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Effect of low-level GaAlAs laser irradiation on the proliferation rate of human periodontal ligament fibroblasts: an in vitro study

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Abstract

Aim: The aim of this in vitro study was to evaluate a potential stimulatory effect of low-level laser irradiation on the proliferation of human periodontal ligament fibroblasts (PDLF).

Materials and Methods: PDLF obtained from third molar periodontal ligaments were cultured under standard conditions and spread on 96-well tissue culture plates. Subconfluent monolayers were irradiated with an 809-nm diode laser operated at a power output of 10 mW in the continuos wave (cw) mode at energy fluences of 1.96–7.84 Jcm^{-2}. The variable irradiation parameters were the time of exposure (75–300 s per well) and the number of irradiations (1–3). After laser treatment, the cultures were incubated for 24 h. The proliferation rate of the lased and control cultures was determined by means of fluorescence activity of a reduction–oxidation (REDOX) indicator (Alamar Blue® Assay) added to the cell culture. Proliferation, expressed in relative fluorescence units (RFU), was determined 24, 48 and 72 h after irradiation.

Results: The irradiated cells revealed a considerably higher proliferation activity than the controls. The differences were significant up to 72 h after irradiation (Mann–Whitney *U*-test, $p < 0.05$).

Conclusion: A cellular effect of the soft laser application is clearly discernible. Clinical studies are needed to evaluate whether the application of low-level laser therapy might be beneficial in regenerative periodontal therapy.

Key words: diode laser; low-level laser therapy; periodontal ligament fibroblasts; proliferation

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Low-level laser therapy (LLLT) has become popular in a variety of clinical applications (Basford 1995, Conlan et al. 1996, Walsh 1997a, b). Unlike dental hard lasers, soft lasers emit light of very low energy density and presumably promote certain biochemical reactions without inducing thermal effects. Two laser types are used in the field of LLLT: helium neon (HeNe) lasers with a wavelength of 633 nm and semiconductor lasers emitting light in the range of 780–950 nm. Low-energy laser light is supposed to reduce pain, to accelerate wound healing and to have a positive effect on inflammatory processes. The mechanism of action of soft laser irradiation, however, is not fully understood. Considerable basic research has been undertaken in the 1970s and 1980s. Critical analysis of published investigations, however, showed that especially early studies by eastern European research groups contained methodical errors and were hardly reproducible (Tunér and Hode 1998, Visser et al. 1990). Experiments with cultures of connective tissue cells showed that low-power irradiation can enhance DNA synthesis (Loevschall & Arenholt-Bindslev 1994), collagen (Balboni et al. 1986) and procollagen (Abergel et al. 1987, Skinner et al. 1996) production, and increase proliferation rate (Bednarska et al. 1998, Webb et al. 1998) and cell migration (Noble et al. 1992). These experiments were mainly carried out with skin and embryonal fibroblasts. In contrast, only few data on laser effects on oral fibroblasts and in particular on periodontal ligament fibroblasts (PDLF) are available. Being responsible for collagen production, PDLF have a key function in periodontal regeneration. Stimulatory effects on the proliferation of these cells could therefore be beneficial for the reestablishment of connective tissue attachment. The aim of the present study was to investigate the effect of low-level 809 nm diode laser irradiation on the proliferation rate of PDLF.

Materials and Methods

Cell cultures

Periodontal ligament fibroblasts were derived from individuals (both male and female) undergoing lower third molar extractions. Informed consent was obtained from the patients prior to surgery. Explant cultures were started and maintained under standard conditions. The roots were cut into pieces and placed on a Petri dish with 2 mL of Eagle basal medium (BME) supplemented with penicillin G (100 U/mL), streptomycin (100 µg/mL), L-glutamin (1.16 g/L) and 10% fetal bovine serum. Incubation was at 37°C in an atmosphere of 5% CO_2/95% air, in 100% humidity. Medium was changed every 5–6 days. When emigrating fibroblast-like cells became confluent around most the tissue fragments, medium was removed and the cell layer washed with phosphate-buffered saline, then 0.25% trypsin in EDTA buffer was added, and incubation continued for 2–4 min. The cells detached by this procedure were stored in liquid nitrogen for use in our experiment. For the experiments, fibroblasts between the 3rd and the 8th passages were used. A solution with a concentration of 1.5×10^4 cell per mL was prepared. 0.2 mL of the cell solution was added to each well of 96-well tissue culture plates. Cell cultures in their logarithmic proliferative phase were irradiated at different energy fluences (see below).

