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Effect of low-level laser on proliferation, angiogenic and dentinogenic differentiation of human dental pulp stem cells

Fatemeh Rezaei[1], Shahrzad Shakoori[1], Mahta Fazlyab[1*], Ehsan Esnaashari[1] and Sohrab Tour Savadkouhi[1]

Abstract

Background The aim was to evaluate the effect of single and double doses of low-level laser irradiation on proliferation of human dental pulp stem cells (DPSC) and expression of vascular endothelial growth factor (VEGF) and dentine sialoprotein (DSP).

Methods In this experimental in vitro study, after confirming the stemness of DPSCs, the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) for MTT assay and VEGF-ELISA and osteogenic medium for DSP-ELISA. The wells containing DPSCs were divided into three main groups and 9 subgroups ($n=7$). In groups with single low-level laser, 660-nm diode laser was irradiated at 100 mW and 3 J/cm^2 energy density for 15 s. In groups with double doses of low-level laser the second identical irradiation was after 48 h. The MTT-assay and ELISA for DSP/VEGF (dentinogenic/angiogenic differentiation) were performed at 1, 7 and 14 days post irradiation. Using the SPSS software 20 (SPSS, Chicago, Ill, USA) with 95% confidence interval ($P=0.05$), a two-way ANOVA test with Tukey's post hoc test was used for the effect of LLLI on VEGF and DSP. The One-Way ANOVA was used for of cell proliferation.

Results Higher proliferation rate in both single and double low-level laser was reported. The difference was statistically significant for double doses of low-level laser ($P=0.001$, $P=0.020$ and $P=0.000$ for 1, 7 and 14 days, respectively). Also after one, 7 and 14 days, cells in significant increase in DSP ($P>0.05$) and VEGF ($P>0.05$) was observed that was significantly higher for double doses of low-level laser.

Conclusions Low level laser enhanced the mitochondrial activity and proliferation of DPSCs. Increased production of DSP/VEGF indicates dentinogenic/angiogenic activity.

Clinical relevance Low level laser increases the proliferation of DPSCs, elevates the production of VEGF (which means better angiogenesis in regenerative treatments) and increases the production of DSP (which means better dentinogenesis in vital pulp treatments).

Keywords Dentine sialoprotein, Dental pulp stem cells, Low-level laser therapy, Regenerative endodontic procedure, Vascular endothelial growth factor, Vital pulp treatment

*Correspondence:
Mahta Fazlyab
Dr.Mfazlyab@gmail.com
[1]Department of Endodontics, Faculty of Dentistry, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

Introduction

Pulp involvement in immature teeth becomes a clinical challenge due to thin canal walls and short roots and if pulp necrosis occurs, chemomechanical debridement and placement of an effective apical seal becomes a double challenge [1, 2]. Besides placing calcium silicate apical plug, regenerative endodontic procedure (REP) is another biological treatment option with clinically acceptable outcomes [1, 3]. Pulp is a rich source of mesenchymal stem cells and banking of dental pulp stem cells (DPSCs) has increased substantially in recent years [4]. DPSCs are capable of stimulating important cellular responses during pulp regeneration and dentinal repair [5]. DPSCs produce many bioactive molecules such as bone morphogenetic proteins (BMPs), transforming growth factor-β (TGFβ), fibroblast growth factor-2 (FGF-2), insulin growth factor (IGF), and pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [6, 7]. In immature teeth, dentinogenic differentiation of dental stem cells is important in two treatments; vital pulp therapy (VPT) of vital teeth and REP of nonvital teeth. For these purposes two bioactive molecules, dentine sialoprotein (DSP) and VEGF, can be measured and marked as signs of dentinogenic and angiogenic activity, respectively [8–11].

In VPT, reparative dentinogenesis is very critical. Identification of factors that enhance the proliferation and dentinogenic activity of DMSCs is extremely important [12]. DPSCs can differentiate into odontoblast-like cells and secrete reparative dentine [13]. Signs of dentinogenic activity are acquiring the specific morphology of dentine with tubular structures and secondary odontoblasts-lining [8, 10, 14], specific molecular contents and cellular functions [10]. Expression of a wide range of differentiation markers such as DSP (member of the SIBLING family- Small Integrin-Binding Ligand N-linked Glycoprotein) has been suggested to characterize an odontoblast phenotype [8, 10, 14]. DSP is specific marker for dentine secretion and odontoblast differentiation [8, 10, 14–16]. It was reported that the presence of DSP, stimulated odontoblastic differentiation in DPSCs [17]. Detection of DSP can be assumed as a sign of dentinogenic activity and odontoblastic differentiation.

In REP of immature nonvital teeth, angiogenesis is a fundamental step which is influenced by multiple factors [9, 13, 18]. VEGF plays crucial roles in angiogenic and odontogenic differentiation of DPSCs [19]. VEGF is an extremely potent pro-angiogenic and mitogen factor that promotes endothelial cell survival and angiogenesis [9], formation of new blood vessels [20] increasing vascular permeability which contributes to a greater blood supply, oxygen supply, and transport of stem cells to the injured tissue, leading to tissue repair [11]. VEGF is fossilized in dentine matrix and can be released by demineralization, in very low levels [21]. Also it is expressed in normal healthy dental pulp and irreversibly inflamed pulps [13, 18]. VEGF is an important growth factor for evaluating regenerative endodontic procedure through measuring angiogenesis [11].

Besides the exogenous growth factors used to enhance the proliferation of DMSCs [3] another method for uplifting proliferation and dentinogenic activity is low-level laser irradiation *aka.* low-intensity laser radiation (LLLI) [12]. LLLI is a non-invasive procedure that induces athermic, nondestructive photobiological processes [22]. LLLI is effective in pain reduction, wound healing, bone repair and remodeling, nerve repair and angiogenesis [12, 22]. LLLI modulates the process of tissue repair by stimulation of cellular reaction such as migration, proliferation, apoptosis, and cell differentiation and induction of the angiogenesis process through secretion of VEGF [20, 23, 24]. LLLI has been applied directly on pulp tissue with different capping materials with promising results [25]. Many in vitro and in vivo studies have shown that LLLI at different wavelengths and dosimetries, modulates physiological and biochemical processes and stimulates production of growth factors such as FGF, IGF and VEGF [26, 27]. Moreover, LLLI of cell cultures exhibited higher levels of mineralization markers, including alkaline phosphatase and dentin sialophosphoprotein [28–30].

