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Photobiomodulation Therapy in the Proliferation and Differentiation of Human Umbilical Cord Mesenchymal Stem Cells: An *In Vitro* Study

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Abstract

Introduction: Since photobiomodulation therapy (PBMT) favors *in vitro* mesenchymal stem cell (MSC) preconditioning before MSC transplantation, increasing the proliferation of these cells without molecular injuries by conserving their characteristics, in the present *in vitro* study we analyzed the effect of PBMT on the proliferation and osteogenic differentiation of human umbilical cord mesenchymal stem cells (hUCMSCs).

Methods: Irradiation with an InGaAlP Laser (660 nm, 10 mW, 2.5 J/cm^2, 0.08 cm^2 spot size, and 10 s) was carried out. The cells were divided into four groups: CONTROL [cells grown in Dulbecco's Modified Eagle Medium (DMEM)], OSTEO (cells grown in an osteogenic medium); PBMT (cells grown in DMEM+PBMT), and OSTEO+PBMT (cells grown in an osteogenic medium plus PBMT). The cell proliferation curve was obtained over periods of 24, 48 and 72 hours using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Osteogenic differentiation was analyzed by the formation of calcium nodules over periods of 7, 14 and 21 days. Morphometric analysis was performed to quantify the total area of nodular calcification.

Results: The highest cell proliferation and cell differentiation occurred in the OSTEO+PBMT group, followed by the PBMT, OSTEO and CONTROL groups respectively, at the observed times ($P<0.05$).

Conclusion: PBMT enhanced the osteogenic proliferation and the differentiation of hUCMSCs during the periods tested, without causing damage to the cells and preserving their specific characteristics, a fact that may represent an innovative pretreatment in the application of stem cells.

Keywords: Photobiomodulation; Lasers, Stem cells; Cell differentiation.

Introduction

Due to their potential application to tissue regeneration and formation, mesenchymal stem cells (MSCs) have been studied in different areas in order to replace conventional treatments.[1] MSCs are undifferentiated and have the potential for application to cell therapy because of their characteristics of self-renewal, proliferation, and differentiation into various types of specialized cells.[2] One of the findings that makes the use of MSCs interesting in the clinical setting, is their ability to migrate to the damaged tissue or toward inflammatory sites after intravenous administration.[3]

The human umbilical cord contains MSCs with a high potential for cell proliferation that can be isolated in a relatively easy, painless, and noninvasive manner. In addition, these cells pose a low risk of infection and show multipotency and more rapid self-renewal properties compared to bone marrow MSCs.[4,5] To be used for cell therapy, MSCs must be expanded *in vitro*. Thus, growth factors can be used in culture media as osteogenic inductors

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in order to promote appropriate cell expansion.[6] Growth factors stimulate and guide stem cells so that they will proliferate and differentiate into osteoblasts, adipocytes or chondroblasts under standard *in vitro* differentiating conditions.[7] Over the last few years, photobiomodulation therapy (PBMT) has been indicated as an effective process in regenerative medicine and dentistry due to its cellular stimuli and biomodulator effects *in vitro* and *in vivo*.[8-12] PBMT enhances MSC proliferation and differentiation by energy absorption by intracellular chromophores, producing adenosine triphosphate (ATP) and increasing DNA activity as well as RNA and protein synthesis.[12]

Since PBMT favors *in vitro* MSC preconditioning before MSC transplantation, increasing the proliferation of these cells without molecular injuries by conserving their characteristics,[13,14] we conducted an *in vitro* study in order to determine the effect of PBMT on the proliferation and osteogenic differentiation of human umbilical cord mesenchymal stem cells (hUCMSCs). Also considering the few studies of this type available in the literature, our objective was to determine whether the irradiation parameters employed contributed in a positive manner to cell proliferation and differentiation.

