UNESP - Universidade Estadual Paulista

“Júlio de Mesquita Filho”

Faculdade de Odontologia de Araraquara

ANA PAULA SILVEIRA TURRIONI

EFEITO DIRETO E TRANSDENTINÁRIO DO LED 850 NM SOBRE O METABOLISMO E CAPACIDADE DE DIFERENCIAÇÃO ODONTOBLÁSTICA DE CÉLULAS-TRONCO DA POLPA DENTÁRIA IN VITRO

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“Vocês serão enriquecidos de todas as formas, para que possam ser generosos em qualquer ocasião e, por nosso intermédio, a sua generosidade resulte em ação de graças a Deus.” 2 Coríntios 9:11

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"Ninguém é tão ignorante que não tenha algo a ensinar.

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Turrioni APS. Efeito direto e transdentinário do LED 850 nm sobre o metabolismo e capacidade de diferenciação odontoblástica de células-tronco da polpa dentária in vitro [Tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2015.

RESUMO

O objetivo geral da presente pesquisa foi avaliar o efeito do LED 850 nm sobre diferentes processos metabólicos de células-tronco da polpa dentária in vitro. Para todos os estudos, células originárias da polpa de dentes decíduos humanos hígidos foram caracterizadas por imunofluorescência utilizando os anticorpos STRO-1, CD44, CD146, Nanog e OCT3/4, antes da realização dos protocolos experimentais. No estudo 1, células foram cultivadas em placas de acrílico, estimuladas à diferenciação odontoblástica e irradiadas com LED (850 nm) em diferentes doses de energia (DE - 0, 2, 4, 8, 15 ou 30 J/cm2, 40 mW/cm2), n=9. Após 12 h e 72 h da irradiação, foram avaliados o metabolismo celular (MTT), a formação de nódulos mineralizados (NM – Alizarin Red) e o número de células viáveis (Trypan Blue). Os dados foram submetidos aos testes de Kruskal-Wallis e Mann-Whitney ($p<0,05$). Para o estudo 2, o mesmo protocolo experimental foi realizado, porém utilizando apenas as doses de 2 e 4 J/cm2 e o tempo de avaliação de 72 h pós-irradiação. Foram avaliados a atividade de fosfatase alcalina (ALP), a produção de proteína total (PT), produção de colágeno total (Sircol), bem como a expressão gênica (qPCR) de ALP, colágeno tipo I (Col I), sialoproteína da dentina (DSPP) e proteína da matriz dentinária (DMP-1), n=8. Os mesmos testes estatísticos foram utilizados. Para o estudo 3, as células foram semeadas sobre a

superfície pulpar de discos de dentina com 0,2 mm de espessura (n=72), submetidas ao mesmo protocolo de irradiação do estudo 2, aplicada sobre a superfície oclusal dos discos de dentina (n=8, ação transdentinária). Os testes incluíram viabilidade celular (MTT), atividade de ALP, produção de PT, expressão gênica de ALP, Col I, DSPP e DMP-1 e morfologia celular (MEV) e os dados foram submetidos aos mesmos testes estatísticos. Para o estudo 1, quando comparadas ao grupo controle, as células irradiadas com 2 ou 4 J/cm2 exibiram maior viabilidade celular após 72 h e todas as DEs aumentaram o número de células viáveis após 12 h. Para a formação de NM, os melhores resultados foram encontrados 72 h após a irradiação, para as doses de 8 e 15 J/cm2. Para o estudo 2, um aumento na atividade de ALP e síntese de colágeno, bem como aumento na expressão de DSPP e ALP foi observado para as duas DEs quando comparadas ao controle, sendo que 4 J/cm2 também estimulou a expressão gênica de Col I e DMP-1. No estudo 3, ambas as doses de energia aumentaram a viabilidade celular e a atividade de ALP. Para produção de PT, expressão de ALP e Col I, apenas 4 J/cm2 apresentou maiores valores quando comparados ao grupo controle. De um modo geral, foi possível bioestimular células pulpares com 4 J/cm2 de energia entregues pela luz LED no comprimento de onda 850 nm, tanto na ausência quanto na presença da barreira dentinária.

Palavras-chave: células-tronco, dentina, diferenciação celular, fototerapia, polpa dentária

Turrioni APS. Direct and transdentinal effect of 850 nm LED on the metabolism and odontoblastic differentiation of dental pulp stem-cells [Tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2015

ABSTRACT

The overall aim of this study was to evaluate in vitro the effect of 850 nm LED irradiation on different metabolic processes of stem cells from human exfoliated deciduous teeth (SHEDs). For all studies, pulp cells originated from sound deciduous teeth were characterized by immunofluorescence using STRO-1, CD44, CD146, Nanog and OCT3/4 antibodies, before experimental protocols. In the first experiment, cells were seeded in 24 well-plates, submitted to ondontoblastic differentiation and irradiated with different energy doses (ED - 0, 2, 4, 8, 15 or 30 J/cm^2, 40 mW/cm^2) n=9. After 12 h and 72 h post irradiation, cell viability (MTT), mineralized nodule formation (MN – Alizarin Red) and number of viable cells (Trypan Blue) were evaluated. Data were submitted to Kruskal-Wallis and Mann-Whitney tests ($p<0.05$). In the second experiment, the same protocol was performed, however, using only 2 and 4 J/cm^2, considering 72 h post irradiation as period of evaluation. Alkaline phosphatase (ALP) activity, total protein (TP) production, total collagen production (Sircol Assay), as well as gene expression (qPCR) of ALP, collagen type I (Col I), dentin sialophosphoprotein (DSPP) and dentin matrix acidic phosphoprotein 1 (DMP-1) were assessed, n=8. For the third experiment, cells were seeded on the pulp surface of 0.2 mm-thick dentin discs (n=72) and submitted to the same protocol of irradiation as experiment 2, applied on the occlusal surface of the dentin discs (n=8,

transdentinal action). The assays included cell viability (MTT), ALP activity, TP production, ALP, Col I, DSPP and DMP-1 gene expression and cell morphology (SEM). Again, the same statistical tests were applied to data. In the first study, when compared with the control group, cells submitted to 2 or 4 J/cm^2 showed higher cell viability after 72 h and all EDs increased the number of viable cells after 12 h. For MN formation, the best results were found 72 h post irradiation, for 8 and 15 J/cm^2. In the second study, an increase in ALP activity and collagen production, as well as in DSPP and ALP expression was observed for both EDs when compared to control group, whereas 4 J/cm^2 also stimulated Col I and DMP-1gene expression. For the third study, both EDs increased cell viability and ALP activity. For TP, ALP and Col I gene expression, only 4 J/cm^2 showed higher values when compared to the control group. Overall, it was concluded that pulp cells can be biostimulated by a LED light at 850 nm delivering 4 J/cm^2, both in the absence and presence of dentin barrier.

Keywords: stem cells, dentin, cell differentiation, phototherapy, dental pulp

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1 INTRODUÇÃO

Sinais da resposta do complexo dentino-pulpar frente à instalação da doença cárie podem ser identificados no tecido dentinário mesmo quando a lesão limita-se ao esmalte dentário, o que demonstra o eficiente mecanismo de sinalização deste complexo (Smith45, 2003; Goldberg, Smith16, 2004). Durante a evolução do processo carioso, especialmente quando o tecido dentinário é diretamente envolvido, a polpa é agredida por bactérias e/ou seus bioprodutos, os quais se difundem através dos túbulos dentinários, dando início à modulação da atividade odontoblástica, no sentido de estimular a deposição de matriz dentinária e subseqüente formação de dentina terciária (Hanks et al.18, 1998). O processo inflamatório, o primeiro mecanismo de sinalização para a defesa do complexo dentino-pulpar, exerce função importante na modulação da resposta da polpa dentária (Goldberg, Smith16, 2004; Ferreira et al.14, 2006). Dependendo da intensidade e duração do estímulo (agressão), pode haver comprometimer irreversível das células odontoblásticas relacionadas aos túbulos infectados com subseqüente morte destas células (Goldberg, Smith16, 2004).

Uma vez que o processo carioso não é auto-limitante, os dentes comprometidos, especialmente nos casos de lesões profundas de cárie em dentina, devem receber tratamento com o intuito de remover a grande concentração de bactérias presentes na dentina infectada (Goldberg, Smith16, 2004; Murray et al.38, 2008). Todavia, neste ato, o tecido pulpar que já apresenta um processo inflamatório evidentemente instalado, sofre agressões adicionais decorrentes do

próprio procedimento clínico de preparação da cavidade dentária. Deve ser prevista nesse momento, a possibilidade de exposição do tecido pulpar durante a remoção da dentina cariada, fato este considerado um complicador quanto ao prognóstico da reparação deste tecido. Finalmente, ao término da remoção total ou parcial do tecido cariado, com ou sem exposição pulpar, a cavidade deve ser selada com um material restaurador, o qual pode constituir mais uma fonte de agressão ao tecido já fragilizado. A somatória de danos ao tecido pulpar pode resultar em dor e exacerbar a reação inflamatória (Whitworth et al.[54], 2005; Modena et al.[36], 2009).

Quando o tecido pulpar é exposto a uma agressão de alta intensidade, as células mais próximas ao tecido agredido são afetadas, ocorrendo assim, a morte das células da camada odontoblástica relacionadas com a área da agressão (Smith[45], 2003; Goldberg, Smith[16], 2004). Deste modo, é induzida a diferenciação de células-tronco da polpa em células odontoblastóides, para que seja possível a reposição dos odontoblastos primários perdidos e a produção de matriz dentinária por estas novas células, com subseqüente formação da dentina reparadora (Pashley[42], 1996; Smith[45], 2003; Goldberg, Smith[16], 2004; Chen et al.[8], 2009; Tziafas, Kodonas[51], 2010).

Nos últimos anos, diversos estudos têm avaliado a eficácia de técnicas que promovam a reparação do tecido pulpar por meio do estímulo da síntese e deposição de matriz dentinária (Kikuchi et al.[26], 1996; Mertz-Fairhurst et al.[33], 1998; Duque et al.[13], 2006; Fuks[15], 2008; Murray et al.[38], 2008; Modena et al.[36], 2009; Alves et al.[4], 2010). Uma dessas técnicas é a fototerapia. Tem sido descrito

que a luz interage com diferentes tipos de tecido causando diversas reações celulares (Karu et al.[23], 2010; Holder et al.[20], 2012, Turrioni et al.[49],2014). Pesquisas têm demonstrado que a foto-estimulação pode causar aumento na síntese de ATP (Karu et al.[25], 1995; Karu et al.[23], 2010), efeito analgésico (Karu et al.[25], 1995; Karu et al.[24], 2005), aceleração da cura tecidual (Al-Watban, Andres[5], 2006; Hsieh et al.[21], 2012) e estímulo da proliferação celular (Koutná et al.[28], 2003; Holder et al.[20], 2012; Oliveira et al.[41], 2013; Turrioni et al.[49], 2014).

Dentre as diferentes fontes de luz, aquela gerada por diodos emissores de luz (LED) tem sido amplamente estudada, sendo que diversos efeitos positivos em relação ao reparo tecidual nos diferentes tecidos já foram relatados. Entre estes se destacam: o estímulo da cicatrização de feridas em ratos diabéticos (Al-Watban, Adres[5], 2006; Dall Agnol et al.[10], 2009), de úlceras (Minatel, França[35], 2009) e dermatites (DeLand et al.[11], 2007), bem como a atenuação de mucosite oral (Whelan et al.[53], 2002; Sacono et al.[44], 2008).

A aplicação da luz sobre células da polpa exposta a algum tipo de agressão pode favorecer a reparação deste tecido conjuntivo especializado, caracterizada pela síntese e deposição de matriz dentinária associada ou não ao estímulo da diferenciação de células-tronco em células odontoblastóides (Tate et al.[47], 2006; Turrioni et al.[50], 2014). Tem sido demonstrado que o comprimento de onda infravermelho pode propiciar efeitos celulares positivos, tais como proliferação celular, aumento na síntese de ATP e redução dos danos protéicos em decorrência da produção de agentes antioxidantes (Almeida –Lopes et al.[3], 2001; Vinck et al.[52], 2003; Wong-Riley et al.[55], 2005; Moore et al.[37], 2005). Em um estudo in

vivo, a aplicação do laser infravermelho em molares de rato induziu a formação de dentina terciária, sem causar danos significantes ao tecido pulpar (Tate et al.[47], 2006). Todavia, os possíveis mecanismos de resposta das células pulpares em nível molecular, frente à irradiação LED, ainda permanecem indefinidos.

Estudos específicos avaliando os efeitos da irradiação sobre células-tronco demonstraram a ocorrência de alguns efeitos bioestimuladores interessantes, tais como aumento da proliferação celular (Stein et al.[46], 2005; Li et al.[29], 2006; Li et al.[30], 2010, Holder et al.[20], 2012) e estímulo da diferenciação osteogênica/odontogênica (Kim et al., 2009; Li et al.[30], 2010; Turrioni et al.[50], 2014). Pesquisas com células-tronco tem sido fundamentais para o avanço do conhecimento envolvendo a regeneração tecidual (Huang et al.[22], 2009; Lizier et al.[32], 2012; Hilkens et al.[19], 2013). Desde que a manipulação destas células foi possível, diversos estudos têm apontado a capacidade de diferenciação in vitro em diferentes linhagens celulares, como as linhagens osteogênica, odontogênica, condrogênica, adipogênica, miogênica e neurogênica (Gronthos et al.[17], 2000; About et al.[2], 2005; Huang et al.[22], 2009; Tziafas, Kodonas[51], 2010; Min et al.[34], 2011, Lizier et al.[32], 2012; Hilkens et al.[19], 2013). Especificamente dentro da área Odontológica, Gronthos et al.[17] (2000) relataram que células-tronco isoladas da polpa podem ser induzidas, in vitro, a se diferenciar em células com fenótipo odontoblástico. Todavia, dados científicos que comprovem a propagação transdentinária do LED e sua possível bioestimulação celular, caracterizada pelo aumento na atividade metabólica dos odontoblastos e diferenciação de células-tronco em células odontoblastóides, ainda não foram demonstrados. Assim, torna-

se necessário, neste momento, definir os parâmetros mais adequados de bioestimulação direta de células-tronco quando da irradiação com LED, bem como identificar a possível atividade transdentinária da LEDterapia sobre células-tronco originárias da polpa dentária e sendo assim, determinar, de forma inovadora, a participação deste tipo de irradiação no processo de reparo do tecido pulpar.

2 PROPOSIÇÃO

Proposição Geral

O objetivo geral da presente pesquisa, composta de 3 estudos, foi avaliar o efeito do LED 850 nm, em parâmetros específicos, sobre diferentes eventos metabólicos de células-tronco da polpa dentária.

Proposições Específicas

Estudo 1: Avaliar cinco doses de energia de LED no comprimento de onda do infravermelho (850 nm), avaliadas em dois diferentes momentos sobre células-tronco originárias da polpa dentária, para definir uma curva dose/tempo/resposta baseada na viabilidade celular, número de células viáveis e formação de nódulos mineralizados.

Estudo 2: Avaliar o efeito direto das duas melhores doses do LED 850 nm, obtidas no experimento de irradiação direta (estudo 1) sobre o metabolismo celular, morfologia celular, expressão gênica e síntese de proteínas relacionadas ao reparo pulpar.

Estudo 3: Avaliar o efeito transdentinário das duas melhores doses do LED 850 nm, obtidas no experimento de irradiação direta (estudo 1) sobre a atividade metabólica de células-tronco da polpa dentária e expressão gênica de proteínas relacionadas ao reparo pulpar.

3 MATERIAL E MÉTODO

Os tecidos pulpares utilizados nos estudos que compões a tese foram doados pelos responsáveis dos pacientes da Clínica de Odontopediatria da Faculdade de Odontologia de Araraquara, por meio da assinatura do Termo de Consentimento Livre e esclarecido, após aprovação do Comitê de Ética em Pesquisa da mesma instituição. Os dentes utilizados no estudo 3 foram doados pelo Banco de Dentes da Faculdade de Odontologia de Araraquara e utilizados após aprovação do Comitê de Ética em Pesquisa da mesma instituição (Anexo 1).