There is minimal evidence on effect of LLLI on the function and differentiation of DPSCs. Our objective was to evaluate the impact of the LLLI with 660 nm diode on proliferation of human DPSCs and expression of VEGF (as the angiogenic factor) and DSP (as a marker of dentinogenic differentiation). In-vitro studies carry the inherent limitation of not capturing the complexity and internal environment of organ systems and may not account for interactions between various body procedures and cellular biochemistry. However, the findings of this in-vitro study will allow the design of a cell preconditioning protocol based on LLLI before transplantation of DPSCs or other DMSCs for dental tissue engineering/regeneration. The null hypothesis is that LLLI could promote odontogenic differentiation of DPSCs and angiogenesis, which may contribute to advances in REP and VPT.

Methods and materials

Study design

This experimental in vitro study was approved by the ethics committee at Faculty of Dentistry, Tehran medical sciences, Islamic Azad University, Tehran, Iran.

Sample size calculation

Using the one-way ANOVA option in PASS11 (NCSS, LLC. Kaysville, Utah, USA) and considering $\alpha = 0.05$ and $\beta = 0.2$ the minimum sample size for MTT assay

and ELISA for DSP and VEGF was 7 wells in each study group ($n = 7$).

Characterization and culture of DPSCs

DPSCs were established from a biopsy of dental pulp of a 35-year-old female (cell serial No. IBRC C10896) and were obtained from the Iranian Biological Resource Center (https://ibrc.ir). Phenotyping and differences in the expression levels of major surface markers were confirmed by flow cytometry analysis using the conjugated antibodies according to the manufacturer's recommendations. The stemness of DPSCs was confirmed through expression of mesenchymal stem cell markers (CD44, CD90 and CD105), (monoclonal antibody, Sigma San Diego, CA, USA) and the lacking markers CD34 (eBioscience, Sigma, San Diego, CA, USA) and CD45 (eBioscience, Sigma, San Diego, CA, USA) [3].

The culture medium was Dulbecco's Modified Eagle Medium (DMEM, High glucose, and glutamine, Gibco, Grand Island, USA) supplemented with 5% fetal bovine serum (FBS, Gibco, USA), 0.1 µM L-glutamine (Invitrogen, Gibco, USA) and 1% penicillin/streptomycin (10000 IU Invitrogen, Gibco, USA) at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air [31].

DPSCs in passages 2 to 5 were fully characterized [32]. After maintaining the cells for 24 h, the cells at logarithmic phase were detached by using a mixed solution of 0.25% trypsin/0.02% EDTA (Sigma, St. Louis, MO, USA) for breakdown of the cell-cell and cell-substrate adhesion and then centrifuged in 50-ml Falcon tube at 1200 rpm for 5 min. Afterwards cells were seeded into 96-well tissue culture plates (SPL Life Sciences, Gyeonggi-do, Korea) containing osteogenic or proliferative medium at a concentration of 1×10^4 cells per well [31, 33].

The osteogenic medium was 0.1 µM dexamethasone, 10 mM β glycerophosphate, and 50 µM ascorbic acid and the proliferative medium was Dulbecco's Modified Eagle Medium (DMEM, High glucose, and glutamine, Gibco, Grand Island, USA) supplemented with 5% fetal bovine serum (FBS, Gibco, USA), 0.1 µM L-glutamine (Invitrogen, Gibco, USA) and 1% penicillin/streptomycin (10000 IU Invitrogen, Gibco, USA) at 37 °C in a humidified atmosphere containing 5% CO_2 for adherence.

Table 1 Main study groups for different tests (MTT; MTT colorimetric reaction assay for cell proliferation, ELISA; the enzyme-linked immunosorbent assay, DSP; dentine sialoprotein, and VEGF; vascular endothelial growth factor)

	LLLI	Days	Group name (*N*)
MTT	Single dose LLI	1	MTT-1-1 (7)
		7	MTT-1-7(7)
		14	MTT-1-14 (7)
	Double dose LLLI	1	MTT-2-1 (7)
		7	MTT-2-7 (7)
		14	MTT-2-14 (7)
	Without LLLI	1	MTT-0-1 (7)
		7	MTT-0-7 (7)
		14	MTT-0-14 (7)
ELISA for VEGF	Single dose LLI	1	VEGF-1-1 (7)
		7	VEGF-1-7 (7)
		14	VEGF-1-14 (7)
	Double dose LLLI	1	VEGF-2-1 (7)
		7	VEGF-2-7 (7)
		14	VEGF-2-14 (7)
	Without LLLI	1	VEGF-0-1 (7)
		7	VEGF-0-7 (7)
		14	VEGF-0-14 (7)
ELISA for DSP	Single dose LLI	1	DSP-1-1 (7)
		7	DSP-1-7(7)
		14	DSP-1-14 (7)
	Double dose LLLI	1	DSP-2-1 (7)
		7	DSP-2-7 (7)
		14	DSP-2-14 (7)
	Without LLLI	1	DSP-0-1 (7)
		7	DSP-0-7 (7)
		14	DSP-0-14 (7)

Study groups

As shown in Table 1, based on receiving LLLI and days of evaluation, the total number of wells containing DPSCs were divided into three main groups and 9 subgroups including:

1-The MTT colorimetric reaction assay for cell proliferation.

2- The enzyme-linked immunosorbent assay (ELISA) for production of VEGF.

3- The enzyme-linked immunosorbent assay (ELISA) for production of DSP.