Laser irradiation

The laser device used in this study was the GaAlAs-diode laser oralaser voxx (Oralia GmbH, 78467 Konstanz, Germany) with a wavelength of 809 nm. The light was delivered by a 600-µm optical fibre. Power output was kept constant at 10 mW in the continuous wave (cw) mode. The fiber was positioned at a distance of 9 mm from the monolayer. With an emission angle of 24° at the end of the optical fibre, an equal exposure of the entire well was ensured. A conical application tip sheltered the adjacent wells from scattered laser light. Prior to lasing the average energy at the end of the fibre was determined by means of an energy meter (Field Master GS, Coherent, 64807 Dieburg, Germany). Irradiation durations were 75, 150 and 300 s. As a function of the irradiation time, energy fluence was 1.96, 3.92 and 7.84 J/cm^2, respectively. In order to investigate a possible effect of successive laser treatments (10 mW, 150 s) irradiation was carried out twice and three times at 24-h intervals prior to staining the cell culture. Control cultures were treated equally except for laser irradiation.

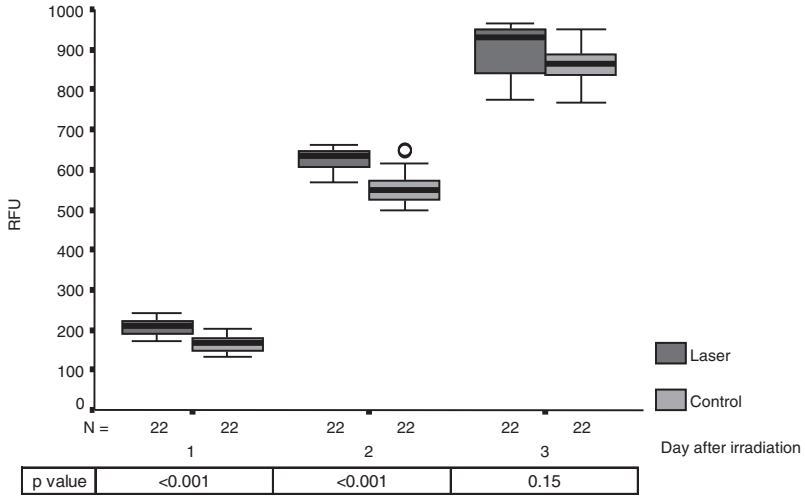

Fig. 1. Proliferation activity expressed in relative fluorescence units (RFU) after a single irradiation at 1.96 J/cm^2. The irradiated cells show a higher proliferation rate up to three days after laser treatment.

Proliferation assay

Proliferation activity was determined by means of the Alamar Blue Assay (Alamar, Sacramento, CA, USA), a simple, non-radioactive assay, used to monitor and determine the proliferation of various cell lines (Ahmed et al. 1994, Breinholt & Larsen 1998, Gloeckner et al. 2001, Kipshidze et al. 2001, Novak et al. 1993, Voytik-Harbin et al. 1998). The assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. After being taken up by the cells, the reduction–oxidation (REDOX) indicator fluoresces and changes colour in response to chemical reduction. Reduction related to cellular growth causes the REDOX indicator to change from oxidized (non-fluorescent, blue) to reduced (fluorescent, red) form. The reduction–oxidation potential of the system Alamar $Blue_{ox}$ + $2H^+ + 2e^- \rightleftarrows$ Alamar $Blue_{red}$ is +380 mV (pH 7; 25°C) and lies inbetween the potential of molecular oxygen ($O_2 + 4H^+ + 4e^- \rightleftarrows 2H_2O$; +820 mV) and the cytochromes ($Cytochromes_{ox} + 1e^- \rightleftarrows Cytochromes_{red}$; +290 to +80 mV). The indicator may substitute for molecular oxygen for any of the oxidoreductases which routinely utilize molecular oxygen as an electron acceptor.

