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Abstract. Photobiomodulation therapy (PBM) has shown positive effects on stem cell differentiation in monolayer cell culture model, but little is known about its effect on three-dimensional (3-D) agarose gel culture. This study evaluated the PBM effect of human dental pulp stem cells (hDPSCs) differentiation and phosphatase alkaline activity (ALP) using an agarose 3-D model under different nutritional conditions. hDPSCs were characterized and seeded on a 0.3% agarose gel layer with different media (osteogenic, adipogenic, chondrogenic) and were assigned into four groups: control 10% fetal bovine serum (FBS), control 5% FBS, PBM 10% FBS, and PBM 5% FBS. Irradiation was performed with continuous-wave InGaAlP laser, 660 nm, 100 mW, 3.3 J/cm², spot size 0.3 cm², 10 s of exposure time, and 1 J of energy per point with 6-h interval between sessions. All groups were evaluated at 7 and 14 days. ALP assay was performed to analyze the deposition of mineralized tissue. At 7 days, PBM 5% FBS group presented better stimulation in osteogenic and adipogenic differentiation compared with control. After 14 days, hDPSCs cultured in 3-D exhibited osteogenic, adipogenic, and chondrogenic differentiation; furthermore, compared to control, PBM significantly stimulated all differentiation processes ($p < 0.05$). It can be concluded that hDPSCs cultured in 3-D agarose associated to PBM could be a promising tool for tissue engineering applications. © 2018 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: [10.1117/1.JBO.23.9.095001](https://doi.org/10.1117/1.JBO.23.9.095001)]

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1 Introduction

Mesenchymal stem cells (MSCs) present important functions, such as capacity of self-renewal and development of tissues compatible with their origin.¹ The interest in the isolation of human dental pulp stem cells (hDPSCs) has increased substantially in recent years and the teeth being considered a relatively rich source of MSCs.² There are many markers to identify MSCs, including the STRO-1 surface marker, which is considered the main positive marker for MSCs and has the characteristic of not marking hematopoietic cells; CD44, a cell adhesion glycoprotein typical of mesenchymal cells; and CD146, a cell adhesion molecule.³ Several studies showed that hDPSCs could be characterized by a combination of these three markers together.^{1,3,4} In addition, it also recommended the analysis of differentiation in multilineage cells to confirm the stemness.⁵ The key to use stem cells is their ability to differentiate into various cell types, according to the stimulus received. MSCs present the potential of differentiation into osteoblasts, chondrocytes, adipocytes, muscle, and neural tissues, according to the microenvironment in which they are located.¹

In this context, photobiomodulation therapy (PBM) is described as a form of light therapy that utilizes nonionizing forms of light sources, including lasers, LEDs, and broadband light, in the visible and infrared spectrum. It is a nonthermal

process involving endogenous chromophores eliciting photo-physical (i.e., linear and nonlinear) and photochemical events at various biological scales.⁶ PBM could improve cell regeneration, stimulate cell growth, accelerate dentin regeneration after pulp exposure, increase cell metabolism, and promote tissue response.^{7,8} Additionally, PBM has been previously investigated and has been shown to promote biomodulation and increase the proliferation and differentiation of hDPSCs when applied in monolayer culture.^{9–11} It represents a beneficial impact on regenerative treatments, such as treatment of dental trauma or necrotic pulp with incomplete apex and periapical lesion, being used as a coadjuvant treatment in protocols of repair and root development, accelerating these processes.^{9–12}

Two systematic reviews demonstrated that irradiation should influence the differentiation effect when the energy density ranges from 0.5 to 4 J/cm² at wavelengths from 600 to 980 nm, and that an energy density higher than 10 J/cm² could damage photoreceptors, reducing the biomodulation effect of laser.^{13,14} Moreover, according to these systematics reviews, the greatest values of proliferation or differentiation were obtained using high power density, low energy density, and short exposure time.^{13,14} Other studies verified a tendency of PBM to improve MSCs viability without deleterious effects; however, considering their method heterogeneity and the limited evidence, it is difficult to obtain a clear conclusion.¹⁵