3.1 ESTUDO 1

DOSE-RESPONSES OF STEM CELLS FROM HUMAN EXFOLIATED TEETH TO INFRARED LED IRRADIATION

Short title: Dose-responses of stem cells to infrared LED

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SUMMARY

Despite the several reports regarding tissue regeneration, including pulp repair induced by different light sources, only limited data have been reported concerning the effects of light-emitting diodes (LED) on stem cells from human exfoliated deciduous teeth (SHEDs). The aim of this study was to evaluate the effects of different energy densities of infrared LED on the cell viability, number of cells and mineralized tissue production by SHEDs. SHEDs were obtained from near-exfoliation primary teeth (n=3), seeded in plain DMEM (10^4 cells/cm^2), and irradiated by a LED prototype (LEDTable 850 nm, 40 mW/cm^2) delivering 0 (control), 2, 4, 8, 15, or 30 J/cm^2 (n=9). Cell viability (MTT assay), viable cell number (trypan blue assay), and mineralized nodule (MN) formation (alizarin red stain) were assessed 12 and 72h post-irradiation. Data were subjected to Kruskal-Wallis and Mann-Whitney tests (α = 0.05). Cells irradiated with 2 or 4 J/cm^2 exhibited higher viability at 72h, and all energy densities promoted an increase in viable cell numbers after 12h. Regarding MN formation, the best results were observed 72h after SHED irradiation with 8 and 15 J/cm^2. It was concluded that the cell viability, cell number and MN formation by pulp cells is enhanced after exposure to infrared LED irradiation. Overall the greater SHED biostimulation was obtained with 4 and 8 J/cm^2.

KEYWORDS

Infrared, phototherapy, pulp cells, metabolism

INTRODUCTION

Bacteria and theirs bioproducts released during caries progression, the clinical procedure of caries removal may cause damage to the pulp-dentin complex. Additionally, some dental materials currently recommended as capping agents or liners might be cytotoxic, resulting in additional injury to the dental pulp (1,2).

To reduce pulp damage or even to improve healing of already-damaged pulp tissue, different techniques, such as phototherapy, have been assessed and recommended (3,4). Low-level light therapy (LLLT), with lasers or light-emitting diodes (LED), has been widely used in dentistry for tissue and cell stimulation (5-11). Concerning the pulp tissue or pulp cells subjected to phototherapy, several authors have reported encouraging scientific data, such as increased deposition of tertiary dentin (5), enhanced cell viability and proliferation (8,11), and biomodulation of inflammatory cytokines and reactive oxygen species (9). Despite these positive results, a major challenge in tissue biostimulation involving phototherapy is to define the optimal window for each cell type and desired action (3) — that is, which parameters of irradiation (energy density, wavelength, power) would be adequate for biostimulation, since it is known that cell and tissue responses may vary according to these physical parameters (3,12,13).

In clinical situations with dentin remaining between the cavity floor and the pulp tissue, the light needs to overcome the dentin barrier to reach and stimulate the subjacent pulp tissue. It has been demonstrated that LED irradiation (blue, red, and infrared spectra) is capable of propagate through a dentin barrier up to 1.0 mm thick (14,15). The authors reported that the smallest scattering of light occurred when the infrared LED was used. Later, it was shown that the light emitted by diodes is able not only to cross the dentin barrier but also to stimulate the underlying pulp cells (11).

Despite the promising cell-biostimulatory results obtained with phototherapy, such as modulation of tissue inflammation (16) and stimulation of cell metabolism of fibroblasts, odontoblast-like cells, and human pulp cells

(10,11,17-19) information about the effects of infrared LED on the metabolism and mineralization ability of stem cells from human exfoliated deciduous teeth is scanty. Therefore, the aim of this study was to evaluate the effects of 850-nm LED irradiation on cultured stem cells from human exfoliated deciduous teeth (SHEDs) at different energy densities.

MATERIALS AND METHODS

Obtaining Primary Cultures of Pulp Cells

Sound primary teeth (n = 3) near exfoliation were obtained during patient care at the Pediatric Dentistry Clinic of Araraquara School of Dentistry - UNESP, after approval by the Ethics Research Committee of the same school (Protocol 63/11). The isolation and characterization of the primary pulp from exfoliated tooth cells used in this study followed a protocol previously described (10). The tests were performed when sufficient cells were obtained for application of the protocol, and 3rd-passage cells were used. All experiments were performed in triplicate.

Irradiation Device

For cell irradiation, a previously described standardized device (LEDtable) containing 24 infrared diodes (850 nm) was used (9-11). The irradiance was fixed at 40 mW/cm^2, and the irradiation times were 50 sec (2 J/cm^2), 1 min and 40 sec (4 J/cm^2), 3 min and 20 sec (8 J/cm^2), 6 min and 15 sec (15 J/cm^2), and 12 min and 30 sec (30 J/cm^2).[9] All cells were irradiated in a dark room. According to a preliminary study, the time that the cells were kept out of the incubator, considering the different irradiation times, did not affect cell viability (data not shown).

Cell Viability Assay (MTT assay)

For cell viability (MTT assay) and cell counting (trypan blue assay), cells were seeded in 24-well plates (10^4 cells/cm^2) and incubated for 12 h in contact with DMEM containing 10% FBS (Gibco, Grand Island, NY, USA). After this period, the medium was aspirated and replaced by DMEM containing only 0.5% FBS, to induce nutritional stress (20). After 12 h, cells were subjected to LED irradiation, and the tests were performed 12 and 72 h post-irradiation.

Cell viability analysis was performed as described by Oliveira et al. (18). Briefly, DMEM was aspirated and replaced by 900 μL of new culture medium (DMEM) plus 100 μL of MTT solution (5 mg/mL of methyl tetrazolium salt in PBS) (Sigma-Aldrich, St. Louis, MO, USA). Cells in contact with the MTT solution were incubated at 37°C for 4 h. After incubation, the MTT solution was aspirated and replaced by 600 μL of an acidified isopropanol solution (0.04 N of HCl). Three aliquots from each sample were analyzed in 96-well plates. Cell viability was assessed in absorbance at 570 nm in a microplate reader (Thermo Plate, Nanshan District, Shenzhen, China).

Viable Cell Counting (trypan blue assay)

After LED irradiation, the medium in contact with the cells was replaced by 0.12% trypsin (Gibco) and kept in contact with the cells for 10 minutes. After cell dissociation, a 50-μL quantity of this cell suspension was aliquoted and added to 50 μL of 0.04% trypan blue solution. The resultant solution was maintained at room temperature for 2 minutes. To perform the cell counts, we placed 10 μL of the solution on a hemocytometer and assessed cells in an automated cell counter (TC 10 Automed Cell Counter 145-0001, BIO-RAD, Hercules, CA, USA). The total cell and non-viable cell counts were performed, and the number of viable cells was obtained by subtracting the non-viable cells from the total cells of the sample.

Mineral Nodule Formation

For the evaluation of mineral nodules (alizarin red stain), cells were seeded (10^4 cells/cm^2) and incubated for 48 h in basal medium. They were then kept in contact with an osteogenic medium containing 5 mM α-glycerophosphate and 100 mM ascorbic acid (Sigma-Aldrich) (21). Irradiation was delivered 12 or 72 h before the time of evaluation. Specifically for this protocol, analysis of nodule formation was performed on the 14th day of culture. Cells were fixed with 70% ethanol for 1 h, then washed with distilled water for 5 minutes followed by staining with 40 mM solution of Alizarin red (Sigma-Aldrich) for 20 minutes, under shaking. After being stained, samples were washed twice with distilled water, for 5 minutes each. Some of the samples were evaluated by optical microscopy for qualitative analysis of mineral nodule formation, and others were solubilized for quantitative analysis. After shaking and checking the homogeneity of the solutions, we transferred three 100-µL aliquots of each sample to a 96-well plate. The absorbance was determined at 562 nm in an ELISA reader (Thermo Plate).

Statistical Analysis

For all tests, absorbance values were transformed into percentages, with the control group (non-irradiated cells, period of 12 h) as 100%. Data were subjected to statistical analysis by the non-parametric Kruskal-Wallis test, complemented by the Mann-Whitney test ($p < 0.05$).

RESULTS

Aiming a better data understanding, Figure 1 shows line graphs based on the medians values, for MTT assay (A), Trypan Blue (B) and Alizarin Red (C). The statistical difference between groups, median, percentile 25 and percentile 75

values for each group can be observed on the Tables 1, 2 and 3 for cell viability, number of viable cells and MN formation respectively.

Cell Viability

Cell viability data, considering energy densities and post-irradiation periods of evaluation, are shown in Table 1. Energy density of 4 J/cm^2 was the only one to increase cell viability compared with the control group ($p < 0.05$), by 27.4% and 27.8% for 12 and 72 h, respectively. Other energy densities did not differ from those of the non-irradiated group ($p > 0.05$). For all irradiated groups, there was a significant increase in cell viability over time.

Analysis of the Number of Viable Cells (Trypan Blue)

Analysis of viable cell-counting data (Table 2) demonstrated that, for the 12-hour period, all energy densities promoted an increase in viable cell numbers, compared with the control group ($p < 0.05$), and better results for this period were obtained with 4 J/cm^2 (50% increase). After 72 h, only the energy density of 4 J/cm^2 showed statistically higher viable cell-counting when compared with the control group (22.4% increase, $p < 0.05$). When the two different evaluation periods were compared, it was noted that, for energy densities of 4, 8, and 15 J/cm^2, the period of 72 h stood out, showing higher values with statistical significance ($p < 0.05$).

Mineralized Nodule Formation

Quantitative data on mineral nodule formation by cultured pulp cells after LED irradiation are shown in Table 3. Comparison of energy densities at each period demonstrated that, for the period of 12 h, there was no statistically significant increase in the irradiated groups when compared with the control group ($p > 0.05$). For the period of 72 h, only energy densities of 8 and 15 J/cm^2 were able to

increase mineral nodule production (20.0% and 21.4%, respectively) when compared with the control group (p < 0.05).

Figure 2 shows the mineral deposition by SHEDs as a function of the different energy densities and post-irradiation periods of evaluation. It was observed that the groups irradiated with 4, 8, and 15 J/cm^2 were those that produced more mineralized nodules.

DISCUSSION

The use of phototherapy for transdentinal biostimulation of pulp cells is an interesting adjuvant treatment for pulpal healing during clinical procedures in operative dentistry. Based on analysis of positive data provided by previous studies, researchers have been encouraged to carry out more investigations to establish the most beneficial physical parameters of LED or laser irradiation of pulp cells (8-11,22).

Current studies have assessed LED energy densities varying from 0.093 J/cm^2 (23) to 162 J/cm^2 (24) and power densities from 0.5 mW/cm^2 (25) to 140 mW/cm^2 (26). According to Alghanmdi et al. (13), power densities from 1 to 500 mW and energy densities from 0.04 to 50 J/cm^2 are considered low-intensity therapy parameters, recommended for the irradiation of cells in culture. Therefore, the irradiation parameters used in this study followed the recommendations of Alghanmdi et al. (13) as well as those of Montoro et al. (9), who observed modulation of reactive oxygen species (ROS) and nitric oxide (NO) formation by human dental pulp cells subjected to infrared LED therapy.

In the present study, it was shown that infrared LED irradiation can increase the viability and number of pulp cells as well as the formation of mineralized nodules, which play an important role in tertiary dentin formation (8). The energy density of 4 J/cm^2 up-regulated the cell viability for all evaluated periods; however, the highest cell viability rate was observed at 72 h post-irradiation. Several studies have assessed the viability of pulp cells exposed to phototherapy (8,11,18,19). Holder et al. (8) showed that the viability of pulp cells

was enhanced after exposure to one irradiation session with red LED. Transdentinal biostimulation of odontoblast-like MDPC-23 cells also occurred when the cells were subjected to three irradiation sessions (19). It is likely that a single short irradiation applied to the dentin remaining between the cavity floor and the pulp would be more appropriate for pre-restoration clinical conditions. Therefore, in the present study, we decided to evaluate the responses of SHEDs exposed to a single LED irradiation. Since there is no consensus about the best irradiation parameters for pulp biostimulation, we varied only the energy densities from 2 to 30 J/cm^2 and assessed the cell responses at 12 and 72 h post-irradiation.

As determined for cell viability, the number of viable SHEDs in the present study was also enhanced after irradiation with infrared LED at selected parameters. The most significant increase in the number of viable cells was observed at 72 h post-irradiation for the energy density of 4 J/cm^2. Holder et al. (8) used the BrdU method to count the number of pulp cells irradiated with LED (653 nm, 3.73 mW/cm^2, 448 mJ/cm^2). The authors observed a higher number of cells in the irradiated groups than in control groups at 3- and 7-day post-irradiation periods. The increased cell proliferation and viability seem to be related to cytochrome c oxidase activation, which enhances levels in the respiratory chain, and adenosine triphosphate (ATP), and these biochemical changes lead to macroscopic effects such as increased cell proliferation (27,28). Analysis of these data confirms that LED therapy can increase the viability and stimulate the proliferation of pulp cells in vitro and in vivo (10,11,22).

The mineral nodule formation by SHEDs was also stimulated by LED irradiation at 8, and 15 J/cm^2. Analysis of these data corroborates those from previous studies in which mesenchymal stem cells (23), fibroblasts (29), odontoblast-like cells (18,19), and rodent pulp cells (8) were subjected to phototherapy. In general, these cells presented increased deposition of mineralized matrix as well as enhanced expression of collagen type I, alkaline phosphatase (ALP), Runx-related transcription factor 2 (Runx2), and osteopontin, which are proteins related to the synthesis and mineralization of the collagen-rich matrix (18,19,23,29). The molecular pathways that cause increased production of these

specific proteins have not been described thus far. However, this macro-effect of phototherapy on pulp cells suggests that light can be an interesting adjuvant treatment to up-regulate the deposition and mineralization of collagen-rich dentin matrix by dental pulp cells during pulpal healing. Seems that different energy doses can be the ideal parameter depending on the outcome. For number of cells and cell viability, 4 J/cm^2 showed better results. On the other hand, for MN formation, doses of 8 and 15 J/cm^2 stood out, suggesting that different doses should be used according with different aims.

Overall, in vitro phototherapy with infrared LED biostimulated all SHED functions assessed in the present study. Therefore, the scientific data obtained in the present study can drive future laboratory investigations or even in vivo studies in animal models to establish irradiation parameters for optimal and friendly clinical phototherapy procedures in pulp tissue regeneration.

SUMMARY IN PORTUGUESE

Apesar de diversos estudos envolvendo regeneração tecidual, incluindo o reparo pulpar induzido por diferentes fontes de luz, dados limitados têm sido reportados a repeito dos efeitos da irradiação com diodos emissores de luz (LED) sobre células-tronco de dentes decíduos esfoliados (SHEDs). O objetivo do presente estudo foi avaliar os efeitos de diferentes doses de energia (DE) do LED infravermelho sobre a viabilidade celular, número de células viáveis e produção de nódulos mineralizados (NM) por SHEDs. As células foram obtidas a partir de dentes decíduos próximos ao período de esfoliação (n=3), semeadas em DMEM completo (10^4 células/cm^2) e irradiadas utilizando um protótipo de LED (LEDTable 850 nm, 40 mW/cm^2) com as doses de 0 (controle), 2, 4, 8, 15 ou 30 J/cm^2 (n=9). A viabilidade celular (MTT), o número de células viáveis (trypan blue assay) e a formação de NM (alizarin red stain) foram realizados 12 e 72 h após a irradiação. Os dados foram avaliados utilizando os testes Kruskal-Wallis e Mann-Whitney (α = 0,05). As células irradiadas com 2 ou 4 J/cm^2 exibiram uma maior viabilidade em 72 h, e todas as DE aumentaram o número de células viáveis

após 12 horas. Para a formação de NM, os melhores resultados foram observados 72 h após a irradição das SHEDs, com as doses de 8 e 15 J/cm^2. Concluiu-se que a viabilidade celular, o número de células e a formação de NM por células pulpares são aumentados após exposição ao LED infravermelho. De um modo geral, a melhor bioestimulação celular (SHEDs) foi obtida com 4 e 8 J/cm^2.