MTT Assay- DPSCs cultured in proliferative medium were divided into 9 subgroups ($n = 7$) including: MTT-1-1; assessed for viability 1 day after single dose LLL irradiation, MTT-1-7; assessed for viability 7 days after single dose LLL irradiation, MTT-1-14; assessed for viability 14 days after single dose LLL irradiation, MTT-2-1; assessed for viability 1 day after double dose LLL irradiation 48 h apart, MTT-2-7; assessed for viability 7 days after double dose LLL irradiation 48 h apart, MTT-2-14; assessed for viability 14 days after double dose LLL irradiation 48 h apart and, negative control (MTT-0-1, MTT-0-7, MTT-0-14) DPSCs cultured in proliferative medium without LLLI that were assessed in days 1, 7 and 14 for viability.

ELISA for expression of VEGF- DPSCs were cultured in proliferative medium and divided into 9 subgroups ($n = 7$) including: VEGF-1-1; DPSCs assessed for VEGF production 1 day after single dose LLL irradiation, VEGF-1-7; DPSCs assessed for VEGF production 7 days after single dose LLL irradiation, VEGF-1-14; DPSCs assessed for VEGF production 14 days after single dose LLL irradiation, VEGF-2-1; DPSCs assessed for VEGF production

1 day after double dose LLL irradiation 48 h apart, VEGF-2-7; DPSCs assessed for VEGF production 7 days after double dose LLL irradiation 48 h apart, VEGF-2-14; DPSCs assessed for VEGF production 14 day after double dose LLL irradiation 48 h apart and, negative control (VEGF-0-1, VEGF-0-7, VEGF-0-14) DPSCs cultured in proliferative medium without LLLI that were assessed in days 1, 7 and 14 for VEGF production.

ELISA for expression of DSP- DPSCs were cultured in osteogenic medium and divided into 9 subgroups ($n=7$) including: DSP-1-1; DPSC assessed for DSP production 1 day after single dose LLL irradiation, DSP-1-7; DPSC assessed for DSP production 7 days after single dose LLL irradiation, DSP-1-14; DPSC assessed for DSP production 14 days after single dose LLL irradiation, DSP-2-1; DPSC assessed for DSP production 1 day after double dose LLL irradiation 48 h apart, DSP-2-7; DPSC assessed for DSP production 7 day after double dose LLL irradiation 48 h apart, DSP-2-14; DPSC assessed for DSP production 14 day after double dose LLL irradiation 48 h apart and, negative control (DSP-0-1, DSP-0-7, DSP-0-14) DPSCs cultured in proliferative medium without LLLI that were assessed in days 1, 7 and 14 for DSP production (Table 1).

LLLI irradiation procedure

Indium-gallium-arsenate-phosphate (InGaAsP) diode laser (AZOR-2 K, Moscow, Russia) was used in this study operating at a continuous wavelength of 660 nm and an adjustable power output from 0 to 200 mW. Initially, we used a digital photometer (Industrial Fiber Optics®, Tempe, AZ, USA) (sensitivity 450–1050 nm) to evaluate the received energy in different diameters of culture well, medium, spot distance, and collateral irradiation conditions. In all experiments, the optical fiber was fixed with a delivery arm to guarantee the irradiation angle of 90° and precise position of 10 mm above the cell monolayer. An aluminum foil lid with 18-mm diameter hole was used to shelter adjacent wells from scattered light. Black backgrounds of irradiated areas were used to minimize light reflections in all experiments.

In the groups with single-dose radiation (MTT-1-1, MTT-1-7, MTT-1-14 and DSP-1-1, DSP-1-7, DSP-1-14 and VEGF-1-1, VEGF-1-7, VEGF-1-14) the laser fiber optic tip was delivered to the top of each well plate and laser was irradiated at power of 100 mW and energy density of 3 J/cm^2 for 15 s. In the groups with double-dose radiation (MTT-2-1, MTT-2-7, MTT-2-14 and DSP-2-1, DSP-2-7, DSP-2-14 and VEGF-2-1, VEGF-2-7, VEGF-2-14) the second irradiation was repeated with identical parameters after 48 h with power of 0.1 watts and energy density of 3 J/cm^2 for 15 s. The irradiation process was done under laminar flow hood (the fluorescent light was switched off) through the aluminum foil lid at room temperature. Between the two laser irradiations the cells were kept in incubator with 5% CO_2 and 95% air at 37°C.

Proliferation assay and dentinogenic/angiogenic differentiation

After the last irradiation cycle the proliferation assay and dentinogenic/angiogenic differentiation were performed as described below at 1, 7 and 14 days post irradiation.

Effect of LLLT on proliferation of DPSCs

The MTT colorimetric reaction assay was used to assess DPSC proliferation and viability. The enzymatic reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (tetrazolium salt) to MTT-formazan is catalyzed by mitochondrial respiration using mitochondrial succinate dehydrogenase. The amount of generated dye due to the activity of dehydrogenase is directly proportional to the number of living cells. DPSCs with concentration of 1×10^4 cells per well were seeded in 96-well plates with 1 mL of DMEM and MTT analysis (Sigma, St. Louis, MO, USA) was performed at 1, 7 and 14 days post-irradiation, following the manufacturer's recommendations [32].

For MTT assay, every three days the culture medium was replaced with 1000 µL of fresh culture medium containing 5 mg/mL of MTT solution (Sigma, St. Louis, MO, USA) in each well. The cells were then incubated at 37°C for 4 h.

After reduction of yellow water-soluble tetrazolium dye (MTT) to purple colored formazan crystals by mitochondrial dehydrogenases, MTT-formazan product was extracted after dissolution in Dimethyl Sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) at 37°C for 15 min at room temperature. Then the plates were wrapped in foil and shake on an orbital shaker for 15 min. Then the absorbance of the formazan solution was read as optical density (OD) using spectrometer (Ocean Optics S2000; Dunedin, Florida, USA) at 450 nm and data were expressed as concentration. The concentration of produced color is proportional to the amount of viable cell that was present in the sample. Results are expressed as Optical Density at 450 nm (OD_{450}) measurements using a microplate reader with a 450 nm filter.

