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Effects of laser irradiation on the release of basic fibroblast growth factor (bFGF), insulin like growth factor-1 (IGF-1), and receptor of IGF-1 (IGFBP3) from gingival fibroblasts

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Abstract Various studies have shown biostimulation effects of laser irradiation by producing metabolic changes within the cells. Little is known about the biological effect of laser irradiation on the oral tissues. Among the many physiological effects, it is important to recognize that low-level laser therapy (LLLT) may affect release of growth factors from fibroblasts. Therefore, the aim of the present study was to determine whether the laser irradiation can enhance the release of basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), and receptor of IGF-1 (IGFBP3) from human gingival fibroblasts (HGF). The number of all samples in the study were 30, and the samples were randomly divided into three equal groups; In the first group (single dose group), HGF were irradiated with laser energy of 685 nm, for 140 s, 2 J/cm^2 for one time, and in the second group, energy at the same dose was applied for two consecutive days (double dose group). The third group served as nonirradiated control group. Proliferation, viability, and bFGF, IGF-1, IGFBP3 analysis of control and irradiated cultures were compared with each other. Both of the irradiated groups revealed higher proliferation and viability in comparison to the control group. Comparison of the single-dose group with the control group revealed statistically significant increases in bFGF ($p<0.01$) and IGF-1 ($p<0.01$), but IGFBP3 increased insignificantly ($p>0.05$). When the double dose group was compared with the control group, significant increases were determined in all of the parameters ($p<0.01$). In the comparison of the differences between the two irradiated groups (one dose and two doses), none of the parameters displayed any statistically significant difference ($p>0.05$). In both of the laser groups, LLLT increased the cell proliferation and cell viability. The results of this study showed that LLLT increased the proliferation of HGF cells and release of bFGF, IGF-1, and IGFBP3 from these cells. LLLT may play an important role in periodontal wound healing and regeneration by enhancing the production of the growth factors.

Keywords Low-level laser therapy · Diode laser · Biostimulation · Insulin-like growth factor-1 · Receptor of Igf-1 · Basic fibroblast growth factor · Fibroblast · Cell culture

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Introduction

Low-level laser has been introduced in 1970s and 1980s, and since then, numerous scientific studies have been carried out to evaluate its effects on various cells including fibroblast, osteoblast, and endothelial cell. Laser enhanced biostimulation has been established to induce intracellular metabolic changes, resulting in faster cell division, rapid matrix production, and cell movement [1]. The reported effects of low-level laser therapy (LLLT) included acceleration of wound healing [2–4], enhanced remodeling and repair of bone [5–7], pain suppression [8, 9], anti-inflammatory functions [10, 11], stimulation of endorphin release and modulation of immune system [12, 13], a faster regeneration of severed nerves [14], and an increase in the formation of new capillaries through the release of growth factors [15].

The growth factors play a role in the control of collagen breakdown, recruitment and formation of new fibroblasts, formation of new collagen and other matrix components, and the formation of new blood vessels. The release of growth factors from both injured cells and inflammatory cells is therefore a critical part of the repair process [16, 17].

The basic fibroblast growth factor (bFGF) has been detected in macrophages and after its release from damaged cells; it could play a crucial role in wound healing processes. bFGF stimulates the proliferation of all cell types involved in the wound healing process both in vitro and in vivo. These include fibroblasts, capillary endothelial cells, vascular smooth muscles, and other cell types such as chondrocytes and myoblasts that are involved in the wound healing of specialized tissues [18–22]. Periodontal regeneration presumably involves several cell types: fibroblast for soft connective tissues, cementoblasts for cementogenesis, osteoblasts for bone, and endothelial cells for angiogenesis.

Insulin-like growth factor (IGF), among other growth factors, may enhance periodontal regeneration by stimulating formation of mesenchymal tissues including soft tissue collagen, bone, and cementum [23]. IGF is chemotactic to fibroblasts and its binding to a membrane bound receptor stimulates the tyrosine kinase receptor, thus, generating a signal which results in a cellular response [24, 25]; for example, periodontal ligament fibroblasts have high affinity receptor for IGF on the cell membrane [26]. The IGFs bind to specific receptors. High-affinity IGF-binding proteins (IGFBPs) function as carrier proteins in various biological fluids, mediate the transport of IGFs from the vascular space, and extend their half-lives [27].

Among the many physiological effects of low level laser irradiation, it is important to recognize that LLLT may affect cells which secrete various growth factors for fibroblast. However, little is known about the biological mechanism of the biostimulation of oral tissues with laser irradiation. Therefore, the aim of this study was to elucidate the molecular mechanisms leading to an increased proliferation of gingival fibroblasts. We also intended to determine whether the effects of laser on gingival fibroblast cells depend on dose. In our study, we investigated effects of LLLT irradiation on the secretion of insulin-like growth factor-1 (IGF-1), receptor of IGF-1, and basic fibroblast growth factor (bFGF) from gingival fibroblast cells.

