ORIGINAL ARTICLE

Effect of Low-level Laser Irradiation on Proliferation of Periodontal Ligament Fibroblasts and Production of Insulin-like Growth Factor 1: An *in vitro* Study

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ABSTRACT

Background and objectives: Low-level laser therapy (LLLT) has been used in dentistry to improve wound healing. The objective of this study is to analyze the proposed role of LLLT on proliferation of periodontal (PDL) fibroblasts and subsequently the release of insulin-like growth factor 1 (IGF-1).

Study design/materials and methods: Periodontal ligament fibroblast obtained from third molars and the teeth advised for orthodontic extractions were cultured under standard conditions and spread on 96-well tissue culture plates. Laser irradiation was carried out with diode lasers with wavelength 660 nm and fluence of 7.5 J/cm^2 for 5 minutes per well thrice after every 24 hours. For growth analysis, after 72 hours cell proliferation rate in control (not irradiated) and treated cultures (irradiated) was counted in Neubauer's chamber. At the same time, IGF-1 release was assessed using enzyme-linked immunosorbent assay (ELISA) test. Along with this, whether rate of cell proliferation and IGF-1 release is dependent on dosage of laser irradiation was evaluated comparing the intragroup results.

Results: The irradiated cells showed a considerably elevated proliferation rate than the controls. The differences were significant up to 24, 48, and 72 hours after irradiation. Comparison of single, double, and triple dose group with the control group revealed increase in rate of proliferation of PDL fibroblasts, and IGF-1 release significantly.

Conclusion: Low-level laser therapy may play an important role in PDL wound healing and regeneration by enhancing the rate of proliferation and production of growth factors in dose-dependent manner.

Keywords: Cell culture, Diode laser, Insulin-like growth factor 1, Low-level laser therapy, Periodontal fibroblast, Periodontal regeneration, Wound healing.

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INTRODUCTION

Laser is the acronym for "Light Amplification by Stimulated Emission of Radiation" named by American physicists Schawlow and Townes.[1] The use of laser (soft and hard) for the management of dental diseases has gained rapid momentum in the recent past. Various lasers are presently available in the market varying from hard tissue laser and soft tissue (combined) to only soft tissue laser. Considering the cost to benefit ratio, diode lasers are smaller, economic, and operate at various power output. Considering its multiple uses, diode lasers seem to be the present choice of economical lasers.

The primary benefits of laser are less bleeding, relatively less edema/inflammation/pain,[2,3] and promoting tissue healing. At higher outputs lasers cause thermomechanical ablation, which is used for hard and soft tissue lesion. However, at lower output at 0.2 to 0.5 W, laser seems to have a stimulating/photobiological or a biomodulatory effect, which promotes healing and reduces inflammation. This phenomenon of photomodulation is referred to as LLLT.[4]

Periodontal wound healing leading to regeneration has to have a well-orchestrated cascade of biological and biochemical events. The PDL fibroblasts seem to be highly important in producing extracellular matrix as well as connective tissue by forming collagen. The role of fibroblast seems to be unchallenged in PDL regeneration as not only collagen but also elastin, fibronectin, and proteoglycan are produced by fibroblasts.

As the understanding of cellular processes and events in repair/regeneration of PDL tissues are becoming clearer, the role of growth factors in mitogenesis, migration, and metabolism involved in tissue engineering is fairly understood. Growth factor is a term used to denote a class of naturally occurring proteins that function to promote proliferation, migration, and alteration in metabolic activity (increased/decreased) of cells.[5,6] Numerous growth factors like platelet-derived growth factor (PDGF), transforming growth factor (TGF-α,β), fibroblast growth factor (FGF), and IGF have been identified to have greater ability to alter repair/regeneration.

Insulin-like growth factor 1 is a mitogenic protein. It is found in substantial levels in platelets and is released

during clotting along with the other growth factors present in platelets. For vascular endothelial cells, IGF-1 is a potent chemotactic agent. In addition, IGF-1 released from platelets or produced by fibroblasts may promote migration of vascular endothelial cells into the wound area, resulting in increased neovascularization. It also stimulates mitosis of many cells *in vitro*, such as fibroblasts, chondrocytes,[7] and osteoblast.[8]

It is an established fact from previously conducted studies that fibroblast and growth factor release plays important role in wound healing. This study was conducted to understand the possible mechanism of adjunctive role of LLLT on the rate of proliferation of PDL fibroblasts and release of IGF-1 and further substantiate the facts that growth factor release might enhance the process PDL regeneration.