After laser irradiation 0.02 mL of the indicator was added to the each well and incubated at 37°C. The proliferation rate was determined 24, 48 and 72 h after irradiation. Data were collected using fluorescence-based instrumentation (Fluoroscan Ascent FL, Labsystems, Helsinki, Finnland). Fluorescence was monitored at 530 nm excitation wavelength and 590 nm emission wavelength, and expressed as relative fluorescence units (RFU).

Statistical methods

A total of 220 cultures were investigated. At each irradiation regimen 22 cultures were laser treated and 22 served as controls. The box-plot design was used to present the data. Differences between the laser and the control groups were evaluated by means of the Mann–Whitney *U*-test and differences were considered to be significant when $p < 0.05$.

Results

Figures 1–3 show the proliferation activity of the cell cultures expressed in relative fluorescence units (RFU). The measured metabolic activity increased with the duration of incubation. For all irradiation regimens, the lased cell cultures showed a higher activity than the controls. The differences were highly significant (Mann–Whitney *U*-test, $p < 0.001$) on days 1 and 2 after irradiation and decreased slightly on day 3. After 72 h of incubation, cell

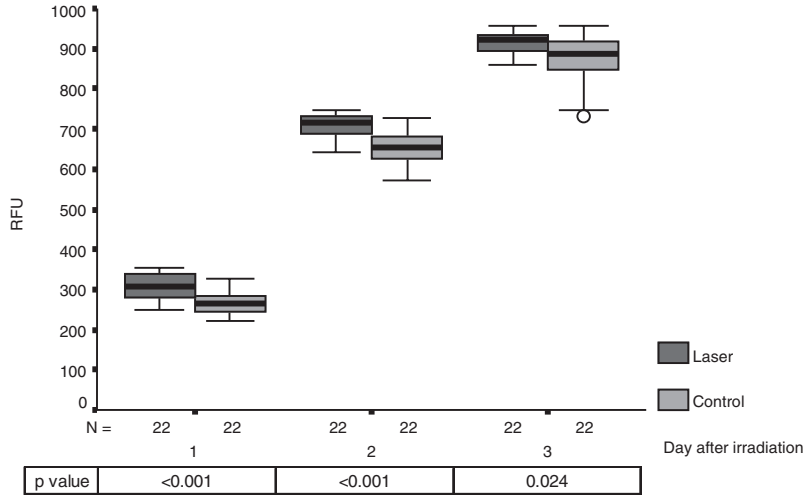

Fig. 2. Proliferation activity after a single irradiation at 3.92 J/cm^2.

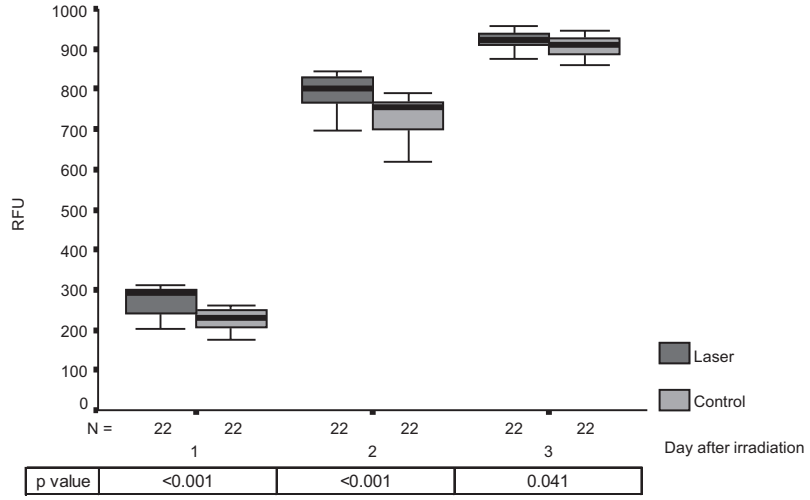

Fig. 3. Proliferation activity after a single irradiation at 7.84 J/cm^2.

activity reached a peak of approximately 900 RFU, irrespective of the incubation time and irradiation regimen. Energy fluence in the range of 1.96–7.84 J/cm^2 was revealed to have similar effects on the cells. When laser treatment was repeated 24 and 48 h after the first irradiation, the RFU values at days 1 and 2 were on a considerably higher level (Figs 4 and 5). This, however, should not be attributed to the repeated treatments, but rather to the fact that the cultures were incubated for a longer time prior to the first measurement.