Materials and methods

Human gingival fibroblasts separation and cell culture

Human gingival fibroblasts (hGF) were obtained from a human gingival connective tissue explant of healthy gingival tissues removed from volunteer adolescent patients whose first premolar teeth were being extracted for orthodontic treatment, and the procedure described by Somerman et al. [28] was performed. Gingival tissue was removed with a surgical scalpel, and the tissue was placed in Hanks Balanced Salt Solution (HBSS) containing 100 U/ml penicillin and 100 μg/ml streptomycin. Biopsy specimens were minced into 2 to 3 mm cubes and transferred to 35 mm tissue culture dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin G, 100 μg/ml of streptomycin, 2 mmol/l L-glutamine, and 10% fetal calf serum (fcs) (Biological Industries, Israel). The cultures were incubated at 37°C in a humidified atmosphere in the presence of 5% CO_2. When the cells that had grown out from the explants reached 80 to 90% confluence, they were detached by 5 min exposure to 0.05% trypsin containing 0.02% ethylenediaminetetraacetic acid (EDTA) then subcultured in culture plates.

Laser irradiation

Portable low-level laser therapy unit wide choice of probes are: red probes of 685 nm wavelength and 30–50 mw output power, infrared probes of 830 nm wavelength and 50–400 mw output power.

Laser irradiation was carried out with a diode laser (BTL-2000 Benešov Made in Czech Republic) which had a continuous wavelength of 685 nm and the power output of 25 mW. The laser beam was delivered by an optical fiber and irradiated a circular area of 1 cm^2. The duration of exposure was 140 s, and the total energy was 2 J/cm^2.

The study comprised three groups: two study groups and a control group. The variable parameter was the number of laser irradiation. In the first study group, cells were irradiated only one time (single-dose group), but the second group was irradiated twice with 24 h interval (double dose

group). Control group was not irradiated with laser. All media in culture plates were collected 24 h after laser irradiation and were used to determine the cell viability, cell proliferation, and the release of bFGF, IGF-1, and IGFBP3.

Cell viability and proliferation

After irradiation, the degree of cell proliferation was determined by trypsinizing the fibroblast cells and resuspending them in phosphate-buffered saline. The cell numbers were counted with a coulter counter.

Assay for bFGF, IGF-1, and IGFBP3

Human bFGF ELISA kit (RayBiotech, GA, USA) was used for the quantitative measurement of human bFGF. This assay employs an antibody specific for human bFGF coated on a 96-well plate. Standards and samples are pipetted into the wells, and bFGF present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated antihuman bFGF antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells, and color develops in proportion to the amount of bFGF bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. A set of bFGF standards is used to plot a standard curve of absorbance vs bFGF concentration from which unknown bFGF concentrations were calculated.

Measurement of IGF-I was used by Non-Extraction IGF-I ELISA kit, an enzymatically amplified "two-step" sandwich-type immunoassay (Diagnostic Systems Laboratories, Texas, USA). In the assay, Standards, Controls and pre-diluted unknowns were incubated in microtitration wells, which have been coated with anti-IGF-I antibody. After incubation and washing, the wells were treated with another anti-IGF-I detection antibody labeled with the enzyme horseradish peroxidase (HRP). After a second incubation step and washing, the wells were incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution is then added, and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 and 620 nm. The absorbance measured is directly proportional to the concentration of IGF-I present. A set of IGF-I standards was used to plot a standard curve of absorbance vs IGF-I concentration from which the IGF-I concentrations in the unknowns were be calculated.

IGFBP-3 ELISA (DSL-10-6600 ACTIVE®, Oxon OX25 5HD UK) is an enzymatically amplified "two-step" sandwich-type immunoassay. In the assay, Standards, Controls and samples are incubated in microtitration wells coated with anti-IGFBP-3 polyclonal antibody. After incubation and washing, the wells are treated with another anti-IGFBP-3 polyclonal antibody labeled with the enzyme horseradish peroxidase (HRP). After a second incubation and washing step, the wells are incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution is then added, and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm. The absorbance measured is directly proportional to the concentration of IGFBP-3 present. A set of IGFBP-3 Standards is used to plot a standard curve of absorbance vs IGFBP-3 concentration from which the IGFBP-3 concentrations in the Controls and unknowns can be calculated.

Statistical analysis

The statistical analysis was performed by SPSS 10.0 (SPSSFW, SPSS, Chicago, IL, USA) statistical program. The differences between the groups were evaluated by means of Mann–Whitney *U* test. *P* values less than or equal to 0.05 were considered to be statistically significant.