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Despite the important knowledge obtained from studies using monolayer cell culture, they do not accurately recreate the natural tissue architecture¹⁶ and some cell functions can be inhibited, thereby limiting their clinical application and relevance.¹⁷ Three-dimensional (3-D) cell culture analysis, which can better simulate *in vivo* the cellular conditions, can be a more physiologically relevant and predictive model, presenting greater stability and longer life expectancy than cells in monolayer culture.¹⁷ Moreover, the morphology of cells and intercellular signaling are more physiological in 3-D cultures than in monolayer culture, thereby promoting better knowledge about cellular function and differentiation *in vivo*.¹⁸ Thus, 3-D culture analysis seems to be more suitable for viability, cytocompatibility, and bioactivity assays.^{12,17}

Additionally, in 3-D culture, when the PBM reaches biological tissues during irradiation, the laser light can be reflected, scattered, absorbed, or transmitted to the surrounding tissues.¹⁹ Furthermore, these interactions are also influenced by several specific laser characteristics, such as power output, oscillation mode (continuous wave or pulsed wave), or application mode (contact or noncontact). Among various 3-D culture models, the agarose gel (a polysaccharide of D-galactose and 3,6-anhydro-L-galactopyranose derived from the cell walls of red algae) presents favorable characteristics in relation to implantation: it is bioinert, nontoxic, and decreases the potential immune rejection in rats.²⁰

Another important aspect is the positive influence of cultures under nutritional deficit conditions in the response of hDPSCs to PBM, improving cell growth.⁹ This happens because the nutritional deficit condition promotes an alteration of the redox state toward cell oxidation, and the cellular response to PBM is near optimal and stronger when potential redox is shifted.^{21,22} Moreover, cells with lower pH are considered more sensitive to the laser action than those with neutral pH.⁹ The nutritional deficit aims to simulate a clinical situation in which the tissue is in a stress process.²³ Therefore, the present study evaluated the PBM effect on osteogenic, adipogenic, and chondrogenic differentiation and phosphatase alkaline activity (ALP) of hDPSCs using an agarose gel 3-D model, under different nutritional conditions.

2 Materials and Methods

2.1 Subjects

The study was approved by the Institutional Review Board of the Federal University of Rio Grande do Sul, Brazil (CAAE 4545961 5.8.0000.5347). Human DPSCs were obtained from four permanent third molars with incomplete root formation, indicated for extraction due to orthodontic reasons, from two healthy patients aged 18 years old.

2.2 Stem Cell Culture

Freshly extracted teeth were immersed in alpha modification minimum essential Eagle's medium (α -MEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies) and 1% penicillin and streptomycin (PenStrep, Gibco), and then transported to the laboratory for processing. After extraction, the apical papilla was removed and the pulp tissue was isolated from apical foramen using Hedstrom files (Maillefer, Switzerland). After pulp removal, it was sectioned into fragments of $\sim 1 \text{ mm}^3$ and kept in

supplemented α -MEM at 37°C, 95% humidity, and 5% CO₂. The hDPSCs were obtained by explant culture in dish (35 × 10 mm) containing α -MEM. The culture medium was changed 24 h after pulp collection and at every 48 h to obtain explants of hDPSCs. When reaching the confluence (70% to 90% of cells) of the plate, the cells were trypsinized (Trypsin, Sigma-Aldrich) to the next passage.

2.3 Stem Cell Characterization

In the second passage (P2), the hDPSCs were analyzed by flow cytometry to confirm their stem cell nature. Briefly, an aliquot of cells was evaluated by staining for surface markers of MSCs (STRO1, CD146, and CD105) and for markers of hematopoietic stem cells (CD45 and CD14) (all from Santa Cruz Biotechnology). Additionally, the multilineage differentiation potential of hDPSCs, up to 14 days, was confirmed by culturing the cells in varied differentiation media:

