Lasers in Medical Science (2019) 34:1689–1698
https://doi.org/10.1007/s10103-019-02804-6

ORIGINAL ARTICLE

Low-level laser therapy affects dentinogenesis and angiogenesis of in vitro 3D cultures of dentin-pulp complex

Hisham El Nawam [1] · Rania El Backly [1,2] · Amira Zaky [3] · Amr Abdallah [1]

Received: 8 October 2018 / Accepted: 7 May 2019 / Published online: 27 May 2019

Abstract

To investigate the effects of gallium-aluminum-arsenide (GaAlAs) diode laser low-level laser therapy (LLLT) on angiogenesis and dentinogenesis of the dentin-pulp complex in a human tooth slice-based in vitro model. Forty tooth slices were prepared from 31 human third molars. Slices were cultured at 37 °C, 5% CO_2, and 95% humidity and randomly assigned to one of the following groups: group I: no laser treatment, group II: 660-nm diode laser; energy density = 1 J/cm^2, group III: 660-nm diode laser; energy density = 3 J/cm^2, group IV: 810-nm diode laser; energy density = 1 J/cm^2 and group V: 810-nm diode laser; energy density = 3 J/cm^2. LLLT was applied on the third and fifth days of culture. After 7 days, tissues were retrieved for real-time RT-PCR analysis to investigate the expression of VEGF, VEGFR2, DSPP, DMP-1, and BSP in respect to controls. Lower energy density (1 J/cm^2) with the 660 nm wavelength showed a statistically significant up-regulation of both angiogenic (VEGF: 15.3-folds and VEGFR2: 3.8-folds) and odontogenic genes (DSPP: 6.1-folds, DMP-1: 3-fold, and BSP: 6.7-folds). *While* the higher energy density (3 J/cm^2) with the 810 nm wavelength resulted in statistically significant up-regulation of odontogenic genes (DSPP: 2.5-folds, DMP-1: 17.7-folds, and BSP: 7.1-folds), however, the angiogenic genes had variable results where VEGF was up-regulated while VEGFR2 was down-regulated. Low-level laser therapy could be a useful tool to promote angiogenesis and dentinogenesis of the dentin-pulp complex when parameters are optimized.

Keywords Low-level laser therapy · Tooth slice model · Dentin-pulp complex · Angiogenesis · Dentinogenesis

Introduction

Complete removal of pulp tissue followed by conventional root canal treatment is the routine treatment of choice in clinical practice once complete pulp tissue necrosis is diagnosed [1]. However, it has been reported that root canal treatment reduces the strength of teeth and increases the risk of tooth fracture [2]. Moreover, with the recent improvements in understanding the regenerative capacity of the dentin-pulp complex and the advancement of new materials and techniques, attention has been given to more conservative and minimally invasive treatment options [3]. Photobiomodulation (PBM), also known as low-level laser therapy (LLLT), has been used in many branches of medicine and dentistry for the last three decades where it was found to enhance wound healing [4, 5], reduce pain and inflammation [4], and promote tissue repair [6]. LLLT refers to the use of red or near infrared lasers, with wavelengths ranging between 600 and 1100 nm and an output power from 1 to 500 mW [7]. This irradiation is either in a continuous wave mode or pulsed irradiation and these parameters result in a laser beam with a relatively low energy density ranging from 0.04 to 50 J/cm^2 [7–9]. The transmitted energy beam has been shown to have no significant thermal effects on tissues [10].

It has been found in various studies that LLLT can promote cellular proliferation [11–13]; differentiation [11, 14, 15]; and odontogenic, osteogenic, and angiogenic gene expression [16,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10103-019-02804-6) contains supplementary material, which is available to authorized users.

✉ Hisham El Nawam
hisham_m_2011@hotmail.com

[1] Conservative Dentistry Department, Endodontics, Faculty of dentistry, Alexandria University, Champolion Street, Azarita, Alexandria, Egypt

[2] Tissue engineering labs, Faculty of dentistry, Alexandria University, Alexandria, Egypt

[3] Department of biochemistry, Faculty of science, Alexandria University, Alexandria, Egypt

17]. However, there is a clear heterogeneity of the parameters of laser irradiation in current literature, making it necessary for further research in order to identify the optimal parameters of LLLT regarding the wavelength, energy density, power output, duration of irradiation, time intervals between irradiations, and distance from the irradiated tissues [18, 19].

Despite the positive influence that LLLT is reported to possess on dental cells, it has also been shown that the response to LLLT may vary according to the nature of the cells and tissues [7]. Additionally, most studies have focused on the use of in vitro cell culture models which represent single-cell components that is different from the complex nature of the clinical situation [19]. Therefore, it is crucial to employ more sophisticated in vitro models such as the tooth-slice 3D culture model to better simulate the clinical condition by allowing the dental pulp cells to remain in their original niche [20].

Hence, the aim of this study was to evaluate the expression of angiogenic and odontogenic genes following low-level laser therapy (LLLT), using gallium-aluminum-arsenide (GaAlAs) diode laser with two different wavelengths, 660 nm and 810 nm, in a human tooth slice-based model.

Materials and methods

Preparation of tooth slices

A total of 31 non-carious maxillary or mandibular third molars requiring extraction for orthodontic reasons or due to impaction were collected from the outpatient clinic of the Oral Surgery Department, Faculty of Dentistry, Alexandria University and other private hospitals.

To minimize the heterogeneity of samples, age range was restricted from 20 to 35 (mean age was 24.4). Moreover, before extraction, teeth were evaluated clinically and radiographically to be free from caries, periodontal involvement, or calcification. Additionally, none of the teeth had been subjected to trauma and none displayed any genetic morphological anomalies.

All procedures were done in accordance with the Ethics Research Committee, Faculty of Dentistry, Alexandria University (IRB NO: 00010556–IORG: 0008839) and in accordance with the 1964 Declaration of Helsinki. Informed consent was obtained from all patients.

Following the protocol described by Murray et al. [21] and Goncalves et al. [22], immediately after extraction, teeth were placed in sterile wash/transport medium composed of Dulbecco's Modified Eagle's Medium (DMEM) with 0.584 g/l L-glutamine, 10,000 IU/ml penicillin, 10,000 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and 2.5 μg/ml Amphotericin B (Lonza Walkersville Inc., Walkersville, MD, USA) at room temperature.