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TABLES

Table 1. Cell viability (% of control) of stem cells from human exfoliated deciduous teeth (SHEDs) 12 and 72 h after irradiation with infrared LED (850 nm) delivering different energy densities.

	Post-irradiation period of evaluation (h)		
Energy density (J/cm^2)	12	p-value	72
0 (control)	100.32 (91.71-118.42) B	Sig.	204.52 (143.87-206.47) B
2	112.6 (99.0-126.8) AB	Sig.	227.6 (190.0-242.3) AB
4	127.7 (109.6-133.3) A	Sig.	231.6 (223.3-242.9) A
8	116.5 (94.6-123.6) AB	Sig.	203.0 (188.6-235.9) AB
15	111.3 (89.7-117.6) B	Sig.	204.8 (164.2-248.3) AB
30	102.2 (93.0-114.8) B	Sig.	198.2 (192.3-215.8) B

Values are medians (25th to 75th percentiles), n = 9. [p-value] Allows comparisons between post-irradiation periods (rows). Sig. = statistically significant difference (p>0.05). [A] Letters allow comparisons among energy doses (columns). Values identified by the same letter are not statistically significantly different (Mann-Whitney, ***p*** > 0.05).

Table 2. Percentages of viable stem cells from human exfoliated deciduous teeth (SHEDs) 12 and 72 h after irradiation with infrared LED (850 nm) delivering different energy densities.

	Period of evaluation (h)		
Energy density (J/cm^2)	12	p-value	72
0 (control)	100.0 (90.5-111.7) C	Sig.	152.2 (143.7-161.7) B
2	124.4 (114.5-150.7) AB	N.s.	163.4 (152.2-170.0) B
4	150.0 (144.5-154.0) A	Sig.	174.6 (166.7-180.0) A
8	130.0 (105.9-140.4) B	Sig.	150.0 (146.7-175.3) AB
15	130.0 (114.7-137.2) AB	Sig.	135.8 (135.0- 158.7) B
30	143.0 (117.2-161.2) AB	N.s.	145.6 (130.0-155.6) B

Values are medians (25th to 75th percentiles), n = 9. $^{\text{p-value}}$ Allows comparisons between post-irradiation periods (rows). Sig. = statistically significant difference (p>0.05); N.s. = not statistically different (p<0.05). ^{A} Letters allow comparisons among energy doses (columns). Values identified by the same letter are not statistically significantly different (Mann-Whitney, ***p*** > 0.05).

Table 3. Mineralized nodule formation (% of control) by stem cells from human exfoliated deciduous teeth (SHEDs) 12 and 72 h after irradiation with infrared LED (850 nm) delivering different energy densities.

	Period of evaluation (h)		
Energy density (J/cm^2)	12	p-value	72
0 (control)	100.8 (91.1-116.8) AB	Sig.	120.0 (99.0-131.0) B
2	111.8 (97.2-116.0) AB	N.s.	111.8 (105.5-118.7) B
4	102.5 (100.5-104.3) A	N.s.	118.5 (98.8-125.9) B
8	107.5 (102.0-108.5) A	Sig.	140.0 (123.5-163.3) A
15	106.0 (102.6-111.9) A	Sig.	141.4 (134.8-236.9) A
30	98.4 (91.4-108.2) B	Sig.	123.1 (105.0-127.1) B

Values are medians (25th to 75th percentiles), n = 9. $^{p\text{-value}}$ Allows comparisons between post-irradiation periods (rows). Sig. = statistically significant difference (p>0.05); N.s. = not statistically different (p<0.05). ^{A} Letters allow comparisons among energy doses (columns). Values identified by the same letter are not statistically significantly different (Mann-Whitney, ***p*** > 0.05).

FIGURE CAPTIONS

Fig. 1 Line graphs based on the medians values, for MTT assay (A), Trypan Blue (B) and Alizarin Red (C), 12 and 72 h after SHEDs irradiation with infrared LED (850 nm) delivering different energy densities (0, 2, 4, 8, 15, and 30 J/cm^2).

Fig. 2 Panel of inverted microscope images representative of mineral deposition by SHEDs, 12 and 72 h after irradiation with infrared LED (850 nm) delivering different energy densities (0, 2, 4, 8, 15, and 30 J/cm^2). Original magnification, 4x

FIGURE 1

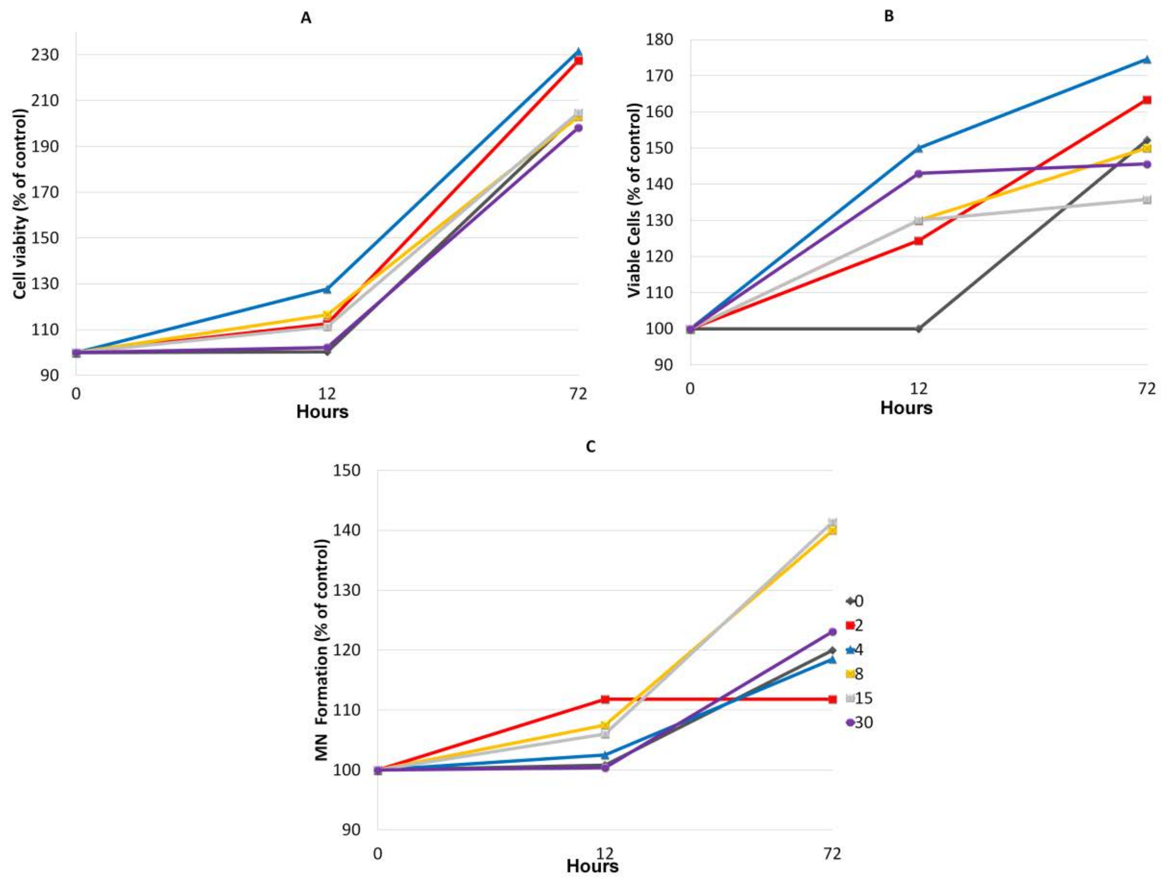

Fig. 1 Line graphs based on the medians values, for cell viability (A), number of viable cells (B) and mineralized nodules production (C), 12 and 72 h after SHEDs irradiation with infrared LED (850 nm) delivering different energy densities (0, 2, 4, 8, 15, and 30 J/cm^2).

FIGURE 2

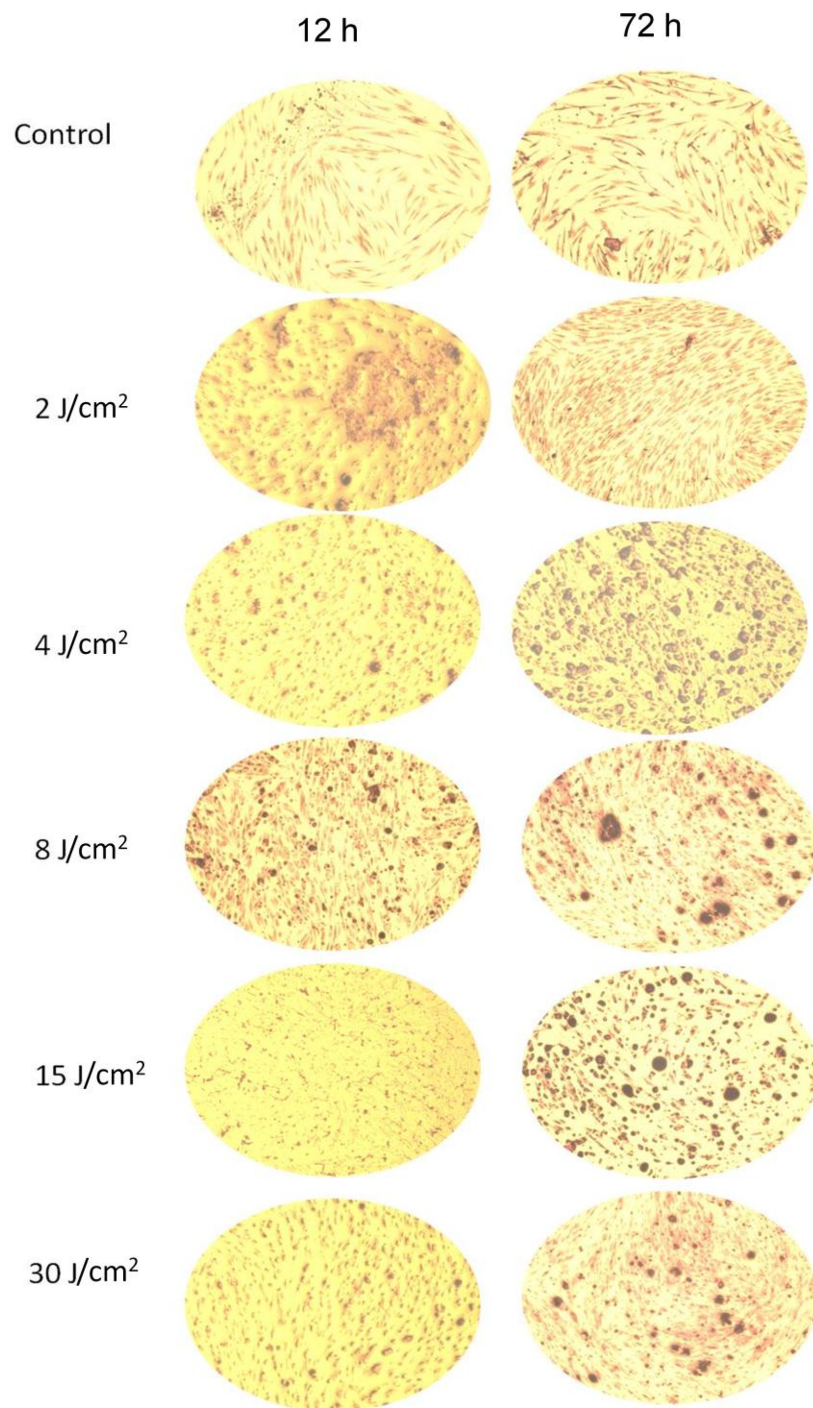

Fig. 2 Panel of inverted microscope images representative of mineral deposition by SHEDs, 12 and 72 h after irradiation with infrared LED (850 nm) delivering different energy densities (0, 2, 4, 8, 15, and 30 J/cm^2). Original magnification, 4x

3.2 ESTUDO 2

PHOTOTHERAPY UP-REGULATES DENTIN MATRIX PROTEINS EXPRESSION AND SYNTHESIS BY STEM CELLS FROM HUMAN-EXFOLIATED DECIDUOUS TEETH

Short title: Phototherapy Up-regulates Dentin Matrix Proteins

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ABSTRACT

Objectives: The aim of this study was to evaluate the effects of infrared LED (850 nm) irradiation on dentin matrix proteins gene expression and synthesis by cultured stem cells from human exfoliated deciduous teeth (SHED).

Methods: Near-exfoliation primary teeth were extracted (n = 3), and SHED cultures were characterized by immunofluorescence using STRO-1, CD44, CD146, Nanog and OCT3/4 antibodies, before experimental protocol. The SHEDs were seeded (3×10^4 cells/cm^2) with DMEM containing 10% FBS. After 24-hour incubation, the culture medium was replaced by osteogenic differentiation medium, and the cells were irradiated with LED light at energy densities (EDs) of 0 (control), 2, or 4 J/cm^2 (n = 8). The irradiated SHEDs were then evaluated for alkaline phosphatase (ALP) activity, total protein (TP) production, and collagen synthesis (SIRCOL^{TM} Assay), as well as ALP, collagen type I (Col I), dentin sialophosphoprotein (DSPP), and dentin matrix acidic phosphoprotein (DMP-1) gene expression (qPCR). Data were analyzed by Kruskal-Wallis and Mann-Whitney tests ($\alpha = 0.05$).

Results: Increased ALP activity and collagen synthesis, as well as gene expression of DSPP and ALP, were observed for both EDs compared with non-irradiated cells. The ED of 4 J/cm^2 also increased gene expression of COL I and DMP-1.

Conclusions: In conclusion, infrared LED irradiation was capable of biostimulating SHEDs by increasing the expression and synthesis of proteins

related with mineralized tissue formation, with overall better results for the energy dose of 4 J/cm^2.

Clinical Significance: Phototherapy is an additional approach for the clinical application of LED in Restorative Dentistry. Infrared LED irradiation of the cavity´s floor could biostimulate subjacent pulp cells, improving local tissue healing.

Key Words: dentin, odontoblasts, phototherapy, protein expression, pulp biology, stem cells

1. Introduction

Pulp tissue inflammation may be exacerbated during cavity preparation and following cavity restoration with non-biocompatible dental materials[1]. Therefore, specific procedures and biomaterials should be clinically applied to enhance pulpal healing by up-regulating collagen-rich dentin matrix production and its mineralization by pulp cells[1]. It has been shown that light-emitting diode (LED) irradiation may improve the repair of different tissues, including pulp[2,3,4]. Furthermore, previous studies have demonstrated that LED irradiation can biostimulate cultured odontoblast-like MDPC-23 cells[5] and human dental pulp cells (HDPCs)[6].

It has been reported that LED irradiation in the infrared range of the spectrum (from 700 nm to 1 mm wavelength) is able to pass through the dentin barrier[7] and photobiostimulate underlying cells[8], increasing the cell viability and alkaline phosphatase activity of odontoblast-like cells as well as up-regulating

collagen type I (Col I) expression[5,9,10]. Additionally, infrared light can improve healing of bone defects mechanically created in rats[11] and reduce pain in temporomandibular disorders[12].

Based on these data, it is reasonable to consider that phototherapy technology with LED represents an interesting alternative for clinical application in restorative dentistry, particularly for stimulation of pulp cells subjacent to the cavity floor. Therefore, the authors hypothesized that infrared LED irradiation, at specific parameters, could enhance dentin matrix and dentin mineralization protein expression/synthesis by pulp cells. The aim of this study was to evaluate the effects of infrared LED (850 nm) irradiation on dentin matrix protein gene expression and synthesis by cultured SHEDs.

