



Influence of photobiomodulation and tricalcium silicate-based cement on viability and dentinogenic activity of cultured human dental pulp stem cells

Influência da fotobiomodulação e cimento de silicato tricálcio na viabilidade e atividade dentinogênica de células tronco cultivadas da polpa de dente humano

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ABSTRACT

Objective: To assess the effect of application of Biodentine (BD), Photobiomodulation (PBM) using 810 nm diode laser and both on the proliferation and odontogenic differentiation of human dental pulp stem cells (HDPSCs). **Material and Methods:** HDPSCs were collected, isolated, and characterized and then divided into six groups: groups 1, control; groups 2, biodentine (BD); group 3, irradiation at 1 J/cm² of 810-nm diode laser; group 4, irradiation at 1 J/cm² and culture with BD; group 5, irradiation at 2 J/cm², and group 6, irradiation at 2 J/cm² and culture with BD. Viability assay was measured through MTT assay and Alkaline phosphatase (ALP) enzyme activity and mRNA levels of RUNX2, collagen 1 (Col-1) and BMP2 were also assessed. **Results:** Photobiomodulation at 1 and 2 J/cm² combined with biodentine significantly promoted HDPSCs proliferation (in MTT assay results) and odontogenic differentiation (through the gene expression of RUNX2, Col-1 and BMP2 levels (p < 0.05)). **Conclusion:** Photobiomodulation at 2 J/cm² combined with biodentine enhanced proliferation and odontogenic differentiation of cultured HDPSCs and thus could further be beneficial for dentin regeneration.

KEYWORDS

Human dental pulp stem cells (HDPSCs); PBM; BMP2; RUNX2; COL1.

RESUMO

Objetivo: Avaliar o efeito da aplicação de Biodentina (BD), Fotobiomodulação (PBM) usando diodo de laser de 810 nm e ambos na proliferação e diferenciação odontogênica de células tronco cultivadas da polpa dental (HDPSCs). **Material e Métodos:** HDPSCs foram coletadas, isoladas, caracterizadas e então divididas em seis grupos: grupo 1, controle; grupo 2, biodentina (BD); grupo 3, irradiação com diodo de laser a 1 J/cm² de 810-nm; grupo 4, irradiação a 1 J/cm² e cultivo com BD; grupo 5, irradiação a 2 J/cm², e grupo 6, irradiação a 2 J/cm² e cultivo com BD. A viabilidade foi mensurada através do teste MTT e a atividade da enzima Fosfatase alcalina (ALP), e níveis de RNAm de RUNX2, de colágeno 1 (Col-1) e de BMP2 foram também mensurados. **Resultados:** Fotobiomodulação a 1 e 2 J/cm² combinada com biodentina promoveu significativa proliferação de HDPSCs (nos resultados do teste MTT) e diferenciação odontogênica (através da expressão genética dos níveis de RUNX2, Col-1 e BMP2 (p < 0.05)). **Conclusão:** Fotobiomodulação a 2 J/cm² combinada com biodentina aumentou a proliferação e diferenciação odontogênica de HDPSCs cultivadas e dessa forma poderia ser benéfica para a regeneração dentinária.

PALAVRAS-CHAVE

Células tronco da polpa dental humana (HDPSCs); PBM; BMP2; RUNX2; COL1.

INTRODUCTION

Regenerative dentistry is a promising solution to standard therapies for preserving pulp vitality and avoiding more extensive treatments such as endodontic therapy and tooth extraction [1]. Conservatively the pulp exposure managed by vital pulp therapy (VPT), like pulp capping or pulpectomy, which promote the regenerative ability of the pulp [2]. The vital pulp therapy objective is to utilize the environments that optimize the regeneration of dentin barrier and pulp like complex to preserve the functionality and ensure the long-term remaining of natural tooth in oral cavity [3].

Currently, there are several clinically available pulp capping materials, such as calcium hydroxide (Ca(OH)₂), or hydraulic calcium-silicate cements (HCSCs), such as mineral trioxide aggregates (MTA) Biodentine (BD) and other silicate-based cements [4]. BD (Septodont, Saint Maurdes-Fosses, France) is calcium silicate material with excellent biocompatibility, bioactive behavior and mechanical properties [4,5]. As compared to other capping materials, it has excellent sealing, high compressive strength, short setting time, easy to deal with and high PH of 12 that make it excellent antimicrobial dental material. Therefore, Biodentine have been used safely and effectively in VPT [5].