Discussion

The application of low-level lasers in medicine was introduced in the 1970s and 1980s. Since then considerable scientific work including the use of cell cultures, animal models and clinical studies has been undertaken to evaluate its potentially beneficial effects. The application of low–level laser therapy (LLLT) has become popular in a variety of clinical applications, including promotion of wound healing and reduction of pain. The latest investigations, in particular animal studies (Allendorf et al. 1997, Breverman et al. 1989, Dyson & Young 1986, Hall et al. 1994, Lyons et al. 1987, Neiburger 1999, Walker et al. 2000, Wanderer et al. 1994) and well-controlled clinical studies in the field of dentistry (Carillo et al. 1990, Fernando et al. 1993, Gerschman et al. 1994, Khullar et al. 1996, Lim et al. 1995, Masse et al. 1993, Schindl & Neumann 1999), however, were often contradictory. Difficulties in measuring operating variables related to pain and tissue repair in an animal model or in a clinical setting emphasize the need for basic cellular research on laser biology.

The exact mechanism of laser–cell interaction is still to be investigated. The stimulation of photoreceptors in the mitochondrial respiratory chain, changes in cellular ATP levels and cell membrane stabilization have been discussed (Conlan et al. 1996). It is generally accepted that laser effects on cells are wavelength- and dose-dependent. The existence of a 'window-specificity' at certain wavelengths and energy dosages has been postulated (Karu 1990). Molecular absorption of laser light is a prerequisite for any cellular effect. The absorption spectrum in fibroblast monolayers was investigated and it was shown that the cytochrome oxidases in particular are expected to absorb in the 800–830 nm range (Lubart et al. 1992, van Breugel and Bär 1992).

The responsiveness of PDLF to low-level laser energy in vitro has been demonstrated by Shimizu et al. (1995). 830–nm GaAlAs laser irradiation of stretched fibroblasts significantly inhibited the production of PGE_2 and IL-1β, an effect that might be responsible for the LLLT-related pain reduction during orthodontic treatment (Lim et al. 1995). Ozawa et al. (1997) described a laser-induced inhibition of the plasminogen activator (PA) plasmin proteolytic system, concluding that laser irradiation may reduce collagen breakdown around the PDLF associated with traumatic occlusion. Surprisingly, data on the effect of low-power laser irradiation on the proliferation rate of PDLF cell cultures are not yet available.

Potential low-level laser effects on the proliferation of connective tissue cells have been described using staining methods in conjunction with photospectrometric analysis (Agaiby et al. 2000, Bednarska et al. 1998, Fedoseyeva et al. 1988, Webb et al. 1998) and radioactive assays based on the incorporation of [^{3}H]thymidine or [^{3}H]proline (Abergel et al. 1987, Balboni et al. 1986). In the present study cell proliferation was determined by means of a simple one-step, non-radioactive assay containing a REDOX indicator. When cellular growth induces the chemical reduction of media, the indicator changes its colour. Proliferation can be assessed using a plate reader. Previous

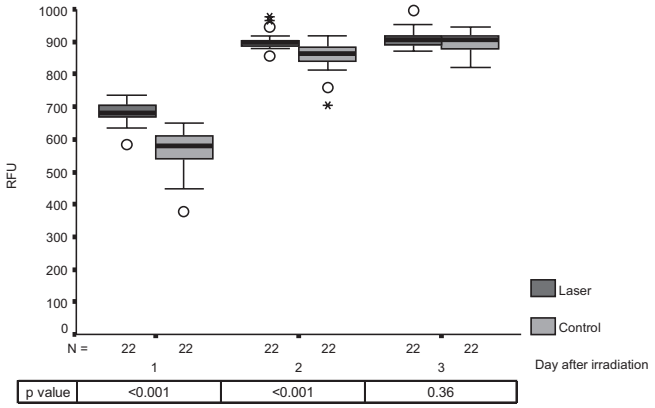

Fig. 4. Proliferation activity after two laser treatments at 3.92 J/cm^2 repeated at a 24-h interval. Measured fluorescence activity on day 2 was at a considerably higher level compared with single irradiation.