2. Materials and methods

2.1. Primary Culture Obtained from Deciduous Teeth

The present study was approved by the Ethical Committee of UNESP - Univ. Estadual Paulista (protocol 63/11). Three near-exfoliation sound primary teeth were donated by the child's legal guardian, who provided a signed consent form. Pulp cells were isolated by enzymatic digestion with collagenase type I and dispase (Worthington Biochemical Corp., Lakewood, NJ, USA)[13]. Cells were cultured in 75 cm^2 flasks in monolayer on DMEM supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY, USA), 100 IU/mL and 100 μg/mL, respectively, of penicillin and streptomycin, and 2 mmol/L of glutamine (GIBCO, Grand Island, NY, USA). The medium was refreshed every 2 days, and the

culture was maintained semiconfluent. All cultures were incubated at 37°C in a 5% CO_2 high-humidity environment. Cells at passages 3 and 4 were used in this study.

2.2. Irradiation Device and Experimental Protocol

For cell irradiation, a standardized device (LEDtable) containing 24 infrared diodes (850 ± 10 nm) was used[6]. Each diode was accompanied by a collimator lens that limited the area of irradiation to the area of one single well of a 24-well culture plate (2 cm^2). Therefore, each diode was able to irradiate the cells adhering to the bottom of each well individually, with no interference from the adjacent well. The irradiance used was 40 mW/cm^2, and the energy densities (EDs) investigated were 0 (control group), 2 J/cm^2 (time of irradiation, 50 sec), and 4 J/cm^2 (time of irradiation, 1 min and 40 sec).

The irradiation protocols used in this study were validated by preliminary tests that monitored temperature change from the moment the diodes were activated up to 2 min without interruption, and the possible interference of the culture medium color (phenol-red DMEM) with absorbance. The results of these preliminary studies showed no temperature increase and no interference of medium color with infrared light absorbance (data not shown).

SHEDs in DMEM with 10% FBS were seeded (3×10^4 $cells/cm^2$) in wells of sterilized 24-well plates and maintained for 24 hrs in a humidified incubator with 5% CO_2 and 95% air at 37°C (Isotemp, Fisher Scientific, Bellefonte, PA, USA). Then, DMEM was replaced by an odontoblastic/osteogenic differentiation

medium (DMEM with 5 mM β-glycerophosphate and 100 μM ascorbic acid; Sigma/Aldrich, St. Louis MO, USA) containing 0.5% FSB (nutritional stress induction)[9,10,14]. After 12-hour incubation, the irradiations were delivered in a dark room, and protein gene expression and synthesis by SHEDs cells were assessed after 72 hrs. The time-point of 72 h after irradiation was selected based on previous studies[5, 9, 10].

2.3. Cell Characterization by Immunofluorescence

An immunofluorescence assay for stem cell markers was performed before the differentiation and irradiation experiments. Cells from three individuals (P1, P2, and P3) were seeded (8 x 10^4cells/ml) in wells of a 96 well-plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and incubated for 24 hrs. Then, cells were fixed in 4% buffered formalin at 4°C overnight, washed 3 times with PBS, and subjected to immunolabeling. Samples seeded for Nanog and OCT3/4 identification (cytoplasm markers; Santa Cruz Biotechnology, Dallas, TX, USA) were permeabilized with Triton 0.1% for 10 min. For CD44, CD146, and STRO-1 identification (membrane markers; Santa Cruz Biotechnology), no permeabilization was performed, and samples were in contact with PBS during this period. After primary antibody incubation, 3 more washing cycles were performed, and samples were placed in contact with 30 μL of TBST solution (Tris-buffered saline and Tween 20) with 5% BSA (bovine serum albumin) for 30 min. The samples were then incubated with 30 μL of a solution containing primary antibodies (CD44 - dilution 1:100; CD146 - dilution 5:1,000; STRO-1 -

dilution 1:10; Nanog - dilution 1:10; and OCT3/4 - dilution 1:10) for 1 hr. After being washed in PBS, samples were in contact with secondary antibodies conjugated with FITC (IgM - dilution 1:400, and IgG - dilution 1:400; Santa Cruz Biotechnology) for an additional 1 hr. Negative controls treated only with secondary antibodies were evaluated. After new washing, sample nuclei were labeled with Hoechst (dilution 1:5,000; Invitrogen, Carlsbad, CA, USA) for 15 min. Cells were then assessed on IN Cell Analyzer 2000 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), and 9 selected areas were considered for quantitative data analysis with IN Cell Investigator Software v1.6.

2.4. Total Protein Production

Total protein production was performed for each experimental and control group according to the protocol of Read and Northcote (1981)[15], which has been widely used[5,9,10]. Briefly, cell lysis was performed by the addition of 1 mL of sodium lauryl sulfate 0.1% (Sigma-Aldrich) to each well for 40 min at room temperature. The samples were homogenized, and then 1 mL of Lowry Reagent Solution (Sigma-Aldrich) was added to all samples, which were then incubated for 20 min at room temperature. After this period, each sample received 0.5 mL of Folin-Ciocalteu's Phenol Reagent (Sigma-Aldrich) followed by incubation for 30 min. Three 0.1-mL aliquots from each sample were transferred to a 96-well plate and were evaluated at a 655-nm wavelength in an ELISA reader (Thermo Plate, Nanshan District, Shenzhen, China). The average reading for the 3 aliquots was computed, and the data were used in the statistical analysis. The measurement of

total protein was calculated according to a standard curve with known albumin concentrations: 0, 16, 32, 64 and 80 µg/mL (BSA, 2mg/mL; Sigma-Aldrich, St. Louis MO, USA).

2.5. Alkaline Phosphatase Activity

The analysis for alkaline phosphatase activity was carried out following the protocol of the Alkaline Phosphatase Kit-Test Endpoint (Labtest Diagnostics SA, Lagoa Santa, MG, Brazil). Cell lysis was performed as for total protein production samples. In each sample, a 50-µL quantity of substrate was added (thymolphthalein monophosphate, 22 mmol L) in addition to 500 µL of buffer (300 mmol/L, pH 10.1). After 10 min of incubation, a 2-mL quantity of color reagent (sodium carbonate, 94 mmol/L; sodium hydroxide, 250 mmol/L) was added. The absorbance of samples was determined by ELISA (Thermo Plate) at a wavelength of 590 nm. We calculated alkaline phosphatase activity by normalizing the absorbance values according to the production values of total protein found. A standard curve containing known concentrations of alkaline phosphatase was also used to calculate the values of ALP activity.

2.6. Collagen Synthesis (Sircol Red)

Collagen synthesis was determined by use of the SIRCOL™ Collagen Assay (Biocolor, County Antrim, Northern Ireland, UK). First, we determined a standard curve by placing 5, 10, 15, 25, 50, and 75 mg/mL collagen reference in 1.5-mL tubes. For tubes containing the samples, 90 µL of cell supernatant and 10

μL of 0.5 M acetic acid were placed. Subsequently, 1 mL of Sircol Red was placed in each tube, and the samples were incubated for 30 min at 37˚C under shaking. The samples were centrifuged at 12,000 rpm for 10 min, and the supernatant solution was discarded. Then, 750 μL of ice-cold Acid-Salt Wash Reagent solution were applied, followed by centrifugation. Supernatant was discarded, and 1 mL of alkali reagent was added to solubilize the pellet. After complete solubilization, 0.1 mL of the solution was collected and applied in duplicate in wells of a 96-well plate for reading by ELISA (Thermo Plate) at 555 nm. The measurement of collagen was calculated according to a standard curve.

2.7. qPCR (ALP, Col I, DSPP, and DMP-1 gene expression)

Total RNA was extracted by the Trizol method (Invitrogen, Carlsbad, CA, USA), and RNA samples were quantified by biophotometry (RS-232C, Eppendorf, Hamburg, Germany). cDNA synthesis was performed with a High Capacity cDNA Reverse Transcriptions Kit (Applied Biosystems, Foster City, CA, USA), as recommended by the manufacturer. After cDNA synthesis, the effects of LED irradiation on the expression of genes encoding alkaline phosphatase (Taqman Assay ID Hs00240993_mL; RefSeq NM_000478.4), dentin matrix protein (Taqman Assay ID Hs01010903_mL; RefSeq NM_004407.3), dentin sialophosphoprotein (Taqman Assay ID Hs00171962_mL; RefSeq NM_014208.3), type I collagen (Taqman Assay ID Hs00164004_mL; RefSeq NM_000088.3), and endogenous control β-actin (Taqman Assay ID Hs01060665_mL; RefSeq NM_001101.3) were assessed.

The reactions were prepared with standard reagents for real-time PCR plus the primer/probe set for each gene. Fluorescence readings were made with Step One Plus equipment (Applied Biosystems) at each amplification cycle, and subsequently analyzed by Step One 2.1 software (Applied Biosystems). All reactions were subjected to the same conditions of analysis and normalized by the signal of the passive reference dye ROX to correct the reading fluctuations due to changes in volume and evaporation along the reaction. The result, expressed in CT value, was related to the number of PCR cycles required for the fluorescence signal to reach the threshold of detection. Individual results expressed as CT values were subsequently transferred to sheets and grouped according to the experimental groups and then normalized to the expression of the selected endogenous gene (β-actin). The concentrations of each target gene mRNA were assessed statistically.

2.8. Statistical Analysis

Raw data from TP production, ALP activity, Col synthesis, and gene expression for ALP, Col I, DMP-1, and DSPP were transformed to percentage of the control by a single rule of 3 (control = 100%). Lack of normal distribution demanded the application of non-parametric Kruskal-Wallis tests, complemented by Mann-Whitney tests for pairwise comparison, at a 5% level of significance.

3. Results

3.1. Cell Characterization by Immunofluorescence

Representative immunofluorescence images of SHEDs from primary teeth identified by CD 146, CD 44, STRO-1, Nanog, and OCT 3/4 antibodies, for the three individuals participating in the experiments (P1, P2, and P3) are shown in Fig. 1. The percentages of labeled cells for P1, P2, and P3 were: (CD146) 84.2%, 71.4%, and 69.0%; (CD 44) 81.8%, 89.2%, and 92.6%; (STRO-1)76.6%, 76.0%, and 75.1%; (Nanog) 78.8%, 80.5%, and 86.2%; and (OCT ¾) 83.5%; 82.2%, and 83.4%, respectively.

3.2. TP Production, ALP Activity, and Collagen Synthesis

Percentages of TP production, ALP activity, and collagen synthesis by SHEDs irradiated with infrared LED according to the EDs used in this study are shown in Figs. 2a, 2b, and 2c, respectively. Cells irradiated with both EDs 2 and 4 J/cm^2 showed significant increases in TP production (*ca.* 10.0%) and ALP activity (*ca.* 69.0%) compared with non-irradiated cells (control), with no difference between EDs (Figs. 2a, 2b). Collagen synthesis was also biostimulated when SHEDs were irradiated with 2 J/cm^2 (14.0%) and 4 J/cm^2 (50.8%). However, the latter ED was statistically higher (Fig. 2c).

3.3. ALP, Col I, DSPP, and DMP-1 Expression

Data for ALP, Col I, DSPP, and DMP-1 gene expression are shown in Fig. 3. The ED of 4 J/cm^2 was capable of increasing the gene expression of all

proteins, while 2 J/cm^2 biostimulated the gene expression of ALP (Fig. 3a) and DSPP (Fig. 3d). For the latter ED, although gene expression for Col I (Fig. 3b) and DMP-1 (Fig. 3c) was greater than the expression in non-irradiated cells, the difference did not reach statistical significance.

4. Discussion

Both gene expression and synthesis of proteins involved in tertiary dentin formation were up-regulated in SHEDs irradiated with an infrared LED source delivering 2 or 4 J/cm^2 (40 mW/cm^2), with an overall greater biostimulation for the latter. A major struggle in the phototherapy field is to find the optimal irradiation set of parameters for each different clinical treatment and specific tissue[16], because there are many factors involved in phototherapy success, such as wavelength, energy density, power density, and the number of irradiations[17]. In addition, there is an optimal energy density of light for any specific application, termed the 'biphasic dose-response'[18]. EDs lower than the optimum value will have a diminished therapeutic outcome, while higher EDs of light may even cause a detrimental outcome[18]. In the present study, 2 and 4 J/cm^2 EDs and 40 mW/cm^2 power density were selected based on previous studies in which the authors demonstrated encouraging results of biomodulation of cultured pulp cells subjected to phototherapy[5,6].

The literature has not been forthcoming regarding the possible mechanism of action of infrared LED irradiation on pulp cells. It is known that wavelengths in the red and infrared bands of the spectrum can be absorbed by chromophores

related to complex IV of the mitochondrial respiratory chain (cytochrome C oxidase), resulting in electron transfer among molecules that increase ATP synthesis[19,20]. Therefore, one may hypothesize that the increased ATP concentration in irradiated pulp cells can induce the expression of specific proteins, such as those of the dentin matrix, enhancing pulpal repair mechanisms.

LED irradiation of cultured mesenchymal cells from bone marrow of rats has been reported to enhance cell proliferation and osteogenic potential[21]. The immunofluorescence results of this study showed that, before the differentiation induction and irradiation protocols, the SHEDs obtained from the three individuals had a high percentage of labeled stem cells. Three days after LED irradiation, the cultured pulp cells expressed and synthesized proteins compatible with odontoblast/osteoblast-like cells, indicating that the irradiation protocols used in this study induced differentiation of stem cells into cells with an odontoblast phenotype.

Previous studies evaluating the effects of light on cells from pulp tissue, as well as results from the present study, found encouraging results, such as an increased deposition of mineralized tissue[4], ALP activity[9,10], and expression of ALP and Col I[10]. In addition, it has been demonstrated that infrared LED therapy can modulate the levels of NO and ROS production by pulp cells pre-treated with LPS, suggesting that infrared light can modulate both the inflammatory process and the formation of mineralized tissue[6]. These interesting previous data, associated with the greater gene expression and synthesis of proteins observed in the present study, when SHEDs were irradiated with specific LED parameters,

confirm the biostimulatory effects of infrared LED phototherapy and encourage further studies concerning the cellular pathways involved in the action of infrared light on dental pulp cells.

In a recent study[5], it has been demonstrated that the infrared LED can improve ALP activity and Col-I expression by odontoblast-like cells. Therefore, both ondontoblast-like cells and pulp stem cells can be biostimulated by infrared LED, suggesting that this kind of therapy may stimulate tertiary dentinogenesis[22] at different ways and could also represent a broader shift in thinking about how to trigger the body's natural regenerative capacity.

Infrared light has the advantage of further penetration into dentin, being able to pass through 1-mm-thick dentin discs[8]. In clinical conditions, this may favor the absorption of light by the pulp cells subjacent to the cavity floor. The infrared LED ability to penetrate dentin, combined with its positive biological effect on pulp-repair-related protein synthesis, makes this light source a valuable tool for inducing pulp repair and regeneration. Moreover, this technology shows additional advantages, such as the low cost of diodes and their long lifetime[23].

5. Conclusion

In conclusion, infrared LED irradiation, at selected parameters, is able to up-regulate gene expression and synthesis of proteins related to the formation and mineralization of dentin matrix by human dental pulp cells. Further *in vivo* studies should be developed to ensure the clinical use of this phototherapy.