Materials and Methods

Cell Culture

Patients' anonymity was guaranteed according to the Helsinki Declaration. Human umbilical cords were obtained from cesarean deliveries carried out at a private hospital in Recife, Brazil. Wharton's jelly cells of umbilical cord were isolated and placed in culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM, Gibco, CA, USA) supplemented with 15% fetal bovine serum (FBS, Gibco, CA, USA), 20% Ham F-12 (Gibco, CA, USA), penicillin (10 000 unit/mL) and streptomycin. The flasks were kept in a 5% CO_2 atmosphere at 37°C in an incubator and the cells were used in the third passage when they reached 80% confluence.

Phenotypization

hUCMSCs were phenotyped in order to confirm their nature using a BDFAC Scalibur™ flow cytometer (Bioscience, San Jose, CA, USA). The cells were incubated with fluorescent monoclonal antibodies CD90 (EXBIO Antibodies, Prague, Czech Republic), CD44 and CD29 (EXBIO Antibodies) associated with fluorescein isothiocyanate (FITC), and CD45, CD34 and CD31 associated with phycoerythrin (PE) (diluted: 1:2000), for 60 minutes at 4°C.

Experimental Conditions

Cells were seeded into 24- and 96-well plates, kept in an incubator with 5% CO_2 at 37°C and divided at random into four groups: CONTROL (cells grown in DMEM), OSTEO (cells grown in an osteogenic medium), PBMT (cells grown in DMEM+PBMT), and OSTEO+PBMT (cells grown in an osteogenic medium plus PBMT).

The inductors of differentiation were inserted after 24 hours of plating. The osteogenic differentiation medium was prepared with DMEM (Gibco, CA, USA) supplemented with 10% FBS (Gibco, CA, USA), penicillin (10 000 unit/mL) (Gibco, CA, USA) with streptomycin (Gibco, CA, USA), 50 µg/mL ascorbic acid (Sigma Aldrich, SP, Brazil), 1 mM β-glycerophosphate (Sigma Aldrich, SP, Brazil), and 10 nM dexamethasone (Sigma Aldrich, SP, Brazil). The cell culture medium was changed every 72 hours during the 21 days of the experiment.

After 24 hours of plating, PBMT and OSTEO+PBMT cells were irradiated with the InGaAIP laser (660 nm) (MMOptics®, Equipamentos Ltd., São Carlos, SP, Brazil) as follows: continuous mode, 10 mW, 2.5 J/cm^2, 0.08 cm^2 spot size and 10 seconds. Before irradiation, the plates were covered with black cardboard with only one orifice perforated according to the diameter of the wells due to the scattering characteristics of irradiation. The experiments were carried out in a standardized manner using a claw-shaped adapter attached to the tip of the equipment positioned 6 cm below the center of the well of the culture plates.[15] In view of the scattering characteristics of laser radiation, the wells were intercalated so that no energy accumulation would occur.

MTT Assay

A solution containing 0.5 mg/mL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma Aldrich) was added to the wells and the plates protected from light were incubated at 37°C for 4 hours. DMEM combined with MTT was then removed from the wells and dimethyl sulfoxide (DMSO, Sigma, SP, Brazil) was added in order to solubilize the crystals formed. Absorbance at 590 nm was read with a spectrophotometer (Flx 800, Fluorescence Microplate Reader software, version 2.06.10, BIOTEK, Winooski, VT, USA) and the cells were analyzed at intervals of 24, 48 and 72 hours in order to construct the cell proliferation curve.

Alizarin Red Stain

The analysis was carried out 7, 14 and 21 days after the beginning of induction. After culture in inductor and control media with and without irradiation, each well was washed three times with PBS for the complete removal of the media. The cells were fixed in 300 µL 10% paraformaldehyde for 20 minutes at room temperature and then treated with 300 µL Alizarin Red (Sigma) for 15 minutes for the detection of nodular calcium formation. The wells were then abundantly washed with deionized water in order to remove excess dye and the wells of each experimental group were photographed with a camera coupled to an inverted light microscope at 40× magnification (Leica DM1000, Leica Microsystems Wetzlar GmbH, Germany). The images obtained were analyzed with LAS Interactive Measurement software

(Leica, Wetzlar, Germany) and the area (pixel^2) of osteogenic differentiation was delimited by a duly calibrated operator (J.M.M.). All experiments were carried out in triplicate.

