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ORIGINAL ARTICLE

Effect of single and multiple doses of low-level laser therapy on viability and proliferation of stem cells from human exfoliated deciduous teeth (SHED)

Luciano Aparecido Almeida-Junior[1] · Nádia Carolina Teixeira Marques[2] · Mariel Tavares de Oliveira Prado[3] · Thais Marchini Oliveira[3] · Vivien Thiemy Sakai[1]

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Abstract

The present study aimed to evaluate in vitro whether the low-level laser (LLL) delivering fractionated total energy (multiple irradiation) or single irradiation stimulates regeneration-associated events (viability and proliferation) in stem cells from human exfoliated deciduous teeth (SHED). Cells received LLL irradiation (InGaAlP–660 nm), according to the following experimental groups: G1 (single irradiation 2.5 J/cm^2, 10 mW, 10 s, 0.10 J), G2 (single irradiation 5.0 J/cm^2, 10 mW, 20 s, 0.20 J), G3 (single irradiation 7.5 J/cm^2, 10 mW, 30 s, 0.30 J), G4 (two irradiations 2.5 J/cm^2, 10 mW, 10 s; total energy 0.20 J), G5 (three irradiations 2.5 J/cm^2, 10 mW, 10 s; total energy 0.30 J), and G6 (non-irradiated). Cell viability was assessed by MTT and trypan blue exclusion (TBE) methods, while cell proliferation was evaluated by crystal violet (CV) and sulforhodamine B (SRB) assays after 24, 48, and 72 h after the first irradiation. By MTT, there was no difference between groups at 24 and 72 h. At 48 h, the groups subjected to multiple irradiation (G4 and G5) presented higher cell viability rates. The average percentages of viable cells for all groups by TBE method were 91.04%, 96.63%, and 97.48% at 24, 48, and 72 h, respectively. By CV, there was no significant difference between groups at 24 and 48 h; at 72 h, G2, G3, and G4 presented higher cell proliferation. By SRB, G1 and G4 presented lower proliferation rates in all the periods. When the groups presenting the same total energy were compared, G2 (0.20 J) presented lower cell viability rates and higher cell proliferation rates in comparison with G4; G3 (0.30 J) presented similar results to those of G5, with higher cell viability and proliferation. The application of laser delivering fractionated total energy (two or three applications of 2.5 J/cm^2) induced higher cell viability at 48 h, while the single irradiation with 2.5 J/cm^2 did not stimulate metabolic activity in such period and the proliferation over time. The 5.0 and 7.5 J/cm^2 single doses and the three applications of 2.5 J/cm^2 maintained cell viability and stimulated proliferation of SHED at 72 h.

Keywords Cell culture techniques · Cell survival · Cell proliferation · Low-level light therapy · Stem cells

Introduction

Photobiomodulation therapy (PBMT) corresponds to the light therapy with the use of a low-level laser (LLL) or light-emitting diodes (LED) that results in the inhibition or stimulation of biological process in the absence of significant heating [1], with beneficial therapeutic outcomes, such as alleviation of pain and inflammation, increased collagen synthesis, promotion of wound healing, and tissue regeneration [2–5]. LLL works mostly through non-thermal photobiological mechanisms eliciting photophysical and photochemical events [6–10]. The most accepted mechanism relies on the modulation of cytochrome c oxidase activity in unhealthy tissues. Cytochorme c oxidase (the terminal enzyme in the mitochondrial respiratory chain) catalyzes the reduction of oxygen for energy metabolism

✉ Vivien Thiemy Sakai
vivienodonto@yahoo.com

[1] Department of Clinics and Surgery, School of Dentistry, Federal University of Alfenas (UNIFAL-MG), Rua Gabriel Monteiro da Silva, 700, Alfenas, MG, Brazil

[2] Department of Pediatric Dentistry, School of Dentistry, José do Rosário Vellano University (UNIFENAS), Alfenas, Minas Gerais, Brazil

[3] Department of Pediatric Dentistry, Orthodontics and Public Health, Bauru School of Dentistry, University of São Paulo (USP), Bauru, São Paulo, Brazil

[6–8]. Another hypothesis concerns light-sensitive ion channels that can be activated allowing calcium to enter the cell [11]. Following laser irradiation, primary and secondary reactions take place. The primary reaction occurs under irradiation (in the presence of light), and results in molecular and functional alterations in the photoreceptors. Secondary reactions occur hours or days after irradiation, altering signal transduction and causing biological responses [3, 9, 12, 13].

