Cultured Epithelial Cells Response to Phototherapy With Low Intensity Laser

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Background and Objectives: Little is known about the intracellular response of epithelial cells to phototherapy. The aim of this in vitro study was to analyze the effect of phototherapy with low-energy lasers with different wavelengths and powers on cultured epithelial cell growth under different nutritional conditions.

Study Design/Materials and Methods: Epithelial cell cultures (Vero cell line) grown in nutritional deficit in culture medium supplemented with 2% fetal bovine serum (FBS) were irradiated with low-energy laser from one to three times with a GaAlAs laser (660 nm) and InGaAlP (780 nm), 40 and 70 mW, respectively, with 3 or 5 J/cm2. Cell growth was indirectly assessed by measuring the cell mitochondrial activity.

Results: Nonirradiated cell cultures grown in nutritional regular medium supplemented with 10% FBS produced higher cell growth than all cultures grown in nutritional deficit irradiated or not. The overall cell growth of cultures grown under nutritionally deficit conditions was significantly improved especially when irradiated with 780 nm for three times.

Conclusions: Phototherapy with the laser parameters tested increases epithelial cell growth rate for cells stressed by growth under nutritionally deficient states. This cell growth improvement is directly proportional to the number of irradiations; however, was not enough to reach the full cell growth potential rate of Vero epithelial cell line observed when growing under nutritional regular condition.

Key words: cell culture; epithelial cells; phototherapy

INTRODUCTION

Phototherapy with low-energy lasers has been used in medicine to treat pathological tissues conditions to reduce or prevent inflammatory processes or promote tissue healing [1]. We have been studying the effect of phototherapy on cultured cells [2–6]. Our studies showed that this therapy increases cell growth of mesenchymal cells such as fibroblasts [2–5], and osteoblasts [6]. We undertook further studies to investigate the effect of laser phototherapy on epithelial cells grown under different nutritional states using the same parameters we had employed in our previous works [2,3].

Wound healing has been reported to be accelerated as result of low intensity laser irradiation [7]. With laser exposure, an acceleration of growth of mesenchymal cells, such as fibroblasts and osteoblasts has been well demonstrated [2–6,8–10]. Besides fibroblasts activation, re-epithelization has also been noted that contributes to the wound-healing process; however, little is known about the intracellular responses of epithelial cells to phototherapy [11,12]. Moreover, oral manifestations of some infectious diseases, such as recurrent herpes simplex virus infections, where the aggression to epithelial cells is observed, the phototherapy has shown effects that included, prevention of lesion formation, acceleration of the healing of lesions, and decreasing the frequency of recurrent lesions [13]. Despite numerous examples of potential clinical benefits of phototherapy, the intracellular mechanisms responsible for these sorts of described benefits have not been totally determined. Thus, it becomes critical to analyze the response of epithelial cells in culture to phototherapy using different wavelengths and energy densities under various growth situations in an effort to identify processes responsible for observed benefits.
MATERIALS AND METHODS

Cell Culture

Vero cells (monkey kidney epithelial cell line—ATCC CRL 1587) were grown at 37°C in Minimum Eagle Medium (MEM, Cultilab, Campinas, SP, Brazil) supplemented by 10% FBS (Cultilab) and 100 U/ml of Penicillin and Streptomycin (nutritional regular culture medium). Cells were maintained in a humidified air–5% CO2 atmosphere.

Effect of Serum Concentration on Cell Growth

Effects of phototherapy on cultured cell growth are noticeably observed when the cultures are grown in nutritional deficit [2]. This in vitro situation produces stresses that are potentially similar to in vivo stress conditions where the phototherapy has shown effect. In previous studies, we have demonstrated that when the cells are grown in culture medium supplemented with reduced FBS concentrations (nutritional deficient culture media), the growth rate of the cell cultures is diminished [2,3,5,6]. To find a FBS concentration for cell culture medium supplementation that allows cell growth, however in a rate lower than that characteristic of cells grown on nutritional regular culture medium, we carried out a study where 1×10^4 cells/well were plated into four 96-well-microtitration plates (12 wells/plate). Growth in 48 wells was enough to analyze, in quadruplicate, the cell mitochondrial activity of three experimental groups (10, 5, and 2% FBS) at four different experimental times (20, 24, 48, and 72 hours after plating) and the cell mitochondrial activity analysis for these cells was used to infer the cell viability and to plot the cell growth curves.