Data Analysis

The histomorphometry data were analyzed by one-way ANOVA complemented with the Tukey test, with the level of significance set at 5% (P<0.05), using Oringi (Pro) software, version 8 (Origin Lab Corp., Northampton, MA, USA).

Results

Immunophenotyping on hUCMSCs

hUCMSCs were found to be positive for adhesion molecules (CD44, 51.3%), marker protein of integrin (CD29, 54.4%) and extracellular matrix proteins (CD90, 84.1%), whereas they were found to be negative for hematopoietic markers (CD34, 91.3% and CD45, 90.9%) and the endothelial marker (CD31, 92.3%).

MTT Assay

The cell proliferation curves based on optical density values obtained from absorbance reading with a spectrophotometer after the MTT assay according to time of induction (24, 48 and 72 hours) for each group are shown in Figure 1. We observed that the highest concentration of cell proliferation was achieved in the irradiated groups OSTEO+PBMT, followed by PBMT. No statistically significant difference (P>0.05) was observed between the groups 24 hours after induction. After 48 and 72 hours of induction, there was no statistically significant difference (P>0.05) between the CONTROL and OSTEO groups, although there was a significant difference (P<0.05) between the CONTROL group and the PBMT and OSTEO+PBMT groups, as well as between all experimental groups.

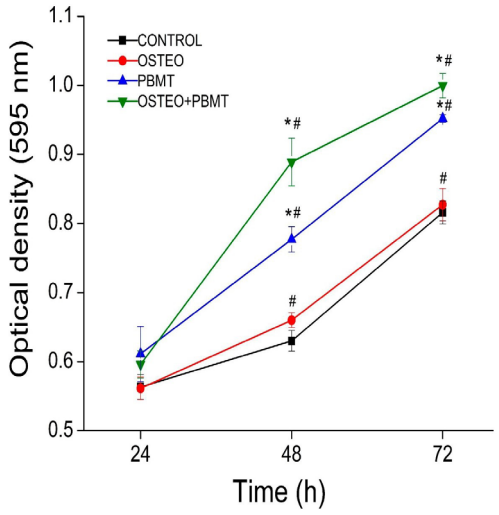

Figure 1. Growth Curves of Human Umbilical Cord Mesenchymal Stem Cells Grown in DMEM (CONTROL), in an Osteogenic Medium (OSTEO), in DMEM Plus Photobiomodulation Therapy (DMEM+PBMT), and in an Osteogenic Medium Plus PBMT (OSTEO+PBMT) for Periods of 24, 28 and 72 hours.

Morphological and Morphometric Features

Morphological analysis of the culture plate wells agreed with the morphometric analysis. Osteogenic activity was observed according to the formation of calcium nodules. Seven days after induction there was an increase in osteogenic activity in the OSTEO+PBMT group, followed by the PBMT, OSTEO and CONTROL groups (Figure 2). The increase in activity was maintained among the groups 14 and 21 days after induction, with the following groups showing the greatest osteogenic activity in increasing order: CONTROL, OSTEO, PBMT followed by OSTEO+PBMT.

Morphometric analysis revealed a significant difference (P<0.05) between all groups at all induction times compared to CONTROL, except for the time of seven days for the OSTEO and PBMT groups. However, these groups differed significantly (P<0.05) when compared to the OSTEO+PBMT group, which was the group with the highest area (pixel^2) values of osteogenic differentiation (Figure 3).

Discussion

Stem cell therapy is a promising treatment for the induction of bone regeneration.[16,17] In addition, several studies have confirmed that PBMT increases the viability, migration, proliferation and induction of differentiation of stem cells from different tissues,[7,13,15,18-20] favoring the preconditioning of these cells before their transplantation.[7] In the present study, we investigated the effect of PBMT on the proliferation and osteogenic differentiation of hUCMSCs and demonstrated a synergism of the association of PBMT with the osteogenic medium, increasing the proliferation and osteogenic differentiation of these cells.