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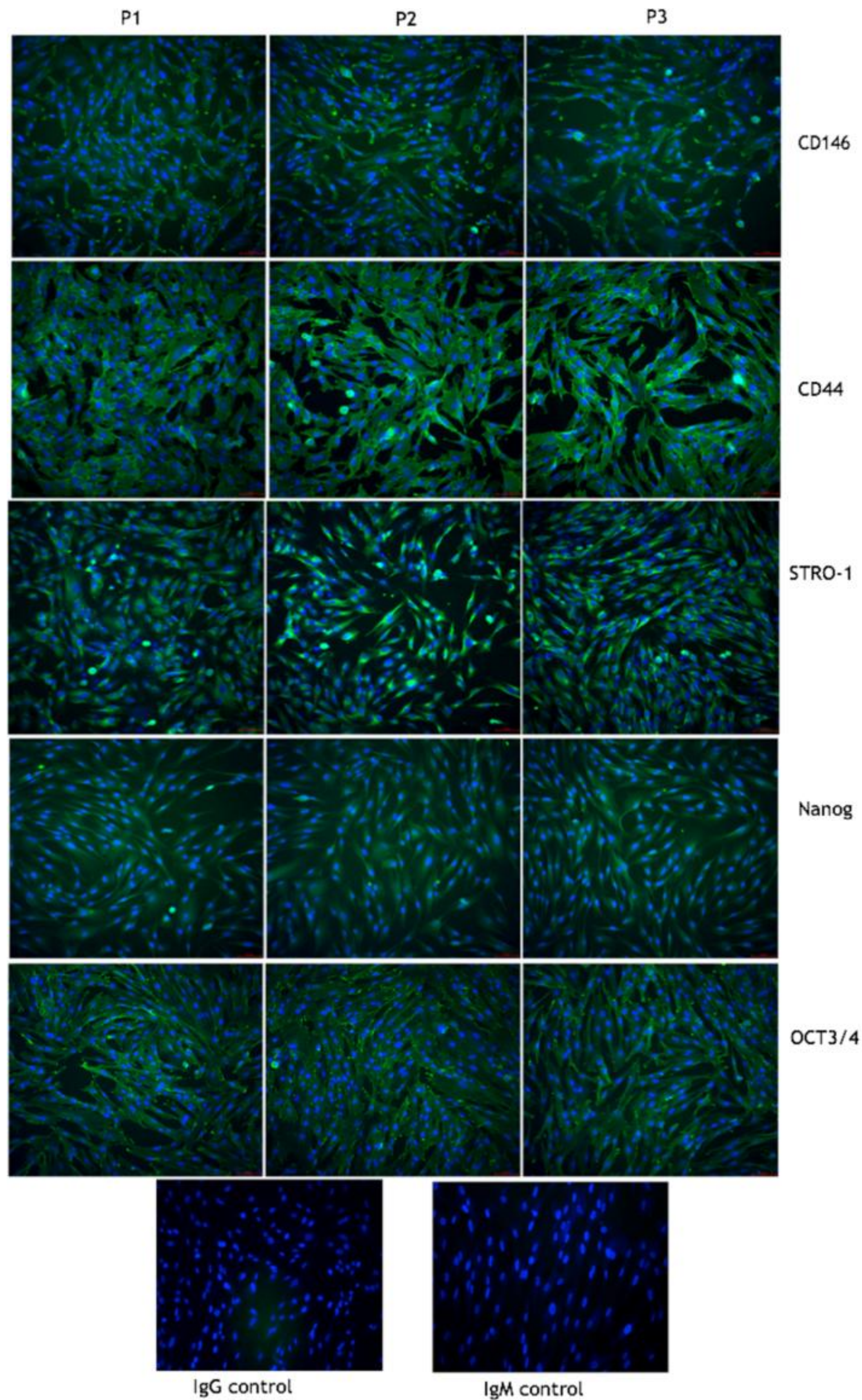

Figure 1. Immunofluorescence images of cells from human-exfoliated deciduous teeth (SHEDs) collected from human primary teeth, characterizing the population of stem cells. Stem cells were marked with CD 146, CD 44, STRO-1, Nanog, and OCT 3/4 antibodies, for the three individuals (P1, P2, and P3) who participated in the experiments, and IgG and IgM were used as controls. Stem cell nuclei are stained in blue, while cytoplasm is stained in green. X20.

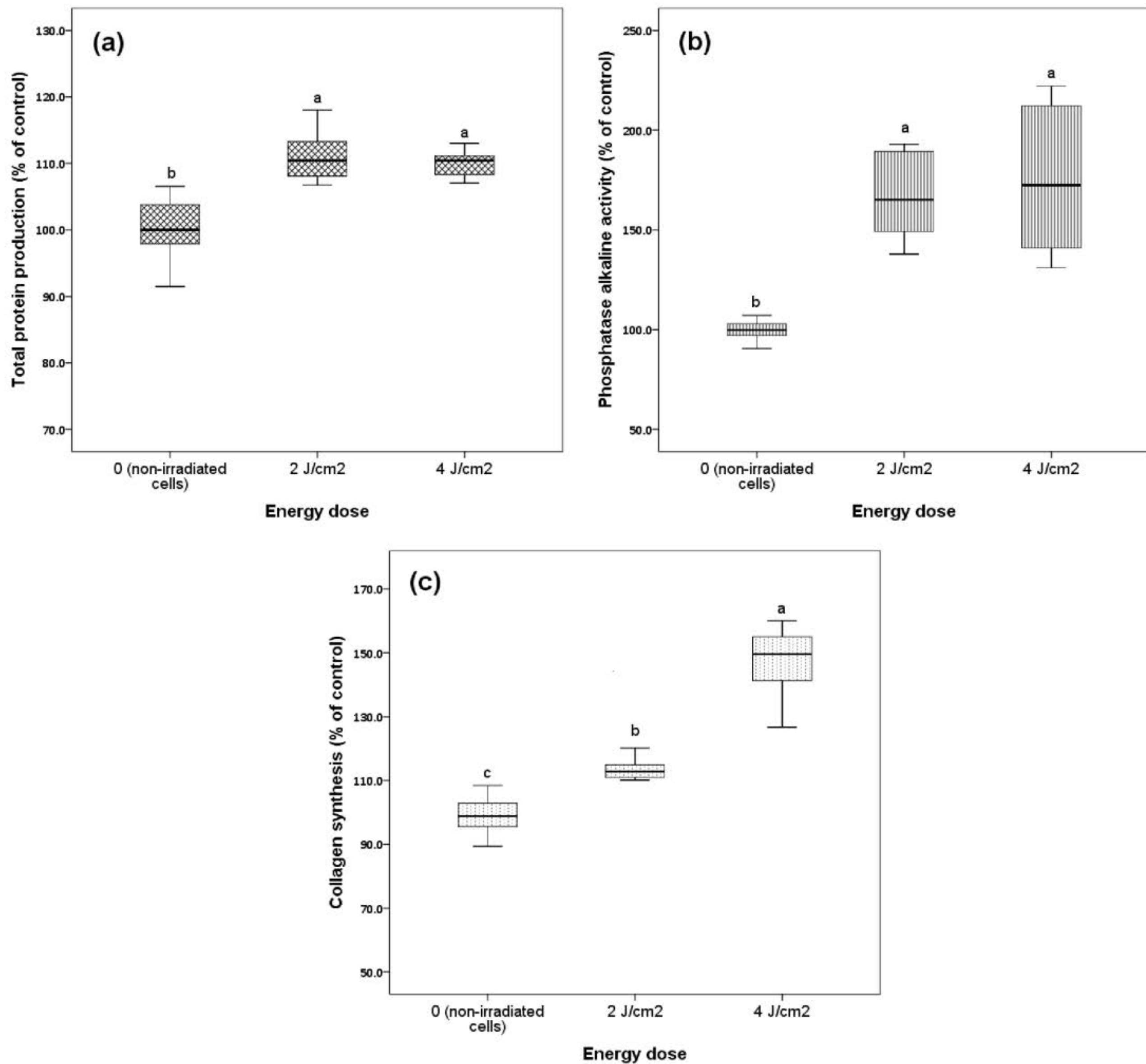

Figure 2. Total protein production (a), phosphatase alkaline activity (b), and collagen synthesis (c) by SHEDs cells irradiated with an infrared LED source delivering an energy dose of 2 or 4 J/cm^2. Different letters indicate statistically significant difference (Mann-Whitney, $p < .05$). The box contains 50% of the datapoints, and the middle line of the box is the median. The tips of the projecting bars show minimum and maximum values, n = 12.

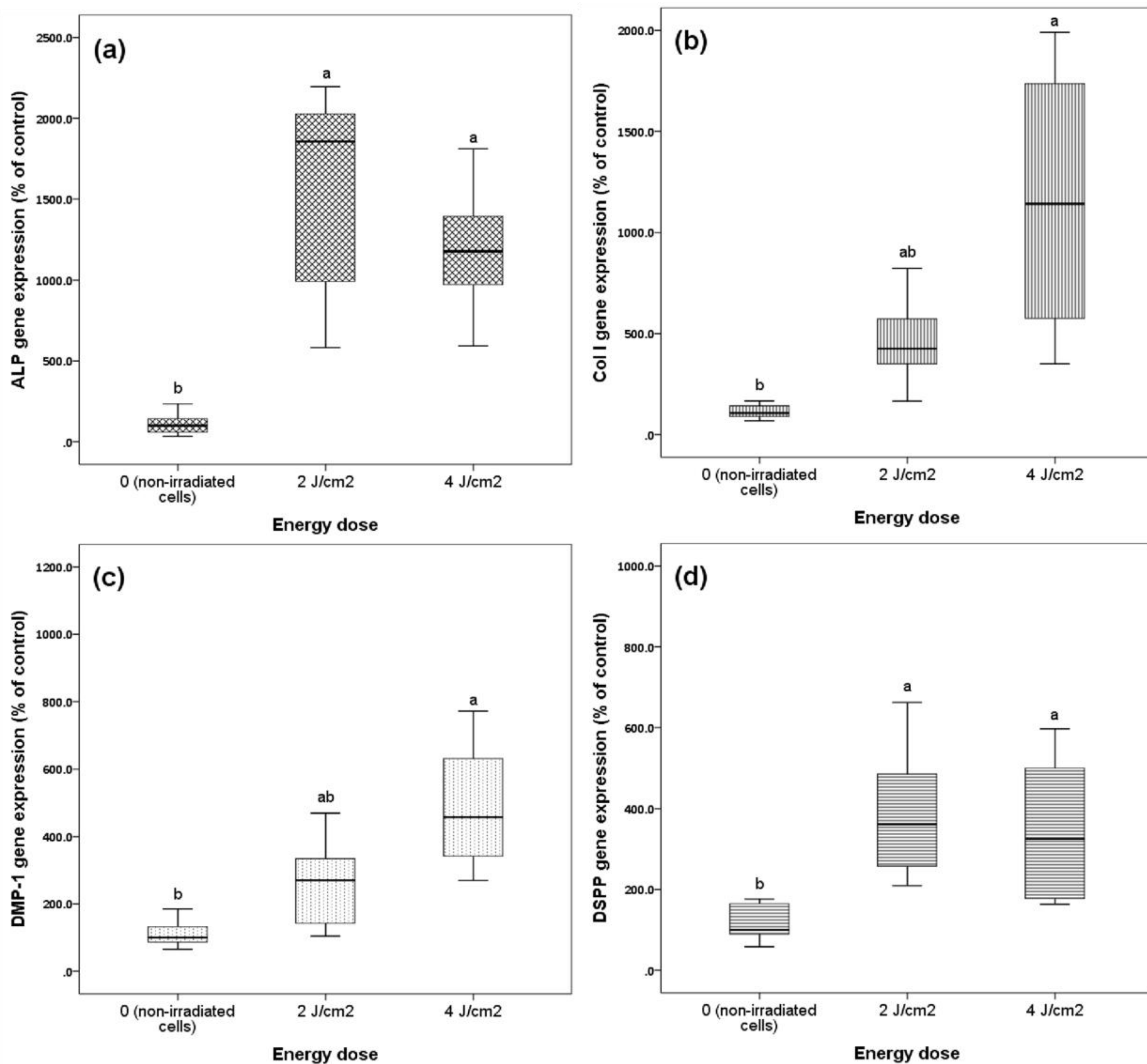

Figure 3. Percentage of gene expression for ALP (a), Col I (b), DSPP (c), and DMP-1 (d) in SHEDs obtained from primary teeth pulp after irradiation with an infrared LED source delivering 2 J/cm^2 or 4 J/cm^2. Non-irradiated cells served as control. All protein gene expression was normalized by β-actin expression. Different letters indicate statistically significant differences (Mann-Whitney, $p < .05$). The box contains 50% of the datapoints, and the middle line of the box is the median. The tips of the projecting bars show minimum and maximum values, n = 9.

3.3 ESTUDO 3

TRANSDENTINAL PHOTOBIOSTIMULATION OF STEM CELLS FROM HUMAN EXFOLIATED TEETH

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Running title: Transdentinal Photobiostimulation of Pulp Stem Cells

Keywords: Dentin, Odontoblasts, Phototherapy, Protein expression, Pulp biology, Stem cells

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Abstract

Aim: The aim of this study was to evaluate the effects of infrared LED (850 nm) irradiation on stem cells from human exfoliated deciduous teeth (SHEDs). **Methodology**: Primary teeth were obtained (n=3) and SHEDs were seeded on the pulp surface of 0.2 mm-thick dentin discs produced from permanent molars, using plain DMEM. After 24-h incubation, the DMEM was replaced by a new culture medium for odontoblast differentiation. After 12 h, SHEDs were irradiated (80 mW/cm^2) with a infrared LED delivering energy doses of: 0 (control), 2 or 4 J/cm^2 (n=6) and the tests were performed 72 h post–irradiation. The tests included cell viability (MTT), alkaline phosphatase (ALP) activity, total protein (TP) production, scanning electron microscopy (SEM), as well as gene expression for ALP, Col I, DSPP and DMP-1. Data were submitted to Kruskal-Wallis and Mann-Whitney tests (α=0.05).

Results*:* Both energy doses (2 and 4 J/cm^2) increased the cell viability and the ALP activity. For TP, ALP and Col I gene expression, only 4 J/cm^2 showed higher values when compared to the control group. Cells morphology was not affect by the irradiation.

Conclusion*:* Infrared LED irradiation was capable of biostimulating SHEDs through the dentin barrier and the best results were obtained when the cells received 4 J/cm^2.

Introduction

Tissue regeneration has been an important target of investigation in the last years. There is an evident progression on treatments and techniques in different areas, involving tissues such as bone (Vimalraj and Selvamurugan, 2014), muscle (Montoya *et al*. 2015), neuronal tissue (Feng *et al*. 2014) and others. However, only scanty data is found with respect to the pulp tissue, what is still a challenge. The current materials recommended for direct application on the pulp tissue are calcium hydroxide or mineral trioxide aggregate (MTA), which are not completely biocompatible (Paranjpe *et al.* 2010). In situations where the pulp tissue is injured by trauma or deep caries, there are few conservative treatment options. In most cases, a invasive treatment is performed such as pulpotomies and root canal treatment (Maltz *et al.* 2011). It would be interesting to have alternatives to improve pulp tissue repair, avoiding invasive treatments while keeping a healthy functional tissue.

Phototherapy has been reported as an interesting alternative for tissue repair, showing promising results in *in vitro* studies (Oliveira *et al.* 2011, Holder *et al* 2012, Oliveira *et al.* 2013, Turrioni *et al.* 2014a, Turrioni *et al.* 2014b, Teuschl *et al.* 2015) and animal models (Ferreira *et al.* 2006, de Mattos et al. 2015). Despite these positive results, the effects of light on pulp tissue are little investigated. In a recent review (Carrol *et al.* 2014), it was reported only six studies related to the use of light for pulp tissue repair/mineralized tissue formation. Moreover, the authors highlighted the lack of information concerning irradiation parameters on the literature (Carrol *et al.* 2014). The phototherapy using infrared wavelength (700 to 1000 nm) has the highest capacity to penetrate the tissue, especially hard tissues. Turrioni *et al.* (2013) demonstrated that the attenuation for infrared LED light in 1 mm-thick dentin (47% loss) is much smaller than the red light (58% loss) or the blue light (70% loss). This information suggests that the infrared light could be more effective for pulp stimulation through dentin barrier and that if could be delivered on the pulp floor of deep cavities before applying the restorative material.

