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Pre-odontoblast proliferation induced by near-infrared laser stimulation

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Abstract. – OBJECTIVE: Laser therapy is known to stimulate cell proliferation and differentiation, an effect called "biostimulation". Although many clinical applications of laser therapy take advantage from such positive effect, the underlying molecular mechanisms are not fully understood. The aim of this work was to investigate the effect of near-infrared laser stimulation on rat pre-odontoblast cells (MDPC-23 cells) and the molecular mechanism/s involved.

MATERIALS AND METHODS: MDPC-23 cells were stimulated with a near-infrared (980 nm) laser source with different energy settings (1-50 J, corresponding to 0.65-32.47 J/cm^2) and cell proliferation was evaluated by manual count. ERK 1/2 pathway activation was evaluated by Western blot analysis.

RESULTS: 1-10 J stimulation (corresponding to 0.65-6.5 J/cm^2) significantly increase MDPC-23 cell proliferation and such effect seems to be mediated by ERK 1/2 signalling pathway activation, showing a key role of ERK 1/2 pathway in mediating the proliferative response induced by laser stimulation.

CONCLUSIONS: Near infrared laser stimulation with low energies (1-10 J) is able to increase cell proliferation through ERK 1/2 signalling pathway activation. At the same time, higher energy stimulation (25-50 J) induces an initial toxic effect, probably activating pro-apoptotic signalling molecules, downstream ERK 1/2 kinase. Such results foster the application of this therapeutic approach in different clinical settings in which a regenerative tissue response is needed.

Key Words:
Biostimulation, MAPKs, Odontoblasts, 980 nm laser light.

Introduction

Diode lasers are characterized by a solid active medium (the semiconductor) transforming electric energy in light energy. Diode lasers used for odontoiatric purposes have a wavelength spanning between visible and infrared portion of the light spectrum and can work both in continuous or gated mode. Based on their wavelength, energy is transported to the target tissue by an optic fiber which diameter can vary between 200 µm and 600 µm. Diode laser emission and the parameters characterizing the stimulation (power, wavelength, energy) can be wisely tuned in order to obtain different clinical effects. The most important effect for odontoiatric procedures is the photothermic effect, mainly exploited for surgical purposes, along with the photochemical effect, mainly exploited to induce biomodulatory responses[1]. As energy absorption varies depending on target tissue and wavelength used, energy settings have to be carefully controlled in order to avoid adverse effects[2].

Laser therapy has been shown to modulate cellular metabolism in different cellular models, thus resulting in an enhancement of cell proliferation and differentiation, an effect currently referred as "biostimulation".

In dentistry, biostimulating effects of laser treatment are widely used to enhance healing processes along with the treatment of dentine hypersensitivity[4,8].

Being located at the dentin-pulp interface, odontoblasts represent one of the first cellular populations attained by odontoiatric laser stimulation[9]. Odontoblasts are the cell lineage responsible for dentine deposition: in mammalian teeth these cells are organized in a monolayer underlying dentine tissue, where they contribute to such mineralized tissue homeostasis by new dentine deposition in physiological and pathological conditions[8-11].

The use of laser treatment to induce cell proliferation and differentiation in different cell lines is quite common, even if the underlying molecular mechanisms are poorly understood[3-7].

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Cellular responses such as proliferation, differentiation and death undergo a careful tuning: a key role in their regulation is played by MAPKs (mitogen activated protein kinases)[12]. In particular, the timing of ERK (extra-cellular signaling regulated kinase) signaling pathway activation is critical in regulating cell mitogenesis and differentiation as its transient activation leads to cell proliferation, while persistent activation results in growth arrest and cell differentiation[13].

The biological effect of laser stimulation on a number of cell types of different embryological origin has been well studied: nevertheless, only few studies focused on odontoblastic *in vitro* models[8,14,15].

The aim of this study was to evaluate the in vitro biostimolatory ability of a near infrared (980 nm) diode laser treatment on a rat pre-odontoblast cell line (MDPC-23). In particular, the effect of increasing laser stimulation intensities (0-50 J) on cell proliferation has been studied, along with its ability to induce ERK signaling pathway activation.