It is known that the molecular mechanism promoted in cells by PBMT is associated with increased gene expression of anti-inflammatory cytokines, including interleukin (IL) 1-alpha and IL-6, as well as inhibition of IL-1β by human keratinocytes following laser irradiation.[21] Thus, these cell responses are believed to occur through the synthesis of ATP, the increased potential of the mitochondrial membranes and the levels of cyclic adenosine monophosphate.[22-24] In this regard, in order to use PBMT, it is important to establish the parameters of irradiation since different standards may produce different effects, especially regarding cell proliferation,[25,26] inducing stimulatory or inhibitory responses.[27,28] Indeed, when we compared the irradiation parameters used, we observed ample divergence between these variables, demonstrating the lack of protocol standardization and the consequent modification of the results.

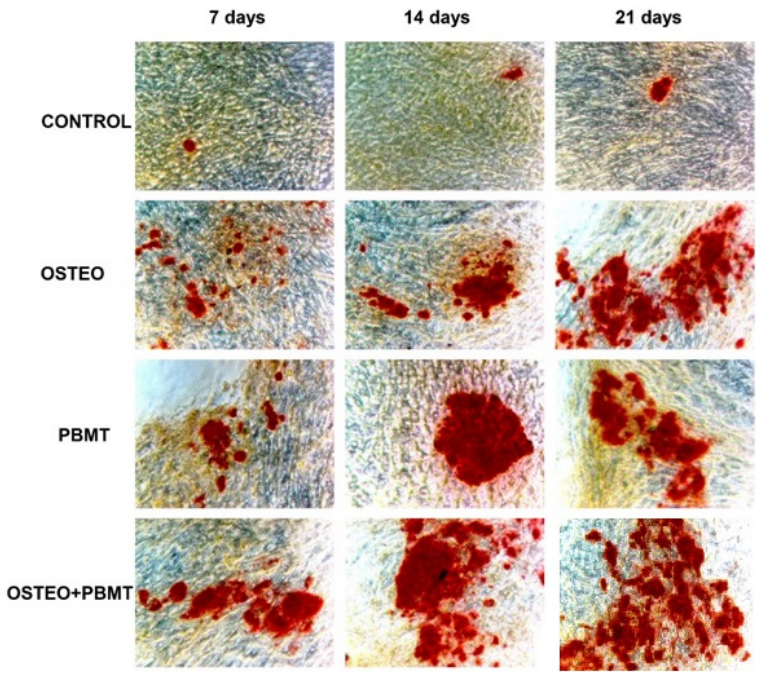

Figure 2. Photomicrographs of Osteogenic Differentiation on Days 7, 14 and 21 After the Beginning of Induction. (A) CONTROL group (cells grown in DMEM); (B) OSTEO groups (cells grown in osteogenic medium); (C) DMEM plus PBMT group (cells grown in DMEM plus photobiomodulation therapy); (D) OSTEO+PBMT group (cells grown in an osteogenic medium plus PBMT). Note the higher osteogenic activity of the group in which the osteogenic medium was associated with PBMT. Alizarin Red, 40× magnification.

Accordingly, we used a laser with a 660 nm wavelength that was able to induce positive biomodulatory effects.[10,15] In line with this, Fekrazad et al[7] reported promising results about MSC proliferation using a laser with a wavelength in the 600-700 nm range. In contrast, other studies[28,29] applying a wavelength of 808 nm to one of their experimental groups did not obtain positive biomodulatory effects regarding MSC proliferation and viability, which is probably due to the high energy density (20 mW/cm^2) used. Indeed, it has been observed that low energy power and density values do not damage the cellular photoreceptors and consequently contribute to the biomodulatory effect of a laser.[10,15,29-31]