AIMS AND OBJECTIVES

This study was aimed to evaluate the effect of LLLT on proliferation of PDL fibroblast with its effect on secretion of IGF-1 factor release and whether IGF-1 release is dependent on dosage of laser irradiation.

MATERIALS AND METHODS

Source of Data

This study was conducted in the Department of Periodontics, at MA. Rangoonwala College of Dental Sciences & Research Centre, Pune, Maharashtra, India, utilizing the outpatient departmental facilities.

The total sample size was 60 teeth and cells derived from them were divided into study (laser group) and control group.

Periodontally healthy teeth from patients in the age group of 18 to 30 years; freshly extracted teeth, e.g., premolars, that were indicated for orthodontic extraction and impacted third molars were included in the study.

Carious, fractured, or periodontally involved teeth as well as teeth from patients with any systemic disease like diabetes mellitus or those undergoing steroid therapy were excluded from the study.

Method of Collection of Data

After fulfilling inclusion and exclusion criteria, teeth were extracted with aseptic, atraumatic precautions. Within 4 to 6 hours after extraction, teeth were transferred to the laboratory in phosphate buffer solution. Then the roots were sectioned into pieces and were placed on a Petri dish with 2 mL of Dulbecco's Modified Eagle Media supplemented with Penicillin G (100 U/mL), Streptomycin (100 gm/mL), Gentamycin (100 gm/mL), and 10% fetal bovine serum. Cultures were incubated at 37°C in atmosphere of 5% CO_2/95% air, in 100% humidity. Change of medium was carried out after every 4 to 5 days. Once fibroblast-like cells start emigrating and reached about 10% confluent around the tooth fragments (3–4 weeks), then the fragments were washed with phosphate buffer saline, 0.25% trypsin in ethylenediaminetetraacetic acid buffer was added and incubated for 3 to 5 minutes. Then the cells were detached, spread on a tissue culture plate, and reincubated till the cells reached 80 to 90% of confluence (4–6 weeks).

After 6 weeks the cells were separated by trypsinization and counted in Neubauer counting chamber. A 0.2 mL of the cell solution was added to each well of 96 well tissue culture plate. Cell counting was done before irradiation.

Laser Irradiation

The study comprised three test groups and a control group. Frequency of irradiation was a variable parameter which was categorized as follows:

- D_1—24 hours
- D_2—48 hours
- D_3—72 hours

Samples were divided into control group and test groups as follows:

- T0: Control group, not subjected to laser irradiation.

Test group "T"

- T1: Received single dose of LLLT and was evaluated at D_1, D_2, and D_3.
- T2: Group was irradiated at D_1, D_2 interval and was evaluated at D_1, D_2, and D_3 interval.
- T3: Group was irradiated at D_1, D_2, D_3 interval and was evaluated at D_1, D_2, and D_3 interval.

The irradiation parameters of the laser units were used as follows:

- Wavelength—660 nm
- Power output—25 MW
- Time—5 minutes (300 seconds) per well
- Energy—7.5 J
- Energy density (fluence)—7.5 J/cm^2
- Exposure—3 times (after every 24 hours)
- Distance—11 mm apart from monolayer

Irradiation was carried out as shown in Figure 1

Cell viability, cell proliferation, and the release of IGF-1 factor after 72 hours were determined. (Cell viability and proliferation: To investigate whether the presence of viable cells along with irradiation led to the proliferation of PDL fibroblasts. 72 hours after irradiation the degree of cell proliferation was determined by trypsinizing the fibroblast cells and resuspending them in phosphate buffer saline. The cell number was counted in Neubauer's chamber.)