Materials and Methods

Cell Culture

MDPC-23 odontoblast-like cells were a kind gift of Prof. Jacques Nör (University of Michigan Dental School, Ann Arbor, MI, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Euroclone, Milan, Italy) supplemented with 10% heat inactivated foetal bovine serum (FBS, Euroclone), 100 U/ml penicillin (Euroclone), 100 mg/l streptomycin (Euroclone) and 2 mM glutamine (Euroclone) in a humidified incubator with 5% CO_2 and 95% air at 37°C.

Laser Irradiation

Cells were irradiated using a DMT Giotto near-infrared (980 nm) laser equipment (DMT Srl, Lissone, Italy). Laser stimulation was performed in the continuous mode, with the light source positioned vertically above each well (distance between the light source and the bottom of the well = 9.7 cm). All experiments were performed in polystyrene tissue culture 24 wells plates (round wells) working at room temperature (~25°C) with no additional light sources other than environmental light. Polystyrene plates are characterized by a light transmittance of near to 90% when irradiated at 980 nm[16]. Whole well area (1.54 cm^2) coverage by the laser light has been evaluated thanks to the instrument pilot light (635 nm, maximum power output 5 mW). Laser irradiation was performed using the biostimulation frame equipped with a 600 μm optical fibre, setting the instrument's power output to 1 W. Before irradiation the medium was removed from each culture well and the culture multiwells, with the lids off, were irradiated for 0, 1, 5, 10, 25, 50 sec, corresponding to an energy stimulation of 0, 1, 5, 10, 25, 50 Joules and to an energy density (spatial average energy fluence) of 0, 0.65, 3.25, 6.50, 16.23, 32.47 J/cm^2 respectively. After laser stimulation the medium was immediately added again into each treated well and the plate was incubated again at 37°C. Laser stimulation was performed twice at 24 h intervals and 24 h after the last irradiation cells were fixed and analysed.

Cell Proliferation

MDPC-23 were seeded at an initial density of $1.5x10^4$ cells/well in 24 wells cell culture plates and allowed to adhere overnight. Non-adherent cells were removed by gentle wash in phosphate buffer (PBS, pH = 7.4). Laser stimulation was performed in the absence of cell culture medium, which was added again immediately after laser stimulation. 24 hours after the last stimulation cells were fixed overnight at 4°C in 3.7% formaldehyde/3% sucrose solution and stained with 0.1% crystal violet alcoholic solution (20% methanol). Stained cells were photographed at 10X magnification using an optical microscope (Leica ICC50HD, Weitzlar, Germany). Laser stimulation effects on cell proliferation were evaluated by manual cell counting. Each experiment was performed three times in triplicate. The counting procedure has been performed by two different researchers blinded to experimental groups to assess reproducibility of the analysis. Interindividual variation was less than or equal to 20% so counting data from both researchers were analyzed. To minimize the effect of the gaussian profile of the laser source, cell counts were performed on at least 5 randomly chosen microscopic fields for each experimental condition. Cell density was expressed as % on control ± standard error of the mean (SEM).

Western Blot

For time course experiments, $2x10^5$ cells were irradiated using the experimental settings giving the maximum proliferative response (10 J, 6.5 J/cm^2) and then incubated for different times (0 min, 5 min, 15 min, 30 min, 60 min). In order to

confirm the specificity of ERK activation following laser stimulation, in some experiments cells were laser-stimulated in presence and absence of a specific ERK inhibitor (U0126). U0126 was added to cell culture medium at a 10 μM concentration[12,17] 10 min before laser stimulation. Cells were lysed in PBS containing 0.5% Triton X-100 and protease inhibitors cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Total protein content of each sample was determined by means of BCA assay (Pierce, Rockford, IL, USA). 15 μg of proteins were separated onto a 12% SDS-PAGE gel under reducing conditions and blotted onto a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) using standard methods. Membranes were blocked in 7% milk in PBS with 0.1% Tween-20 at room temperature for 2 hour. Membranes were then incubated with primary specific anti-ERK1/2 (1:1000, Cell Signaling Technology cat #9102, Cell Signalling Inc, Beverly, MA, USA) or anti-phospho-ERK1/2 (1:5000, Cell Signaling Technology cat #9106) antibodies overnight at 4°C. Signals were revealed using the appropriate secondary peroxidase-conjugated antibodies, and the bands were visualized by chemoluminescence (Amersham Biosciences, Little Chalfont, UK). Western blot experiments were quantified by densitometric analysis using ImageJ software.

