficient culture conditions. This occurred because cell response to low-level laser biostimulation depends on a combination of the following factors: wavelength, energy density, power density, exposure time and the type of cell subjected to the irradiation. Studies on cell cultures are important for determining the best combination of parameters for different types of cells. Further studies should be conducted in order to understand the mechanisms of laser biostimulation on skeletal muscle cells. Such studies may also determine the correct laser parameters for optimizing the biostimulation of myoblasts.
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