Ideally, pulp capping material induct preexisting odontoblast to form tertiary dentin and utilize dental pulp stem cells to form reparative dentin [6].

HDPSCs are so promising source of stem cells in bioengineering strategies due to their greater accessibility and lower cost as compared to other type of adult stem cells (ASCs) as their isolation techniques are costly and invasive. because HDPSCs obtained from third molars, orthodontically unnecessary teeth and supernumerary ones [7].

Biodentine has already known for proliferation induction of HDPSCs and the initiation of mineralization through releasing transforming growth factor beta from pulp cells [8].

The interaction of HDPSCs with biodentine during vital pulp therapy affects the proliferation and differentiation of dental pulp cells has already approved [9].

Several studies assessed the biocompatibility of biodentine (in vitro) [10]. They examined the cell proliferation, viability, survival rates or proliferation assays like MTT, XTT, crystal violet, MTS or fluorescent dyes via spectrophotometer or the flow cytometry [10].

As compared to the widely known biologic active material MTA, Biodentine revealed highest cell viability than MTA in one study, and similar biocompatibility to MTA [11]

Most of the time the odontogenic effects of biodentine on HDPSCs were similar to that of MTA bioactive material, however, there were some differences in terms of odontogenesis expression genes, mineralization ability in-vitro [4,10] or dentin bridge thickness in-vivo [12].

Laser application on VPT has been already evaluated in clinical studies, aiming at two different effects: (A) to produce hemostasis in vital pulp therapy before application of the capping materials. This has been achieved by means of different types of laser (Erbium family, diode, Nd:YAG, or CO₂ laser [13]. (B) To enhance pulp wound healing potential before pulp capping and stimulate dentin regeneration. For the last point, photobiomodulation (PBM) has been suggested as the modality of choice [14].

PBM also known by alternative terms, such as low-level laser irradiation, low-intensity laser therapy, low-energy laser therapy, cold laser therapy, biostimulation laser therapy, or phototherapy [15]. PBM defined by an output power less than 500 mW and is mainly applied on the red or near infrared electromagnetic spectrum from (630–980 nm) [16].

PBM is one of the strategies that can be safely used to stimulate the proliferation of cells. The concept of PBM is to directly supply photobiostimulative energy to the cells which causes stimulation of atoms and molecules of the cells [17].

PBM has been reported to be able to stimulate and promote proliferation in vitro and in vivo [18-20] enhance cell growing, development and regeneration in addition to an anti-inflammatory effect. The positive effect of PBM on the proliferation of certain cells was studied before but PBM effect on HDPSCs need more studies as there is no definite parameters or protocols for this [17].

It remains unclear which of the available biodentine, low-level laser or both are the most

conducive to the proliferation and odontogenic differentiation of HDPSCs.

Based on the above, the present study highlighted on the effects of photobiomodulation and biodentine on human dental pulp stem cells proliferation and odontogenic differentiation.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Ethics Committee of National Institute of Laser Enhanced Sciences, Cairo University, Egypt numbered (019021). All procedures were performed according to the declaration of Helsinki 2008 and the patients gave their informed written consent for their enrollment in the research study. The current research was conducted in collaboration with the Medical Biochemistry and Molecular Biology Department (Tissue culture unit) of the faculty of Medicine, Cairo University.

Human dental pulp stem cells (HDPSCs) isolation and culturing

- Ten sound impacted third molars were extracted from healthy adult male patients (21 and 22 years old) at the outpatients' clinic, Cairo University, after their informed consent. Therefore, a relatively narrow age range of patients was chosen to reduce the aging effects of HDPSCs. The extracted teeth were placed on ice in phosphate buffer saline (PBS) (ThermoScientific®, USA), and were then transmitted to the tissue culture unit directly.
- Under the sterile condition, working within a biohazard laminar flow hood (Thermo Fisher Scientific®, USA). Teeth were washed with phosphate buffer saline (BioWhittaker®, Lonza, USA cat#: 17-516F). The crowns of the collected teeth were cut at the cemento-enamel junction and the pulp tissue was collected from the pulp chamber and the root canal.
- Then, the collected pulp tissues were incubated into collagenase type II (Sigma Aldrich, USA) for 2 h at 37°C, centrifuged, filtered, and resuspended until the pellet was formed. The cell pellet was plated and cultured in a density of 15–30 × 10³ cells/cm² in basic medium and incubated in the culture at 37°C with 5.5% CO₂ and 90%–95%

humidity. The culture medium was changed every three days until 80%–95% confluence of the cell was achieved [21].