Regarding the pulp cells/tissue subjected to PBMT, researches have been conducted to achieve beneficial therapeutic outcomes for pulp repair after conservative procedures (pulpotomy and pulp-capping), resulting in increased tertiary dentin deposition, and increased cell viability and proliferation rates [14, 15], or as an alternative method for root canal treatment, such as pulp tissue engineering (the design and manufacture of a healthy and functionally competent dental pulp tissue using a combination of cells, scaffolds, and morphogenic factors) [16, 17], mainly for young patients who have suffered pulp necrosis in immature permanent incisors as consequence of trauma [18].

Stem cells appear to be particularly susceptible to PBMT [11]. In addition, the use of stem cells might bring advantages for dental pulp tissue engineering. Stem cells from human exfoliated deciduous teeth (SHED) are useful candidates for such purpose due to the high proliferation rate and increased cell population doublings [19, 20], despite the ability to differentiate into odontoblasts and endothelial cells if properly stimulated [16, 21–24]. Although several studies have been conducted to assess the effect of LLL on SHED focusing on the stimulation of their regenerative potential [4, 5, 12, 15, 16, 18, 19, 25–29], very little is known about the use of fractioned irradiation energy (multiple irradiation) instead of a single irradiation of SHED. The health of SHED after irradiation can be determined by quantifying distinct characteristics, such as cell viability and proliferation. Cell viability assays enumerate the ratio of live and dead cells in a population by staining and counting live or dead cells or by measuring cell metabolic activity [30], whereas cell proliferation assays assess dividing cells through the direct measurement of DNA synthesis [31]. Therefore, the purpose of the present study was to evaluate the effect of LLL on the viability and proliferation of SHED subjected to multiple or single irradiation protocols with different energy densities and total energy in order to help define laser parameters that would improve the potential of SHED for dental tissue regeneration.

Material and methods

Cell culture

SHED, kindly provided by Dr. Thais M. Oliveira (DDS, MSc, PhD, Bauru School of Dentistry, University of São Paulo, SP, Brazil), were isolated by a standard enzymatic digestion protocol, and characterized according to Miura et al. [19]. All procedures involving human samples were approved by the Institutional Review Board (protocol no. CAAE 88330218.6.0000.5417). Cells were maintained in Eagle's minimum essential medium alpha modification (MEMα; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco, Invitrogen, Grand Island, NY, USA) and 1% penicillin and streptomycin solution (penicillin–streptomycin, Gibco, Invitrogen) at 37 °C and 5% CO_2, and split at a ratio of 1:3 when they reached 80% confluence. The culture medium was changed every two days. SHED at passages five to seven were used in all experiments.

Cells were seeded in 24-well plates (4×10^4 cells per well) for the trypan blue exclusion (TBE) assay, or in 96-well plates (1×10^4 cells per well) for the 3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl -2H- tetrazolium bromide (MTT), crystal violet (CV), and sulforhodamine B (SRB) assays, with MEMα supplemented with 10% FBS and allowed to attach overnight. The medium in all wells was replaced by fresh culture medium supplemented with 1% FBS (serum starvation) for 1 h [5, 29].

Low-level laser therapy conditions

SHED were subjected to different laser irradiation parameters (groups 1 to 5) or remained non-irradiated (group 6), as described in Table 1. In the multiple irradiated groups (groups 4 and 5), laser was applied every 6 h. Laser irradiation was carried out in the dark, using an indium-gallium-aluminum phosphide (InGaAlP) red laser handpiece (MMOptics, São Carlos, São Paulo, Brazil) at 660-nm wavelength, 10-mW output power, 0.04-cm^2 laser tip area, and 0.225-cm tip diameter. The irradiation was punctual, in continuous mode, in contact with the bottom of the wells and with the laser beam at a 90° angle in relation to bottom of the 96-well plates, which were positioned in a stable supporting structure [5, 25, 29]; for the 24-well plates, five irradiation points were defined in order to cover the total area of the wells.