- Osteogenic differentiation: α MEM supplemented with 1% penicillin and streptomycin, 10% FBS, 100 nM dexamethasone (Sigma-Aldrich, USA), 0.05 μM ascorbate-2-phosphate (Sigma-Aldrich, USA) and 10 mM β -glycerophosphate (Sigma-Aldrich);²⁴
- Adipogenic differentiation: α MEM supplemented with 1% penicillin, 10% FBS, 1 mmol/L dexamethasone, 5 $\mu\text{g}/\text{mL}^{-1}$ bovine insulin (Sigma-Aldrich), 0.5 mmol/L isobutylmethylxanthine (Sigma-Aldrich), and 60 mmol/L indomethacin (Sigma-Aldrich);²⁵ and
- Chondrogenic differentiation: α MEM supplemented with 1% penicillin and streptomycin, 10% FBS, 50 nmol/L ascorbic acid 2-phosphate, 6.25 mg/mL bovine insulin, and 10 ng/mL transforming growth factor-beta 1 (TGF- β 1/ Sigma-Aldrich).²⁶

2.4 Three-Dimensional Culture and hDPSC Differentiation

A pilot study was conducted to confirm the possibility of analysis of osteogenic, adipogenic, and chondrogenic differentiation of hDPSC in three-dimensional (3-D) culture. To obtain the 3-D culture, a 0.3% agarose solution (Sigma-Aldrich, St. Louis, MO) was prepared in α MEM and 100 μL of this solution were pipetted into each well of a 96-well plate (TPP, Trasadingen, Switzerland). After agarose solidification, 1×10^4 of hDPSCs (third passage) cultured in 150 μL of α MEM supplemented with 1% penicillin and streptomycin and regular 10% FBS were seeded in each well. During 14 days, the cells were maintained at 37°C, 95% humidity, and 5% CO₂ in osteogenic, adipogenic, and chondrogenic differentiation media, and after fixation, they were stained with alizarin red, oil red, and alcian blue, respectively. Homogeneous cell embedding in agarose was confirmed by light microscopy (100 $\times$).

2.5 Osteogenic, Adipogenic, and Chondrogenic Differentiation in 3-D Culture

Table 1 shows the distribution of all groups according to PBM and nutritional status: regular (10% FBS) or deficit (5% FBS). The 3-D culture preparation and seeding of hDPSCs were performed as previously described in the pilot study. After 24 h of cell adhesion, the cells were induced to different differentiation

Table 1 Experimental groups according to the PBM and nutritional condition (regular: 10% FBS or deficit: 5% FBS) for each differentiation analysis (osteogenic, adipogenic, and chondrogenic).

Groups	PBM	Nutritional condition
Control 10%	–	Regular
Control 5%	–	Deficit
PBM 10%	+	Regular
PBM 5%	+	Deficit

medium with 5% or 10% FBS according with the group. For that purpose, they were maintained in osteogenic, adipogenic, or chondrogenic media during 14 days. The medium was totally changed at every 48 h for 14 days.

2.6 Photobiomodulation Therapy

PBM was applied with a continuous-wave indium–gallium–aluminum–phosphide (InGaAlP) diode laser (MM Optics Ltd., São Carlos, Brazil) using the following parameters: wavelength of 660 nm, power of 100 mW, spot size of 0.3 cm², energy density of 3.3 J/cm², 10 s of exposure time, 1 J of energy per point of application, 6-h interval,²⁷ during 7 and 14 days consecutively, totalizing 28 and 56 J of energy in each point, respectively.

The laser probe was fixed perpendicular to each plate and in contact with the tissue culture plates. The cells were plated keeping empty wells between seeded wells, to minimize the unintentional dispersion of light between wells during laser application, and the output power of the equipment was tested using a power meter (Laser Check; MM Optics LTDA, São Paulo, Brazil). The control group was treated under identical conditions but with the laser device switched off.

2.7 Alizarin Red Staining

Cells were plated in triplicates according to the experimental groups described in Table 1. To evaluate the cells for their mineralized matrix synthesis potential, after 7 and 14 days, the cells were fixed in 10% formaldehyde solution (Sigma-Aldrich) washed with phosphate-buffered saline (PBS) (3×) and mineralized extracellular matrix stained with 2% alizarin red (Sigma-Aldrich), pH 4.2, for 20 min (24). Quantitative analysis of the nodules was performed, as proposed by Hessle et al.²⁸ For this, 1.0 mL of cetylpyridinium chloride (Sigma-Aldrich) was added to each well for 15 min for solubilization of nodules under agitation, followed by quantification in an ELISA reader (Thermo Fischer Scientific in, Waltham, Massachusetts) at 570-nm wavelength. The measuring absorbance of a well containing only agarose was subtracted from the absorbance of test groups for the three differentiations below.