After several washes, attached gingival and periodontal ligament tissues were removed using sterile periodontal curettes (Hu-Friedy Co., Chicago, USA), followed by surface disinfection by 70% ethanol. (Fig. 1a).

Each tooth received two to three parallel transverse cuts at the level of the cemento-enamel junction (CEJ). The second and third cuts were immediately apical to the first where the first cut was made 1 mm coronal to the CEJ. The level of cutting was done according to a measurement done on each tooth by measuring the distance between the cemento-enamel junction (CEJ) and the coronal portion of root furcation on the trunk of the tooth. If the distance between the CEJ and the furcation was 2 mm, only one slice was obtained by making two transverse cuts. On the other hand, if the distance was 3 mm or more, two slices were obtained from the tooth by making three transverse cuts. Therefore, the slices were obtained only from the area of the CEJ and immediately apical or coronal to it in order to minimize any variability that may arise due to anatomical differences in the dentinal tubules and pulp tissue further down apically in the tooth. Cutting was performed using a low speed diamond disk (Horico Dental, Berlin, Germany) mounted on a low speed micro-motor (Saeshin Precision Co. Ltd., Daegu, Korea) and cooled by cold phosphate-buffered saline (PBS) (Lonza Walkersville Inc., Walkersville, MD, USA) (Fig. 1b). Each slice was verified to be 1 mm in thickness.

Representative slices were evaluated by light microscopy to confirm that the cutting procedure did not cause tissue detachment at the dentin-pulp interface (Fig. 1c). The slices that were further processed in the study were not evaluated under light microscopy to maintain sterility of the culture conditions prior to testing.

Preparation of 3D organ cultures

Each tooth slice was immediately placed into 1 well of a 6-well plate containing 3 ml of wash/transport medium of Dulbecco's Modified Eagle's Medium (DMEM) containing 0.584 g/l L glutamine, 10,000 IU/ml penicillin, 10,000 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and 2.5 μg/ml Amphotericin B (Lonza Walkersville Inc., Walkersville, MD, USA). Washing was repeated three times for each slice for 5 min each time [21, 22].

Each tooth slice was then placed in 1 well of a 24-well plate, containing 1 ml of the previously mentioned culture medium additionally supplemented with 15% fetal bovine serum (Lonza Walkersville Inc., Walkersville, MD, USA) (Fig. 1d).

Tooth slices were cultured for a total of 7 days at 37 °C in an atmosphere of 5% CO_2 in air, in a humidified incubator, and the medium changed after 24 h and every 2 days afterwards [21].

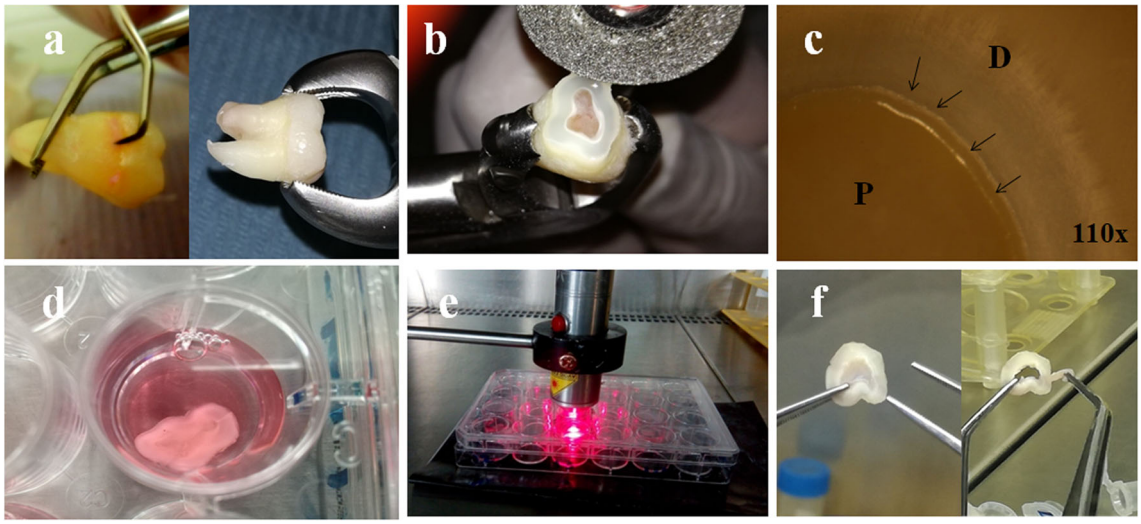

Fig. 1 Preparation of tooth slices and 3D cultures, low-level laser therapy, and tissue retrieval for qRT-PCR. **a** Cleaning the tooth immediately after extraction using sterile periodontal curettes followed by surface disinfection using 70% ethanol, then firmly grapping the tooth and preparing it for slicing. **b** Horizontally cutting the tooth to obtain 1-mm-thick slice just apical to CEJ, using a diamond disk mounted on a low speed micromotor, while cold PBS was used for cooling. **c** Inspection of tooth slice under stereomicroscope with 110 magnification to its original size to verify the integrity of pulp "P" and dentin "D" and the interface between them (arrows). **d** Each slice was placed into a well of 24-well plate containing 1 ml of culture medium, slices were plated in such a way leaving an empty well between cultured slices to prevent unintentional delivery of laser between wells during irradiation. **e** Laser irradiation was carried out in partial darkness under safety cabinet in sterile conditions, a black paper was placed underneath the culture plate to decrease backscattering of laser beam and laser probe was fixed at 2 cm distance from irradiated samples. **f** Retrieval of tissues from all groups after 7 days of incubation followed by separating pulp tissue from dentin using sterile tweezers and preparation for qRT-PCR

On the third day of culture and again on the fifth day, tooth slices received low-level laser treatments (LLLT) as explained below [12, 14]. After 7 days of culture, samples were retrieved for gene expression analysis.

Demographic data of all samples analyzed in the current study are provided in Table 3 including the age and sex of patients, tooth number and number of slices obtained from each tooth sample.

Low-level laser irradiation

The LLL probe was held perpendicular to each plate at a fixed distance of 2 cm away from the irradiated sample. Tooth slices were plated in such a way that one well, between the experimental wells, was left empty to prevent the unintentional delivery of laser between wells during irradiation [12] (Fig. 1e).

The evaluation of power output was assessed before each experiment using a power meter (Melles Griot, Albuquerque, NM, USA) by measuring the actual power delivered 2 cm away from sample and after passing through the plastic cover of the 24-well plate.