Cells returned to regular nutritional conditions (10% FBS) immediately after the first irradiation, and subjected to viability and proliferation assays after 24, 48, and 72 h. Three independent experiments were performed.

Cell viability by MTT assay

At the end of the incubation periods (24, 48, and 72 h), the supernatants were discarded, cells were washed with phosphate-buffered saline (PBS), and 110 μl of MTT solution was added to each well, for a final concentration of 0.5 mg ml^{-1}. After an additional 4-h incubation period at room temperature, the supernatant was discarded, and 200-μL dimethyl sulfoxide (DMSO, Fisher Scientific, Hampton, NH, USA) was added to each well to solubilize the formazan

Table 1 Experimental groups divided according to the low-intensity laser irradiation parameters and number of laser applications

Groups	Energy density (J/cm^2)	Output Power (mW)	Time (s)	Number of laser applications	Total time (s)	Total energy (J)
G1	2.5	10	10	1×	10	0.10
G2	5.0	10	20	1×	20	0.20
G3	7.5	10	30	1×	30	0.30
G4	2.5	10	10	2×	20	0.20
G5	2.5	10	10	3×	30	0.30
G6	Non-irradiated	–	–	–	–	–

crystals. Immediately, the absorbance was read in a microplate reader (Zenyth, 200 RT, Anthos) at 570 nm [29, 32]. Data were obtained from three wells per condition.

Cell viability by TBE assay

At the end of the incubation periods, the supernatants were collected in microcentrifuge tubes. Cells were washed with PBS and dissociated with 250 μl of 0.25% trypsin-EDTA exposure for five min. Then, trypsin neutralization was performed with the reserved supernatants. Cells were centrifuged at 100×*g* for five min; supernatants were discarded and the pellet was resuspended in a mixture of 100 μl of MEMα and 100 μl of 0.4% trypan blue. After three min, viable and non-viable cells were counted in hemocytometer. The calculation of percentage of viable cells was performed using the following formula: Viable cells (%) = (total number of viable cells per mL/ total number of cells per mL) x 100 [33]. Data were obtained from two wells per condition.

Cell proliferation by CV assay

At the end of the incubation periods, the culture medium was removed and each well was washed with PBS. Following, 100% methanol was added for 10 min. Methanol was removed and CV solution was added to the wells for 3 min. Then, this solution was discarded and each well was washed with PBS twice. Sodium citrate 0.05 mol L was added for 10 min. Immediately, the absorbance was read in a microplate reader (Zenyth, 200 RT, Anthos) at 540 nm. In this experiment, the volume of each solution was 200 μL [25]. Data were obtained from three wells per condition.

Cell proliferation by SRB assay

At the end of the incubation periods, cells were fixed by the addition of 50 μL of 10% trichloroacetic acid and incubated for 1 h at 4 °C. Plates were washed in tap water five times and allowed to dry. Cellular protein was stained by adding 50 μL of 4% SRB in 1% acetic acid and incubated at room temperature for 30 min. Excess SRB was removed by washing the wells with copious irrigation with 1% acetic acid and remaining SRB was solubilized in 100 μL of 10 mM Tris-base unbuffered. Absorbance was determined on a spectrophotometer at a wavelength of 565 nm [5]. Data were obtained from three wells per condition.

Statistical analysis

All data obtained by absorbance measurements (MTT, CV, and SRB assays) were blank corrected. Data were tested for normality using the Shapiro-Wilk test. For MTT, CV, and TBE results, data were directly analyzed by two-way ANOVA, while for SRB, the analysis of variance was performed using chi-square transformation. All data were submitted to Scott Knott's post hoc test. Statistical analyses were performed using R statistical software (version 3.4.3), considering the level of significance of $P < 0.05$.

Results

Cell viability by MTT e TBE assays

By MTT assay, there were no significant differences in the viability of cells among the groups after 24 h. At 48 h, G4 and G5 had higher absorbance values than G2, G3, and G6 ($P < 0.001$). Cells in G1 were significantly less viable than those in the remaining groups ($P < 0.001$). At 72 h, there were no significant differences among the groups. All the groups presented a significant increase in the mitochondrial metabolic activity over the experimental period ($P < 0.001$), except for G1, in which cell viability was similar between 24 and 48 h ($P > 0.05$), but significantly higher at 72 h ($P < 0.001$, Fig. 1).