2.8 Oil Red O Staining

After culture in adipogenic medium, as previously described, staining with oil red O dye (Sigma-Aldrich) was performed to visualize lipid vesicles in the cells after 7 and 14 days of

culture. The cells were fixed with 2% paraformaldehyde (Sigma-Aldrich) for 30 min, washed with PBS, and stained with the dye diluted in PBS (6:4) for 1 h. After this, the cells were washed with PBS and observed under a light microscope. Stained oil red O was also eluted with 4% Nonidet P-40 (Sigma-Aldrich) in isopropanol (vol/vol) for further quantification by spectrophotometry at 490 nm.²⁹

2.9 Alcian Blue Staining

To evaluate the chondrogenic potential of cells, alcian blue (Sigma-Aldrich) dye staining was performed in the cultures after 7 and 14 days. The dye was prepared using 1 g of alcian blue dissolved in 100 mL of 3% acetic acid (Sigma-Aldrich). The cells were fixed with 2% paraformaldehyde (Sigma-Aldrich) for 30 min, washed with PBS, and stained with the diluted dye in PBS (6:4) for 1 h. Stained alcian blue was also eluted with 4% Nonidet P-40 (Sigma-Aldrich) in isopropanol (vol/vol) for further quantification by spectrophotometry at 490 nm.²⁹

2.10 Alkaline Phosphatase Activity in 3-D Culture

ALP enzyme was used as a marker of osteoblastic activity (Labtest Diagnostica, Lagoa Santa, MG, Brazil) and was measured in hDPSCs cultured in 3-D agarose after 7 and 14 days in all groups described in Table 1. The cells were lysed with TRIS 1% (Sigma-Aldrich) for 50 min at room temperature and the experiment was performed according to the manufacturer's instructions. The absorbance was measured in triplicate by 96-well plate (TPP) spectrophotometry at 590 nm in a spectrophotometer, the results were calculated and data were expressed as ALP enzyme activity (U/L).

2.11 Statistical Analysis

All experiments were performed in quadruplicate. Differences between test and control groups were analyzed by Kruskal–Wallis followed by Dunn tests on the software GraphPad Prism 5 (GraphPad Software Inc., San Diego, California), considering a significance level of 5% ($p < 0.05$).

3 Results

3.1 hDPSCs Characterization

The hDPSCs expressed typical levels of MSC surface markers (Fig. 1). The cultures expressed positivity for MSC markers (CD105, CD146, and STRO-1), whereas the hematopoietic cell markers (CD45 and CD14) were absent or minimally expressed. The percentage of positive cells for the CD105 was 98.8%, for CD146 was 32.2%, and STRO-1 was 3.9% for the hDPSC population. The percentage of cells for the CD45 was 0.3% and CD14 was 0.2% for the hDPSC population.

Moreover, those cells cultured in monolayer during 14 days showed a deposition of mineralized matrix as stained by alizarin red, lipid vesicles stained by oil red O, and glycosaminoglycans deposits stained by alcian blue, indicating, respectively, the osteoblastic, adipose, and chondroblastic differentiation (Fig. 2). Thus, according to these results, the cells analyzed could be defined as hDPSCs.

Fig. 1 Immunophenotyping of hDPSCs by flow cytometry. Cultures expressed positivity for MSC markers (a) CD105, (b) CD146, and (c) STRO-1, whereas the hematopoietic cell markers (d) CD45 and (e) CD14 were absent or minimally expressed.

Fig. 2 Characterization of hDPSCs for their *in vitro* ability for osteogenic, adipogenic and chondrogenic differentiation. (a) hDPSCs cultured without induction of differentiation as negative control. (b) Calcium deposits stained with alizarin red. (c) Lipid vacuoles stained by oil red. (d) Alcian blue staining of glycosaminoglycans deposits (original magnification, $\times 100$).