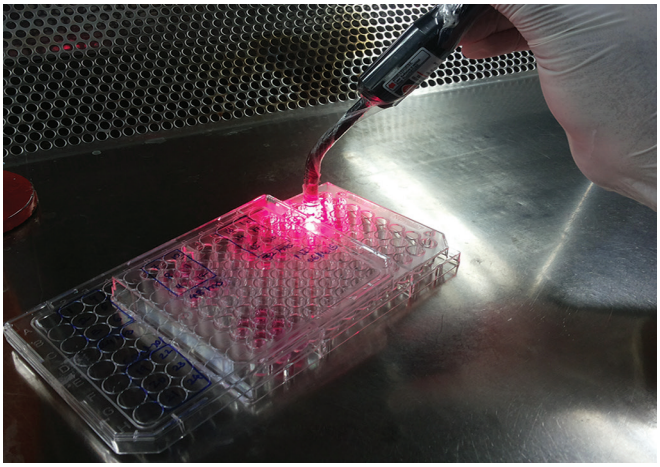


Fig. 1: Low level laser application

Table 1: The inter-group and intra-group comparison of cell count

Cell count (x10^4) (cells/mL)	*Laser group (n = 20)*	*Control group (n = 20)*	*P-value [Inter-group]*
Single dose	10.95 ± 0.19	6.45 ± 0.09	0.001*** HS
Double dose	19.60 ± 0.29	7.54 ± 0.10	0.001*** HS
Triple dose	27.90 ± 0.37	8.66 ± 0.13	0.001*** HS
P-value [Intra-group]			
Single dose v double dose	0.001*** HS	0.001*** HS	–
Single dose v triple dose	0.001*** HS	0.001*** HS	–
Double dose v triple dose	0.001*** HS	0.001*** HS	–

HS: Highly significant; ***(P-value<0.001) in table 1 indicates it is highly significant indicating that in laser as compared to control grp the cell proliferation was pronouncedly increased.Similarly triple dose showed excess cell proliferation as compared to single and double dose group.

Proliferation Assay

Proliferation activity was determined by means of sulforhodamine B assay. It is a simple, nonradioactive assay used to monitor and determine the proliferation of various cell lines and the cell viability.[9,10]

After being taken up by the cells, the reduction–oxidation (REDOX) indicator fluoresces and changes color in response to chemical reduction. Reduction related to cellular growth causes the REDOX indicator to change from oxidized (nonfluorescent blue) to reduced form (fluorescent red). The proliferation activity was measured by the color change.

Assay for IGF-1

Measurement of IGF was done by ELISA kit (Krishgen) with a two-step enzymatically amplified sandwich-type immunoassay. In this assay standard, control, and per-diluted unknowns were incubated in microtitration wells, which were coated with anti-IGF-1 antibody. After incubation and washing wells were treated with another anti-IGF-1 detection antibody labeled with the enzyme horseradish peroxidase. After a second incubation step and washing, the wells were incubated with the substrate tetramethylbenzidine. 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-1 present. A set of IGF-1 standards was used to plot a standard curve of absorbance *vs* IGF-1 concentration with the unknowns to be calculated.

STATISTICAL ANALYSIS

Mann–Whitney "U" test was applied to these groups, and U value was obtained as 5.2873 with p-value <0.001. Thus, the results were statistically highly significant.

RESULTS

Comparison of Cell Count in Control *vs* Test Group at Different Intervals of Time

Comments

Firstly, we compared the effect of lasers on cell proliferation with different doses, i.e., T1 *vs* T2 *vs* T3 by counting cells on D_1, D_2, and D_3 respectively. The mean cell count on three consecutive days is represented in Table 1, and the results are as follows:

- Intragroup Comparisons

In control (Fig. 2) and laser groups, the mean cell count after single dose (T1) represented in Figure 3 was significantly lesser compared with the mean cell count after double (T2) and triple doses (T3), as shown in Figures 4 and 5 respectively (p-value <0.001 for both; Table 1).

At the same time, the difference in cell count in control (nonirradiated) and test (irradiated) groups was compared, which showed the following results.

- Intergroup Comparisons

In control group, T_0 is represented as in Figure 2. The mean cell count was significantly lesser than the laser group represented in Figures 3 to 5 (p-value <0.001; Table 1).