Angiogenic activity of DPSCs; secretion of VEGF

The culture medium was Dulbecco's Modified Eagle Medium (DMEM, High glucose, and glutamine, Gibco, Grand Island, USA) supplemented with 5% fetal bovine serum (FBS, Gibco, USA), 0.1 µM L-glutamine (Invitrogen, Gibco, USA) and 1% penicillin/streptomycin (10000 IU Invitrogen, Gibco, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. The cellular suspension with density of 10^4 cells per mL was made by adding PBS to the plates. After 24-hour incubation in

culture medium laser was irradiated. Secretion levels of VEGF was determined using the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Zellbio ELISA kit for VEGF ZellBio GmbH, Berlin, Germany) on days 1, 7 and 14 post laser irradiation.

For preparation of cell culture supernatant, cell culture media was pipetted into a centrifuge tube and centrifuge at 2000 rpm for 10 min at 4 °C. The supernatants were aliquot to clean, chilled tubes on ice and stored at -80 °C.

For cell content extraction, the tissue culture plates were paced on ice, the medium was aspirated and cells were gently wash once with ice cold PBS-Tween (PBST) three times. Then PBS was aspirated and 0.5 mL per 100 mm plate of complete extraction buffer (PBST with polyvinyl pyrrolidone) was added. The cells were scraped to collect in tilted plate and vortexed briefly then were incubated on ice for 15–30 min. The samples were then centrifuge at 13,000 rpm for 10 min at 4 °C. The supernatant was collected and 40 µL per each well was used for ELISA.

Buffered concentrates were brought to room temperature and diluted before starting the test procedure. The entire contents was poured into a clean 100 mL graduated cylinder with distilled water. Then a 1:100 dilution of the concentrated biotin-conjugate solution was made in a clean plastic tube. Then the Streptavidin-HRP solution was added to all wells. The wells were covered with an adhesive film and incubate at room temperature (18 °C to 25 °C) for 1 h on a microplate shaker. Then the adhesive film was removed. Then 100 µl of tetramethyl benzidine (TMB) substrate solution was added. Samples were kept at room temperature (18 °C to 25 °C) for about 30 min.

When the highest standard has developed a dark blue color and reached an OD of 0.90–0.95, 100 µL stop solution was added into each well. Results were read 10 min after addition of the stop solution. According to the manufacturer's recommendations the absorbance of each well was read by visual observation and spectrophotometric measurement on ELISA reader using 450 nm as the primary wave length. The OD of the samples was converted to ng/mL. The conversion factor of OD_{450} to concentration (ng/mL) was performed using a calibration curve based on the standard protein concentration and the following equation: Concentration of sample (ng/ml) = $OD_{450} \times (0.84 \pm 0.073)/50$.

Table 2 MTT colorimetric reaction assay in different study groups: the mean (SD) of cell viability

	Groups (*N*)	Mean (SD)	*P*-value
Day 1	MTT single dose LLLI (7)	1.40 (0.18)	0.001
	MTT double dose LLLI (7)	1.73 (0.43)	
	MTT no LLLI (7)	1.05 (0.04)	
	Total (21)	1.39 (0.38)	
Day 7	MTT single dose LLLI (7)	1.51 (0.49)	0.025
	MTT double dose LLLI (7)	1.85 (0.67)	
	MTT no LLLI (7)	1.06 (0.14)	
	Total (21)	1.47 (0.57)	
Day 14	MTT single dose LLLI (7)	2.43 (0.33)	0.000
	MTT double dose LLLI (7)	2.68 (0.24)	
	MTT no LLLI (7)	1.32 (0.33)	
	Total (21)	2.15 (0.67)	

Dentinogenic differentiation of DPSCs; secretion of DSP

A number of 1×10^4 DPSCs/mL were seeded in 1 mL of fresh osteogenic medium (0.1 µM dexamethasone, 10 mM β glycerophosphate, and 50 µM ascorbic acid) medium into 96-well plates in two aleatory groups (control and LLLT) and incubated for 24 h at 37°C in a humid atmosphere with 5% CO_2 for adherence. The ELISA procedure identical to VEGF measurement was followed for DSP using the double antibody sandwich enzyme-linked immunosorbent assay (DSP-ELISA) (Zellbio ELISA kit for DSP ZellBio GmbH, Berlin, Germany) on days 1, 7 and 14 post laser irradiation.

Statistical analysis

The proliferation and osteogenic/angiogenic differentiation data were expressed as means with Standard Deviation (±) of at least three independent experiments. A two-way ANOVA test with Tukey's post hoc test was used for comparing the effect of LLLI and time on production of angiogenic (VEGF) and osteogenic (DSP) factors. The One-Way ANOVA was also used for comparison of cell proliferation. All the tests were done using the SPSS software version 20 (SPSS, Inc, Chicago, Ill, USA) with 95% confidence interval ($P = 0.05$).

Results

Effect of LLLT on proliferation of DPSCs

Cell proliferation data from MTT analysis was assessed at one, seven and 14 days after the final session of laser irradiation. Data were analyzed for each group individually and in between groups. For the individual group analysis, the statistical tests were used to compare the different experimental groups at each time point (Tables 2 and 3). From days one to 14, in all study groups with or without LLLI, an increase in cell proliferation over time was observed. However, in groups without LLLI, the increase in cell viability was minimal (Table 2). Analysis of the mean number of cells showed a higher proliferation rate in the single or double irradiated groups when compared to the control group without LLLI, with a statistically significant difference for double LLLI ($P = 0.001$, $P = 0.020$ and $P = 0.000$ for 1, 7 and 14 days, respectively) (Tables 2 and 3).

Briefly, single and double doses of LLLI caused an increased proliferative activity of DPSCs.