Laser Irradiation

Laser irradiation was delivered with GaAlAs/InGaAlP lasers (MM Optics Ltd., São Carlos-SP-Brazil). Irradiations were done in contact using the punctual irradiation mode in a 3.6 mm² area. Two wavelengths were used, 660 and 780 nm with powers of 40 and 70 mW (at the display), respectively. Two energy densities settings were used; for 660 nm: 3 J/cm² (2.8 seconds) and 5 J/cm² (3.8 seconds), and for 780 nm: 3 J/cm² (1.9 seconds) and 5 J/cm² (2.5 seconds).

We set up the experiments to standardize the procedures as previously described [3,4]. Briefly, knowing that the distance between the laser source and the surface of application is critical, the laser application was done through the bottom of the optically clear 96-well-microtitration plates. Therefore, the laser beam did not transpose the culture medium being applied straight on the cell instead. Additionally, the distance between the laser beam and the cell monolayer was held constant at 1 mm. Finally, the laser irradiation was carried out in a partial darkness without other light influence than laser. The LaserCheck power meter (Coherent, Inc., Santa Clara, CA) verified the output power of the laser equipment. The control groups were treated under identical conditions except that the laser equipment was kept off.

Experimental Groups

- Control: no laser irradiation
- Group 1: 660 nm, 3 J/cm²
- Group 2: 660 nm, 5 J/cm²
- Group 3: 780 nm, 3 J/cm²
- Group 4: 780 nm, 5 J/cm²

Effect of Laser Irradiation on Cell Growth

For cell growth analysis, control (nonirradiated) and treated cultures (irradiated) were plated on 96-well-microtitration plates. The cultures were incubated in humidified air–5% CO2 atmosphere for 6 hours before the irradiation. Before irradiation, the culture medium was replaced by the nutritional deficient culture medium supplemented with 2% FBS. Then, one, two, or three irradiations were applied. The second and the third irradiations were successively done with 6-hour intervals. Samples from each group were taken for mitochondrial activity analysis 20, 24, 48, and 72 hours after the first irradiation. The cell mitochondrial activity analysis was also used to infer the cell viability and to plot the cell growth curves. All the experiments were done in triplicate.

Cell Mitochondrial Activity Analysis

Cell mitochondrial activity was analyzed using the MTT-based cytotoxicity assay. The MTT assay involves the conversion of the water soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to an insoluble formazan. The formazan is then solubilized and the concentration determined by optical density at 570 nm. A MTT reduction analysis kit (Vybrant MTT, Molecular Probes, Eugene, OR) was used. Immediately after the end of the assay procedures the absorbance was read in a micro plate reader (Biotrak II, Biochrom Ltd., Eugendorf, Austria) using a 562 nm filter. The absorbance data were used to plot the cell growth curves.

Statistical Analysis

The optical density data, corresponding to the cell viability, obtained in quadruplicate for the serum concentration determination experiment and in triplicate for the irradiation experiments, are presented as mean ± the standard error of mean. The data were compared by the Kolgomorov–Smirnoff (Lilliefors) test followed by ANOVA test. The level of significance was 5% (P < 0.05).

RESULTS

Effect of Serum Concentration on Cell Growth

The growth of Vero cells in culture medium supplemented with different FBS concentrations (2, 5, and 10%) was compared in order to determine the best serum concentration for the proposed laser experiments. The growth curves are presented in Figure 1.

Growth was observed in cultures grown in culture media supplemented with all FBS concentrations tested. The
growth of cultures treated with culture medium supplemented with 2 and 5% FBS was similar and, significantly less than that for cultures grown in nutritional regular culture medium, which contains a FBS concentration of 10%.

**Effect of Laser Irradiation on Cell Growth**

The effect of the laser irradiation was analyzed in Vero cells grown in nutritional deficient culture medium supplemented with 2% FBS. For each laser parameter tested comparative cell growth curves were plotted and are presented in Figures 2–5.