Similarly, variation in IGF-1 release was assessed and showed the following results:

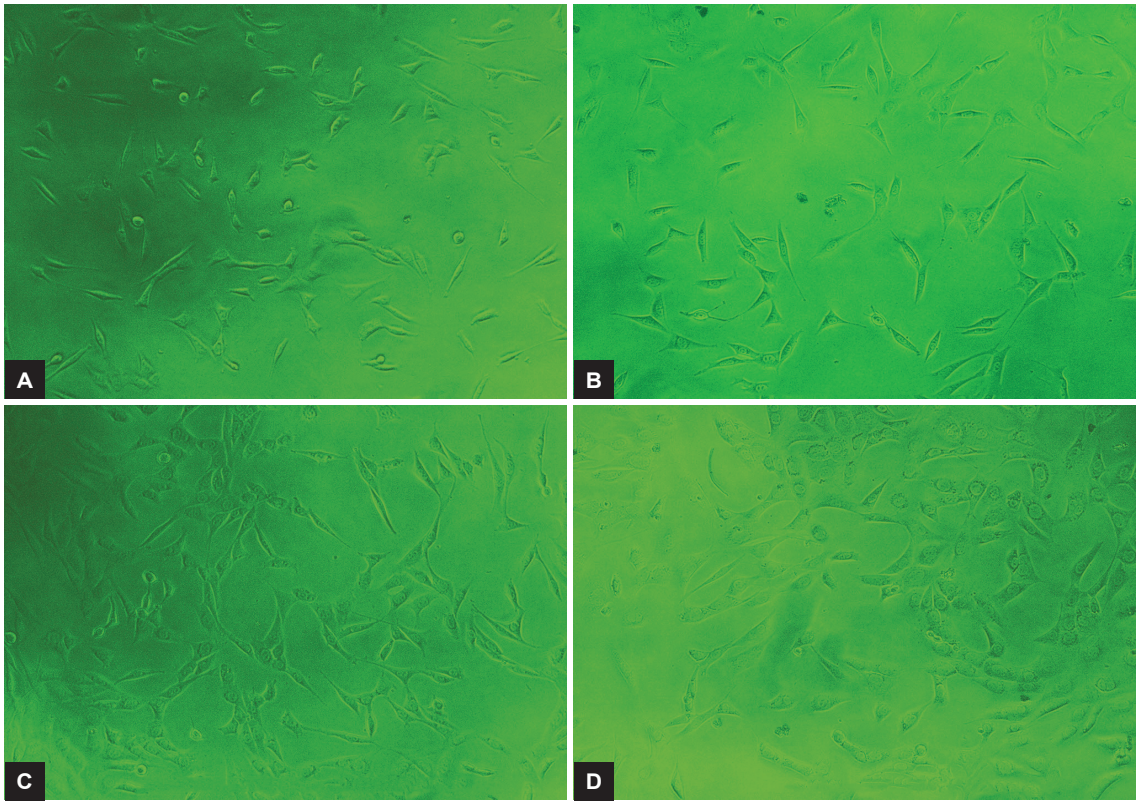
Comparison of IGF-1 Release in Control *vs* Laser Group at Different Intervals of Time