Table 3 Two by two comparison of cell viability (MTT assay) in study groups after different days (with Post Hoc tests) (* indicates significant differences)

	Groups (*N*)		Mean Difference (SD)	*P*-value
Day 1	MTT no LLLI	MTT single dose LLLI (7)	-33.59 (13.89)	0.065
		MTT double dose LLLI (7)	-64.78 (13.89)*	0.001
	MTT single dose LLLI	MTT no LLLI (7)	33.59 (13.89)	0.065
		MTT double dose LLLI (7)	-31.19 (13.89)	0.090
	MTT double dose LLLI	MTT no LLLI (7)	64.78 (13.89) *	0.001
		MTT single dose LLLI (7)	31.19 (13.89)	0.090
Day 7	MTT no LLLI	MTT single dose LLLI (7)	-41.96 (24.48)	0.227
		MTT double dose LLLI (7)	-73.67 (24.48) *	0.020
	MTT single dose LLLI	MTT no LLLI (7)	41.96 (24.48)	0.227
		MTT double dose LLLI (7)	-31.70 (24.48)	0.416
	MTT double dose LLLI	MTT no LLLI (7)	73.67 (24.48) *	0.020
		MTT single dose LLLI (7)	31.70 (24.48)	0.416
Day 14	MTT no LLLI	MTT single dose LLLI (7)	-82.96 (12.33) *	0.000
		MTT double dose LLLI (7)	-102.14 (12.33) *	0.000
	MTT single dose LLLI	MTT no LLLI (7)	82.96* (12.33)	0.000
		MTT double dose LLLI (7)	-19.18 (12.33)	0.290
	MTT double dose LLLI	MTT no LLLI (7)	102.14 (12.33) *	0.000
		MTT single dose LLLI (7)	19.18 (12.33)	0.290

Table 4 DSP production in study groups after different days (with Post Hoc tests)

	Groups (*N*)	Mean (SD)	*P*-value
Day 1	DSP no LLLI (7)	1.76 (0.56)	0.000
	DSP single dose LLLI (7)	3.33 (0.88)	
	DSP double dose LLLI (7)	4.56 (0.93)	
	DSP in proliferative medium no LLLI (7)	0.82 (0.07)	
	Total (28)	2.62 (1.60)	
Day 7	DSP no LLLI (7)	2.90 (0.75)	0.000
	DSP single dose LLLI (7)	4.18 (0.63)	
	DSP double dose LLLI (7)	7.46 (1.20)	
	DSP in proliferative medium no LLLI (7)	1.47 (0.66)	
	Total (28)	4.00 (2.39)	
Day 14	DSP no LLLI (7)	4.20 (1.12)	0.000
	DSP single dose LLLI (7)	5.75 (0.66)	
	DSP double dose LLLI (7)	8.45 (0.98)	
	DSP in proliferative medium no LLLI (7)	2.15 (0.59)	
	Total (28)	5.14 (2.48)	

Table 5 Two by two comparison of DSP production in study groups after different days (with Post Hoc tests). (* indicates significant differences)

Groups			Mean Difference (SD)	*P*-value
Day 1	DSP no LLLI	DSP single dose LLLI	-2.50 (0.37) *	0.000
		DSP double dose LLLI	-3.73 (0.37) *	0.000
		DSP in proliferative medium no LLLI	-0.94 (0.37)	0.085
	DSP single dose LLLI	DSP no LLLI	2.50 (0.37) *	0.000
		DSP double dose LLLI	-1.22 (0.37)*	0.016
		DSP in proliferative medium no LLLI	1.56 (0.37) *	0.002
	DSP double dose LLLI	DSP no LLLI	3.73 (0.37) *	0.000
		DSP single dose LLLI	1.22 (0.37) *	0.016
		DSP in proliferative medium no LLLI	2.79 (0.37) *	0.000
	DSP in proliferative medium no LLLI	DSP no LLLI	0.94 (0.37)	0.085
		DSP single dose LLLI	-1.56 (0.37) *	0.002
		DSP double dose LLLI	-2.79 (0.37) *	0.000
Day 7	DSP no LLLI	DSP single dose LLLI	-2.71 (0.45) *	0.000
		DSP double dose LLLI	-5.99 (0.45) *	0.000
		DSP in proliferative medium no LLLI	-1.42 (0.45) *	0.021
	DSP single dose LLLI	DSP no LLLI	2.71 (0.45) *	0.000
		DSP double dose LLLI	-3.28 (0.45) *	0.000
		DSP in proliferative medium no LLLI	1.28 (0.45) *	0.042
	DSP double dose LLLI	DSP no LLLI	5.99 (0.45) *	0.000
		DSP single dose LLLI	3.28 (0.45) *	0.000
		DSP in proliferative medium no LLLI	4.56 (0.45) *	0.000
	DSP in proliferative medium no LLLI	DSP no LLLI	1.42 (0.45) *	0.021
		DSP single dose LLLI	-1.28 (0.45) *	0.042
		DSP double dose LLLI	-4.56 (0.45) *	0.000
Day 14	DSP no LLLI	DSP single dose LLLI	-3.60 (0.46) *	0.000
		DSP double dose LLLI	-6.30 (0.46) *	0.000
		DSP in proliferative medium no LLLI	-2.05 (0.46) *	0.001
	DSP single dose LLLI	DSP no LLLI	3.60 (0.46) *	0.000
		DSP double dose LLLI	-2.69 (0.46) *	0.000
		DSP in proliferative medium no LLLI	1.55 (0.46) *	0.013
	DSP double dose LLLI	DSP no LLLI	6.30 (0.46) *	0.000
		DSP single dose LLLI	2.69 (0.46) *	0.000
		DSP in proliferative medium no LLLI	4.25 (0.46) *	0.000
	DSP in proliferative medium no LLLI	DSP no LLLI	2.05 (0.46) *	0.001
		DSP single dose LLLI	-1.55 (0.46) *	0.013
		DSP double dose LLLI	-4.25 (0.46) *	0.000

Dentinogenic differentiation of DPSCs by secretion of DSP

Dentinogenic differentiation of DPSCs through production of DSP was assessed with ELISA at one, seven and 14 days with or without laser irradiation (Tables 4, 5 and 6). In all of the studied time points and all study groups, there was an increase in DSP. In groups without LLLI, after 7 and 14 days increased amount of DSP was observed which was significant between 1 and 14 days ($P=0.00$). Mere presence of DPSCs in osteogenic

Table 6 Comparison of DSP production in study groups in different days (Tukey HSD). (* indicates significant differences)