To date, it has been demonstrated that a red light is able to stimulate the deposition of mineralized tissue by primary pulp cells from rodent (Holder *et al.* 2012),

to induce reactional dentinogenesis on human teeth (Ferreira *et al.* 2006), to modulate the oxidative stress in human dental pulp cells (Montoro *et al.* 2014), to stimulate the metabolism of odontoblast-like cells (MDPC-23) (Oliveira *et al.* 2011, Oliveira *et al.* 2013; Turrioni *et al.* 2014a) and to up-regulate dentin matrix protein synthesis and production by human pulp cells (Turrioni et *al.* 2014b). In some of these studies, a LED (light emitting diode) source was used to generate a red or infrared light (Montoro et al., 2014; Turrioni et al., 2014b). Despite these results, additional studies are still needed to investigate irradiation parameters and the ideal condition for clinical application of this technique. There are many studies evaluating the direct effect of light on cells, but only few studies evaluating the effect of light on cells seeded on dentin discs (Oliveira *et al.* 2013, Turrioni *et al.* 2014a). This is the first study aiming to evaluate the effect of infrared LED irradiation (850 nm) on human pulp cells seeded in human dentin.

Material and Methods

Pulp cells obtaining and characterization

Cells were obtained from dental pulp of exfoliated deciduous teeth (SHEDs) (n=3) using the enzymatic digestion technique (Gronthos *et al.* 2000, Turrioni *et al.* 2014b). The protocol was approved by the Ethics Research Committee from Araraquara School of Dentistry - UNESP (Protocol 63/11). Briefly, cells were kept in contact with a solution containing 4 mg/mL dispase and 3mg/mL type I colagenase (Worthingthon, Lakewood, NJ, USA) diluted in DMEM (Lifetechnology, Grand Island, NY, USA) for one hour and seeded in 75 cm^2 flasks. Cells from the fourth passage were used for the experiments.

Before the experiments, the cells were characterized by an immunofluorescence protocol using STRO-1 (1:100), CD44 (1:50), CD146 (1:50), Nanog (1:100) and OCT3/4 (1:100) antibodies (Lifetechnology). The secondary antibodies used were IgM (dilution 1:400) for STRO-1 and IgG (dilution 1:400) for the other markers (Santa Cruz Biotechnology, Dallas, TX, USA). For nuclei staining, it was used Hoechst (dilution 1:5000, Invitrogen, Carlsbad, CA, USA) for 5 minutes. The immunofluorescence protocol is detailed described by Turrioni *et al.* (2014). Cells were assessed using IN Cell Analyzer 2000 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), and 9 selected

areas were considered for quantitative data analysis with IN Cell Investigator Software v1.6.

Dentin discs obtaining

Thirty-six sound third molars were obtained, after approval protocol by the Research Ethics Committee of Araraquara Dental School - UNESP (63/11). The teeth were immersed in 70% ethanol for 5 days and subjected to removal of periodontal ligament remnants and debris adhered to the surface. Two 0.2 mm-thick dentin discs were obtained from the central region of the crown of each tooth and the thickness was determined by using a digital tachometer-PA (500-144B, Mitutoyo South America Ltda., Santo Amaro, SP, Brazil).

To obtain the dentin discs, it was used a diamond blade (11-4254, 4 "x 0.012"/ 15LC series, Buehler Ltda., Lake Bluf, IL, USA) mounted on a metallographic cutter (ISOMET 1000, Buehler Ltda.). After obtaining the discs, they were analyzed with a magnifying glass (SZ2-ILST - Olympus Comporation, Tokyo, Japan). The discs that presented "islands" of enamel or depressions representing the uppermost portion of the pulp horns were discarded. Subsequently, the surfaces of dentin discs were sanded and regularized with 400 and 600 water sandpapers (T469-SF-Noton, Saint-Gobam Abrasives Ltda., Jundiaí, SP, Brazil), and during this procedure the discs were frequently evaluated using a digital caliper (500-144B, Mitutoyo South America Ltda.), in order to make sure about the final thickness of 0.2 mm.

The total area of the selected dentin discs was reduced using a cylindrical diamond tip (number 1095 - KG Sorensen, Cotia, SP, Brazil) at high speed, finished in low speed, in such way that the final diameter of each disc was 8 mm. This procedure was performed in order to create conditions for adapting these discs with the pulp surface facing up, in the center of a metallic device. Each metallic device with the adapted dentin disc allowed the application of LED on the occlusal surface (face down), while the pulp surface (where the cells were seeded) remained in contact with the culture medium (face up).

After reduction of the discs area, a 0.5 M EDTA solution (pH 7.2) was applied for 2 minutes on both sides of the dentin disc with the purpose of surface cleaning and removal of the smear layer by preparing the specimens for the power-measuring test. After conditioning the dentin, the EDTA solution was rinsed off the surfaces of the

discs by applying sterile deionized water for 60 seconds. Finally, the dentin discs were individually submitted to the light attenuation measuring.

Light attenuation measuring

The light attenuation measuring (Turrioni *et al.* 2012) aimed to calculate the power loss that may occur during irradiation in the presence of the discs. For this, a power detector was positioned on the pulp surface of the dentin disc, while the light beam was applied on the occlusal surface of the same disc. The procedure was performed in Biophotonics Laboratory of IFSC (Institute of Physics of São Carlos - USP - São Carlos, SP). To carry out these measurements, the discs were placed with the occlusal surface facing up, in a small compartment containing a power detector (Field Master, Coherent, Santa Clara, CA, USA), which was directly connected to a potentiometer (LM-2 - Coherent).

Each disc was irradiated using a device containing an acrylic cylinder, which function was collimating the beam and direct it to the area where the discs were adapted. The device was supported by a metal shaft, allowing stability at the time of irradiation. The power loss in each disc was calculated by the difference between power obtained by the irradiation chamber in the absence and presence of discs, respectively, and converted into a percentage. By the results obtained with this test, it was possible to evenly distribute the dentin discs among the different experimental and control groups.

Experimental conditions

After homogenous division of the discs in experimental and control groups through the light attenuation measurement test, the discs were individually packed and sterilized by ethylene oxide gas (Lanza *et al.* 2009, Turrioni *et al*. 2014a). In addition, the metal devices and silicon rings were individually autoclaved in glass vials containing deionized water, for 15 minutes, at 120 ° C and pressure of 1 kgf.

Within a laminar flow hood (Bio Protector Plus 12, Veco of Brazil - Ind. Equipment Ltda, Campinas, SP, Brazil), the sets dentin disc/metal device were inserted in compartments of 24 well-plates. SHEDs were seeded (60,000 cells/disc) on the pulpal surface of the discs, using DMEM with 10% FBS and maintained for 24 h in a humidified incubator with 5% CO_2 and 95% air at 37 ^{o}C (Isotemp, Fisher Scientific, Bellefonte, PA, USA). The DMEM was than replaced by a differentiation medium

(DMEM with 5 mM b-glycerophosphate and 100 mM ascorbic acid; Sigma/Aldrich, St. Louis MO, USA) containing 0.5% FSB (nutritional stress induction) (Turrioni *et al.* 2014b). After 24 hours the irradiations were deliverd. For irradiation procedure, the 24-well plates were placed on the LEDTable (850 nm, 80 mW/cm^2) for specific periods, according to the energy dose (50 sec for 2 J/cm^2 and 1 min and 40 sec for 4 J/cm^2), in a dark room. All tests were performed 72 hours after irradiation.

Cell Viability (MTT Assay)

Cell viability was performed using the metiltetrazolium colorimetric method (MTT Assay, Sigma/Aldrich). To prepare the MTT solution, 25 mg of metiltetrazolium salt were weighed on an analytical balance precision (AG 2000 Gehaka Diadema, SP, Brazil) and subsequently added to 5 ml of phosphate buffered saline solution (PBS), resulting in a final mixture concentration of 5mg/ml. In each well, 900 uL of medium associated with 100 uL of MTT solution (5 mg/mL in PBS metiltetrazolium salt) were applied to the culture. The cells in contact with the MTT solution were incubated in a humidified incubator at 37 ° C with a time of 4 hours. After this time, the MTT solution was carefully aspirated and replaced by 700 uL of the acidified isopropanol solution (0.04 N HCl). Three aliquots of 100 uL of each compartment were transferred to a 96 wells- plate (Costar Corp., Cambridge, MA, USA). Cell viability was assessed in proportion to the absorbance determined at 570 nm in an ELISA reader (Thermo Plate, Nanshan District, Shenzhen, China).

Total protein production

The levels of total protein was performed for each experimental and control group, using the Read protocol, Northcote (1981) which has been widely used (Oliveira *et al.* 2011, Turrioni *et al.* 2014 a,b). The culture medium was aspirated and the samples were washed three times with 1 ml PBS heated to 37 ° C. Cell lysis was performed by adding 1.2 ml of sodium lauryl sulfate, 0.1% (Sigma - Aldrich) in each well for 40 minutes at room temperature. The samples were homogenized and then 1 ml of each compartment was transferred to Falcon tubes (Corning Incorporated, Corning, NY) properly identified (test tubes), and for representing the blank tube was added 1 mL of distilled water. Then, 1 ml of Lowry reagent solution (Sigma - Aldrich) was added to all tubes and shaken for 10 seconds on a shaker (Phoenix AP 56, Labstore, Curitiba, Brazil)

20 minutes at room temperature. After that period, 500 uL of Folin Phenol Reagent Solution and Ciocalteau's (Sigma - Aldrich) were added to each tube, and again shaken for 10 seconds. Thirty minutes after, three 100 uL aliquots from each tube were transferred to a 96-compartment culture plate and the absorbance of the test tubes was read at a wavelength of 655 nm in an ELISA reader (Thermo Plate) against blank. The average reading of the three fractions was computed. The level of total protein was calculated using a standard curve with known concentrations of protein (0, 16, 32, 64 and 80 mg/mL - BSA, 2 mg/mL, Sigma-Aldrich).

Alkaline phosphatase (ALP) activity

Alkaline phosphatase activity was performed according to protocol Alkaline Phosphatase Kit - Endpoint Assay (Labtest Diagnostics SA, Lagoa Santa, MG, Brazil). After washing and lysis as described for total protein protocol, Falcon tubes (tests, and blank) were adequately identified and 50 uL substrate (thymolphthalein monophosphate 22 mmol/L – Kit Reagent #1) and 500 uL buffer (300 mmol/l, pH 10.1 - Kit Reagent #2) were added to each tube. Only on the standard tube was added 50 uL of the standard solution 45 U/L (Reagent 4). The tubes were placed in a water bath (Fanem, Guarulhos, SP, Brazil) at 37 ° C for 2 minutes. The samples were then homogenized and 50 uL of each well were transferred to test tubes, kept in a water bath and gently shaken. After 10 minutes incubation, was added 2 mL of color reagent (sodium carbonate 94mmol/L and sodium hydroxide 250 mmol/L – Kit Reagent #3). The absorbance of samples was determined by spectrophotometer (Thermo Plate), at a wavelength of 590 nm. Alkaline phosphatase activity was calculated by normalizing the absorbance values for total protein production values found. A standard curve with known concentrations of alkaline phosphatase was also used to calculate the values of ALP activity.

ALP, Col I, DSPP, and DMP-1 gene expression

Matrix proteins gene expression was assessed acording to Turrioni *et al.* (2014). Briefly, Trizol was used to extract the total RNA (Invitrogen), RNA samples were quantified by biophotometry (RS–232C, Eppendorf, Hamburg, Germany), cDNA synthesis was performed with a High Capacity cDNA Reverse Transcriptions Kit (Applied Biosystems, Foster City, CA, USA) and the effects of the transdentinal LED irradiation on the expression of genes encoding alkaline phosphatase (Taqman Assay ID

Hs00240993_mL; RefSeq NM_000478.4), dentin matrix protein (Taqman Assay ID Hs01010903_mL; RefSeq NM_004407.3), dentin sialophosphoprotein (Taqman Assay ID Hs00171962_mL; RefSeq NM_014208.3), type I collagen (Taq-man Assay ID Hs00164004_mL; RefSeq NM_000088.3), and endogenous control b-actin (Taqman Assay ID Hs01060665_mL; RefSeq NM_001101.3) were assessed. The reactions were prepared with standard reagents for real-time PCR plus the primer/probe set for each gene, the readings were made with Step One Plus equipment (Applied Biosystems) and subsequently analyzed by Step One 2.1 software (Applied Biosystems). Individual results expressed as CT values were transferred to sheets and grouped according to the experimental groups and then normalized to the expression of the b-actin.

Scanning electron microscope

For cell morphology analysis, the cells adhered to the dentin were first subjected to the fixing process (2.5% glutaraldehyde for 2 hours). After fixing the cells, they were washed three times with 1 ml PBS (5 minutes each wash) and then post-fixed for 60 minutes in 200 uL of 1% osmium tetroxide (Sigma/Aldrich). The samples were dehydrated in ascending ethanol trade solutions (30%, 50%, 70%, 95% 2x and 2x 100% - 30 min in each solution) and then subjected to drying by means of low surface tension solvent 1,1,1,3,3,3-hexamethyldisilazane (HMDS - ACROS Organics, Rutherford, NJ) and kept in a desiccator for 12 hours. Finally, the samples were fixed in "stubs", metallized with gold and examined in a scanning electron microscope (SEM, JEOL-JMS-T33A Scanning Microscope, JEOL - USA Inc., Peabody, MA, USA).

Statistical analysis

The data set of each response variable was evaluated for its distribution. Since there was no adherence to the normal curve, the non-parametric Kruskal-Wallis and Mann-Whitney tests were applied, considering an independent variable (“energy dose”). Statistical tests were considered at the pre-determined significance of 5%.

Results

Characterization by immunofluorescence

SHEDs presented a percentage of 77,6%, 87,4%, 72,6%, 88,2% and 75,9% for CD146, CD44, STRO-1, Nanog and OCT 3/4 respectively. The images representing the marked cells are shown in Figure 1.

Cell Viability, TP production and ALP activity

Percentages of cell viability, TP production, ALP activity by SHEDs irradiated with infrared LED according to the EDs used in this study are shown in Fig. 2a, b, and c, respectively. Both doses caused an increase in cell viability when compared to the control group, with statistical difference ($p < 0.05$), and the increases were 15% for the dose of 2 J/cm^2 and 33% for 4 J/cm^2. The dose of 4 J/cm^2 showed higher values when compared to the dose of 2 J / cm2 ($p < 0.05$).

Only 4 J/cm^2 caused an increase in the production of total protein when compared to the control group, which was statistically significant ($p < 0.05$), and the increase was 12%. The dose of 2 J/cm^2 did not differ from the control group ($p > 0.05$) and showed lower values than 4 J / cm^2 ($p < 0.05$).

For ALP activity, both ED showed higher values when compared to control group (21% and 23% increase for 2 and 4 J/cm^2 respectively, $p<0.05$).

ALP, Col I, DSPP, and DMP-1 expression

Percentages of ALP, type I collagen, DSPP and DMP-1 gene expression by SHEDs irradiated with infrared LED according to the EDs used are shown in Fig. 3a, b, c and d respectively. For ALP and Col I gene expression, only 4 J/cm^2 showed higher values (45% and 122% respectively) when compared with the control group. For DSPP and DMP-1 gene expression, there was no difference between irradiated and control group ($p>0.05$).

Cell morphology (SEM)

For the experimental and control groups a large number of pulp cells covering the dentin discs was observed, and a slight increase in the number of cells for the experimental groups was observed when compared to the control group. In all groups

the cells showed no morphological changes (Figure 4). In Figure 5 is shown the detail of the matrix covering the dentin disc, highlighting the projections of the collagen matrix.