**Effect of Laser Irradiation on Cell Growth (660 nm and 3 J/cm²)**

The comparison of Vero cell growth after one, two, and three irradiations with 660 nm and 3 J/cm² and nonirradiated control cultures grown in nutritional deficient (2% FBS) and regular culture medium (10% FBS) is shown in Figure 2. The overall growth of cultures irradiated once and twice was similar to that of nonirradiated control cultures grown in nutritional deficient medium. The number of viable cells for cultures irradiated three times was higher than that of nonirradiated control cultures grown in nutritional deficient medium. This difference was statistically significant at the first experimental time (24 hours; \( P < 0.01 \)). The nonirradiated control cultures grown in nutritional regular medium produced significantly higher number of viable cells (\( P < 0.01 \)) than all other groups throughout the whole experimental time, except the group irradiated three times. At the second experimental time (24 hours) the number of viable cells for this group was similar to the control group grown in nutritional regular medium.

**Effect of Laser Irradiation on Cell Growth (660 nm and 5 J/cm²)**

The comparison of Vero cell growth after one, two, and three irradiations with 660 nm and 5 J/cm² and nonirradiated control cultures grown in nutritional deficient (2% FBS) and regular culture medium (10% FBS) is shown in Figure 3. The overall growth of cultures irradiated once and twice was similar to that of nonirradiated control cultures grown in nutritional deficient medium. The number of viable cells for cultures irradiated three times was higher than that of nonirradiated control cultures grown in nutritional deficient medium. This difference was statistically significant for the second (24 hours; \( P < 0.01 \)) and third (48 hours; \( P < 0.01 \)) experimental times. The nonirradiated control cultures grown in nutritional regular medium produced significantly higher number of viable cells (\( P < 0.01 \)) than all other groups throughout the whole experimental time, except the group irradiated three times. At the second experimental time (24 hours) the number of viable cells for this group was similar to the control group grown in nutritional regular medium.

**Effect of Laser Irradiation on Cell Growth (780 nm and 3 J/cm²)**

The comparison of Vero cell growth after one, two, and three irradiations with 780 nm and 3 J/cm² and nonirradiated control cultures grown in nutritional deficient (2% FBS) and regular culture medium (10% FBS) is shown in Figure 4. The overall growth of cultures irradiated once and twice was similar to that of nonirradiated control cultures
Fig. 2. Growth curves of Vero cells irradiated with the diode laser with the same wavelength and energy density (660 nm, 3 J/cm²) for one, two, or three times. Small caps indicate significant differences between the control (10%) and the other groups; capital letters indicate differences between three irradiations groups and the control (2%). a, significantly higher than all the other groups; b, significantly higher than the other groups except the group treated with three irradiations; A, significantly higher than the control (2%).

Fig. 3. Growth curves of Vero cells irradiated with the diode laser with the same wavelength and energy density (660 nm, 5 J/cm²) for one, two, or three times. Small caps indicate significant differences between the control (10%) and the other groups; capital letters indicate differences between three irradiations groups and the other groups; a, significantly higher than all the other groups; b, significantly higher than the other groups except the group treated with three irradiations; A, significantly higher than the control (2%).
Fig. 4. Growth curves of Vero cells irradiated with the diode laser with the same wavelength and energy density (780 nm, 3 J/cm²) for one, two, or three times. Small caps indicate significant differences between the control (10%) and the other groups; capital letters indicate differences between two and three irradiations groups and the control (2%). a, significantly higher than all the other groups; b, significantly higher than the other groups except the group treated with three irradiations; A, significantly higher than the control (2%).

Fig. 5. Growth curves of Vero cells irradiated with the diode laser with the same wavelength and energy density (780 nm, 5 J/cm²) for one, two, or three times. Small caps indicate significant differences between the control (10%) and the other groups; capital letters indicate differences between three irradiations groups and the other groups. a, significantly higher than all the other groups; b, significantly higher than the other groups except the group treated with three irradiations; A, significantly higher than the control (2%).
grown in nutritional deficient medium. The number of viable cells for cultures irradiated three times was higher than that of nonirradiated control cultures grown in nutritional deficient medium. This difference was statistically significant in the second (24 hours; $P < 0.01$) and third (48 hours; $P < 0.01$) experimental times. The nonirradiated control cultures grown in nutritional regular medium presented significantly higher numbers of viable cells ($P < 0.01$) when compared to all other groups throughout the entire experimental time, except for the group irradiated three times. At the second experimental time (24 hours) the number of viable cells for this group was similar to the control group grown in nutritional regular medium.