By TBE method, the average percentages of viable cells in the groups were 91.04%, 96.63%, and 97.48% at 24, 48, and 72 h, respectively, with significant difference among 24 h and the other periods ($P < 0.001$). No significant difference was found in the viability of SHED among the groups in each experimental period ($P > 0.05$, Table 2).

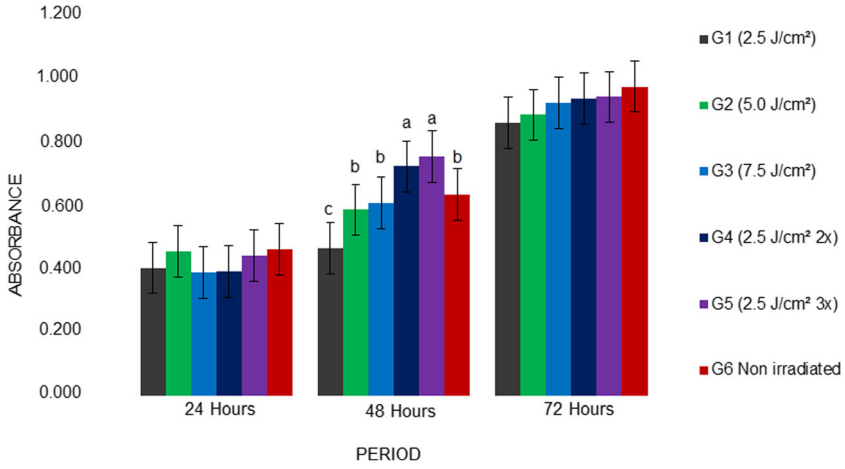

Fig. 1 Viability, by MTT assay, of SHED subjected to different parameters of laser irradiation 24, 48, and 72 h after the first irradiation. Lower case letters indicate statistical differences among groups for the same experimental period ($P < 0.001$).

Cell proliferation by CV e SRB assays

By CV assay, there were no significant differences among groups at 24 and 48 h after irradiation ($P > 0.05$). At 72 h, G1, G4, and G6 had lower proliferation rates in comparison with G2, G3, and G5 ($P < 0.05$). In all groups, a continuous cell growth was observed over the experimental period, with significant differences in the absorbance rates of G1, G4, and G6 at 24 h in comparison with the other periods ($P < 0.001$), of G2 at 72 h in comparison with the other periods ($P < 0.001$) and of G3 and G5 among all the periods of evaluation ($P < 0.001$, Fig. 2).

By SRB, statistical analysis revealed absence of interaction among groups and experimental periods ($P < 0.05$). Taken the data of the three periods together, there was lower cell proliferation in G1 and G4 in comparison with G2, G3, G5, and G6 ($P < 0.05$). Taken the data of all the groups together, a continuous increase in cell proliferation was observed, with significant differences among the periods of evaluation ($P < 0.001$, Fig. 3).

Discussion

Nowadays, LLL therapy has emerged as a tool to accelerate tissue repair and to evoke analgesia and edema regression after dental procedures, providing more comfort to the patients as a consequence [4, 5, 34]. In order to achieve beneficial and predictable therapeutic outcomes with the use of such technology, researches are needed to establish irradiation protocols to the several clinical purposes.

Considering that PBMT depends on the irradiation parameters and that little is known about the effect of laser therapy on SHED [4, 5, 12, 15, 16, 18, 19, 25–29], in the present study, InGaAlP 660-nm visible red laser, operating at 10 mW, was employed, varying the energy density and the number of laser application (G1, 2.5 J/cm^2; G2, 5.0 J/cm^2; and G3 7.5 J/cm^2 in a single irradiation; G4, two irradiations of 2.5 J/cm^2; and G5, three irradiations of 2.5 J/cm^2), which also resulted in distinct total energies (G1, 0.10 J; G2 and G4, 0.20 J; G3 and G5, 0.30 J). The choice of the parameters was based on a previous study with pulp fibroblasts [35], with the main purpose to compare both the effect of similar energy