- Intragroup Comparisons

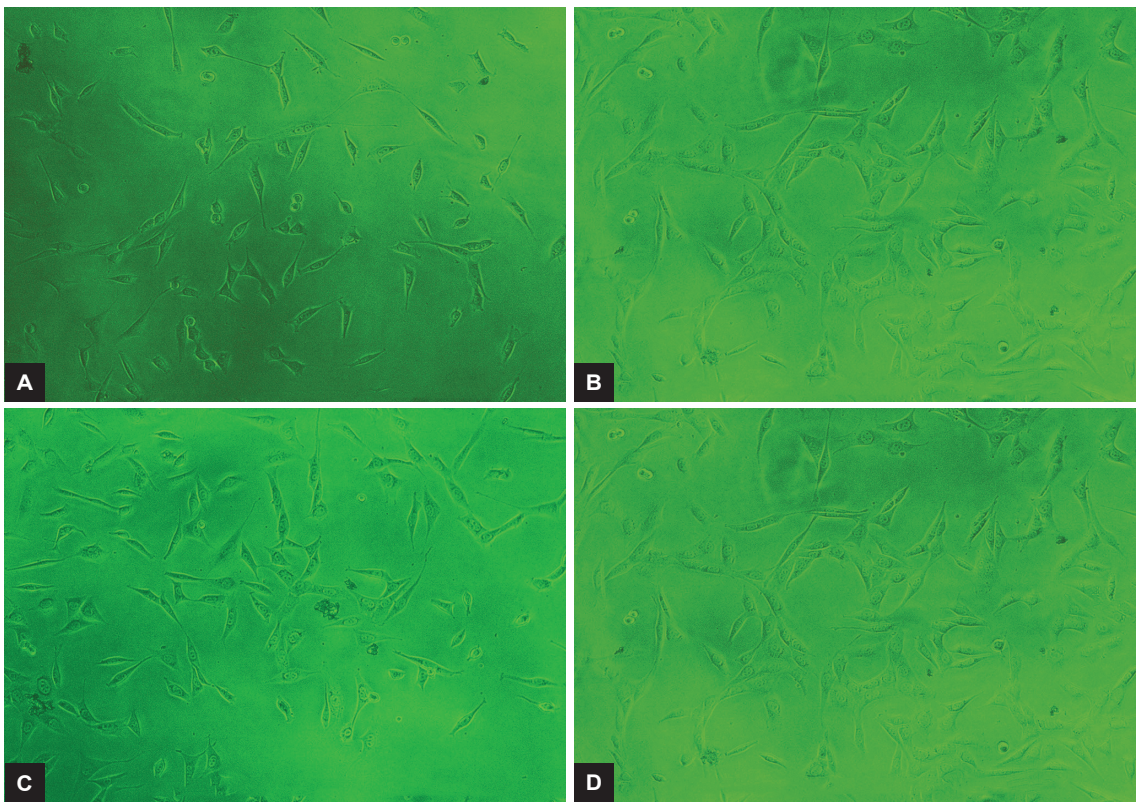
The mean IGF level in control and laser groups after single dose (T1) was significantly lesser compared with the mean IGF level after double (T2) and triple doses (T3) (p-value <0.001 for both) (Table 2).

- Intergroup Comparisons

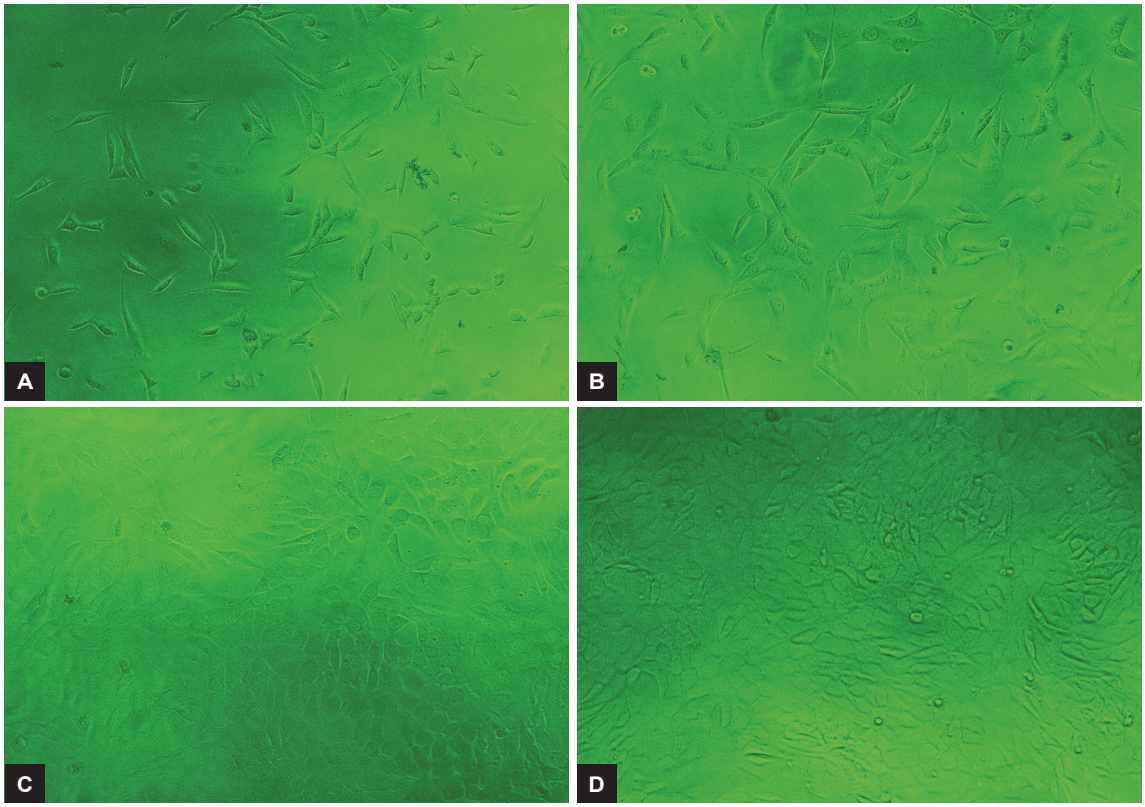
The mean IGF level was significantly lesser in control group compared with the laser group (p-value <0.001; Table 2).