Discussion

The ability to stimulate pulp cells into odontoblastic differentiation is of major relevance in pulp tissue repair. Therefore, finding an applicable technique that can be safely used, without causing tissue damage is utterly important, especially when dealing with deep cavities where pulp is already affected (Goldberg and Smith, 2004, Tziafas *et al.* 2004). In the present study, it was evaluated the capacity of infrared LED irradiation to stimulate SHEDs into ondotoblastic differentiation in the presence of dentin barrier. It was seen that 4 J/cm^2 was the best energy density to promote that differentiation.

Several studies have assessed cell viability, total protein production or expression of dentin matrix proteins after LED or laser irradiation, on pulp cells (Oliveira *et al.* 2011, Hsieh *et al.* 2012, Holder *et al.* 2012, Oliveira *et al.* 2013, Turrioni *et al.* 2014a,b). Concerning the odontoblast-like cells (MDPC-23), it can be found in the literature, the evaluation of the direct effect using infrared laser irradiation (Oliveira *et al.* 2011), the transdentinal effect using infrared laser irradiation (Oliveira *et al.* 2013) and the transdentinal effect using blue, red or infrared LED irradiation (Turrioni *et al.* 2014a). Results from these studies reveal that infrared laser irradiation can promote cell stimulation using doses of 2, 10, 15 or 25 J/cm^2 (for direct effect, 20mW/cm^2 and transdentinal effect, 50 mW/cm^2) and overall 25 J/cm^2 showed the best results. For LED irradiation, 4 and 25 J/cm^2 promoted cell stimulation, mainly for red and infrared wavelengths (transdentinal effect, 80 mW/cm^2). Therefore, phototherapy with both LED and laser irradiation can biostimulate cells but the results can vary according to the set of physical parameters used, including the energy dose.

In the same way, previous study evaluated the same pulp cell type used in the present study (SHEDs), although directly irradiating the cells with infrared LED (Turrioni et al, 2014b). According to Turrioni *et al*. (2014b), energy doses of 2 and 4 J/cm^2 (20 mW/cm^2) were able to stimulate SHEDs to differentiate into odontoblast-like cells and the best results were found using 4 J/cm^2. Our data shows similar finds, however in an attenuated way. While the direct effect of 4 J/cm^2 promoted an increase

on ALP activity (130%), ALP and COL I gene expression (1000% for both) and DSPP and DMP-1 gene expression (400%), in this study it was found 23% increase on ALP activity, and 45% and 122% increase on Col I and ALP gene expression respectively. For DSPP and DMP-1 gene expression, there was no effect, even though cell viability increased after irradiation. These finds suggest that dentin barrier can play an important role on pulp cells biostimulation and that the biostimulatory effects are attenuated on the presence of dentin, even for 0.2 mm-thick. The scattering of LED light on dentin tissue is not only related with the dentin tubules but with the total internal reflection through the peritubular dentin (Turrioni et al., 2012). Consequently, light scattering might exert an important effect on the attenuation of cell biostimulatory effect through dentin barrier.

DSPP and DMP-1 are non-collagenous extracellular matrix proteins secreted by mature odontoblats or osteoblasts. Indeed, these two proteins are key factors on mineralized dentin formation (Goldberg and Smith, 2004, Tziafas *et al*. 2004). The results of the present study indicate that the dentin barrier can interfere on the complete SHEDs differentiation, and maybe, a longer period is required for detecting the effective presence of these two proteins in vitro, after differentiation induction and transdentinal LED irradiation. It is important to bare in mind that the pathways activated by laser or LED irradiation are not completed elucidate. The only known pathway is associated with the activation of citocromo c oxidase cascade and increase of ATP synthesis (Karu *et al.* 2010).

Therefore, it can be concluded that 4 J/cm^2 is a suitable energy dose for transdentinal cell biostimulation and SHEDs initial differentiation into odontoblast-like cells, when combined with the other physical parameters used in this study. Further studies are necessary to understand the pathways involved with the effects of light on dentin tissue formation. Based on the positive results found in this study, we conjecture about the clinical applicability of LEDtherapy on the treatment of deep caries lesions and the possibility of incorporating this therapy as a conservative approach to stimulate the pulp repair. These assumptions should be further investigated in in vivo and clinical studies.

Acknowledgments

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Figure legends

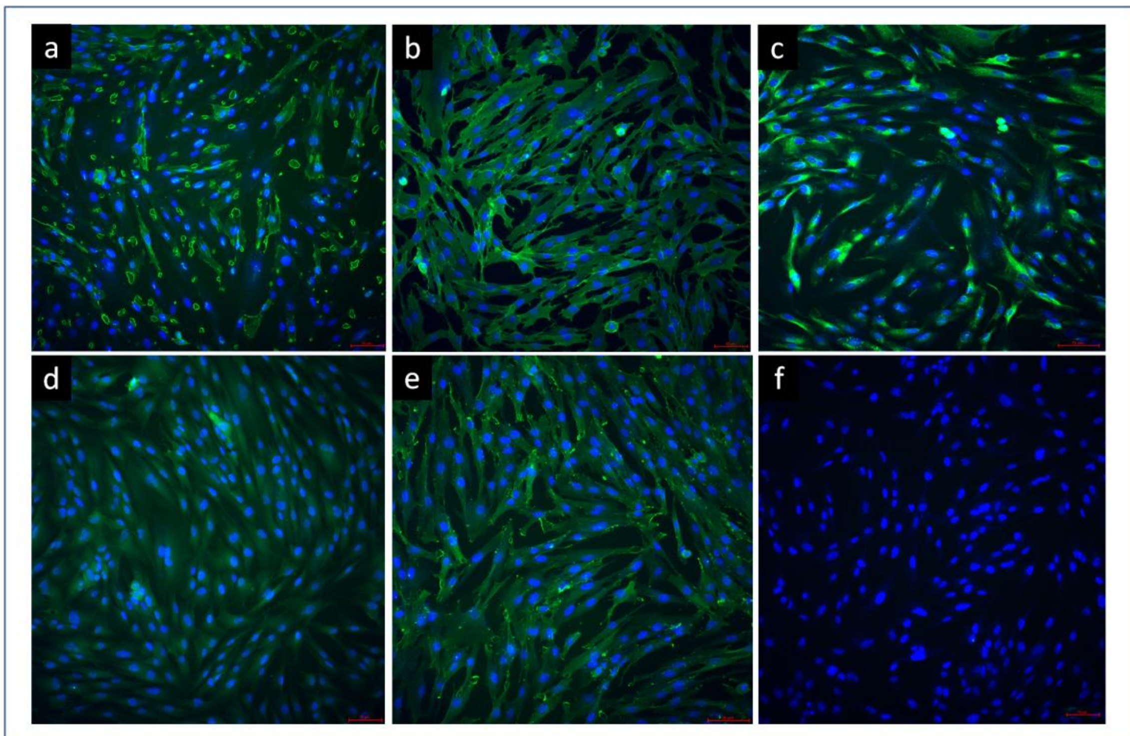
Figure 1: Representative images of the SHEDs staining, using CD146 (a), CD44 (b), STRO-1 (c), Nanog (d), OCT 3/3 (e) and negative control (f). Cell nuclei (marked in blue) accompanied by the respective cytoplasm (green) characterize stem cells. X20.

Figure 2: Representative graphs of a - cell viability (%), b - TP production (%) and c - alkaline phosphatase activity (%) in pulp cells undergoing transdentinal irradiation with infrared LED, comparing different doses of energy used.

Figure 3: Representative graphs of a - ALP gene expression (%), b - Col I gene expression (%), c - DSPP gene expression (%) and d- DMP-1 gene expression (%) by pulp cells undergoing transdentinal irradiation with infrared LED, comparing different doses of energy used.

Figure 4: SEM images showing the overall aspect of cells and extracellular matrix covering the dentin discs for for the experimental and control groups. a, b, c and d represent conditioned dentin disc EDTA (no cells), control group, 2 J / cm^2 and 4 J / cm^2, respectively, X500 .

Figure 5: Details of the cell morphology with a large pulp cytoplasm and numerous cytoplasmic processes (arrows) adhering to the tubular dentin (D), X10.000.



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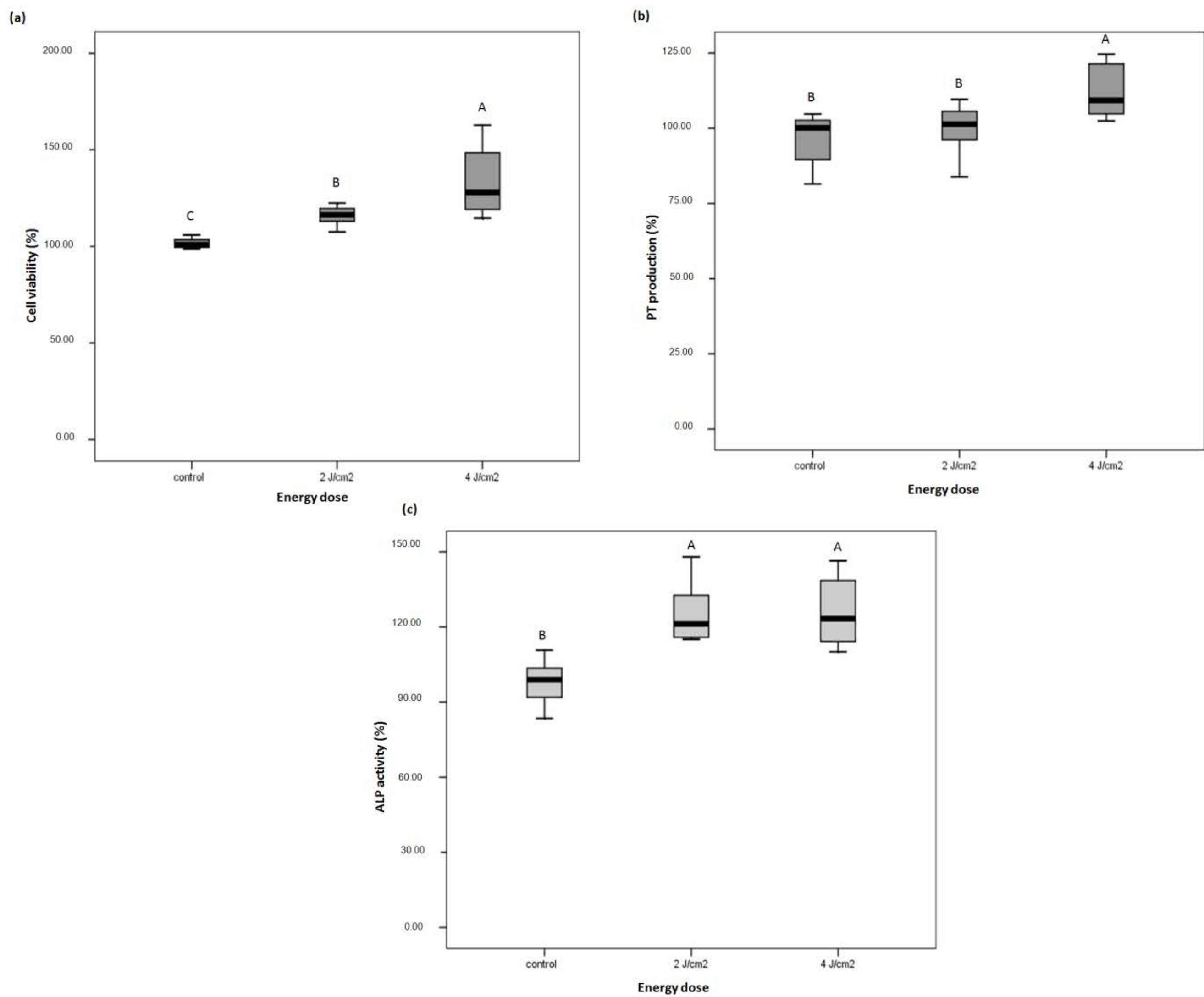

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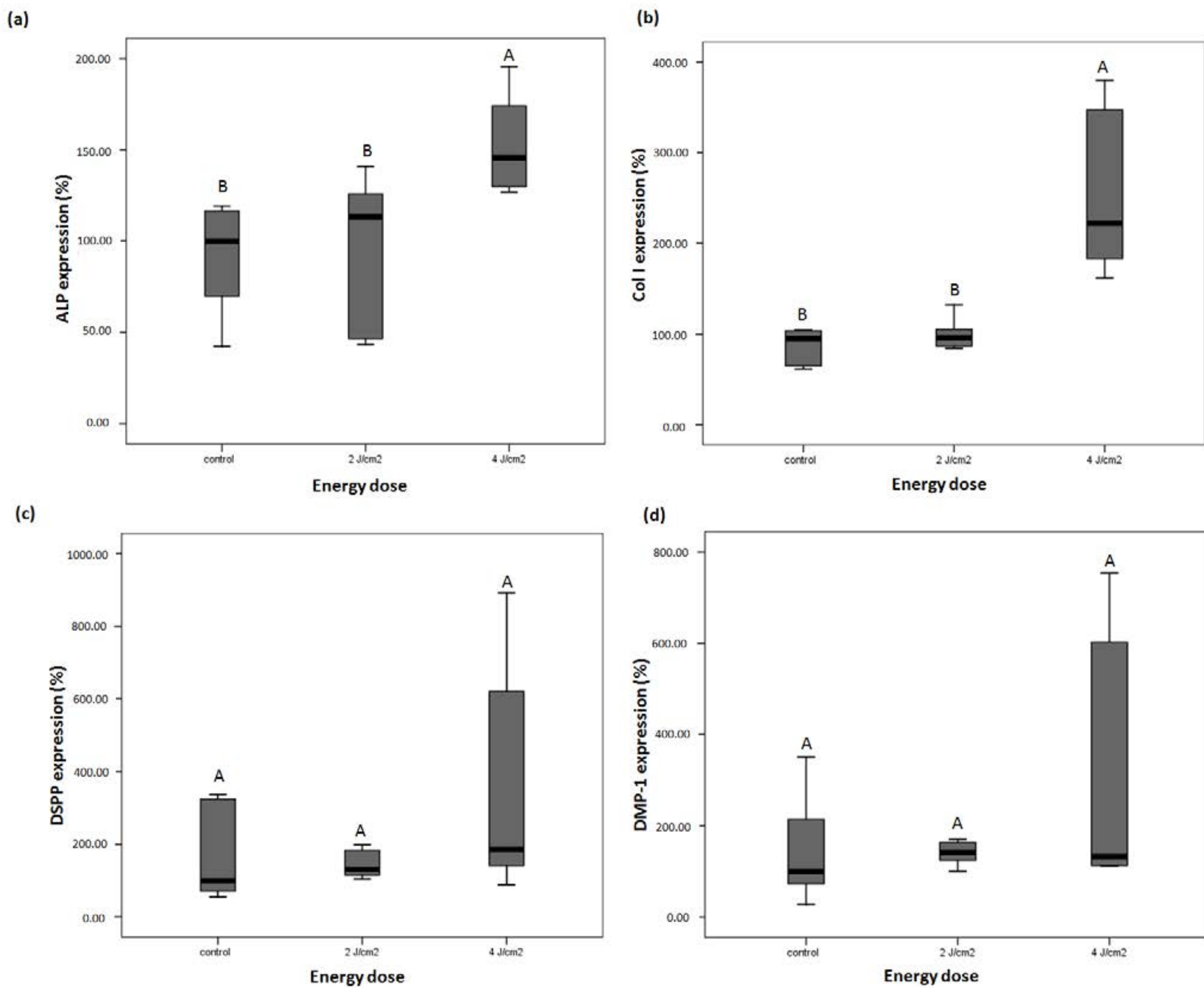
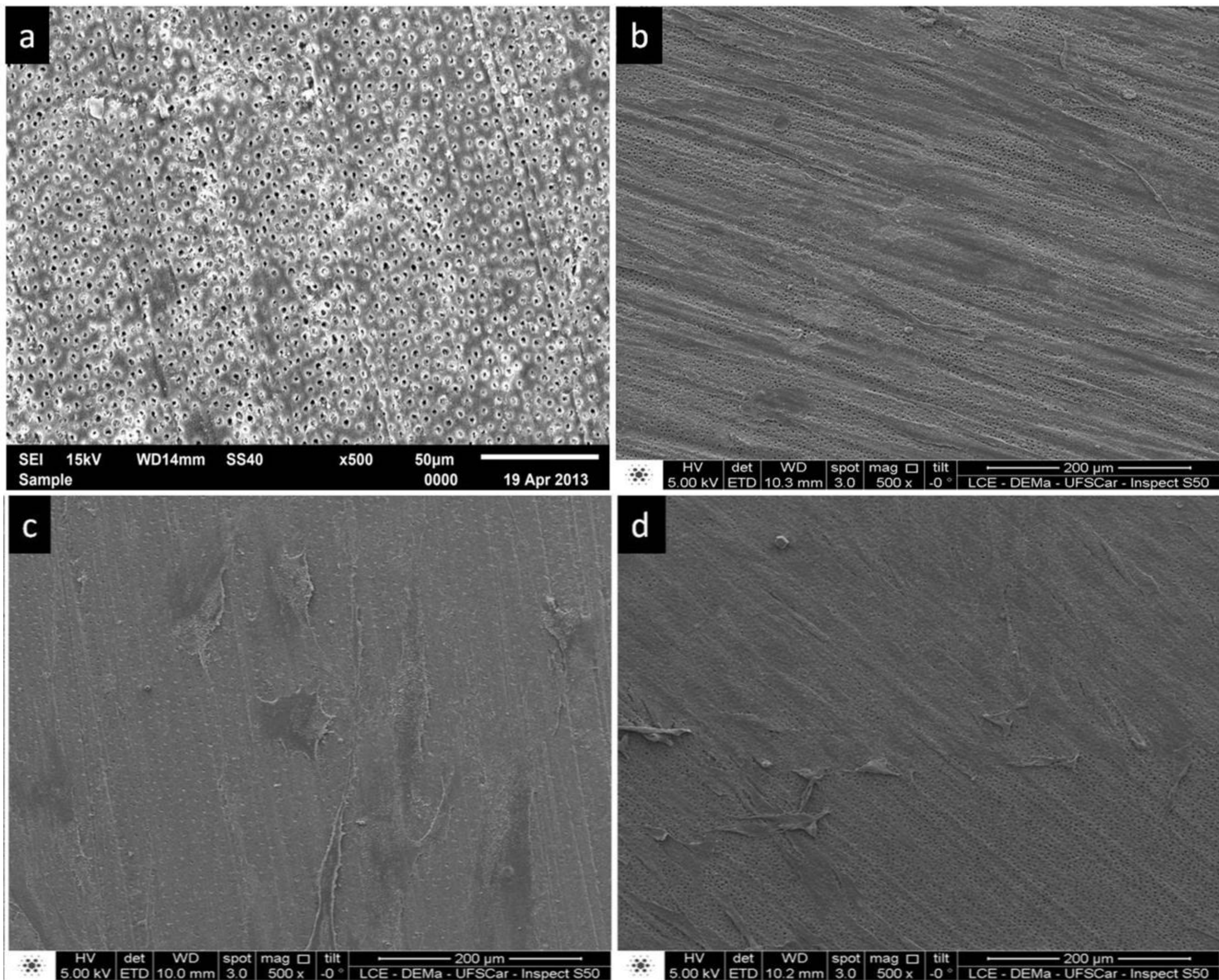