Table 2 Percentage of viable cells by trypan blue exclusion method, in the periods of 24, 48, and 72 h after the first application of low-level laser with different parameters of irradiation ($P < 0.05$)

	24 h		48 h		72 h	
Groups	Viable (%)	Standard deviation	Viable (%)	Standard deviation	Viable (%)	Standard deviation
G1	86.25	1.77	93.58	0.83	97.22	0.16
G2	89.16	7.47	95.16	2.15	98.55	1.79
G3	92.82	3.41	97.17	0.58	97.16	0.18
G4	91.82	1.90	96.99	0.33	97.77	0.45
G5	90.14	6.77	96.92	0.52	97.19	0.52
G6	91.26	1.89	96.93	0.23	96.71	0.23
Average	91.04[b]		96.63[a]		97.48[a]	

Lower case letters indicate statistical differences between the average percentages of viable cells at 24, 48, and 72 h. The analysis of variance was performed with the use of arcsine transformation to attend the presuppositions of the analysis

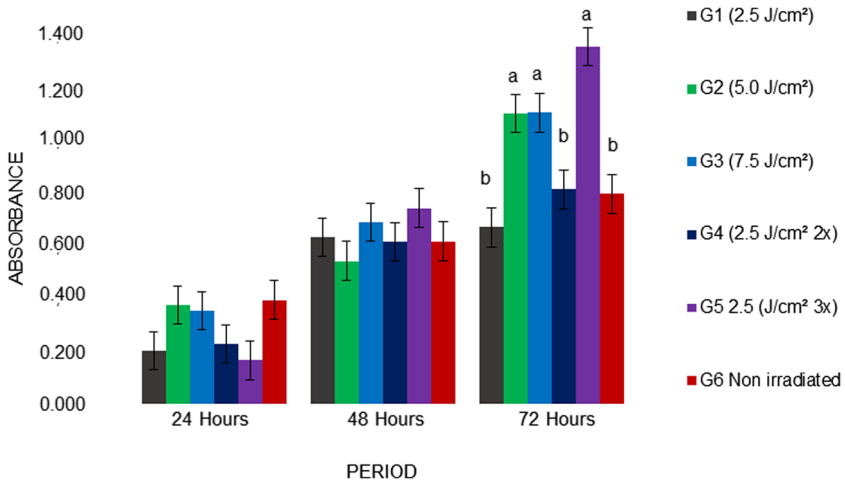

Fig. 2 Proliferation, by CV assay, of SHED subjected to different parameters of laser irradiation 24, 48, and 72 h after the first irradiation. Lower case letters indicate statistical differences among groups for the same experimental period ($P < 0.05$).

densities with different number of applications (comparison among G1, G4, and G5) and the effect of similar total energies, varying the energy density and the number of applications (comparison between G2 and G4 and between G3 and G5) in SHED. The experimental periods were established at 24, 48, and 72 h, allowing evaluating the biological responses resulting from the secondary reactions of LLL on SHED, such as cell viability and proliferation, important in the process of tissue repair and regeneration.

Each of these biological responses was evaluated through the use of two different methods; cell viability was assessed by MTT and TBE assays, while cell proliferation was assessed by CV and SRB assays. Such methods present distinct principles, and the interpretation of their results may bring complementary or confirmatory information. MTT assay is used to determine the rate of metabolic activity in eukaryotic cells; it is dependent of mitochondrial respiration and indirectly serves to evaluate the energy capacity of a cell [35–37]. TBE method is used to determine the number of viable cells in a cell suspension; it is based on the principle that live cells have intact cell membrane that excludes some dyes, such as trypan blue [26]. CV is a non-enzymatic assay, whose dye has affinity by the external surface of the DNA double helix. The amount of dye absorbed depends on the total DNA content in the culture, being directly proportional to cell biomass [5, 25, 35, 37]. Finally, SRB assay relies on the property of the dye, which binds stoichiometrically to proteins under mild acidic conditions and then can be extracted using basic conditions; thus, the amount of bound dye can be used as a representation of the cell biomass, which can then be extrapolated to measure cell proliferation [16, 38]. Since the phototherapy may be related to increase of cell viability and proliferation [39], the performed assays may be considered adequate to the purpose of our study [5, 29, 35].