Slices were randomly assigned to four experimental groups and one control group as follows:

Group I ($n = 8$) served as the control group which received no laser treatment but were removed from the incubator and exposed to the same environment for the same duration as the laser-treated samples.

Group II ($n = 8$): LLLT using gallium-aluminum-arsenide (GaAlAs) diode laser (Changchun New Industries Co. Ltd. (CNI), China) with a wavelength of 660 nm, delivering an energy density of 1 J/cm^2 [12, 13, 17].

Group III ($n = 8$): LLLT with the same device but with energy density of 3 J/cm^2 [12, 13, 17].

Group IV ($n = 8$): LLLT using GaAlAs diode laser (Changchun New Industries Co. Ltd. (CNI), China) that has a wavelength of 810 nm, delivering an energy density of 1/cm^2 [14, 16, 23].

Group V ($n = 8$): LLLT again using the same 810-nm diode laser but with energy density of 3 J/cm^2 [14, 16, 23].

Laser irradiation was performed in dark conditions in a biological safety cabinet and was in continuous irradiation mode; a black paper was placed underneath the plate to absorb laser beam and decrease back scattering. During irradiation, the laser probe was fixed on top of the plastic cover of the 24-well plate to standardize the distance. The irradiation was done with no medium being present in the well to decrease any chance of absorption of energy by the medium; however, samples were never allowed to dry-out (Fig. 1e).

Energy densities were calculated using the following formula:

$$\text{Energy density (fluence)}\ \left(\text{J/cm}^2\right) = [\text{power (W)} \times \text{time (s)}]/\text{area}\ \left(\text{cm}^2\right).$$

Spot sizes of laser beams over the areas being irradiated were 0.283 cm^2 and 0.18 cm^2 for the 660-nm and 810-nm lasers, respectively. These spot sizes were corresponding to the irradiated areas within the slices taking into consideration that the average pulp tissue area in the slices was 0.12 cm^2 (± 0.02). Power output of both laser devices was adjusted at 30 mW. The irradiation time was calculated accordingly, to deliver these energy densities over the irradiated areas. Laser parameters are displayed in Table 1.

Total RNA isolation and quantitative reverse transcriptase polymerase chain reaction

After 7 days, tissues from tooth slices were collected and processed for real-time PCR to evaluate the expression of angiogenic and odontogenic/osteogenic markers including vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor-2 (VEGFR2), dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP1), and bone sialoprotein (BSP) (Fig. 1f).

Total ribonucleic acid RNAs were extracted using Biozol extraction kit according to the manufacturer's guidelines [24]. A total of 400 μl of Biozol (Hangzhou Bioer Technology Co., Ltd., China) was added to each sample for homogenization, incubated for 15 min in ice; then, 100 μl of chloroform was added, incubated for another 15 min in ice. It was then centrifuged at 12,000 rpm for 15 min at 4 °C using Hettich Mikro 200R refrigerated centrifuge (DJB Labcar Ltd., Buckinghamshire, England).

The upper aqueous layer was transferred to a fresh tube and the same volume of cold isopropyl alcohol was added and the tube incubated at − 20 °C for 25 min. The mix was centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was discarded. Washing with 400–700 μl of cold 75% ethanol and centrifuged at 12,000 rpm for 5 min at 4 °C. The last two steps were repeated for three times. RNA pellets were left to dry for 30 min then dissolved with 50 μl of RNase-free water.

A spectrophotometer (Optizen, Mecasys Co., Ltd., Daejeon, Korea) was used to quantify RNA concentrations by measuring the absorbance at 260 nm and 280 nm for detection of protein contamination. First-strand complementary deoxyribonucleic acid cDNA was then reverse transcribed according to the manufacturer's instructions for cDNA synthesis kit (HiSenScriptRH[-], Intron biotechnology, Gyeonggi-do, Korea).

Quantitative real-time PCR was performed using Eco Real-Time PCR system (Illumina Inc., San Diego, CA, USA). PCR reactions were performed in 10-μl solution containing cDNA, forward and reverse primers, and EvaGreen qPCR Mix (Solis BioDyne OÜ Riia, Tartu, Estonia) (Fig. 2i).

The sequences of primers used are specified in Table 2.

Quantitative real-time polymerase chain reaction was carried out in duplicate for all groups. Threshold cycle (C_t) values of all samples were retrieved from Eco Real-Time PCR Software v4.1.2.0. Data was calculated based on the C_t values and normalized to a housekeeping gene (β-actin).

Afterwards, fold change (relative expression) was calculated using the following formula: ((2 ^ (−ΔΔCt))) in relation to the control group (Table 3).

Statistical analysis

Data was statistically analyzed by a statistician who was blinded to the allocation of samples within different groups. The two-tailed Student's *t* test was performed using graphpad software (https://www.graphpad.com/). The results were expressed as the mean ± standard deviation. *P* value less than 0.05 was considered statistically significant (Supplementary Table 1).

Results

Evaluation of angiogenic potential

Samples treated by 660 nm wavelength showed more significant up-regulation of angiogenic genes where VEGF expression was increased by 15.3-folds using the lower energy density (1 J/cm^2) which was statistically significant (*P* value < 0.0001), while the higher energy density (3 J/cm^2) caused only a 3.2-fold increase. However, it was also statistically

Table 1 Low-level laser parameters used in the study and sample grouping

Group	Number of slices obtained	Number of slices processed	Wavelength (nm)	Energy density (J/cm^2)	Power (mW)	Time (s)
Group I (control)	11	8	–	–	–	–
Group II	10	8	660	1	30	9.4
Group III	10	8	660	3	30	28.3
Group IV	11	8	810	1	30	6
Group V	10	8	810	3	30	18