Figure 3: Representative graphs of a - ALP gene expression (%), b - Col I gene expression (%), c - DSPP gene expression (%) and d- DMP-1 gene expression (%) by pulp cells undergoing transdentinal irradiation with infrared LED, comparing the different doses of energy used.



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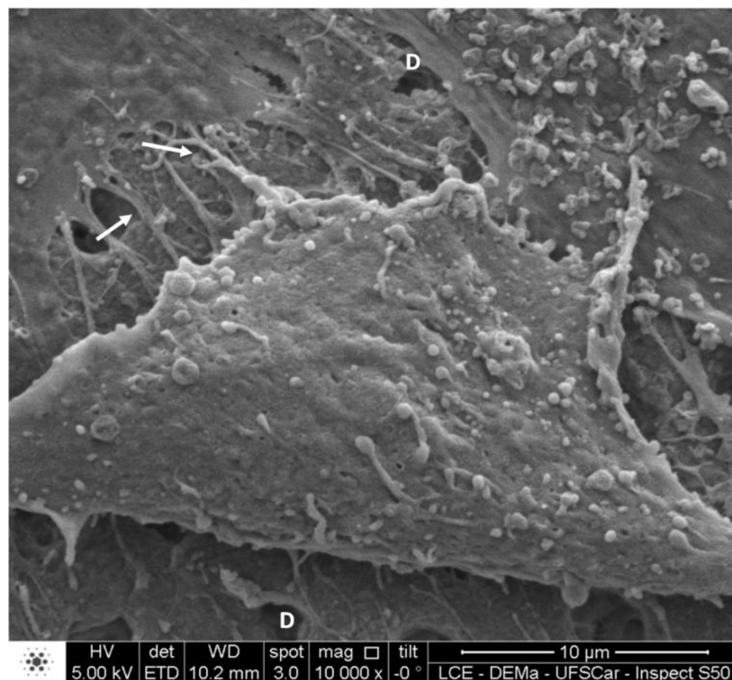

Figure 5: Details of the cell morphology showing a large cytoplasm and numerous cytoplasmic processes (arrows) responsible for attaching the cell to the dentin substrate (D). SEM 10.000x.

4 CONSIDERAÇÕES FINAIS

Encontrar parâmetros ideais da irradiação de baixa intensidade para o estímulo do reparo pulpar na aplicação direta ou transdentinária tem sido alvo de muitos estudos. Diferentes protocolos e parâmetros físicos têm sido testados em estudos in vitro (Oliveira et al.[40], 2011; Holder et al.[20], 2012; Oliveira et al.[41], 2013; Turrioni et al.[49], 2014; Turrioni et al.[50], 2014) e in vivo (Ferreira et al.[14], 2006; Tate et al.[47], 2006).

Dentre as opções de fontes de luz, a utilização de lasers e LEDs tem sido vislumbrada para aplicação clínica em enfermidades como dermatites (Deland at al.[11], 2007; Ablon,[1] 2010; Dogra, Mahajan,[12] 2015) e doenças neuronais (Wong-Riley et al.[55], 2005; Li et al.[31], 2014), bem como na cicatrização de feridas (Romanos et al.[43], 1995; Corazza et al.[9], 2007; Dall Agnol et al.[10], 2009; Chaves et al.[7], 2014). Entretanto, poucos estudos na literatura abordam a aplicação da fototerapia de baixa intensidade para o reparo do tecido pulpar (Carrol et al.[6], 2014).

Os estudos pertencentes a este trabalho procuraram definir parâmetros apropriados para a irradiação LED, objetivando a bioestimulação de células pulpares e a diferenciação odontoblástica in vitro. Devido à falta de parâmetros definidos para a utilização da fototerapia sobre células pulpares, inicialmente buscou-se determinar uma curva dose/tempo resposta baseada na viabilidade celular, número de células viáveis e formação de nódulos mineralizados (Estudo 1). Uma vez definidas as melhores densidades de energia para bioestimulação das células pulpares in vitro, foi avaliada a ação do LED na produção e expressão de proteínas relacionadas à diferenciação odontoblástica de forma direta (Estudo 2) ou na presença da barreira dentinária, ação transdentinária (Estudo 3).

De uma forma geral, os resultados sugerem que a aplicação da dose de energia de 4 J/cm^2, utilizando uma irradiância de 40 mW/cm^2, no comprimento de 850 nm foi o melhor conjunto de parâmetros para estimulação direta das células pulpares. Ao testarmos as diferentes DEs, a irradiação direta com 4 J/cm^2 foi capaz de estimular a viabilidade celular (aumento de 27%) e o número de células viáveis (aumento 22%) após 72 horas da irradiação. Além disso, este parâmetro do LED causou um aumento da produção de proteína total (10%), atividade de ALP (69%), produção de colágeno total (51%), e expressão gênica de todas as proteínas avaliadas (ALP, Col I, DSPP e DMP-1).

Ao investigarmos a ação do LED infravermelho na presença do tecido dentinário com espessura de 0,2 mm, foram encontrados resultados positivos. As células foram bioestimuladas, embora diferindo quanto a intensidade da resposta em função da atividade metabólica avaliada (aumento de 33% na viabilidade celular, 12% da produção de PT, 23% na atividade de ALP e estímulo na expressão de ALP e Col I). Estes dados indicam que, mesmo utilizando um comprimento de onda com alta penetrância em tecidos (Neupane et al.[39], 2012; Turrioni et al.[48], 2013) e uma irradiância de 80 mW/cm^2, o efeito do LED infravermelho pode sofrer interferência na presença da barreira dentinária. Mesmo apresentando efeitos atenuados, a dose de 4 J/cm^2, no comprimento de 850 nm apresentou os melhores resultados para bioestimulação e diferenciação de células-tronco pulpares.

Os resultados positivos encontrados com os três estudos sugerem que a fototerapia com LED infravermelho, na dose de 4 J/cm^2 pode ser um método inovador e clinicamente viável, o qual não apresenta efeito deletério às células-tronco pulpares in vitro e estimula a viabilidade e a capacidade de diferenciação odontoblástica de células-tronco pulpares in vitro. Entretanto, a efetividade desta terapia no reparo do complexo

dentino-pulpar e os possíveis efeitos quando aplicada clinicamente, direta ou indiretamente, sobre o tecido pulpar são questões que necessitam ser abordadas em estudos futuros.

Com o presente estudo, esperou-se encontrar um parâmetro, utilizando a irradiação infravermelha emitida por LED, que seja capaz de estimular o metabolismo, proliferação e principalmente, a diferenciação de células-tronco, por meio do aumento da atividade da fosfatase alcalina (ALP), da formação de nódulos de mineralização, bem como da expressão de proteínas envolvidas na formação e mineralização da matriz dentinária. A partir dos resultados positivos na presente pesquisa, vislumbra-se a aplicabilidade clínica da LEDterapia em casos de tratamento de lesões profundas de cárie, sem ou, especialmente, com exposição pulpar e a possibilidade de indicação de procedimentos mais conservadores do tecido pulpar em dentes senis, sejam eles permanentes ou decíduos. Obviamente, essas teorizações deverão ser futuramente investigadas em estudos in vivo e clínicos.

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*De acordo com o manual da FOAr/UNESP, adaptadas das normas Vancouver. Disponível no site: http://www.foar.unesp.br/#!/biblioteca/manual

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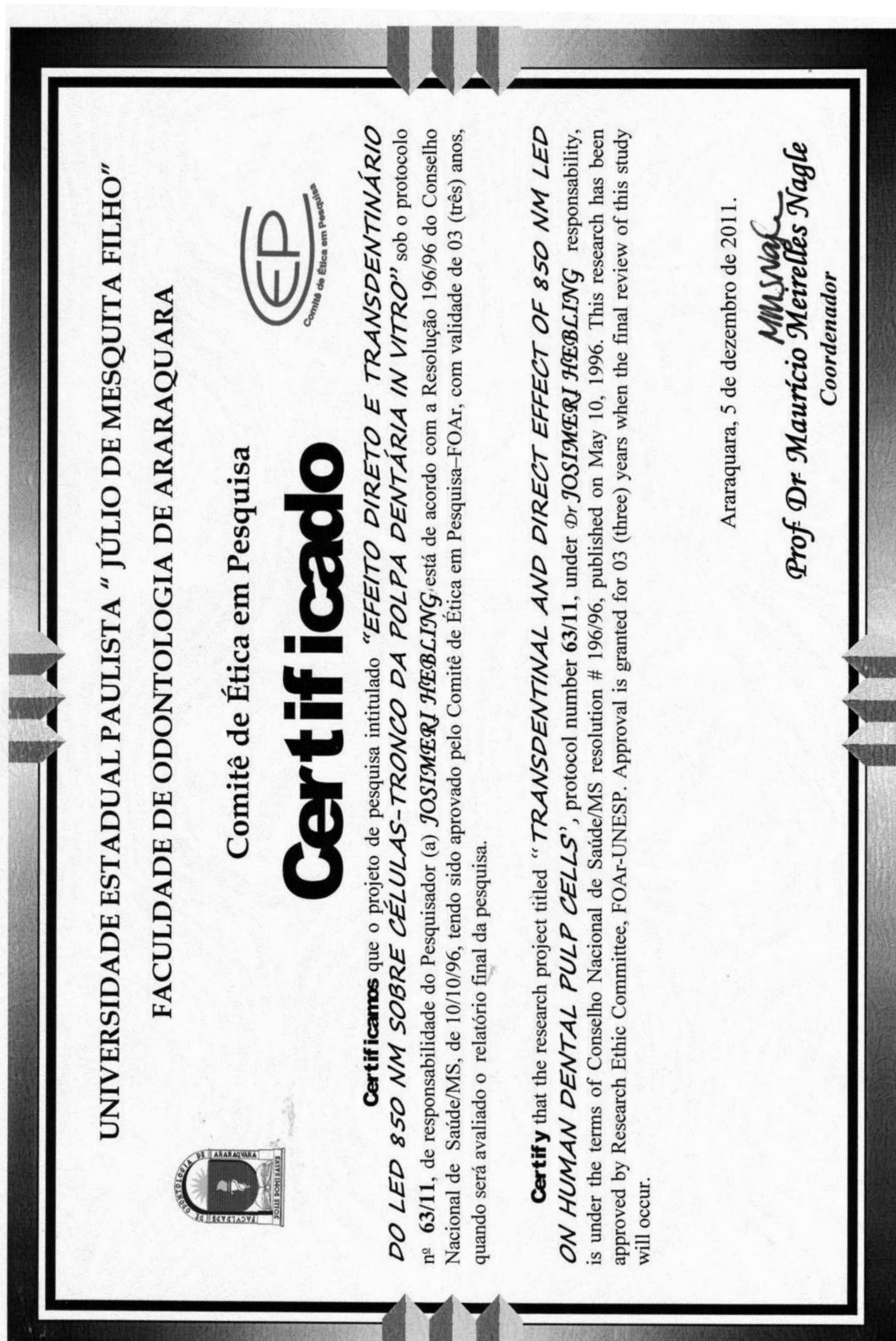
6 ANEXO

Certificado de aprovação do Comitê de Ética em Pesquisa (CEP) da Faculdade de Odontologia de Araraquara.



UNIVERSIDADE ESTADUAL PAULISTA " JÚLIO DE MESQUITA FILHO"

FACULDADE DE ODONTOLOGIA DE ARARAQUARA

Comitê de Ética em Pesquisa

CEP Comitê de Ética em Pesquisa

Certificado

Certificamos que o projeto de pesquisa intitulado *"EFEITO DIRETO E TRANSDENTINÁRIO DO LED 850 NM SOBRE CÉLULAS-TRONCO DA POLPA DENTÁRIA IN VITRO"* sob o protocolo nº **63/11**, de responsabilidade do Pesquisador (a) *JOSIMERI HEBLING*, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa–FOAr, com validade de 03 (três) anos, quando será avaliado o relatório final da pesquisa.

Certify that the research project titled *" TRANSDENTINAL AND DIRECT EFFECT OF 850 NM LED ON HUMAN DENTAL PULP CELLS"*, protocol number **63/11**, under Dr *JOSIMERI HEBLING* responsability, is under the terms of Conselho Nacional de Saúde/MS resolution # 196/96, published on May 10, 1996. This research has been approved by Research Ethic Committee, FOAr-UNESP. Approval is granted for 03 (three) years when the final review of this study will occur.

Araraquara, 5 de dezembro de 2011.

Prof Dr Maurício Meirelles Nagle

Coordenador

Autorizo a reprodução deste trabalho

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Araraquara, 07 de abril de 2015.

ANA PAULA SILVEIRA TURRIONI