Immunological characterization of HDPSCs

Fluorescence-activated cell sorting analysis was used to confirm surface antigens specific to CD105 (R&D Systems, USA; cat #, FAB1320F-025), and stem cells marker as CD 90 (R&D Systems, USA cat #FAB2405P-025), CD 34 (R&D Systems, USA cat #FAB2405P-025) and was negative to CD 45 (R&D Systems, USA; cat # FAB114A), excluding hematopoietic, endothelial cells [22].

Odontogenic differentiation media

Additionally, HDPSCs were recultured in odontogenic differentiation media (Sigma®, USA), supplemented with 10 mM b-glycerophosphate, 0.05 mM ascorbic acid, and 100 mM dexamethasone for 21 days [21].

Biodentine preparation

Under aseptic conditions, BD capsules were prepared according to manufacturer instructions by a ratio of 5 drops per capsule of Biodentine (Septodont, Saint-Maurdes-Fossés, France) [8]. BD like tablets were created using amalgam carrier and each tablet weighted 50 micrograms (using Precisa XB-220A balance). Then BD tablets were incubated for a couple of days in a CO₂ incubator at 37 °C and 5% humidity for complete setting then added to the well plates of HDPSCs [8].

Study design

Ninety well tissue culture plates were equally divided into six groups as follows when passage 4 of HDPSCs was achieved: group 1, a control group without irradiation; group 2, HDPSCs with biodentine alone without irradiation; group 3, HDPSCs with laser irradiation at 1 J/cm²; group 4, HDPSCs cultured with biodentine and laser irradiation at 1 J/cm²; group 5, irradiation at 2 J/cm²; and group 6, biodentine and laser irradiation at 2 J/cm².

Photobiomodulation

Cells were irradiated with a diode laser (Denlase, Daheng Group, Inc, China) in continuous wave mode parameters: 100 mW, wavelength of 810 nm, spot area of 0.5 cm²,

exposure time for 5 seconds and 10 seconds and irradiation at 1 and 2 J/cm². Cells were double irradiated (at 0 and 48 h) with laser probe fixed perpendicular to each plate, and irradiation was conducted in dark conditions [23].

To minimize the unintentional dispersion of light between wells during laser application, the culture dishes were wrapped within the dark paper sheets with a hole of a diameter corresponding to the diameter of the laser spot area of the headpiece and the cells were distributed as there were empty wells between seeded well cells [17,24].

Methods for evaluation

A) Evaluation of Cell viability

MTT assay was used in the cell viability analysis at 96 hours after the second laser application, which was used to assess cell metabolic activity through the number of viable cells, and absorbance was directly proportional to the number of living cells in culture. Cells were cultured in 96-well plates at a density of 1x10³ cells/well, using MTT reagent (Sigma-Aldrich Co.), and absorbance of the samples was monitored in an ELISA reader (Dynatech MRX 5000; Dynex, Chantilly, VA) at 490-630nm [4].

B) Odontogenic differentiation of HDPSCs

1) Quantitative RT-PCR for odontogenic genes (RUNX2, BMP-2 and Col-1)

Total cellular RNA for RUNX2, BMP-2 and Col-1 were extracted using GF-1 Nucleic Acid Extraction Kits (Vivantis® Technologies, USA, cat#GF-TR-050) and reverse transcribed using a complementary DNA master (SensiFAST™ One-Step Kit, USA, cat # PI-50217 V).

PCR assays were conducted according to the manufacturer protocol in real-time PCR device (Step One Applied Biosystems, USA).

The human-specific PCR primers of RUNX2, BMP-2 and Col-1 were used (TaqMan Gene, Applied Biosystems, CA, USA). PCR primers of genes that were used are presented in Table I.

The RT reaction was followed by a real-time PCR with a SYBR Green assay performed on an Applied Biosystems (Step One System, SDS software version 2.1 and RQ Manager 1.2). GAPDH was used as an endogenous reference control gene for normalization control [8,18].