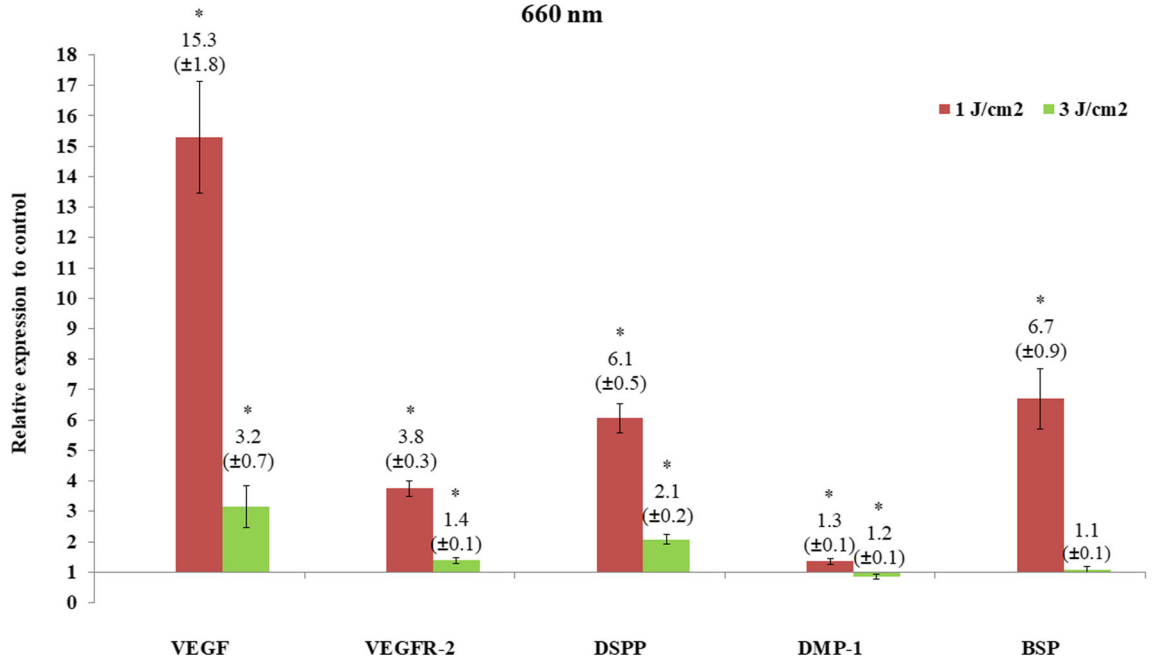

Fig. 2 Real-time PCR analysis of samples treated with 660-nm laser values are means (±SD) representing the fold change in angiogenic and odontogenic/osteogenic gene expression in relation to control. Asterisks indicate statistically significant differences in fold change relative to control group

significant (P value < 0.0001). VEGFR-2 was also up-regulated by the lower energy density (1 J/cm^2) by 3.8-folds. As for the higher energy density (3 J/cm^2), this gene was also up-regulated by 1.4-folds. Both of them were increased to a statistically significant level (P value < 0.0001) (Figs. 2 and 4).

On the other hand, LLLT of samples with 810 nm resulted in less satisfactory results where VEGF expression had a statistically significant up-regulation by 3-folds and 2.8-folds for the lower (1 J/cm^2) and higher (3 J/cm^2) energy densities, respectively. However, VEGFR-2 was down-regulated by 2- and 1.8-folds in the 1 J/cm^2 and the 3 J/cm^2 groups, respectively. This down-regulation was statistically significant (Figs. 3 and 4).

Evaluation of odontogenic/osteogenic potential

Samples treated by the wavelength of 660 nm showed higher up-regulation with the lower energy density (1 J/cm^2) where DSPP expression showed a statistically significant increase (P value < 0.0001) by 6.1-folds and 2-folds with the use of the lower energy density (1 J/cm^2) and the higher energy density (3 J/cm^2), respectively. DMP-1 was up-regulated by 1.3-folds when the lower energy density (1 J/cm^2) was used, which was found to be statistically significant (P value < 0.0001). On the other hand, the higher energy density (3 J/cm^2) was found to down-regulate its expression (1.2-fold decrease) which was statistically significant (P value = 0.0011). BSP was up-

Table 2 Sequences of primers of the analyzed genes with the corresponding accession numbers

Gene	Primer sequence	Accession number
VEGF	Forward: 5′-CCCACTGAGGAGTCCAACAT-3′ Reverse: 3′-TTTCTTGCGCTTTCGTTTTT-5′	NM_001171623.1
VEGFR2	Forward: 5′-CGGTCAACAAAGTCGGGAGA-3′ Reverse: 3′-CAGTGCACCACAAAGACACG-5′	EU826563
DSPP	Forward: 5′-CGGTCAACAAAGTCGGGAGA-3′ Reverse: 3′-CAGTGCACCACAAAGACACG-5′	NM_014208.3
DMP1	Forward: 5′-GCAACTCCACGGAGAGCAAA-3′ Reverse: 3′-TAGCCGTCTTGGCAGTCATT-5′	U34037
BSP	Forward: 5′-AAGGGCACCTCGAAGACAAC-3′ Reverse: 3′-CCCTCGTATTCAACGGTGGT-5′	NM_004967
β-actin	Forward: 5′-GCC GATAGTGATGACCTGAC-3′ Reverse: 3′-GGCATCCTGACCCTGAAGTA-5′	AB196465.1

Table 3 Demographics of samples used in the study

Patient number	Age	Sex	Tooth location	Obtained slices	Processed slices
1	35	Female	18	2	2
2	26	Female	38	2[a]	1
3	25	Male	48	1[a]	1
4	29	Male	38	2[a]	1
5	23	Female	38	1	1
6	23	Male	48	2[a]	1
7	23	Female	28	1	1
8	21	Male	48	1	1
9	21	Female	48	2[a]	1
10	26	Female	38	3[a]	2
11	25	Male	18	2	2
12	26	Female	38	1	1
13	20	Female	28	1	1
14	21	Female	38	2[a]	1
15	21	Female	28	1	1
16	26	Female	48	3[a]	2
17	26	Male	18	1	1
18	21	Male	48	3[a]	2
19	25	Female	38	1	1
20	23	Female	38	2	2
21	21	Male	38	2[a]	1
22	21	Female	48	1	1
23	25	Male	18	2[a]	1
24	24	Male	48	2	2
25	26	Female	38	1	1
26	27	Female	28	2	2
27	25	Female	38	2	2
28	26	Male	48	2[a]	1
29	28	Female	28	1	1
30	24	Male	48	2[a]	1
31	23	Female	48	1	1

[a] Sample was lost during processing due to infection or insufficient size of retrieved pulp tissue

regulated to a statistically significant level (P value < 0.0001), where it showed a 6.7-fold increase when the lower energy density (1 J/cm^2) was applied. However, a non-statistically significant increase by 1.1-fold was noted when the higher energy density (3 J/cm^2) (P value = 0.0693) (Figs. 2 and 4).