Statistical Analysis

One-way ANOVA followed by Bonferroni's post hoc tests were done for statistical analysis. Statistical procedures were performed with the Prism 4.0 statistical software (GraphPad Software Inc., CA, USA). Probability values of $p<0.05$ were considered statistically significant.

Results

Laser Effect on MDPC-23 Cells Proliferation

Laser stimulation was able to increase MDPC-23 cell proliferation only when performed at low energy, while higher energies did not affect cell proliferation. As shown in Figure 1, cells stimulated at 1, 5, 10 Joules, corresponding to 0.65, 3.25, 6.5 J/cm^2 respectively, display a statistically significant increase in cell proliferation compared to control levels. On the other hand, 25 and 50 Joules stimulation, corresponding to 16.23 and 32.47 J/cm^2 respectively, did not alter significantly cellular proliferation.

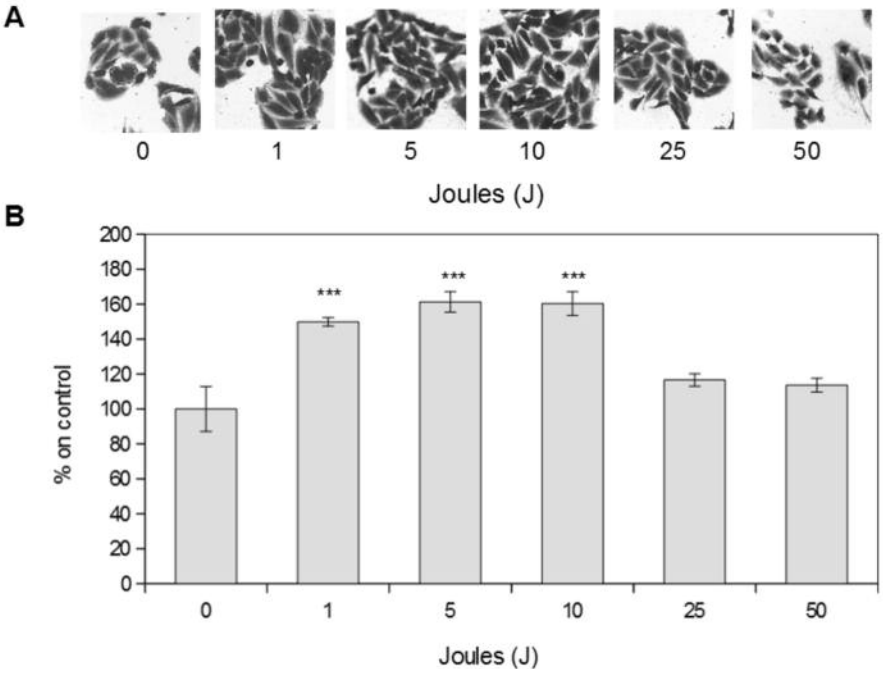

Figure 1. Laser effect on MDPC-23 cells proliferation ***A)*** representative images of MDPC-23 cells stimulated with near-infrared (980 nm) laser every 24 hours for two days. Magnification 10X. ***B)*** Cellular proliferation quantification. Results are expressed as mean values ± standard error of the mean. $^{***}p<0.01$ compared to control.