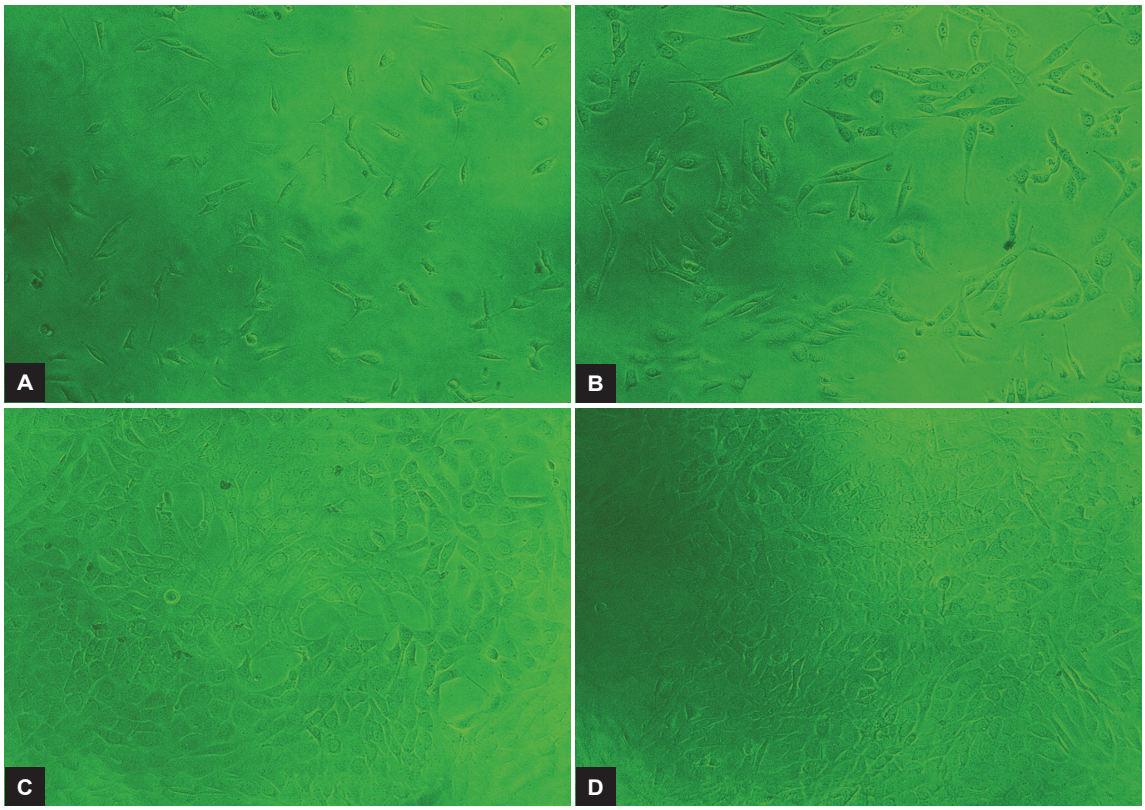
Figs 2A to D: (A) Cells before irradiation in control group; (B) cell proliferation 24 hr after LLLT irradiation in control group; (C) cell proliferation 48 hr after LLLT irradiation in control group; and (D) cell proliferation 72 hr after LLLT irradiation in control group



Figs 3A to D: (A) Cells in single dose application i.e.T1 group before LLLT irradiation; (B) cells in single dose application i.e.T1 group 24hr after LLLT irradiation; (C) cells in single dose application i.e.T1 group 48 hr after LLLT irradiation; and (D) cells in single dose application i.e.T1 group 72 hr after LLLT irradiation