However, when the 810-nm laser was used, there was a statistically significant up-regulation in the higher energy density group (3 J/cm^2) where DSPP expression was relatively unchanged in the lower energy density group (1 J/cm^2) with no statistical significance ($P = 0.9478$) while it was up-regulated with a statistical significance ($P < 0.0001$) by 2.5-folds when the higher energy density (3 J/cm^2) was used. DMP-1 had a 7.9-fold up-regulation with the lower energy density (1 J/cm^2) and a 17.7-fold up-regulation with the higher energy density (3 J/cm^2) which were both statistically significant ($P < 0.0001$). BSP gene was also up-regulated with a statistical significance, by 2-folds with the lower energy density and by 7.1-folds by the higher one ($P < 0.0001$). (Figs. 3 and 4).

Discussion

An optimal goal in modern endodontics is to restore a healthy dentin-pulp complex both functionally and anatomically [25]. Low-level laser therapy has been recently shown to have biostimulatory effects that could be utilized to promote the natural repair processes of dental tissues [8, 15].

The primary focus of the current study was to shed more light on the role of LLLT on two basic processes of tooth repair and regeneration: dentinogenesis and angiogenesis. To our knowledge, this is the first study to use a 3D in vitro culture model to test the direct effects of LLLT on the dentin-pulp complex as a tissue.

Most of the studies in the literature evaluated the effects of LLLT on mesenchymal stem cells of the pulp regarding their proliferation and viability, while some other studies evaluated their differentiation potential [19]. In the current study, the tooth slice-based model was chosen for more realistic simulation of the clinical conditions and to keep the pulpal cells in their natural niche within the dentin-pulp complex [20].

It should also be noted that when applying LLLT in the in vivo studies, not only the cells within the dentin-pulp complex are affected by the laser dose but also cells and release of mediators from the surrounding tissues; hence, the documented effects could also extend to influence the recruitment of circulating cells in the blood stream. This accentuates the need for in vitro studies that can explain the direct effects of LLLT on the dentin-pulp complex as a whole [26].

In this study, specific odontogenic/osteogenic and angiogenic genes were selected for analysis. LLLT has been found to directly increase the expression of one of the most crucial molecules in dental tissue regeneration, which is transforming growth factor beta (TGF-β) [16]. TGF-β1 is a key regulator for the expression of various non collagenous dentin matrix proteins which are found to be essential for proper mineralization and maturation of dentin [27, 28]. Among these non-collagenous dentin matrix proteins are dentin matrix protein (DMP-1), dentin sialophosphoprotein (DSPP), and bone sialoprotein (BSP).

DMP-1 is essential for proper mineralization and organization of dentinal tubules and inducing the differentiation of pulp's mesenchymal stem cells into odontoblast-like cells [29]. DSPP, which is highly expressed by odontoblasts [30], is also involved in mineralization and maturation of dentin [31]. BSP expression has been also shown to be increased during reparative dentinogenesis, suggesting that reparative

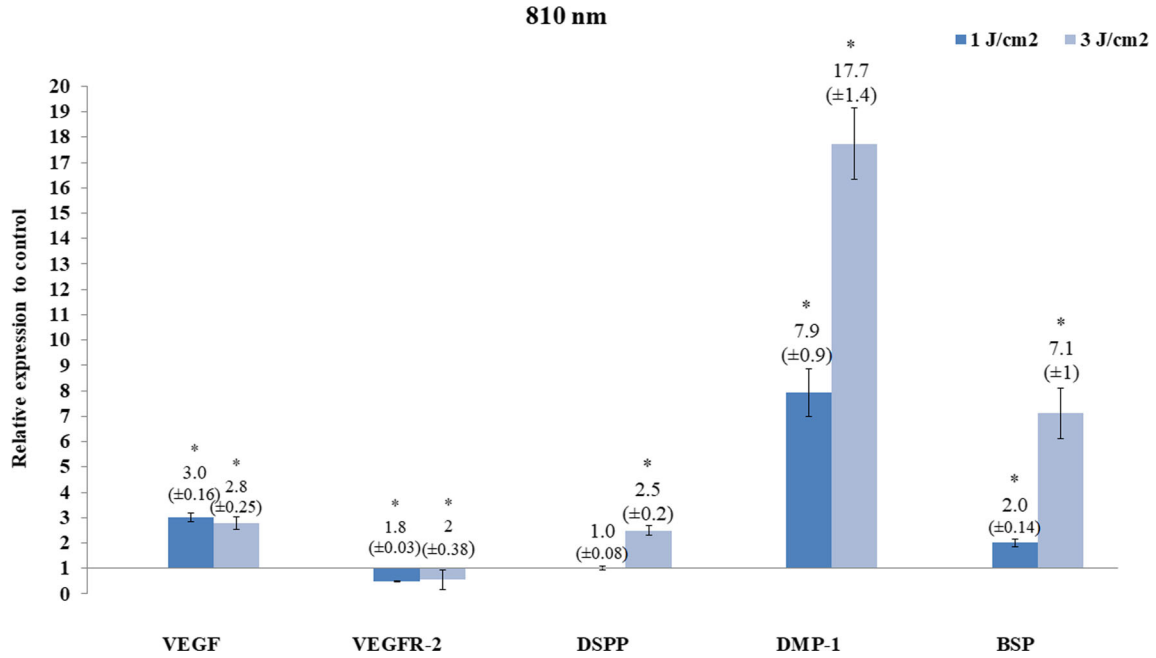

Fig. 3 Real-time PCR analysis of samples treated with 810-nm laser values are means (±SD) representing the fold change in angiogenic and odontogenic/osteogenic gene expression in relation to control. Asterisks indicate statistically significant differences in fold change relative to control group

dentin could have both dentinogenic and osteogenic characteristics [32].

LLLT was also found to enhance the expression of both vascular endothelial growth factor (VEGF) and its receptor (VEGFR2) [17]. VEGF is a crucial pro-angiogenic factor which binds to its receptor on cell surface and triggers signaling cascades that greatly influence the process of neovascularization [33].

In the present study, some of the used LLLT parameters appeared to have a positive influence on the biological processes occurring within the dentin-pulp complex. The results suggested that the effects of LLLT on dentin-pulp complex are dose dependent and are also affected by the change in wavelength.