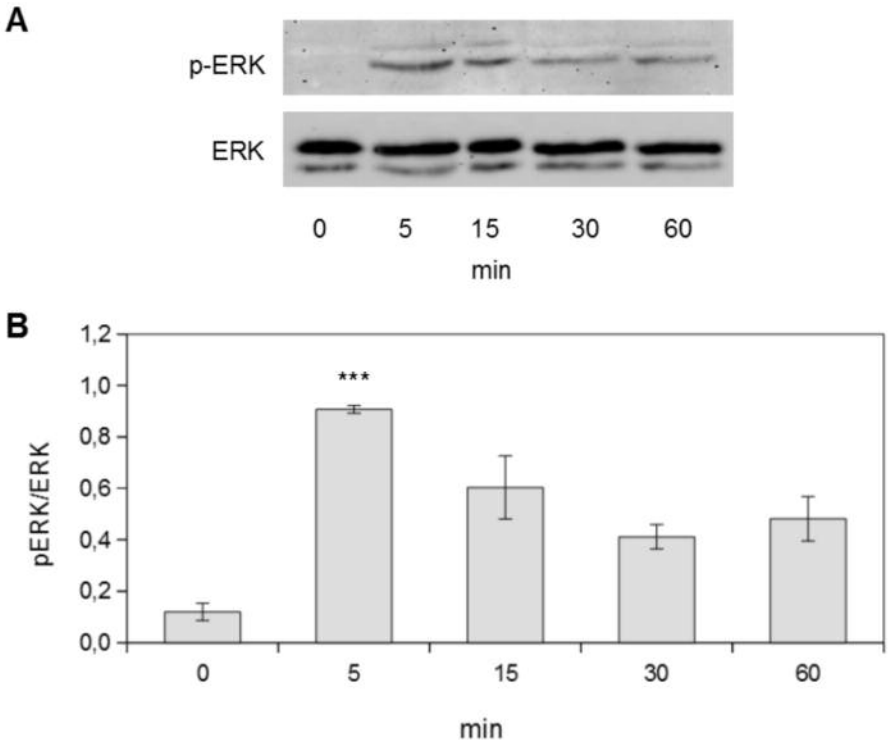

Figure 2. Laser stimulation induces ERK 1/2 signaling pathway activation ***A)*** representative Western blot images of MDPC-23 cells stimulated with 10 J energy. Cells were lysed at different time points. ***B)*** Densitometric quantification of ERK phosphorylation. Results represent the mean values obtained from three independent experiments and are expressed as mean values ± standard error of the mean. $^{***}p<0.01$ compared to control.

Laser Effect on ERK 1/2 Signaling Pathway Activation

In order to evaluate the role of ERK (extracellular signaling regulated kinase) 1/2 signaling pathway in the observed increase in cell proliferation, time course experiments on MDPC-23 cells stimulated with the energy (10 J, 6.5 J/cm^2) giving the maximum proliferation response were carried out. As shown in Figure 2, 10 J (6.5 J/cm^2) laser stimulation resulted in a transient increase in ERK 1/2 activation. In particular, this signaling pathway activation showed a maximum response 5 minutes after laser stimulation, as highlighted by phospho-ERK/ERK ratio.

In order to confirm the direct correlation between laser stimulation and ERK signaling pathway activation, some experiments were performed in presence of a U0126, a highly selective ERK inhibitor. As shown in Figure 3, U0126 pre-treatment completely abrogated laser induced ERK signaling activation.

Once identified the time point corresponding to the maximum ERK activation, the effect on ERK phosphorylation of the whole stimulation settings used in the study has been studied. As shown in Figure 4, cell proliferation increase at 5 and 10 J (3.25 and 6.5 J/cm^2) corresponds to an increase in ERK phosphorylation. The increase in cell proliferation after 1 J (0.65 J/cm^2) stimulation observed at the end of the experiment does not correspond to an increase in ERK activation, probably because the time point evaluated is too short for such experimental condition. When considering 25 and 50 J stimulation cell proliferation decreases: also ERK phosphorylation decreases compared to the peak level, without disappearing completely. It is conceivable that such residual ERK activity results in the activation of pro-apoptotic, rather than proliferative downstream signals.