Results

The comparison of the single dose group with the control group revealed statistically significant increases in bFGF ($p<0.01$) and IGF-1 ($p<0.01$), but the increase in IGFBP3 was not significant ($p>0.05$). When the double dose group was compared with the control group, significant increases

Table 1 Comparison of the single dose group with the control group

Groups	bFGF (pg/ml)	IGF-1(pg/ml)	IGPB3(pg/ml)
Single dose; $n=10$	789.9 (636.25–886.4)	159.75 (146.28–166.55)	3.2 (2.78–4.38)
Control; $n=10$	124 (115.8–125,5)	105.2 (100,7–110.5)	2.9 (2.5–3.1)
P	P<0.01	P<0.01	*P*>0.05

Values in the parenthesis show the 25–75 percentiles (quarter)

Table 2 Comparison of the double dose group with the control group

Groups	bFGF(pg/ml)	IGF-1(pg/ml)	IGPB3(pg/ml)
Double dose; n=10	768.5 (686.7–965)	169.1 (141.66–172.95)	4.1 (3.4–4.95)
Control; n=10	124 (115.8–125.5)	105.2 (100.7–110.5)	2.9 (2.5–3.1)
P	P<0.001	P<0.001	P<0.01

Values in the parenthesis show the 25–75 percentiles (quarter)

were found in bFGF, IGF-1, and IGFBP3 ($p<0.01$) (Tables 1 and 2). In the comparison of the two Laser irradiated groups (one dose and two doses), alterations in none of the parameters were statistically significant ($p>0.05$).

The proliferation of fibroblasts increased in all the groups, and it was determined that the increases in the lased groups were more than those in the control group. Cell viability was 119.6% for single dose and 109% for double dose group, revealing that LLLT did not cause any cell damage.

Discussion

Growth factors initiate many of the events associated with the turnover, repair, and regeneration of periodontal tissue [24, 25, 29, 30]. In some studies, bFGF and IGF-1 have been suggested to promote periodontal regeneration by stimulating the formation of mesenchymal tissues including collagen, bone, and cementum [23, 31, 32]. Kasasa et al. [33] reported IGF to be a powerful chemoattractant of fibroblastic cells. Similarly, the mitogenic and angiogenic features of bFGF suggest that it may play an important role in wound healing [19–21]. Thus, in this study, we aimed to determine the effect of LLLT on the expression of bFGF, IGF-1, and IGFBP3 from gingival fibroblasts.

In several investigations, LLL energy was demonstrated to enhance fibroblast proliferation [1–4]. In the literature, there is conflicting results about the number of laser applications adequate for proliferation of fibroblasts. In our study, the effects of single dose and double dose laser irradiation (2 J/cm^2) were compared. Fibroblast proliferation increased in both of the groups in comparison to the control group. Consecutive laser application caused numerically more proliferation, but this increase was not statistically significant when compared with single dose group. The single dose results are consistent with the report of Loevshall and Arenholt [34]. However Khandra et al. [1] found no significant differences between the control group and the sample exposed to a single dose of 3 J/ cm^2 for 360 s. Conflicting results may be explained by the alterations in the parameters such as wavelength of the laser system, power output, irradiation time, multiple exposures, and distance of the fiber from the specimen. Hans et al. [35] found that both stimulation and inhibition of the fibroblast can be caused with the same laser in the same cells. Similarly, Yu et al. [22] reported that fibroblasts irradiated with laser energy at 2.16 J/cm^2 demonstrated increased cell proliferation, whereas laser energy at 3.24 J/cm^2 suppressed cell proliferation compared to the control group. Our results have demonstrated that LLLT has a positive biostimulation effect on fibroblast cells and does not cause cell damage.

The present study revealed that the stimulatory mechanism of laser irradiation in biostimulation could be associated with growth factors (IGF-1, bFGF) and IGFBP3 produced from fibroblastic cells. Some experimental studies suggest that laser irradiation may stimulate the secretion of growth factors [36]. Although Yu et al. [22] demonstrated that laser energy at 2.16 J/cm^2 can stimulate the release of bFGF from fibroblasts; it is not known whether laser irradiation could enhance the secretion of IGF-1 and IGFBP3 or other growth factors from fibroblasts. Our study demonstrated that production of IGF-1 and IGFBP3 from fibroblast can also be increased by laser irradiation at a wavelength of 685 nm, 1.40 s exposure time, and 2 J/cm^2. In this study, IGFBP3 increased numerically in both of the laser groups. But this increase was statistically significant only at the double dose group when compared with the control group.

To our knowledge, in the literature, it has not been reported so far whether LLLT could stimulate the expression of polypeptide growth factors, except Yu et al. [22] who investigated the release of bFGF. Our findings regarding IGF1 and IGFBP3 revealing the effects of laser biostimulation have not been shown before. This study supported the results of other researchers and suggested that LLLT may increase the acceleration of wound healing because of the stimulatory effects on fibroblast proliferation. Our study also obtained some new findings explaining the mechanism of the biostimulation of LLLT. The biostimulatory effect of LLLT may be associated with the enhanced production of growth factors such as IGF-1, bFGF, and IGFBP3. LLLT may play an important part in periodontal wound healing and regeneration by enhancing the production of the growth factors. Further in vivo and in vitro investigations are needed to understand the

mechanism leading to stimulation of HGF and to evaluate whether LLLT might be beneficial in regenerative periodontal therapy.

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