Regarding the 660 nm wavelength, the lower energy density (1 J/cm^2) resulted in significant increase in angiogenic and odontogenic/osteogenic genes. However, the higher energy density (3 J/cm^2) had an up-regulatory influence on the angiogenic genes but not on the overall odontogenic/osteogenic markers. This was consistent with the findings of Wu et al. [34] who reported positive results regarding cell proliferation and expression of osteogenic markers with the same wavelength when low energy densities (1 and 2 J/cm^2) were used while less significant results were obtained with a higher

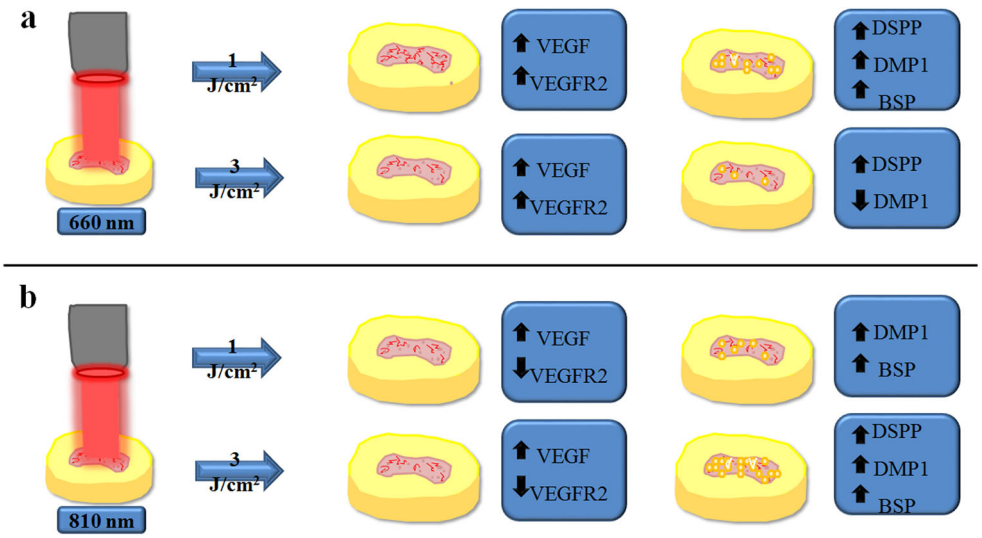

Fig. 4 Diagrammatic representation of the different effects of LLLT according to the used laser parameters

energy density (4 J/cm^2). It was also concurrent with De-Oliveira et al. [17] who also reported higher proliferation and higher gene expression of VEGF and VEGFR2 with the lower energy densities (0.7, 1.5, and 3 J/cm^2) but not with higher ones (9 J/cm^2). Barboza et al. [35], Zaccara et al. [12], and Ginani et al. [13] also reported positive results with 660 nm wavelength and (1 J/cm^2) energy density which was consistent with our results. Godoy et al. [36] and Marques et al. [37] also reported positive outcomes in studies on human teeth (in vivo) using the 660 nm wavelength with energy densities of 2 and 2.5 J/cm^2, respectively, which can be correlated with our findings.

On the contrary, Pereira et al. [38] using 660-nm diode laser with energy densities of 0.05, 0.30, 7, or 42 J/cm^2, Pacheco et al. [39] using 660 wavelength and energy density of 90 or 150 J/cm^2 and Bidar et al. [40] using a 630 nm wavelength and energy density of 7.5 J/cm^2 have all reported no significant difference after LLLT. These findings may be attributed to the use of either too low or too high energy densities [18].

In the current study, the difference in tissue response to different energy densities can be correlated with the biphasic dose response phenomenon, where the low doses may produce better results when compared to higher doses using the same wavelength. However, if insufficient energy was used and the threshold was not reached, there will be no biological effect on targeted cells or tissues [8].

It was noticeable that the odontogenic/osteogenic potential was not significantly up-regulated when the higher energy density (3 J/cm^2) was used with the 660 nm wavelength, while the angiogenic markers were significantly up-regulated. This may suggest that, at the end point of the current study, this energy density triggered the angiogenic response which in fact precedes reparative dentinogenesis as stated by Tran-Hung et al. [41]. This can explain the down-regulation of DMP-1 and the unaffected expression of BSP using this energy density.

As for the 810 nm wavelength, the higher energy density (3 J/cm^2) showed a stronger up-regulation regarding the expression of odontogenic/osteogenic markers compared to the lower energy density (1 J/cm^2), while both of them showed almost similar effects on angiogenic genes. This was consistent with Renno et al. [42] and Milward et al. [43] who reported that using higher energy density with this wavelength resulted in more cell growth of osteoblasts and dental pulp stem cells (DPSCs), respectively. Soleimani et al. [14] also reported that 810-nm laser resulted in enhanced differentiation of human bone marrow stem cells (hBMSCs) into both osteoblasts and neurons when higher energy densities were used (4 and 6 J/cm^2, respectively).

This was also in accordance with Arany et al. [16] who reported that LLLT of cultured human DPSCs resulted in a significant up-regulation of odontogenic markers DMP1, dentin sialoprotein (DSP), osteopontin (OPN), and alkaline phosphatase (ALP) when an energy density of 3 J/cm^2 was used rather than lower doses (0.03 and 0.3 J/cm^2) or higher doses (30 J/cm^2). This was also confirmed in vivo. Ateş et al. [23], however, found that energy densities of 0.5, 1, or 2 J/cm^2 had no significant effect on proliferation, differentiation, or osteogenic gene expression of human osteoblasts. These energy densities may not have reached the therapeutic threshold of this wavelength and may be needed to use higher energy densities to achieve positive outcomes.

On the other hand, Bouvet-Gerbettaz et al. [44] reported that using this wavelength with energy density of 4 J/cm^2 and had some inhibitory effects on murine bone marrow stem cell proliferation with no effects on differentiation. However, these negative results may be a result of using a relatively high power output (520 mW) compared to the used power outputs in the other studies ranging between 20 and 50 mW.

In the present study, it was noted that the use of 810 nm wavelength had biostimulatory effects on the overall odontogenic/osteogenic gene expression, with the exception of the lower energy density (1 J/cm^2) having no significant effect on DSPP expression. This may be attributed to the fast cleavage process into DSP and dentin phosphoprotein (DPP) as soon as DSPP protein is formed [45]. However, it could be due to the weaker biostimulatory effect of this energy density under the current experimental conditions. It was also noted that with this wavelength, the expression of VEGFR2 was down-regulated with both energy densities, but at the same time, up-regulation of the odontogenic/osteogenic genes took place, which could be interpreted as decreased endothelial cell activity; and increased cellular differentiation into odontoblast-like cells together with increased cellular activity of the pre-existing odontoblasts. However, this needs to be confirmed by further histological studies.