3.2 Photobiomodulation Therapy Promotes Increase in hDPSCs Differentiation in 3-D Agarose Model

Initially, a pilot study was performed to evaluate the differentiation capacity of hDPSCs cultured in 3-D agarose model. As demonstrated in Fig. 3, hDPSCs cultured in 3-D after 14 days exhibited osteogenic [Fig. 3(a)], adipogenic [Fig. 3(b)], and chondrogenic differentiation [Fig. 3(c)]. These results indicated that agarose did not interfere with the differentiation properties of these stem cells and also in the absorbance measuring process.

Afterward, the effect of PBM on the ability of hDPSCs to differentiate in a 3-D culture model was evaluated (Figs. 4–6). PBM demonstrated positive effects, significantly stimulating both osteogenic, adipogenic, and chondrogenic differentiation processes. All irradiated groups significantly increased the process of the three types of differentiation after 14 days ($p < 0.05$).

Osteogenic differentiation was analyzed by a calcium deposition after 7 and 14 days of cell culture in 3-D and PBM

treatment. After 7 days, only the PBM 5% FBS presented significant increase in osteogenic differentiation compared to control 10% FBS ($p < 0.001$) [Fig. 4(a)]. At 14 days, both groups that received PBM (10% FBS and 5% FBS) exhibited significantly increased mineralization compared to controls ($p < 0.001$) [(Fig. 4(b))].

The ALP assay in 3-D culture confirmed the results of osteogenic differentiation following the same trend in enzyme activity. At 7 days, the highest activity for ALP enzyme was observed in PBM 5% FBS group compared with controls (10% and 5% FBS) [Fig. 4(c)] ($p < 0.05$). At 14 days, all PBM groups (10% and 5% FBS) presented significantly higher ALP compared to controls ($p < 0.05$) (10% and 5% FBS) [Fig. 4(d)].

Regarding the analysis of adipogenic differentiation after 7 days, a decreased capacity was observed in control 5% FBS group compared to PBM groups (10% FBS and 5% FBS) [Fig. 5(a)]. However, at 14 days, all PBM groups (10% FBS and 5% FBS) showed significant increase in lipid vesicles formation compared to control groups (10% FBS and 5% FBS) ($p < 0.05$) [Fig. 5(b)].

Fig. 3 Macroscopic aspect of controls and hDPSCs with osteogenic, adipogenic, and chondrogenic differentiation in 3-D agarose gel cultures. (a) Osteogenic differentiation, (b) adipogenic differentiation, (c) chondrogenic differentiation, (d) osteogenic control, (e) adipogenic control, and (f) chondrogenic control (original magnification, well diameter = 6.4 mm).

Fig. 4 PBM promoted an increase in (a and b) hDPSCs osteogenic differentiation and (c and d) osteoblastic activity. Bars with different letters represent significant differences between groups in each time point ($p < 0.05$).

Fig. 5 Adipogenic differentiation of hDPSCs after (a) 7 and (b) 14 days of 3-D culture model. Bars with different letters represent significant differences between groups in each time point ($p < 0.05$).

Fig. 6 Chondrogenic differentiation of hDPSCs after (a) 7 and (b) 14 days evaluated by the deposition of glycosaminoglycans. Bars with different letters represent significant differences between groups in each time point ($p < 0.05$).

Chondrogenic differentiation evaluated by a deposition of glycosaminoglycan was similar among groups at 7 days ($p > 0.05$) [Fig. 6(a)]. At 14 days, PBM promoted an increase in a deposition of these proteins compared only to the control 10% FBS ($p < 0.05$) [Fig. 6(b)].

4 Discussion

MSCs are characterized by their high proliferative potential, regenerative capability, and ability to differentiate *in vitro* into osteoblasts, chondrocytes, adipocytes, myocytes, and neural cells according to the microenvironment in which they are located.^{1,30} A variety of tissues present MSCs that may be used in tissue engineering and regenerative medicine studies.¹ Among them, the tooth is considered a rich source of these cells, identified by Gronthos et al.¹ as DPSCs. Additionally, PBM enhanced the proliferation and differentiation of DPSCs in monolayer cell culture, which does not accurately recreate the natural tissue architecture. Within that context, the identification of factors that enhance the proliferation and/or differentiation of DPSCs is extremely important. Thus, the present study analyzed (a) the capacity of hDPSCs to differentiate in three lineages using 3-D agarose culture model and (b) the effect of PBM on the differentiation potential of hDPSCs in 3-D agarose culture model under different nutritional conditions. The PBM demonstrated positive effects, significantly stimulating the osteogenic, adipogenic, and chondrogenic differentiation process in 3-D model.