Groups	Days		Mean Difference (SD)	*P*-value
DSP no LLLI	Day 1	Day 7	-0.64 (0.27)	0.077
		Day 14	-1.32 (0.27) *	0.000
	Day 7	Day 1	0.64 (0.27)	0.077
		Day 14	-0.67 (0.27)	0.062
	Day 14	Day 1	1.32 (0.27) *	0.000
		Day 7	0.67 (0.27)	0.062
DSP single dose LLLI	Day 1	Day 7	-0.85 (0.39)	0.107
		Day 14	-2.42 (0.39) *	0.000
	Day 7	Day 1	0.85 (0.39)	0.107
		Day 14	-1.57 (0.39) *	0.002
	Day 14	Day 1	2.42 (0.39) *	0.000
		Day 7	1.57 (0.39) *	0.002
DSP double dose LLLI	Day 1	Day 7	-2.90 (0.55) *	0.000
		Day 14	-3.89 (0.55) *	0.000
	Day 7	Day 1	2.90 (0.55) *	0.000
		Day 14	-0.98 (0.55)	0.209
	Day 14	Day 1	3.89 (0.55) *	0.000
		Day 7	0.98 (0.55)	0.209
DSP in proliferative medium no LLLI	Day 1	Day 7	-1.13 (0.45)	0.054
		Day 14	-2.43 (0.45) *	0.000
	Day 7	Day 1	1.13 (0.45)	0.054
		Day 14	-1.30 (0.45) *	0.026
	Day 14	Day 1	2.43 (0.45) *	0.000
		Day 7	1.30 (0.45) *	0.026

Table 7 VEGF production in study groups after different days (with Post Hoc tests)

	Groups (*N*)	Mean (SD)	*P*-value
Day 1	VEGF no LLLI (7)	0.19 (0.01)	0.030
	VEGF single dose LLLI (7)	0.20 (0.01)	
	VEGF double dose LLLI (7)	0.21 (0.01)	
	Total (21)	0.20 (0.01)	
Day 7	VEGF no LLLI (7)	0.22 (0.01)	0.018
	VEGF single dose LLLI (7)	0.24 (0.01)	
	VEGF double dose LLLI (7)	0.27 (0.04)	
	Total (21)	0.24 (0.03)	
Day 14	VEGF no LLLI (7)	0.28 (0.03)	0.000
	VEGF single dose LLLI (7)	0.32 (0.04)	
	VEGF double dose LLLI (7)	0.41 (0.05)	
	Total (21)	0.34 (0.07)	

medium led to an increased level of DSP with or without LLLI in comparison with control cells in proliferative medium albeit being statistically not different ($P = 0.085$).

After one, 7 and 14 days, cells in single and double LLLI showed significant increase in DSP ($P > 0.05$) (Tables 4 and 5).

In groups with single-dose LLLI, similar increased amount of DSP was evident after 7 and 14 days which was significant between 1 and 14 days ($P = 0.00$). In cells with double doses of LLLI the increase in DSP was significant from days 1 to 7 or 14 ($P = 0.00$ for both) (Table 6).

It can be assumed that single and double doses of LLLI caused an increase in dentinogenic differentiation of DPSCs in osteogenic medium.

Table 8 Comparison of VEGF production in study groups in different days (Tukey HSD). (* indicates significant differences)

	Groups		Mean Difference (SD)	*P*-value
Day 1	VEGF no LLLI	VEGF single dose LLLI	-0.00 (0.00)	0.389
		VEGF double dose LLLI	-0.01 (0.00) *	0.023
	VEGF single dose LLLI	VEGF no LLLI	0.00 (0.00)	0.389
		VEGF double dose LLLI	-0.01 (0.00)	0.281
	VEGF double dose LLLI	VEGF no LLLI	0.01 (0.00) *	0.023
		VEGF single dose LLLI	0.01 (0.00)	0.281
Day 7	VEGF no LLLI	VEGF single dose LLLI	-0.02 (0.01)	0.320
		VEGF double dose LLLI	-0.05 (0.01) *	0.014
	VEGF single dose LLLI	VEGF no LLLI	0.02 (0.01)	0.320
		VEGF double dose LLLI	-0.02 (0.01)	0.234
	VEGF double dose LLLI	VEGF no LLLI	0.05 (0.01) *	0.014
		VEGF single dose LLLI	0.02 (0.01)	0.234
Day 14	VEGF no LLLI	VEGF single dose LLLI	-0.03 (0.02)	0.347
		VEGF double dose LLLI	-0.13 (0.02) *	0.000
	VEGF single dose LLLI	VEGF no LLLI	0.03 (0.02)	0.347
		VEGF double dose LLLI	-0.09 (0.02) *	0.003
	VEGF double dose LLLI	VEGF no LLLI	0.13 (0.02) *	0.000
		VEGF single dose LLLI	0.09 (0.02) *	0.003

Angiogenic activity of DPSCs by secretion of VEGF

Angiogenic activity of DPSCs through production of VEGF was assessed with ELISA at one, seven and 14 days with or without laser irradiation. In all of the studied days, increased amount of VEGF in each study group was evident (Tables 7, 8 and 9). After one day, single and double doses of LLLI caused an increase in VEGF which was significant with double doses of LLLI compared with no laser irradiation ($P = 0.02$). After seven days, double-dose LLLI significantly elevated VEGF production in comparison with no LLLI ($P = 0.01$) but the increase was not significant for single dose LLLI ($P = 0.23$). After 14 days, double-dose LLLI showed increased VEGF compared to no LLLI ($P = 0.000$) or single dose LLLI ($P = 0.003$).

As a result, single and double doses of LLLI caused an increase in angiogenic differentiation of DPSCs.