Table I - Sequence of polymerase chain reaction primers

Gene	Primer Sequence
Runx2	forward: CCACCGAGACCAACAGAGTC
	reverse: TCACTGTGCTGAAGAGGCTG
BMP-2	forward: GGAACGGACATTCGGTCTCTT
	reverse: AGTCCGTCTAAGAAGCACG
Collagen type I	forward: BCAGTCGATTCACCTACAGCACG
	reverse: GGGATGGAGGGAGTTTACACG
GAPDH	forward: TATCGTGAAGGACTCA
	reverse: GCAGGGATGAGTTCTGGA

2) Von Kossa Stain (VKS)

Calcium deposition as a final product of the odontogenic differentiation process was assessed using Von Kossa staining on day 21. HDPSCs were stained with a solution of 2% Von Kossa staining (Polysciences, Warrington, PA) at pH 4.5 for 15 min at room temperature and washed with distilled water then observed using an inverted microscope (Leica, USA) [25].

3) Alkaline phosphatase (ALP): enzyme activity

HDPSCs (1x10³ cells /well) were seeded on 96-well culture plates for 21 days, homogenized in 50-μL assay buffer, centrifuged for 3 min. Alkaline Phosphatase Assay Kit (Amplite™ AAT Bioquest, Inc., USA cat# 11950) was used by adding para-nitrophenylphosphate, a chromogenic phosphatase substrate, and then incubated at 37°C for 10–30 min and measured levels of ALP documented in kilo unit (KU)/100 mL at 400nm wave length [26].

Statistical methods

A total sample size of 90 was sufficient to detect the effect size of 0.40, a power (1-β) of 80% and at a significant level of 5% (p<0.05), each group was represented by 15 sample. The sample size was calculated according to G*Power software version 3.1.9.4. Where, *f*S the effect size, α= 0.05, β= 0.2, Power= 1- β = 0.8

Analysis of data was carried out using GraphPad Prism version7. Mean and standard deviation were used to summarize data. Comparisons among groups were done using one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test to compare multivariable in more than 2 groups [24].

RESULTS

Characterization of HDPSCs

Immunological characterization was assessed as HDPSCs were negative to CD34 and CD 45 surface antigen and positive to CD 90 surface antigen and CD105 as shown in Figure 1.

Cell viability was assessed via MTT assay

It was measured at 96 h It showed that, after laser irradiation of 2 J/cm² (group 5) and 2 J/cm² with biodentine (group 6), MTT activity had a statistically significant increase in group 5 and group 6 compared to the control group (p value < 0.0001). Moreover, there was a significant difference between groups 3 and 5 (p value < 0.001) with the highest values of group 5, giving the idea that laser irradiation at 2 J/cm² increases proliferation of HDPSCs compared to laser irradiation at 1 J/cm². These results confirmed a synergistic effect of photobiomodulation and biodentine in enhancing proliferation of HDPSCs in Table II.

RT-PCR analysis for osteogenic genes (RUNX2, BMP-2 and Col-1)

RUNX2

RUNX2 plays important role in odontoblast differentiation and dentin mineralization. When measured on day 21, there was no significantly statistical difference between groups 2,3 and the

control group but groups 4,5 and 6 were significantly statistical difference. As compared to group 1, group 2 and group 3 (p < 0.0001), Figure 2.

It showed a statistically significant difference between groups (p < 0.0001), especially groups irradiated with 2 J/cm² than those that were irradiated at 1 J/cm². The highest significant value in tested genes expression was with group 6 compared to group 1 (control group) (Figure 2).

BMP-2

One of the odontogenic genes as it induces hard tissue formation. there was no difference between group 2,3 and the control but other groups were significantly statistical difference. group 2 and group 3 were not statistically difference from group1 (Figure 2). The highest significant value of the gene expression was with group 6 followed by group 5 (Figure 2).

Col-1

Col-1 gene is the major component of the connective tissue in dental pulp.

There was no significant difference between the group 2 (biodentine) and control group, indicating that biodentine alone does not affect HDPSCs differentiation. However, with groups of laser irradiation at 2 J/cm² (groups 5 & 6) there were statistically different values against the control and biodentine groups (p = 0.0001) (Figure 2).