Zaccara et al[15] used dental pulp stem cells (DPSCs) irradiated with laser (0.5 and 1.0 J/cm^2) and de Andrade et al[32] treated adipose tissue-derived mesenchymal stem cells (AD-MSC) with a 660 nm laser, with 40 mW power and 0.56, 1.96 and 5.04 J energy. Both authors reported a statistically significant difference between the irradiated and control groups after the MTT assay, demonstrating that PBMT promoted the proliferation of both DPSCs and AD-MSC. Our results agree with these previous reports since the hUCMSCs studied here showed increased cell viability and proliferation when associated with PBMT. It is worth mentioning that higher energies such as 5.04 J may be harmful to cell biostimulation.[32]

In the current study, the optical density values obtained showed that cell proliferation was even higher for the 72 hours time point after the beginning of induction, followed by the 48 and 24 hours time points. These results agree with those reported by Yang et al,[33] who observed the highest optical density in the group treated with LED light associated with the osteogenic medium on days five and seven. Although the cited study used LED light (with a central band of 620 nm) in contrast to the present study which used a 660 nm laser, these similar results may be explained by the fact that a laser emits coherent light and LED emits non-coherent light, but both sources have photobiological effects when used with similar irradiation parameters.[34]

Interestingly, we observed that the groups treated with PBMT had a larger area of mineralized nodules, with an even higher osteogenic activity in the group in which this treatment was combined with the medium for osteogenic induction. In line with this, Wang et al[17] used a laser at densities of 2 and 4 J/cm^2 in combination with a medium of osteogenic differentiation and observed that this treatment significantly promoted the proliferation and osteogenesis of bone marrow MSCs on the 7th and 21st days after the beginning of induction. In addition, studies investigating the effects of PBMT on the neural differentiation of hUCMSCs[35] have reported promising results demonstrating that a 635 nm laser increased cell proliferation and that an 808 nm laser combined with cerebrospinal fluid favored neural differentiation in MSCs, showing that the combination of PBMT with the biological inductor contributed to cell differentiation according to the medium to which MSCs were added.[35]

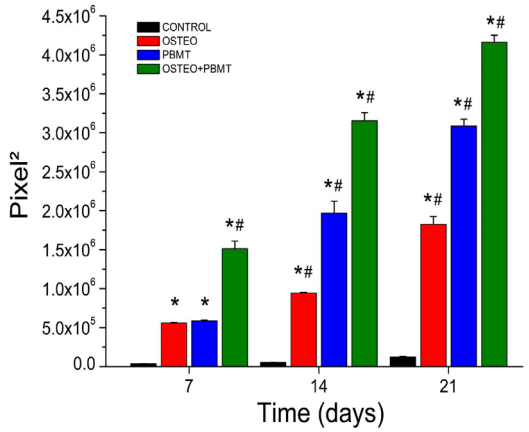

Figure 3. Mean + Standard Deviation of the Area (pixel²) of Osteogenic Differentiation in the Various Groups According to the Various Induction Times.

Despite the difficulty in comparing the experimental results obtained here with PBMT to those obtained in previous studies due to the variation in the irradiation parameters and the different cell tissue used, the present results suggest that the combination of PBMT with hUCMSCs can improve the *in vitro* expansion of these cells, increasing their proliferation and osteogenic differentiation in order to achieve an appropriate quantity of cells for future implantation for bone tissue regeneration.

Conclusion

In summary, the osteogenic proliferation and differentiation of hUCMSCs after induction were higher in the cells submitted to the osteogenic medium associated with PBMT with the parameters employed in this study, a relevant fact that may represent an innovative pretreatment in the application of stem cells, increasing cell proliferation without causing cell damage, maintaining its specific characteristics. Further studies associating UCMSC with PBMT are needed in order to establish the parameters of laser therapy with future clinical applicability.

Ethical Considerations

The study was approved by the Ethics Committee of the University of Pernambuco (No. 49503715.0.0000.5208).

Conflict of Interests

The authors declare no conflict of interest.

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