Figs 4A to D: (A) Cells in double dose application i.e T2 group before LLLT irradiation; (B) cells in double dose application i.e T2 group 24 hr after LLLT irradiation; (C) cells in double dose application i.e T2 group 48 hr after LLLT irradiation; and (D) Cells in double dose application i.e T2 group 72 hr after LLLT irradiation



Figs 5A to D: (A) Cells in triple dose application i.e.T3 group before LLLT irradiation; (B) cells in triple dose application i.e T3 group 24 hr after LLLT irradiation; (C) cells in triple dose application i.e T3 group 48 hr after LLLT irradiation; and (D) cells in triple dose application i.e T3 group 72 hr after LLLT irradiation

Table 2: The inter-group and intra-group comparison of IGF

IGF (pg/mL)	Laser Group (n = 20)	Control Group (n = 20)	P-value [Inter-Group]
Single dose	0.085 ± 0.0017	–	–
Double dose	1.332 ± 0.0189	–	–
Triple dose	1.886 ± 0.0124	0.0282 ± 0.0002	0.001*** HS
P-value [Intra-group]			
Single dose v double dose	0.001*** HS	–	–
Single dose v triple dose	0.001*** HS	–	–
Double dose v triple dose	0.001*** HS	–	–

Values are Mean ± Standard error of mean. P-values for inter-group comparisons are obtained by using independent sample t-test. p-values for intra-group comparisons are obtained by using Paired t-test. p-value < 0.05 is considered to be statistically significant. ***p-value < 0.001.

The graphical representation of comparing cell count and IGF release has been done as shown in Graphs 1 and 2 respectively.

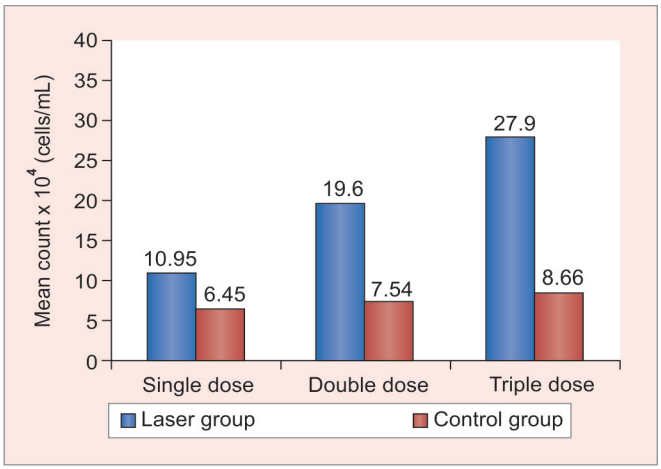
DISCUSSION

Since the late 1970s and early 1980s, LLLT has become a popular mode of treatment modality in medicine and surgery.[11] Considering its significant role in wound healing, and its property of biostimulation, a lot of scientific work at molecular level has been carried out using cell cultures and animal models,[12,13] the results of which have shown its ability to induce faster cell division, rapid matrix production, and cell movement. Clinical trials have also been undertaken to evaluate its potential beneficial effects like pain suppression, anti-inflammatory action, accelerated wound healing, enhanced remodeling, repair of bone,[8] release of growth factors[14] as well as transcriptional gene expression,[15].

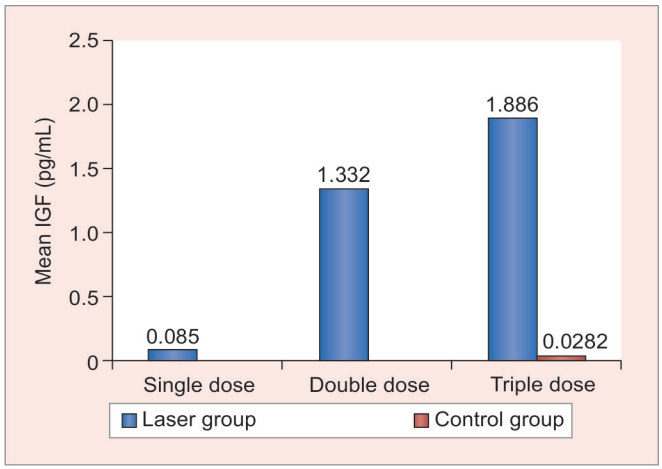
Regeneration of PDL tissues has always been a goal of PDL therapy but it seems to be an elusive goal. The intent of this study was to investigate whether LLLT enhances PDL fibroblastic growth and also increases IGF levels such that it could be used as an adjunct to the current conventional technique for enhancement of PDL regeneration. The present methodologies and measurement of variables in clinical settings and animal models are complex. So quantification of the biostimulatory effect of LLLT at a molecular level is relatively indefinable.