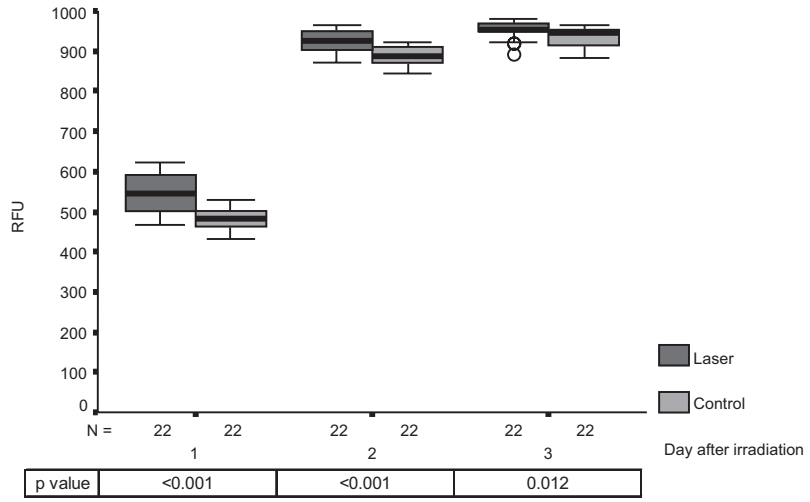

Fig. 5. Proliferation activity after three laser treatments at 3.92 J/cm^2 repeated at 24-h intervals. The irradiated cells show a higher proliferation rate up to 3 days after laser treatment. Measured fluorescence activity on days 1 and 2 was on a considerably higher level compared with single irradiation.

works indicated that that cell proliferation assessed by Alamar Blue closely correlated with that of [^{3}H]thymidine incorporation (Ahmed et al. 1994, Voytik-Harbin et al. 1998). The assay used in our experiment features several advantages over a radioactive assay: it is non-hazardous, simple, less costly, non-labour intensive and rapid in the assessment of large number of samples. It has been used to quantify 633-nm HeNe-laser-stimulated endothelial cell proliferation (Kipshidze et al. 2001).

The results obtained in the present study revealed that 809-nm low-level laser light has a stimulatory effect on the proliferation of periodontal ligament fibroblasts. This finding might be of clinical relevance in the treatment of periodontitis. The attachment of progenitor cells on root surfaces is a prerequisite for the formation of new collagen bundles (Boyko et al. 1980, Fardal et al. 1986). The formation of new connective tissue can therefore reduce probing depths. An increased proliferation rate of PDLF may not be related to increased collagen production, although their potential for enhanced collagen production collectively may be reasonably expected to increase.

In a clinical application, laser light would have to be supplied by an optical fibre inserted into the pocket. Thus laser light would not have to pass overlaying tissue where it can be reflected, scattered or absorbed, but could be applied directly in the periodontal space.

Cell proliferation as a result of stimulation by low-level laser irradiation might be associated with the autocrine production of growth factors. Yu et al. (1993) found increased concentrations of the basic fibroblast growth factor (bFGF) in the supernatant of lased fibroblast cultures. The elevated levels of this polypeptide, however, were induced by 660–nm laser irradiation and it is not clear if the effects can be observed subsequent to 809-nm laser treatment. Further investigations are needed to elucidate the molecular mechanisms leading to an increased proliferation of periodontal ligament fibroblasts induced by laser light of this wavelength. We observed that irradiation of the cell cultures led to highly significant differences between the laser and the control group up to 48 h after laser treatment. Seventy-two hours after irradiation the differences, although still significant, decreased at all irradiation regimens. It is not clear, however, if this was due to a gradually vanishing laser effect or to a saturation point after a 3-day incubation period. A similar effect was observed in an in vitro study investigating the attachment of PDLF on root surfaces (Kreisler et al. 2001). Moreover, it is not fully clear if unknown laser-induced metabolic processes contributed to the differences between the irradiated and the control cell cultures.

Previous studies indicated that laser irradiation at energy densities up to 4 J/cm^2 had stimulating effects whereas higher energy fluences had rather inhibitory characteristics (Walsh 1997a). This phenomenon did not occur in our investigation. Even at an energy dose of 8 J/cm^2 the stimulating effect was comparable to that at 2 and 4 Jcm^{-2}.

A cellular effect of the soft laser application is clearly discernible and the results obtained in this investigation seem interesting. Nonetheless, further laboratory research work is required to understand the mechanisms leading to the stimulation of periodontal fibroblasts. Moreover, clinical studies are needed to evaluate whether the application of low–level laser therapy might be beneficial in regenerative periodontal therapy.