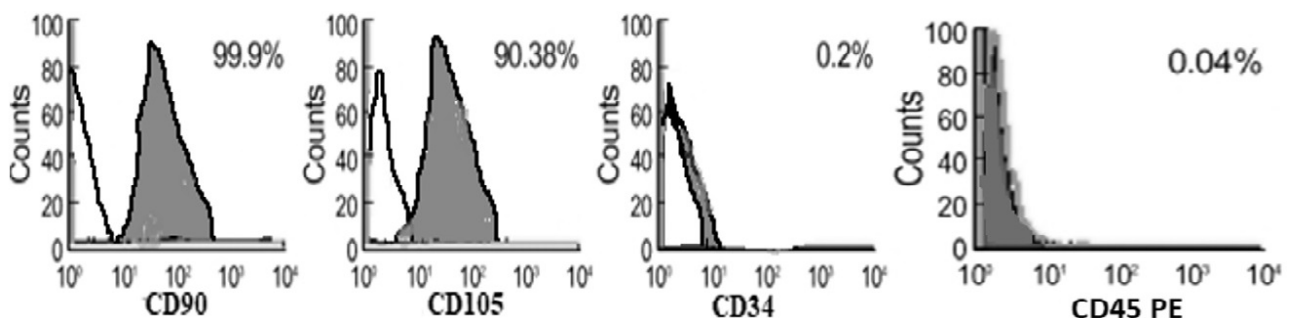


Figure 1 - Characterization of hDPSCs were confirmed by Flow cytometry. They were highly positive for CD105 and CD 90 and negative for CD34 and CD45.

Table II - Showing mean \pm SD and significance of MTT at 96 hours among different studied groups

MTT 96 h	group 1	group 2	group 3	group 4	group 5	group 6
	0.13 \pm 0.015	0.17 \pm 0.03*	0.14 \pm 0.02	0.29 \pm 0.03*#	0.43 \pm 0.04*#\$	0.53 \pm 0.05*#\$\$@&

*Denotes significant difference against control; #Denotes significant difference against group 2; \$Denotes significant difference versus group 3; @Denotes significant difference versus group 4; &Denotes significant difference versus group 5.