In the current study, it was difficult to directly compare the results with that of previous studies due to the differences in study model, as it seems that the response to LLLT varies according to the nature of cells and tissues [7]. Furthermore, despite the promising results of using the tooth slice-based model to evaluate angiogenesis and dentinogenesis following low-level laser therapy, it was not shown in the present study whether LLLT would have positive results when used on inflamed pulps in the same model. Another limitation is the short culture time; longer culture periods and different time intervals for evaluation are recommended. Future work will also focus on analyzing protein expression to allow a more complete picture of the effects of LLLT in this model and perhaps combining the use of both wavelengths together to achieve the optimal benefits. A representation of the potential clinical application of both lasers simultaneously is displayed in supplementary Fig. 1.

Conclusion

In conclusion, the present study suggests that LLLT appears to be a useful tool to enhance angiogenesis and dentinogenesis of dentin-pulp complex which could have several benefits in future clinical applications of regenerative endodontics and vital pulp therapy procedures. Additional studies are needed taking into consideration the limitations of the current study.

Acknowledgments The authors would like to acknowledge the assistance of the tissue engineering lab team at the faculty of dentistry, Alexandria University and the molecular therapeutics lab team at the faculty of science, Alexandria University for their valuable assistance in this work.

Funding No external funding sources to declare.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures of this study were done in accordance with the Ethics Research Committee, Faculty of Dentistry, Alexandria University (IRB NO: 00010556–IORG: 0008839) and in accordance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all patients.

References

1. Raedel M, Hartmann A, Bohm S, Walter MH (2015) Three-year outcomes of root canal treatment: mining an insurance database. J Dent 43(4):412–417
2. PVc S, PCsF SF, Queiroz EC, Arajo TC, Campos RE, Arajo CA, Soares CJ (2008) Fracture resistance and stress distribution in endodontically treated maxillary premolars restored with composite resin. J Prosthodont 17(2):114–119
3. Solomon RV, Faizuddin U, Karunakar P, Deepthi Sarvani G, Sree Soumya S (2015) coronal pulpotomy technique analysis as an alternative to pulpectomy for preserving the tooth vitality, in the context of tissue regeneration: a correlated clinical study across 4 adult permanent molars. Case Rep Dent 2015
4. Woodruff LD, Bounkeo JM, Brannon WM, Dawes KS, Barham CD, Waddell DL, Enwemeka CS (2004) The efficacy of laser therapy in wound repair: a meta-analysis of the literature. Photomed Laser Surg 22(3):241–247
5. Posten W, Wrone DA, Dover JS, Arndt KA, Silapunt S, Alam M (2005) Low-level laser therapy for wound healing: mechanism and efficacy. Dermatol Surg 31(3):334–340
6. da Silva JP, da Silva MA, APF A, IrL J, Matos AP (2010) Laser therapy in the tissue repair process: a literature review. Photomed Laser Surg 28(1):17–21
7. AlGhamdi KM, Kumar A, Moussa NA (2012) Low-level laser therapy: a useful technique for enhancing the proliferation of various cultured cells. Lasers Med Sci 27(1):237–249
8. Huang Y-Y, Chen ACH, Carroll JD, Hamblin MR (2009) Biphasic dose response in low level light therapy. Dose-Response 7(4):dose–response 09-027
9. Pinheiro ALB, Nascimento SC, de Barros Vieira AL, Brugnera A Jr, Zanin FA, Rolim Az B, Soriano da Silva P (2002) Effects of low-level laser therapy on malignant cells: in vitro study. J Clin Laser Med Surg 20(1):23–26
10. Caruso-Davis MK, Guillot TS, Podichetty VK, Mashtalir N, Dhurandhar NV, Dubuisson O, Yu Y, Greenway FL (2011) Efficacy of low-level laser therapy for body contouring and spot fat reduction. Obes Surg 21(6):722–729
11. Elnaghy AM, Murray PE, Bradley P, Marchesan M, Namerow KN, Badr AE, El-Hawary YM, Badria FA (2013) Effects of low intensity laser irradiation phototherapy on dental pulp constructs. World J Stomatol 2(1):12–17
12. Zaccara IM, Ginani F, Mota-Filho HG, Henriques ACG, CAGo B (2015) Effect of low-level laser irradiation on proliferation and viability of human dental pulp stem cells. Lasers Med Sci 30(9): 2259–2264
13. Ginani F, Soares DM, de Oliveira Rocha HA, de Souza LB, Barboza CAG (2018) Low-level laser irradiation induces in vitro proliferation of stem cells from human exfoliated deciduous teeth. Lasers Med Sci 33(1):95–102
14. Soleimani M, Abbasnia E, Fathi M, Sahraei H, Fathi Y, Kaka G (2012) The effects of low-level laser irradiation on differentiation and proliferation of human bone marrow mesenchymal stem cells into neurons and osteoblastsâ€ an in vitro study. Lasers Med Sci 27(2):423–430
15. Theocharidou A, Bakopoulou A, Kontonasaki E, Papachristou E, Hadjichristou C, Bousnaki M, Theodorou G, Papadopoulou L, Kantiranis N, Paraskevopoulos K (2017) Odontogenic differentiation and biomineralization potential of dental pulp stem cells inside mg-based bioceramic scaffolds under low-level laser treatment. Lasers Med Sci 32(1):201–210
16. Arany PR, Cho A, Hunt TD, Sidhu G, Shin K, Hahm E, Huang GX, Weaver J, Chen AC-H, Padwa BL (2014) Photoactivation of endogenous latent transforming growth factor β1 directs dental stem cell differentiation for regeneration. Sci Transl Med 6(238): 238ra269–238ra269
17. de Oliveira TS, Serra AJ, Manchini MT, Bassaneze V, Krieger JE, de Carvalho PTC, Antunes DE, Bocalini DS, PJF T, Silva JA (2015) Effects of low level laser therapy on attachment, proliferation, and gene expression of VEGF and VEGF receptor 2 of adipocyte-derived mesenchymal stem cells cultivated under nutritional deficiency. Lasers Med Sci 30(1):217–223
18. Ginani F, Soares DM, CAGo B (2015) Effect of low-level laser therapy on mesenchymal stem cell proliferation: a systematic review. Lasers Med Sci 30(8):2189–2194
19. Marques MM, Diniz IMA, de Cara SPHM, Pedroni ACF, Abe GL, D'Almeida-Couto RS, Lima PLV, Tedesco TK, Moreira MS (2016) Photobiomodulation of dental derived mesenchymal stem cells: a systematic review. Photomed Laser Surg 34(11):500–508
20. Sloan AJ, Shelton RM, Hann AC, Moxham BJ, Smith AJ (1998) An in vitro approach for the study of dentinogenesis by organ culture of the dentine-pulp complex from rat incisor teeth. Arch Oral Biol 43(6):421–430
21. Murray PE, Lumley PJ, Ross HF, Smith AJ (2000) Tooth slice organ culture for cytotoxicity assessment of dental materials. Biomater 21(16):1711–1721
22. Gon alves SB, Dong Z, Bramante CM, Holland GR, Smith AJ, Nor JE (2007) Tooth slice-based models for the study of human dental pulp angiogenesis. J Endod 33(7):811–814
23. Ateş GB, Can AA, Gülsoy M (2017) Investigation of photobiomodulation potentiality by 635 and 809 nm lasers on human osteoblasts. Lasers Med Sci 32(3):591–599
24. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. AnalytBiochem 162(1):156–159