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Zussamenfassung

Auswirkungen von Niedrigwellen-Laserbestrahlung (GaAIA) auf die Teilungsrate menschlicher periodontaler Ligament-Fibroblaste: eine Invitro-Studie

Das Ziel dieser Invitro-Studie war es, einen potentiellen stimulativen Effekt von Niedrigwellen-Laserbestrahlung auf die Wucherung menschlicher periodontaler Ligament Fibroblaste (PDFL) auszuwerten.

PDFL, das von dritten molaren periodontalen Ligamenten stammte, wurden unter standardisierten Bedingungen kultiviert und auf 96 Petrischalen verteilt.

Untere zusammenfliessende Einzelschichten wurden mit einem 809 nm Diodenlaser bestrahlt, der mit einem Energieausstoss von 10mW (im CW-Modus) und einem Energiefluss von 1,96 bis 7,84 Jcm^{-2} betrieben wurde.

Die variablen Bestrahlungsparameter waren zum einen die Zeit die die kultivierten Gewebe der Strahlung ausgesetzt waren (75 to 300 s pro Kultur), und zum anderen die Anzahl der Bestrahlungsvorgänge (1-3).

Nach der Laser-Behandlung wurden die Kulturen für 24 Stunden im Brutschrank gelagert. Die Teilungsrate der bestrahlten sowie der Kontrollkulturen wurde durch das Zusetzen eines Redox-Indikators (Alamar Blue® Assay) zu den Kulturen bestimmt; dieser löst fluoriszierende Aktivitäten in den Kulturen aus. Die Teilungsrate, ausgedrückt in relativen fluoriszierenden Einheiten (RFU), wurde 24, 48 und 72 Stunden nach der Bestrahlung bestimmt.

Die bestrahlten Zellen offenbarten eine wesentlich höhere Teilungsaktivität als die Kontrollkulturen. Die Unterschiede waren erheblich im Zeitraum bis zu 72 Stunden nach Bestrahlung (Mann Whitney-U-Test, $p < 0.05$).

Eine Zellauswirkung durch die Weichlaser-Anwendung is deutlich festzustellen. Klinische Studien werden nötig sein, um auszuwerten, ob die Anwendung von Niedrigwellen-Lasertherapie bei der regenerativen periodontalen Behandlungstherapie von Nutzen sein kann.

Résumé

Effet de l'irradiation par laser GaAIAs basse énergie sur le taux de prolifération des fibroblastes de ligament parodontal humain : étude in vitro

Le but de cette étude in vitro était d'évaluer l'effet stimulant potentiel de l'irradiation par laser basse énergie sur la prolifération des fibroblastes de ligament parodontal humain.

Les fibroblastes de ligament parodontal obtenus à partir de ligaments parodontaux de troisième molaire furent cultivés dans des conditions normales et furent étalés sur des plaques de culture tissulaire 96 puits. Les couches monocellulaires sous-confluentes furent irradiées à l'aide d'un laser à diode 809 nm opéré à une puissance utile de 10 mW en continu à des fluences énergétiques allant de 1,96 à 7,84 Jcm^{-2}. Les paramètres d'irradiation variables étaient la durée d'exposition (75 à 300 s par puits) et le nombre d'irradiations (1-3). Après le traitement par laser, les cultures furent incubées durant 24 heures. Le taux de prolifération des cultures témoins et celui de celles passées au laser fut déterminé grâce à l'utilisation d'une activité de fluorescence d'un indicateur redox (Analyse Alamar Blue®) ajouté à la culture cellulaire. La prolifération, exprimée en unités relatives de fluorescence fut déterminée 24, 48 et 72 heures après l'irradiation.

Les cellules irradiées révélèrent une activité de prolifération considérablement supérieure à celle des cultures témoins. Les différences étaient toujours importantes 72 heures après l'irradiation (Test U Mann-Whitney, $p < 0{,}05$). Un effet cellulaire de l'application du laser basse énergie, ou 'soft-laser', était clairement visible. Des études cliniques sont requises pour déterminer si l'application d'un traitement par laser basse énergie peut s'avérer bénéfique dans le cadre d'un traitement de régénération parodontale.

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