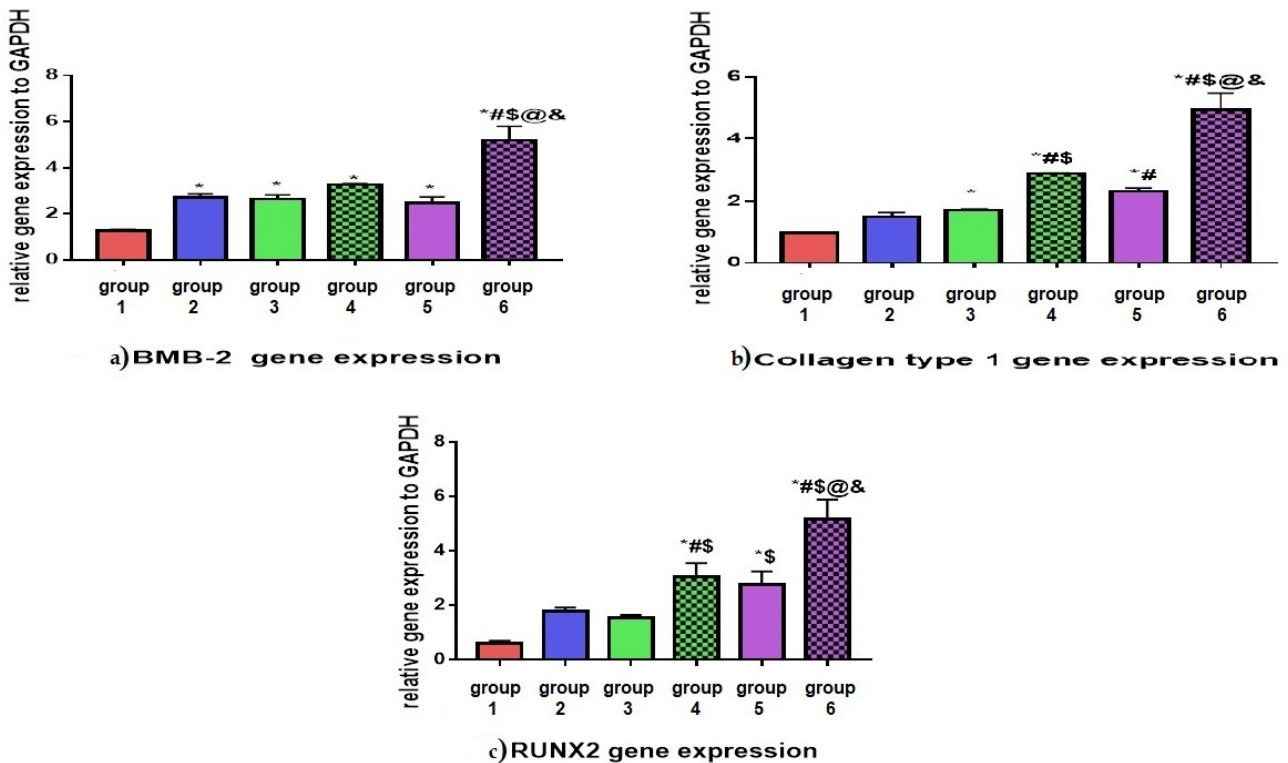


Figure 2 - The effects of BD and 810 LLT on BMP2, COL1 and RUNX2 expression, (a) BMP2 gene expression; (b) COL1 gene expression; (c) RUNX2 gene expression. when *Denotes significant difference against control; #Denotes significant difference against group2; \$Denotes significant difference versus group3; @Denotes significant difference versus group4; &Denotes significant difference versus group5.

Von Kossa stain

A phase-contrast light microscope was used to observe the von Kossa staining of extracellular calcium deposits in the test groups after odontogenic differentiation of **HDPSCs**. The calcified mineralization effect was the highest in the group 6, followed by group (5, 4, 3, 2 respectively) and the lowest in the control group (Figure 3).

ALP activity assay

The level of ALP at 21 days showing a statistically significant difference between different groups of study (p value < 0.0001). When there is a statistically significant difference between all study groups against control group (p value = 0.01, 0.002, < 0.0001 , 0.0004 and < 0.0001 respectively) as in Figure 4.

DISCUSSION

Biodentine used as bioactive dental material which demonstrated more biocompatibility when investigated on many cell lines with better handling properties and shorter setting time as compared with other dental biomaterials [10].

Diode laser with a wavelength 810 nm laser was used in this study as a source for PBM despite the fact that minimal number of studies tested its effect on **HDPSCs** [17]. As PBM cause changes in biochemical cell activity by photobiological effects, the anti-inflammatory effect (mediated by the microvascularization increase on area and by the local vasodilatation), the increase of collagen synthesis and the mesenchymal stem cells proliferation and increase of ATP (adenosine triphosphate) production [17].

First part of this study showed immunological characteristics using flowcytometry to detect specific markers of **HDPSCs**, which was positive expressed to CD90, and CD105 while negative expressed to CD34 and CD45, these findings meet the requirements for the definition of multipotent MSCs by the international society for cellular therapy [22]. Similar findings reported in other studies [27].

In present study, MTT used to evaluate the effect of BD and 810nm PBM with 1 and 2 J/cm² on cell proliferation of **HDPSCs**. At 96 hours, statistically differences were detected between study groups and there was an increase in proliferation but there is no statistically difference between Group3 and