Discussion

Laser therapy has been used as an alternative, noninvasive method to stimulate wound healing for the last 30 years and is also widely applied in different branches of regenerative medicine and

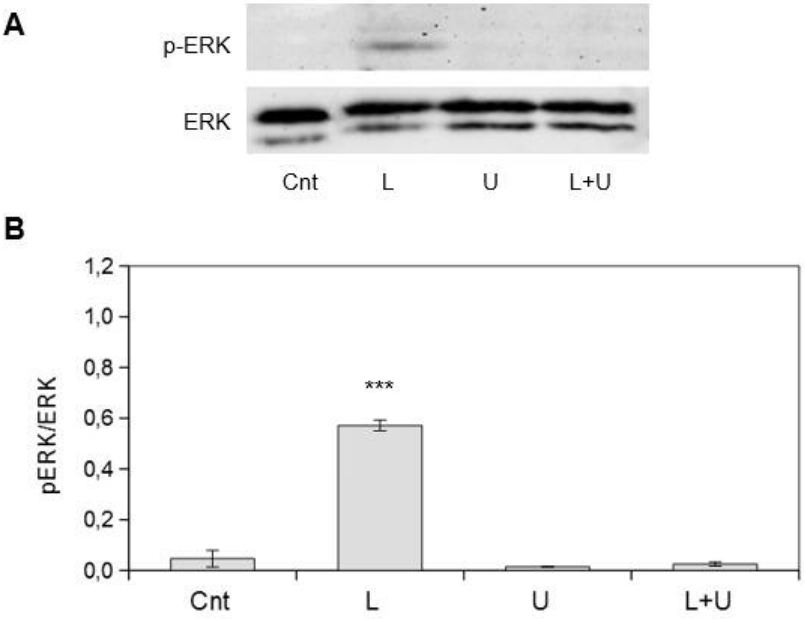

Figure 3. ERK 1/2 pathway inhibition completely abrogates laser-induced ERK 1/2 signaling activation ***A)*** Representative western blot images of MDPC-23 cells stimulated with 10 J energy in presence and absence of U0126 (10 μM). Cells were lysed 5 minutes after laser stimulation. ***B)*** Densitometric quantification of ERK phosphorylation. Results represent the mean values obtained from three independent experiments and are expressed as mean values ± standard error of the mean. $^{***}p<0.01$ compared to control. In both figures: cnt = control (not stimulated); L = 10 J laser stimulation; U = 10 μM U126; L+U = 10 J laser stimulation after 10 μM U0126 pre-treatment (10 minutes).

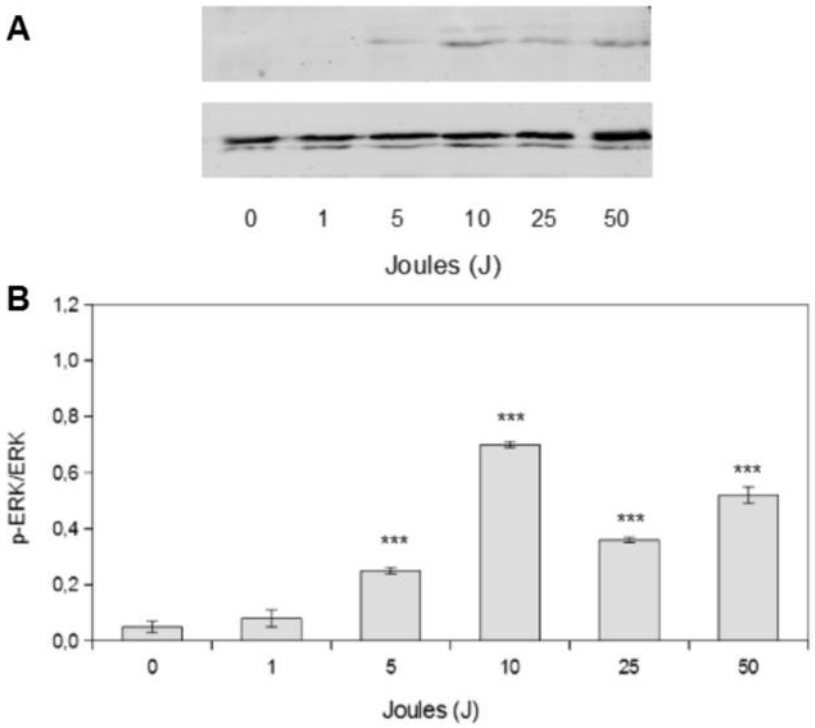

Figure 4. Laser effect on ERK 1/2 signaling pathway activation ***A)*** Representative Western blot images of MDPC-23 cells stimulated with 1-50 J energy. Cells were lysed 5 minutes after laser stimulation. ***B)*** Densitometric quantification of ERK phosphorylation. Results represent the mean values obtained from three independent experiments and are expressed as mean values ± standard error of the mean. $^{***}p<0.01$ compared to control.

dentistry, thanks to its beneficial effects on a variety of pathological conditions including pain relief and inflammation[4,6].