25. Kim SG (2017) Biological molecules for the regeneration of the pulp-dentin complex. Dent Clin N Am 61(1):127–141
26. Schindl A, Heinze G, Schindl M, Pernerstorfer-Schon H, Schindl L (2002) Systemic effects of low-intensity laser irradiation on skin microcirculation in patients with diabetic microangiopathy. Microvasc Res 64(2):240–246
27. Boskey AL (1991) The role of extracellular matrix components in dentin mineralization. Crit Rev Oral Biol Med 2(3):369–387
28. Butler WT (1998) Dentin matrix proteins. Eur J Oral Sci 106(Suppl 1):204–210
29. Almushayt A, Narayanan K, Zaki AE, George A (2006) Dentin matrix protein 1 induces cytodifferentiation of dental pulp stem cells into odontoblasts. Gene Ther 13(7):611–620
30. Be'gue-Kirn C, Krebsbach PH, Bartlett JD, Butler WT (1998) Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin, tooth-specific molecules that are distinctively expressed during murine dental differentiation. Eur J Oral Sc 106: 963–970
31. Suzuki S, Sreenath T, Haruyama N, Honeycutt C, Terse A, Cho A, Kohler T, Müller R, Goldberg M, Kulkarni AB (2009) Dentin sialoprotein and dentin phosphoprotein have distinct roles in dentin mineralization. Matrix Biol 28(4):221–229
32. Hwang YC, Hwang IN, Oh WM, Park JC, Lee DS, Son HH (2008) Influence of TGF-beta1 on the expression of BSP, DSP, TGF-beta1 receptor I and Smad proteins during reparative dentinogenesis. J Mol Histol 39(2):153–160
33. Gerwins P, Skoldenberg E, Claesson-Welsh L (2000) Function of fibroblast growth factors and vascular endothelial growth factors and their receptors in angiogenesis. Crit Rev Oncol Hematol 34(3):185–194
34. Wu J-Y, Chen C-H, Yeh L-Y, Yeh M-L, Ting C-C, Wang Y-H (2013) Low-power laser irradiation promotes the proliferation and osteogenic differentiation of human periodontal ligament cells via cyclic adenosine monophosphate. Int J Oral Sci 5(2):85–91
35. CAGo B, Ginani F, Soares DM, Henriques CG, Freitas RA (2014) Low-level laser irradiation induces in vitro proliferation of mesenchymal stem cells. Einstein (Sao Paulo) 12(1):75–81
36. Godoy BM, Arana-Chavez VE, Nunez SC, MSe R (2007) Effects of low-power red laser on dentine-pulp interface after cavity preparation. An ultrastructural study. Arch Oral Biol 52(9):899–903
37. Marques NCT, Neto NL, de Oliveira Rodini C, Fernandes AP, Sakai VT, Machado MAAM, Oliveira TM (2015) Low-level laser therapy as an alternative for pulpotomy in human primary teeth. Lasers Med Sci 30(7):1815–1822
38. Pereira LO, Longo JPF, Azevedo RB (2012) Laser irradiation did not increase the proliferation or the differentiation of stem cells from normal and inflamed dental pulp. Arch Oral Biol 57(8): 1079–1085
39. Pacheco PS, de Oliveira FA, Oliveira RC, ACP S'A, de Rezende MLR, Greghi SoLA, Damante CA (2013) Laser phototherapy at high energy densities do not stimulate pre-osteoblast growth and differentiation. Photomed Laser Surg 31(5):225–229
40. Bidar M, Moushekhian S, Gharechahi M, Talati A, Ahrari F, Bojarpour M (2016) The effect of low level laser therapy on direct pulp capping in dogs. Lasers Med Sci 7(3):177
41. Tran-Hung L, Laurent P, Camps J, About I (2008) Quantification of angiogenic growth factors released by human dental cells after injury. Arch Oral Biol 53(1):9–13
42. Renno ACM, McDonnell PA, Parizotto NA, Laakso EL (2007) The effects of laser irradiation on osteoblast and osteosarcoma cell proliferation and differentiation in vitro. Photomed Laser Surg 25(4): 275–280
43. Milward MR, Hadis MA, Cooper PR, Gorecki P, Carroll JD, Palin WM Biomodulatory effects of laser irradiation on dental pulp cells in vitro. In: Proc of SPIE Vol, 2015. pp 930908–930901
44. Bouvet-Gerbettaz S, Merigo E, Rocca J-P, Carle GF, Rochet N (2009) Effects of low-level laser therapy on proliferation and differentiation of murine bone marrow cells into osteoblasts and osteoclasts. Lasers Surg Med 41(4):291–297
45. MacDougall M, Simmons D, Luan X, Nydegger J, Feng J, Gu TT (1997) Dentin phosphoprotein and dentin sialoprotein are cleavage products expressed from a single transcript coded by a gene on human chromosome 4 dentin phosphoprotein DNA sequence determination. J Biol Chem 272(2):835–842