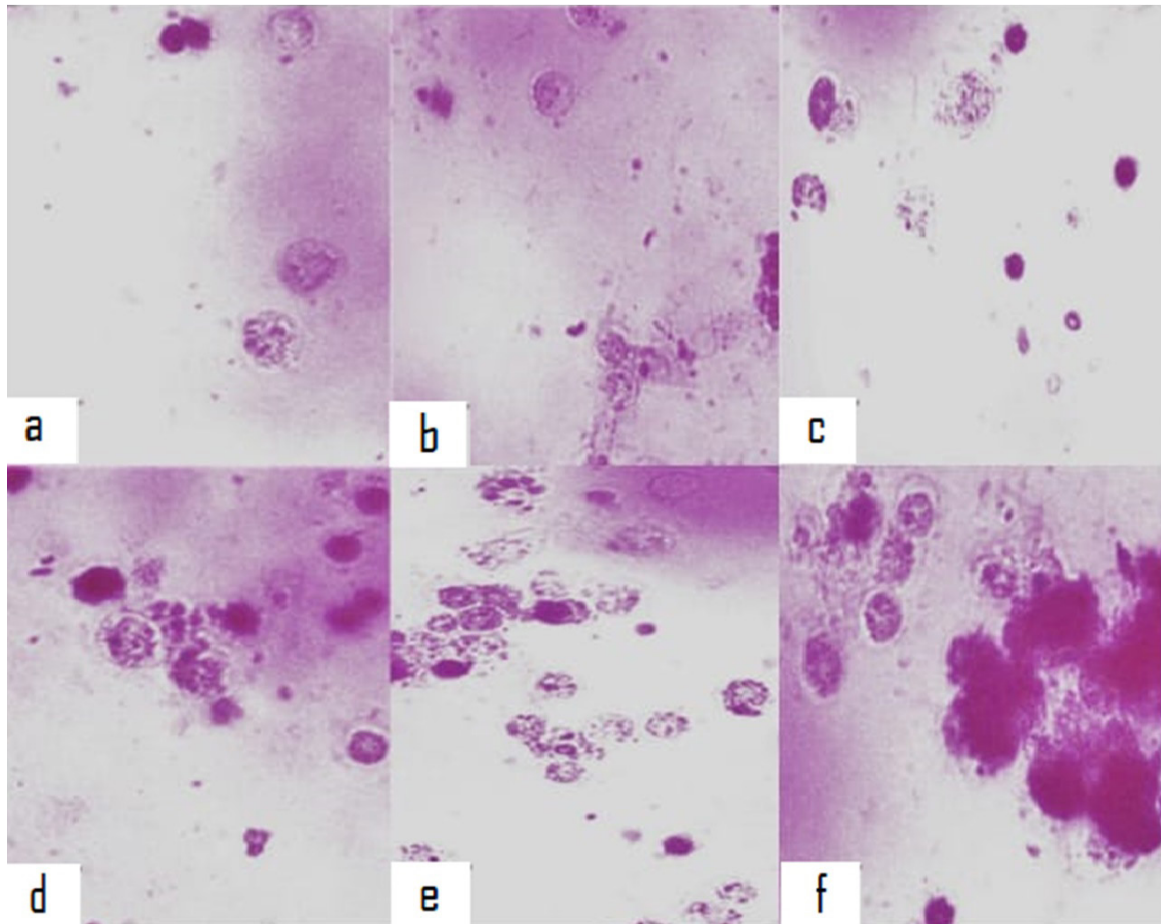


Figure 3 - Microscopic images of Von kossa stain with X200 magnification (scale have, at 100 μ m) of different study groups at day 21; showing (a) group1 (control group); (b) group2 (BD); (c) group3 (1J/cm²); (d) group4 (BD+1J/cm²); (e) group5 (2J/cm²); and (f) group6 (BD+2J/cm²), the highest mineralization and calcium nodules formation (as group6 showed the denser calcified nodules).

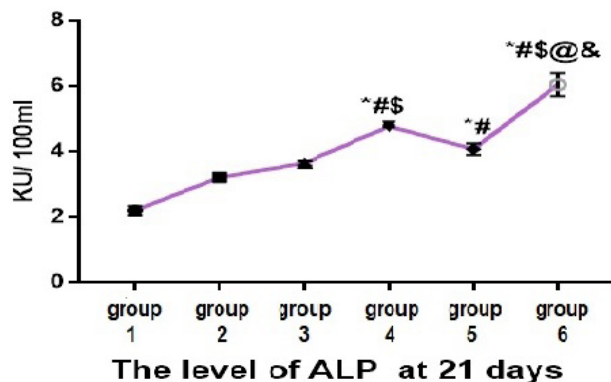


Figure 4 - Line graph showing level of ALP enzyme activity among different studied groups at 21 day: with significant difference between group5 (2J/cm²) against group 2(BD) ($P<0.01$). Also group6 (BD+2J/cm²) with other study groups ($P<0.001$). Data were expressed as Mean \pm SD, p value <0.05 was significant *Denotes significant difference versus control; #Denotes significant difference versus group 2; \$Denotes significant difference versus group 3; @Denotes significant difference versus group 4; &Denotes significant difference versus group 5.

control group despite the fact that there was initially increase. The present results seemed differ from previous study [23] showing that 810nm PBM had inhibitory effect on cell proliferation as mentioned by other study [28].