**Effect of Laser Irradiation on Cell Growth (780 nm and 5 J/cm²)**

The comparison of Vero cell growth after one, two, and three irradiations with 780 nm and 5 J/cm² and nonirradiated control cultures grown in nutritional deficient (2% FBS) and regular culture medium (10% FBS) is shown in Figure 5. The overall growth of cultures irradiated once and twice was similar to that of nonirradiated control cultures grown in nutritional deficient medium. The number of viable cells for cultures irradiated three times was significantly higher ($P < 0.01$) than that for nonirradiated control cultures grown in nutritional deficient medium throughout the experimental time. The nonirradiated control cultures grown in nutritional regular medium presented significant higher numbers of viable cells ($P < 0.01$) than all other groups throughout the whole experimental time, except the group irradiated three times. At the second experimental time (24 hours) the number of viable cells of this group was similar to the control group grown in nutritional regular medium.

**DISCUSSION**

Low intensity laser administration is capable of increasing epithelial cell growth rate for cells stressed by nutritionally deficient conditions. The improvement in cell growth rates is directly proportional to the number of irradiations; however, laser irradiation was not capable of inducing cell cultures to reach their potential cell growth rates for Vero epithelial cell line grown under normal nutritional conditions.

**Effect of Serum Concentration on Cell Growth**

The positive effect of low intensity laser radiation on mesenchymal cells, especially fibroblasts, has been well established [2–5]. Exposure of cells to low intensity laser appears to be capable of stimulating the growth of these types of cells. This effect is particularly evident when the cell functions are compromised [2]. In vitro cells can be compromised by growing them under nutritionally deficient conditions. These nutritional conditions must not abolish the cell growth completely but should only reduce the cell growth rate. Then, the effect on growth rates for cells grown under these conditions can be measured when cells are exposed to low intensity laser. In vitro stress can be achieved for each strain by varying FBS concentration. Some cell types are able to grow with half the FBS concentration of the nutritional regular culture medium that required for their characteristic normal growth rates [2,4–6], others require an accentuated decrease in this concentration to diminish the cell growth rate [3]. As there were no data about Vero cell growth in different FBS concentrations, three concentrations (10, 5, and 2%) were tested for in order to find the appropriate FBS concentration for the subsequent irradiation experiments. We were looking for a FBS concentration that would lead to an accentuated reduction of the Vero cell growth without totally preventing the cell growth. We found that using a culture medium supplemented with 2% FBS produced a condition where Vero cells viability was maintained but growth was significantly lower than that of the cells grown in nutritional regular culture medium supplemented with 10% FBS. Thus, this serum concentration was appropriate for the proposed experiments for exploring the effects of low intensity laser irradiation on cell growth.

**Effect of Multiple Irradiations**

The beneficial effects of phototherapy with low intensity laser depend on the number, timing and frequency of irradiations, and the type of laser used. In some studies, the desired beneficial effect are more obvious when the irradiations are repeated [3,14], while in others excessive repetitions caused undesirable effects, such as pathological changes in the newly formed muscular structures during muscle regeneration [15]. Thus it was decided to perform single irradiation as well as repeated two and three exposures.

In vivo phototherapy protocols have utilized either daily or every other day irradiation schedules. Cells in culture have a much faster cell division rate than that generally observed in vivo, and for this reason we followed an in vitro protocol previously reported in the literature [3] with 6 hours between the irradiations.

Independently of the laser parameter tested (wavelength and energy density), positive cell growth effects were observed primarily when the cells were irradiated for three times. Although, the laser irradiation settings used were not able to stimulate the cell growth enough to produce growth rates similar to those observed when the Vero cells were grown in regular nutritional culture medium, it was noted that repeated irradiations increased the cell growth rate of cells grown under nutritional deficit conditions. This would seem to support the utilization of repeated irradiations with low intensity laser when attempting to impact clinical lesions and conditions.