Initially, hDPSCs were obtained and characterized by the combination of two methods, multilineage differentiation potential (osteogenic, adipogenic, and chondrogenic) and by flow cytometry to assess the expression of STRO-1, CD44, and CD146 markers. These methodologies are corroborated by several studies that used the same markers of MSCs^{1,3,4} and also by three multilineage differentiation potentials.^{5,30} All these results were obtained using the monolayer culture (2-D) model that has been used as standard in several *in vitro* studies with MSCs; however, some authors appoint that this arrangement of cells does not mimic the cell-cell interaction of tissues.³¹ Currently, 3-D culture has become the best way to facilitate cell adhesion and proliferation, and it preserves the physiological function of tissue.^{16,18,32} Therefore, the 3-D culture model could mimic the natural tissue architecture, facilitating analysis of the differentiation potential, since it is influenced by the surrounding microenvironment.^{16,18,32} Then, we decided to investigate if hDPSCs maintain the capacity of osteogenic, adipogenic, and chondrogenic differentiation in three lineages using 3-D culture model. Some previous cell culture investigations used 3-D agarose gel model with different concentrations ranging from 0.9% to 3%.^{33–35} Srikanth et al.³⁵ demonstrated that a low concentration of agarose allows better understanding of the osteogenic differentiation when compared to higher concentrations. In the present study, we selected 0.3% agarose that was a favorable concentration for cell adhesion and differentiation with easy cell manipulation during change of media

and analysis steps. Our results showed that hDPSCs maintained the capacity of multilineage differentiation using 3-D agarose culture model. These results are in accordance with some studies that demonstrated the synthetic and natural-based scaffolds, such as collagen sponges, chitosan, hydroxyapatite, polymer, biomatrix, or platelet lysate, have been used to load hDPSCs and showed that mineralized tissue can be formed when they are employed.^{32,36,37} Also, agarose has been considered a bioinert scaffold with nontoxic properties.²⁰

PBM is an emerging technique in which exposure to low-level laser light (red and near infrared radiation) or LEDs stimulates cellular function by the activation of mitochondrial respiratory chain components, resulting in initiation of a signaling cascade that promotes activation of some signaling circuitries resulting in increased cellular metabolism and proliferation.²¹ Considering that PBM could allow a significant increase in viability, proliferation, migration, and differentiation of stem cells, the 3-D culture associated to its use may be a therapeutic opportunity in regenerative dentistry.¹¹ Then, we tested the effect of PBM on the differentiation potential of hDPSCs in 3-D agarose culture model with different nutritional conditions.

The hDPSCs, in 3-D agarose-based model and irradiated at the energy density tested in the present study, presented early mineral deposition in the cultures (7 days after induction). Recently, Diniz et al.³⁸ showed that PBM is able to improve cell survival of DPSCs encapsulated in an injectable and thermoresponsive cell carrier (Pluronic® F-127) loaded with recombinant human bone morphogenetic protein 4 (rhBMP4). In addition, PBM accelerated odonto/osteogenic differentiation of dental derived MSCs.³⁸

Our main results showed that PBM (10% FBS and 5% FBS) promoted an increase in hDPSCs differentiation (osteogenic, adipogenic and chondrogenic), especially after 14 days, when compared with nonirradiated groups. These findings agree with previous investigations that analyzed the influence of PBM in osteogenic differentiation and ALP expression in monolayer culture using different irradiation parameters.^{38–40} By contrast, other authors also evaluated the PBM effect in hDPSCs using different 3-D cultures and observed that ALP activity increased until 7 days but gradually decreased until 14 days.^{12,32} These contradictory results could be justified by biomaterials used or PBM protocols, including the different time intervals between each application used at the referred studies: 1-day interval, 30 cycles of 30 s only at first day, or 3-day intervals, respectively. Herein, the 6-h interval was used because laser therapy exerts a dose-dependent effect on the biological responses and seems to have a cumulative effect at each new dose applied, and the best result of PBM has been observed in this time interval.²⁷