Discussion

The purpose of this study was to evaluate the effect of single and double doses of LLLT with 660 nm diode on proliferation and cellular activity of human DPSCs through MTT assay, and their angiogenic and dentinogenic differentiation through measuring the amount of secreted VEGF and DSP, respectively. The results indicated an

Table 9 Comparison of VEGF production in study groups in different days (Tukey HSD). (* indicates significant differences)

Groups	Days		Mean Difference (SD)	*P*-value
VEGF no LLLI	Day 1	Day 7	-0.02 (0.01)	0.100
		Day 14	-0.091 (0.01) *	0.000
	Day 7	Day 1	0.02 (0.01)	0.100
		Day 14	-0.06 (0.01) *	0.000
	Day 14	Day 1	0.09 (0.01) *	0.000
		Day 7	0.06 (0.01) *	0.000
VEGF single dose LLLI	Day 1	Day 7	-0.04 (0.01) *	0.029
		Day 14	-0.11 (0.01) *	0.000
	Day 7	Day 1	0.04 (0.01) *	0.029
		Day 14	-0.07 (0.01) *	0.000
	Day 14	Day 1	0.11 (0.01) *	0.000
		Day 7	0.07 (0.01) *	0.000
VEGF double dose LLLI	Day 1	Day 7	-0.05 (0.02)	0.072
		Day 14	-0.20 (0.02) *	0.000
	Day 7	Day 1	0.05 (0.02)	0.072
		Day 14	-0.14 (0.02) *	0.000
	Day 14	Day 1	0.20 (0.02) *	0.000
		Day 7	0.14* (0.02)	0.000

overall increased level of VEGF and DSP production and cell proliferation with double doses of LLLI and with time dependent increasing pattern. Thus the null hypothesis was confirmed.

Tissue engineering has three basic requirements; stem cells, growth factors and scaffold [12]. While most studies on REP have focused on the scaffold and growth factors [34–36], less investigation can be found on pretreatment or conditioning of stem cells. The first requirement can be taken from the DPSCs. Dental pulp is a highly vascularized gelatinous soft connective tissue with mesenchymal origin [3, 13] and high inherent regenerative potential [13]. Dental mesenchymal stem cells (DMSCs) are spindle-shaped cells [37, 38] with characteristic stem cell markers (CD44, CD90 and CD105). Postnatal human DMSCs have high proliferative capacity and unique multi-differentiation potential [38–40] and give rise to both mesenchymal and non-mesenchymal cells like osteoblastic, adipogenic, and chondroblastic linages [11] and neuronal and endothelial cells [41]. DMSCs from dental pulp of adult teeth are called dental pulp stem cells-DPSCs [38]. These cells can also differentiate into functional odontoblasts and be used as a source for dental pulp tissue engineering [42].

In the present study, LLLI increased the amount of VEGF and DSP produced by DPSCs and this increase was more for double doses of LLLI. The results of this experiment indicated that LLLI can be useful in pulp tissue engineering by means of preconditioning of DPSCs before regenerative endodontic procedure and vital pulp therapy. This is due to increased production of VEGF (as an indicator and promoter of vascularization in REP) [3] and DSP (as the phenotypic marker of odontoblastic differentiation of DPSCs in VPT and REP) [8].

In many studies it has been shown that LLLI leads to higher vascularity by causing vasodilation of capillaries, thus promoting local circulation, and raising the level of tissue oxygenation, resulting in a significant increase in tissue metabolism and regeneration [43]. This phenomenon is primarily attributed to mitochondrial photochemical/photophysical process and oxidative change that leads to cell viability. Additionally, increased cell proliferation happens as a consequence of DNA synthesis [44]. LLLI photochemical mechanism of action is not related to thermal effects. LLLI can accelerate tissue remodeling and wound healing by concomitant increase in fibroblast growth factor and decrease in pro-inflammatory mediators [45].

Pioneer studies suggest that LLLI decreases the formation of necrotic tissue in skin flap surgery, which is attributed to enhanced angiogenesis, elevated vascular density, increased number of mast cells, and VEGF [46]. Also LLLI is reported to decrease wound surface area in diabetic patients, suggesting effective improvements in angiogenesis [47, 48]. In periodontics, LLLI caused increased VEGF gene expression on human gingival fibroblasts in-vitro [49]. In an animal study, after LLLI, cell proliferation and VEGF expression were significantly elevated which was due to enhancing angiogenic effect of endothelial cells [50].

In dental pulp, vasculature is the earliest functional system formed by vasculogenesis during embryonic development [51]. In adult life, new blood vessels are mainly formed by angiogenesis, which is the sprouting of new vessels from existing ones [52]. Both embryonic vasculogenesis and adulthood angiogenesis are controlled by angiogenic growth factors [11]. During vasculogenesis of a developing tooth, mesoderm-derived precursors of endothelial cells invade the papilla during bell stage, where they aggregate to form vascular structures. As tissue oxygen diffusion is limited to 100 to 200 µm, vascular network is required to ensure all cells to be within this distance [19]. When tissue grows and exceeds 200 µm in size, hypoxia can be a driving force for DPSCs for secretion of proangiogenic factors that mediates transcription of VEGF [53]. VEGF seems to play a pivotal role in endothelial differentiation of DPSCs. Indeed, supplementing the in vitro culture medium with VEGF, led to endothelial differentiation of DPSCs [54], and mice fibroblast cells [20]. Furthermore, DPSCs express VEGFR-2, the receptor for VEGF [55]. As indicated by different surface markers, DPSCs synergistically differentiate into osteoblast-like and endothelial-like cells [56]. It can be concluded that DPSCs are not only recruited for lost tissue regeneration but may also be involved in this angiogenic

process. As indicated by the results of the current study, LLLI increases the production of VEGF by DPSCs.

In REP and VPT, attempts have been made to mimic dentinogenesis [57]. In Many studies the occurrence of tubular structure in the newly formed hard tissue in teeth with superficial pulp amputation and capping with different biomaterials was considered as dentinogenesis [58–60]. Likewise, the polarized or columnar cells lining the hard tissue are labeled as secondary odontoblasts or odontoblast like cells [61, 62]. However, in order to define dentine and odontoblasts properly, factors such as molecular content of the tissue and functions of the cell should be taken in account [8]. Dentine sialoprotein (DSP) is member of the Small Integrin-Binding LIgand N-linked Glycoprotein, *aka*, SIBLING family. Expression of DSP can be an indicator of an odontoblastic differentiation marker and dentinogenic phenotype [8, 63]. In different studies, DSP is suggested to be a relatively specific marker for dentinogenesis and late odontoblast-like differentiation [10, 14, 63]. Laser application as an adjunct for VPT can express antibacterial, bio-stimulation, hemostatic, and wound healing [64, 65], and caused increased amounts of lectin and collagen [43]. With adequate parameters, LLLI stimulates cell proliferation and differentiation, mitochondrial respiration, protein synthesis, and bone formation in human periodontal ligament stem cells, fibroblasts, and odontoblasts. Also DPSCs respond positively to LLLI [45].