Even if it is widely used for photodynamic therapy, to reduce inflammation and to stimulate cell differentiation and tissue repair, its use is still controversial[6,14], as the mechanisms underlying its beneficial effects are poorly understood. Furthermore, the wide array of possible effects of laser stimulation observed in different cell lines by different research groups could be explained by differences in the irradiation parameters used, as the biological effects of laser irradiation depend on the properties of the light source (i.e. wavelength, output power and energy density).

As cell proliferation is a very important physiological response obtained in clinical practice by laser stimulation, such biological effect has been studied in vitro using different cellular models[5]. Laser irradiation has been shown to be able to increase cell proliferation, among the number of cell lines tested, in fibroblasts, keratinocytes, osteoblasts, mesenchymal and cardiac stem cells, endothelial cells[3-5]. Moreover, it is known that such effects of photodynamic therapy result in reduction of inflammation and tissue repair[15].

In the present study we focused our attention on a pre-odontoblast cell line (MDPC-23 cells): such cells have been chosen as they are the main responsible of the maintenance of tooth integrity by producing new dentin[9-11].

All the experiments were performed using a near infra-red (980 nm) laser equipment with energy output set to 1W in order to obtain a direct correlation between energy (Joules) and stimulation time (seconds). Laser equipment is provided with a pilot red light (635 nm) with a power output of 5 mW. Considering the experimental setting adopted for cell treatment, parameters referred to pilot light did not affect the observed cellular responses (data not shown). Experimental data presented herein show that laser stimulation with energy settings ranging from 1 to 10 J (0.65-6.5 J/cm^2) are able to stimulate MDPC-23 cell proliferation, while higher energies do not have a statistically significant effect on such phenomenon, probably because of an initial toxic response, as previously observed by Migliario et al in a pre-osteoblast cell line[3].

Once proved the ability of laser stimulation to increase cell proliferation, attention has been focused on the underlying molecular mechanism involved, as this item is currently poorly understood. In particular the role of MAPKs (mitogen activated protein kinases) has been investigated. Attention has been focused on MAPKs as this intracellular signaling pathway is known to control many aspects of mammalian cell physiology (i.e. cell proliferation, differentiation and death), acting by phosphorylating downstream transcription factors[12]. Among the components of MAPK family, ERK (extra-cellular signaling regulated kinase) signaling pathway is critical in regulating cell mitogenesis and differentiation: more in detail, transient activation of such pathway leads to cell proliferation, while persistent activation results in growth arrest and cell differentiation[13].

In the present study we demonstrated that the observed increase in cell proliferation after 5 and 10 J (3.25 and 6.5 J/cm^2) stimulation correlated with ERK activation, a result in line with data available from the literature[18]. In our experimental model 1 J (0.65 J/cm^2) laser stimulation was able to stimulate cell proliferation at the end of the experiment but this increase in cell proliferation did not reflect a ERK pathway activation: this is probably due to the timing adopted for ERK activation evaluation that could be too short for such stimulation parameter. Furthermore, our data show that laser stimulation with higher energies (25 and 50 J, corresponding to 16.23 and 32.47 J/cm^2 respectively) does not mediate a statistically significant increase in cell proliferation at the end of the experiment while determining ERK pathway activation. It is conceivable that in such experimental conditions ERK activation finally results in pro-apoptotic rather than prolifertive signaling pathway activation, with laser stimulation starting to cause toxic effects, maybe due to thermal damage or increased production of reactive oxygen species, as previously described for other cell lines[3].

Conclusions

Even if more detailed studies will be necessary to investigate laser stimulation ability to positively influence new dentin deposition, results discussed herein show that near infrared (980 nm) irradiation with energy settings ranging from 1 to 10 J (0.65-6.5 J/cm^2) positively influences odontoblasts proliferation, suggesting useful applications of this therapeutic approach in different clinical settings in which a regenerative tissue response is needed.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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