The regeneration of PDL tissues requires formation of new cementum, PDL ligament, and alveolar bone. The PDL fibroblasts play an important role in regeneration than gingival fibroblasts because of the presence of undifferentiated mesenchymal cells. Stimulatory effect on proliferation of these fibroblastic cells could thus be beneficial for establishment of new connective tissue attachment and regeneration. Majority of these studies have been done on gingival fibroblasts,[14,16,17] while on artificial cell lines only limited data regarding effect of lasers on PDL fibroblasts are available.[18,19] Thus the correlation between LLLT and IGF has not been established.

The agents promoting PDL ligament fibroblast proliferation and migration as well as collagen biosynthesis would appear to be mediators for enhancing new PDL ligament formation. Multiple growth factors, like PDGF, TGF, FGF, IGF, have the potential for playing important role in regeneration. IGF-1 is a potent growth factor since PDL fibroblasts have high affinity receptor for IGF-1 on its cell membrane.[20] Thus, through its chemotactic nature for fibroblasts, it binds to IGF binding protein which is carrier protein in various biological fluids, mediates transport via vascular space, and then binds to specific receptors on fibroblasts. Thus this study was undertaken to shed light on areas which have been hardly or minimally studied, i.e., effect of lasers on PDL fibroblasts and IGF-1 production.



Graph 1: The intergroup and intragroup distribution of cell count



Graph 2: The intergroup and intragroup distribution of IGF

The hypothesis proposed for our study was that along with proliferative effect of LLLT irradiation on PDL fibroblasts, it might also be associated with autocrine production of growth factors. Multiple researches have successfully found increased concentration of FGF from lased fibroblast,[14,16,21] TGF-1, PDGF,[22] but none of the studies have shown release of IGF from PDL fibroblasts.

Potential low-level laser effect on proliferation of PDL fibroblasts cells has been described using radioactive assays based on the incorporation of [3H]thymidine or [3H]proline.[23] In the present study, cell proliferation was determined by simple one-step, nonradioactive assay containing a REDOX indicator. Cellular growth was indicated by change in the color of media. The assay used in our experiment features several advantages that it is nonhazardous, simple, inexpensive, nonlabor involving, rapid, and indicating the quantity of viable cells in percentage.

The primary understanding of LLLT always suggested that wound healing may be due to stimulatory effects on fibroblast proliferation but in our study we would like to hypothesize that LLLT would play an important role in would healing and also accentuate the regeneration process by enhanced production of growth factors.

A quick cursory review of literature concerning LLLT and PDL fibroblasts has revealed conflicting results regarding number of laser applications adequate for proliferation of fibroblasts. On comparing the dose and healing response relationship, i.e., quantity and frequency of the LLLT on the tissue response, it has been shown that effect of single dose does not cause significant increase in the amount of proliferation of the cells. But double and triple doses have shown around three-fold increase in the amount of proliferation of the cells. Single-dose results are consistent with reports of Loevschall et al[24] and Walsh and Murphy,[25] whereas Khadra et al[26] found inhibiting effect with no significant difference between control group and sample group. After a thorough search in the reasons for such conflicting results, the probable reasons for the same could be attributed to variation in parameters like wavelength of laser system, power output, irradiation time, and distance of fiber from the cells. Hence, in an attempt to minimize these errors in our study, we used a standard wavelength of diode laser (660 nm) with a power output of 25 MW.

In conclusion, our study indicated that 660 nm diode laser with power output of 25 MW in noncontact mode with an irradiation time of 5 minutes had a stimulating effect on PDL fibroblasts. The amount of proliferation of these PDL fibroblasts was dose-dependent. Along with stimulatory effect, LLLT also helped in release of IGF-1 factor which could be the result of dose-dependent fibroblastic growth. This finding might be of clinical relevance in the management of PDL destruction as progenitor cells on the root surfaces could be stimulated to form new collagen bundles.[8,27]

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