Some studies attributed the biostimulative effect of PBM on osteoblasts to an increased expression of autocrine factors that regulate osteoblast proliferation and differentiation, such as type 2 bone morphogenetic protein (BMP-2) and TGF- β 1.^{12,39,40} These data suggest that PBM stimulates osteoblast differentiation in the initial proliferative and matrix synthesizing stages. Recently, an investigation using an *in vivo* model showed that PBM therapy allowed the stem cells to be influenced by the growth factors released from the root canal walls, resulting in cell differentiation and tissue formation.⁴¹ Also, molecular analyses indicated that red photobiomodulation therapy in osteoblasts and hMSCs stem cells increased vinculin-rich clusters, osteogenic markers expression (Runx-2, alkaline phosphatase,

osteopontin, Col I, OCN, DSPP, DMP1, and HSP27), and mineralized bone-like nodule structure deposition.^{42–44}

Adipogenic differentiation was previously investigated to characterize the stem cells or evaluate some materials.^{18,25,30} However, until this moment, it was not used to quantify the lipid vesicle deposition induced by PBM. Similarly, in osteogenic differentiation and ALP activity, the adipogenic differentiation was increased in our study by PBM from 7 to 14 days ($p < 0.05$).

The association of PBM and chondrogenic differentiation was previously evaluated by Kushibiki et al.⁴⁵ using a prechondrogenic activated T cell death (ATDC5) cells in monolayer culture. The authors observed that PBM enhanced the total collagen contents 14 days after low level laser irradiation ($p < 0.01$). Agreeing with them, the present investigation observed that hDPSCs induced chondrogenic differentiation in agarose gel, showing a significant increase in the deposition of glycosaminoglycans when PBM was used in comparison with the control 10% ($p < 0.05$). The present findings about the PBM biostimulation on the adipocyte and chondrocyte expression genes and on the modulation of both differentiations must be further elucidated by new investigations.

With the intention to improve the effect of PBM on proliferation and differentiation, previous investigations demonstrated that a culture under nutritional deficit plays a positive influence on cell growth.^{9,43} Some studies evaluated the proliferation of DPSCs, human exfoliated deciduous teeth (SHEDs) and fibroblasts, respectively, under nutritional deficit of 5% FBS in monolayer culture.^{9,23,46} They found that irradiated cells in nutritional deficit presented cell growth similar or higher than the control cells in ideal nutritional condition. No studies have evaluated the effect of PBM in hDPSCs differentiation under nutritional deficit (5% FBS). Thus, the present study compared the osteogenic, adipogenic, and chondrogenic differentiation under nutritional deficit or regular nutrition in 3-D culture. Our results showed that in general, PBM improved the differentiation capacity of hDPSCs. In addition, deficit nutritional condition (5% FBS + PBM) was associated to higher stem cell differentiation. This is a very significant discovery that can be justified by the fact that deficit condition alters the redox state toward oxidation of cells (oxidative stress) and the cellular response to PBM is near optimal and stronger when potential redox is shifted.²¹ Under conditions such as exposure to excessive or prolonged oxidative stress, the increase in ROS may reach a threshold level that triggers the opening of a mitochondrial channel favoring PBM effects.⁴⁷ PBM basic mechanisms have been described and are related to activation of cellular cytochromes that promote a cascade of events leading to increase in ATP production, reduction of oxidative stress, and initiation of secondary cell-signaling pathways. The overall results of PBM are the increase of energy metabolism, improvement of cell viability, and modulation of several cellular processes.⁴⁴ Cells in nutritional deficit present higher oxidative stress than cells in normal condition (10% FBS); therefore, they are more sensible to PBM stimulation. But, the mechanisms involved in the positive effect of PBM on hDPSCs differentiation need to be more deeply investigated. According to Hamblin,⁴⁷ energy provided by PBM induces biomodulation by stimulating membranes, organelles, and modulation of oxidative stress of the target cell to restore its functions normality leading to hDPSCs to have more differentiation or proliferation.

5 Conclusion

PBM therapy positively influenced the differentiation process of hDPSCs cultured in 3-D agarose being a promising tool for tissue engineering applications.

Disclosures

No competing financial interests exist.

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