**Effect of the Wavelengths**

It was interesting that the improvement in Vero cell growth following phototherapy was apparently independent of the wavelength used. However, there was a trend for a more pronounced increase in growth rate when the infrared (780 nm) was used. In fact, when three irradiations were administered, the cultures irradiated with the 780 nm laser presented significantly higher cell growth rates.
compared to nonirradiated cells grown in nutritional deficit conditions.

**Effect of the Fluencies**

When cell cultures were irradiated with 660 nm laser at 3 J/cm², even after three irradiations, cell growth was similar to that of the control cultures grown in nutritional deficit conditions and was significantly lower than that of the nonirradiated groups grown in nutritional regular culture medium. It has been shown that these laser irradiation parameters are capable of stimulating cell growth of fibroblasts in culture [2]. It is possible that the energy delivered by this setting was not sufficient to stimulate Vero cell growth due to specific cell differences between Vero cells and fibroblasts. Our previous study [2] found that the doubling time of fibroblasts grown in nutritional deficit conditions during the log phase of the cell growth curve was less than 24 hours [2], whereas the doubling time of Vero cells in our study under the same growing conditions was 48 hours. This suggests that the laser energy density (or fluency) required by the Vero cells to produce increase cell growth response has to be higher, since by increasing the energy density to 5 J/cm² at the same wavelength, there was a consistent benefit for cells irradiated three times.

In conclusion, under the conditions of this study, the epithelial Vero cells showed positive responses to laser irradiation when grown under nutritionally deficient conditions. While the laser exposure parameters utilized in this study failed to allow nutritionally stressed epithelial cells to achieve growth rates achieved by cells grown under regular nutritional conditions, specific advantages were seen in multiple exposures. Many questions arose from this study, thus new researches must be done in order to understand the mechanisms of laser biostimulation. Moreover, these studies must also determine the correct laser parameters for fully biostimulate epithelial cells.

It is important to determine what intracellular events are responsible for the effects noted in this study. It has long been reported that one of the more likely targets of low intensity lasers that can result in positive effects for the cell is the mitochondria. Accordingly, the effects of laser irradiation on the mitochondria have been previously reported [4,16,17]. Laser irradiation is postulated to intensify the formation of a transmembrane electromechanical proton gradient in mitochondria [16]. Thus, the efficiency of the proton-motive force (pmf) is increased and more calcium is released into the cytoplasm from the mitochondria. At low laser doses, this additional calcium transported into the cytoplasm triggers mitosis and enhances cell proliferation. In addition to the mitogenic action of calcium release into the cytoplasm by the pmf, a short-term rise in the intracellular pH by the creation of the electromechanical proton gradient triggers mitogenic signals in the cells. Moreover, it is well known that the pmf increases ATP production, which activates Na⁺, K⁺-ATPase, and other ions carriers. Thus, the intracellular K⁺ level is increased and the Na⁺ concentration and membrane potential are decreased. These factors also influence cell proliferation [16].

This raises the question of what are the ideal stimulation conditions for mitochondria in order to produce maximal cell growth rates—do cells need to be lased more than once in order to induce a response, or more specifically do several laser exposures over short periods of time provide a distinct benefit over single exposures? What is known is that the effect of laser irradiation is immediate and, the commonly used low intensity laser wavelengths are not ionizing, but then, it would be expected that multiple exposures would have no cumulative effect in the laser irradiated cells. They respond immediately but then return to their original state. On the other hand, Manteifel et al. [17] demonstrated that the effect of laser on the mitochondria is not only physiological, but also morphological, since the presence of giant mitochondria is observed in irradiated cells. We also have previously reported [4] morphological alterations in mitochondria in irradiated cells. Future research directions will need to determine the effect of cumulative laser treatments.

It is critically important that the effects of low intensity lasers on cells be explored under controlled laboratory conditions if we are to understand how to best to utilize these devices in clinical medical situations. This study has explored how varying wavelength and energy density can effect cell growth under nutritionally deficient conditions. Once the various effects of low intensity exposure across various cell types and laser conditions can be demonstrated consistently, we will be in a better position to use low intensity lasers in clinical situations to produce consistent results.

**REFERENCES**