Zaccara et al. [28] mentioned that 810nm has an inhibitory effect according to previous study by Hawkins 2007 who tested 830nm and measured cell viability at one hour and 24 hours post irradiation with high energy density 5 and 16 J/cm² as mentioned by Staffoli et al. [17] that energy density above 10 cause inhibitory effects and also the short-tested time explained the non-statistically difference.

In the present study, group 5 showed increase in MTT against control which indicate a positive effect that may be explained by what mentioned as photobiomodulation is a dose depending therapy [29]. While its already approved that PBM improve cell proliferation in various cell types like mesenchymal cells, epithelial, and osteoblasts [8,18].

The present results were constant with previous studies using either BD or 810 nm PBM [8,18,30], as there was an increase in cell proliferation when high energy density applied in group 5 compared with group3 and also when

mixed groups of PBM with BD used like group 6, which represented the highest cell proliferation. group 6 proved the positive role of 810nm PBM on enhancing **HDPSCs** proliferation as it significantly differs from group 2, that already approved the effect of BD on cell proliferations [8].

In this study, to examine the effects of BD, PBM and the mixed groups on the odontogenic differentiation of **HDPSCs**; the relative expression of target gene markers, BMP2, RUNX2 and collagen type I, in the test groups were detected by a reverse-transcription reaction to the complementary DNA template. Statistically significant differences were detected between the tested groups. Group 6 was the most efficient in increasing the relative expression of mRNA for the odontogenic markers (BMP2, RUNX2 and collagen type I), followed by group 4, group 5, and group 2. The relative expression of the mRNA for these markers in group 4, group 5 and group 6 differed significantly from that in the negative control group; in contrast, there was no significant difference in this respect between the negative control group and the group 3 regarding RUNX2 and COL1 gene expression. This demonstrated that group 3 with laser 1 J/cm² was the least efficient in increasing the relative expression of mRNA for the odontogenic markers, and in subsequent odontogenic differentiation.

Regarding our PCR findings at day 21, The genes expression patterns of mineralized associated proteins BMP2, RUNX2 transcription factor and COL 1 were matched with results of previous studies [8,18,31].

BMP2, one of the odontogenic genes as it induces hard tissue formation [32] its role is important in regulation of odontogenic differentiation and control the mineralization of dentin matrix, gene were significantly upregulated than control and the highest was in Group 6, while an increase in RUNX2 genes expression were significantly up-regulated in Group 6, Group 5 and Group 4. The last 2 groups were different against group 6 p value = 0.001 and 0.002 respectively. The present results were the same as recent studies [27,30] but Previous studies have shown that RUNX2 downregulated in course of odontogenic differentiation, but (RUNX2) is important in odontoblast differentiation and dentin mineralization by regulating the expression of noncollagenous matrix proteins [32]. Widbiller et al. [32] stated that more quantification

of RUNX2 protein level might be important but it require large cell numbers.

At the beginning of dentinogenesis, upregulation of genes for extracellular matrix formation, like COL1A1, takes place [33]. Collagen type I is the major component of the connective tissue in dental pulp and encodes the collagenous protein that provides the framework for inorganic deposition [33]. In this study, the transcription of mRNA for COL1A1 was initially upregulated.

The genes expression patterns of mineralized associated proteins and RUNX2 transcription factor were matched with results of previous studies [27,32,33].

Present study measured the alkaline phosphatase activity at day 21 and showed that the statistically difference appeared only between (group 5, group 4 and group 6) which indicates the superior induction effect of laser in hard tissue formation as compared to previously approved effect of BD in ALP increasing that differ from group 2 in the present study which showed the increase but not significant and that can be explained due to the difference in the amount of BD used [8], also the difference between group 4 and group 6 was explained by the dose dependent mechanism of PBM [17].

In present study and to detect the extracellular mineralization ability VKS was used and the findings showed dense and darker nodules than control group. The darkest nodules appeared at group 6. These findings were the same as previous studies utilizing either BD or PBM (despite the various wavelengths used) even when they used other types of undifferentiated stem cells [26,34,35].

CONCLUSION

Photobiomodulation at 2J/cm² combined with biodentine enhanced the **HDPSCs** proliferation and odontogenic differentiation and thus could be a novel strategy for dentin regeneration.

Acknowledgments

None.

Conflict of Interest

The authors have declared no conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Regulatory Statement

This article approved by ethical committee of NILES numbered (019021).

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Date submitted: 2020 Sept 10
Accept submission: 2021 July 21