In two separate studies using the MTT assay, it was showed that LLLI with 940 nm diode laser increased the osteogenic differentiation and expression of osteogenic/odontogenic genes, including bone sialoprotein, alkaline phosphatase, dentin matrix protein 1, and dentin sialophosphoprotein, in DPSCs [66] and stem cells of apical papilla (SCAP) [67]. In the present in vitro analysis, application of single or double identical doses of LLLI with 660-nm diode and 100 mW power output and energy density of 3 J/cm^2 for 15 s on human dental pulp stem cells, induced secretion of DSP which is an indicator of intentional leading of DPSCs to differentiation to secondary odontoblast and dentinogenesis. Also this enhanced the mitochondrial activity by reduction of MTT of DPSCs which is a sign of cellular proliferation. Also increased production of VEGF was observed. Here, the improvement in stem cell proliferation and upregulation of DSP could be of importance in the future use of these cells for tissue regenerating therapy in dentistry and medicine [68]. However, reduction in survival of hDPSCs according to MTT assay was reported after LLLI with 940 nm diode laser [66], which is in contrast to the results of the present study. Another study reported LLLT as a stimulator of SCAPs cell viability when applied in combination with dental capping agents [69]. This controversial results can be attributed to different LLLI wavelengths. LLTI exerts regenerative effects [22]. However, many parameters influence the effect of LLLI, one of which is the tissue penetration depth [70]. The absorption of laser in any given tissue, depend on its wavelength [43]. Eduardo et al. [68] and Marques et al. [71], demonstrated that lasers with higher energy densities and a shorter period of time, caused greater viability and proliferation of pulp fibroblasts. Applied dose plays a positive role in cell growth in vitro [68, 71]. The reason is that the heating of the chromophore over longer irradiation periods may cause cytochrome-c oxidase inhibition [72].

In this regard, irradiation parameters such as energy density, power density, and irradiation mode (continuous or pulsed) are important but the most detrimental single parameter is the wavelength [4, 32]. Many literature on beam measurement and dose calculation is available but no guidelines are present setting out the minimum standards for reporting beam parameters and dose [73]. Some experiments have been done on DMSCs with diode lasers with different wavelengths of 660 nm [4, 68, 74, 75], 810 nm [33], and 980 nm [76]. The power output ranged from 28 to 100 mW. In most studies the cellular proliferation was measured by mitochondrial activity through the MTT reduction-based assay [4, 68, 74, 75]. Another factor in LLLI is the distance. Different studies have used variable distances between the laser spot and the bottom of the plate containing cells or above the cellular layer: 1.5 mm from the bottom [75], 20 mm [33] and 1 mm [76] above the cell layer. The laser administration regimen is not the same for all studies. Parameters such as energy output, energy density, wavelength, and laser exposure, the type of laser, emission mode and time used can influence the efficacy of LLLI [73]. Due to this heterogeneity, it is not possible to compare the detailed results of these studies. However, it has been shown that LLLI increases cell proliferation in different cell lines including adult stem cells [12]. LLLI modulates the process of tissue repair through stimulation of cellular migration, proliferation, apoptosis, and differentiation and secretion of VEGF [22, 31, 76, 77]. The mechanism by which low intensity lasers induce biomodulation of cell activity has been well described. Laser irradiation intensifies the formation of a transmembrane electromechanical proton gradient in mitochondria [78].

In previous studies, the LLLT has been effective in pain reduction, wound healing, bone repair and remodeling, nerve repair, as well as increased cell proliferation [12]. Moreover, biomodulation for cell lines such as osteoblasts have been reported that increased their proliferation but not adhesion and osteonectin synthesis [79].

DPSCs can clearly be considered an essential part of the angiogenic process during dentin-pulp tissue regeneration. LLLI can improve the desired cellular differentiation

proliferation. More research is, however, required to fully establish the role of LLLI parameters in this process.

Conclusion

According to the results single or double doses of LLLI with 660-nm diode laser, can enhance the mitochondrial activity and proliferation of DPSCs. Also increased production of DSP and VEGF was observed that is an indicator of dentinogenic activity and angiogenesis.

Take-away lessons:

- LLLI increases the proliferation of DPSCs.
- Double identical doses of LLLI increases the production of VEGF by DPSCs.
- Double identical doses of LLLI increases the production of DSP by DPSCs.
- Two doses of LLLI have better results but one dose is also acceptable.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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As the corresponding author, I Dr. Mahta Fazlyab, deny any conflict of interest.

Author contributions

FR and ShSh: Were responsible for the payments and conducted all cellular procedure. MF: Generated the idea of the study, guided the procedure and wrote the manuscript. EE: Provided the laser device and supervised the procedure. SS: Provided the laboratory needs and supervised the procedure. As the corresponding author, I Dr. Mahta Fazlyab declare that all authors have contributed significantly and all authors are in agreement with the manuscript.

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Data availability

Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate

This experimental in vitro study was approved by the Research Ethics Committee at Islamic Azad University, Tehran medical sciences, Dental Branch, Tehran, Iran.

Study protocol approval

After approval of the experimental in vitro study protocol by the ethics committee at Faculty of Dentistry Islamic Azad University and Iran National Committee for Ethics in Biomedical Research (https://ethics.research.ac.ir/IR.IAU.DENTAL.REC.1401.120) and (https://ethics.research.ac.ir/IR.IAU.DENTAL.REC.1401.055) the study was conducted.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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