It is worth mentioning that, in the present study, the interval between the multiple irradiations was established at 6 h, according to previous study [35]. Some authors state that, due to the cumulative laser effect, if the irradiations take place in close intervals, irradiated cells may be damaged; on the other hand, if the intervals are too long, the treatment is likely to be ineffective [10, 40]. Based on the results of cell viability and proliferation, the interval between irradiations seemed to be appropriated, since SHED subjected to multiple irradiations (G4 and G5) presented high metabolic activity at 48 h, and the cells from G5 also presented high proliferation rate at 72 h. The results found in these periods are directly related to the average SHED population doubling time, which occurs at 41.3 h [20].

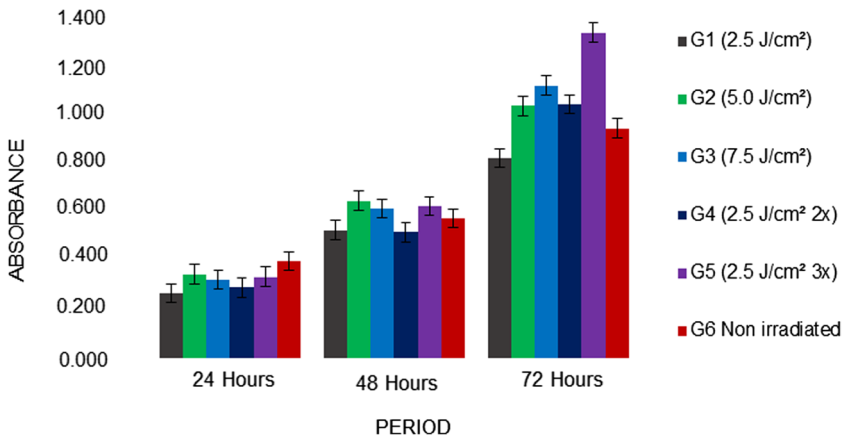

Fig. 3 Proliferation, by SRB assay, of SHED subjected to different parameters of laser irradiation 24, 48, and 72 h after the first irradiation

Mester et al. [41] state that low doses with adequate interval between irradiations are more effective than high doses in a single laser application. For pulp fibroblasts, Meneguzzo et al. [40] found that cell viability was increased in the groups subjected to multiple irradiations than in those subjected to a single irradiation. Fernandes et al. [35] also demonstrated that pulp fibroblasts irradiated with a total energy of 0.30 J, fractionated in three applications of 2.5 J/cm^2, presented higher viability than those irradiated with 7.5 J/cm^2 in a single application. The fact that multiple irradiation (fractioned total energy) is more effective than a single irradiation with the same total energy was also observed in the present study, since the laser delivering a total energy of 0.20 J was more effective to induce SHED metabolic activity when two applications with the energy density of 2.5 J/cm^2 were performed than a single application with 5.0 J/cm^2. Similarly, a total energy of 0.30 J induced higher metabolic activity when three applications of the laser with the energy density of 2.5 J/cm^2 occurred in comparison with a single irradiation with 7.5 J/cm^2.

Regarding cell proliferation evaluated by a CV method, a single irradiation with 5.0 and 7.5 J/cm^2, as well as three applications of 2.5 J/cm^2, induced higher proliferation rates than that observed for the non-irradiated cells and for those subjected to one or two applications of 2.5 J/cm^2 at 72 h. These results corroborate those from Fernandes et al. [35], since the authors concluded that a single application of 5.0 J/cm^2 induced higher proliferation rates of pulp fibroblasts than two irradiations with 2.5 J/cm^2.

It is suggested that the absence of significant difference between cellular responses in G2 and G3 (single irradiation with 5.0 and 7.5 J/cm^2, respectively) in all the experiments may have occurred due to the fact that both energy densities are too close, causing similar biological effects. Although PBMT is dependent on the dose applied, there are different therapeutic windows to the effective photostimulation, in which each cell type has its own ideal irradiation [40, 42–44]. Therefore, in order to detect different cellular responses among groups, it is possibly necessary to irradiate the cells with more discrepant energy densities, as performed by Meneguzzo et al. [40], who assessed the effect of 6.3, 12.6, and 18.9 J/cm^2 energy densities of fibroblasts.

Moreover, the average percentage of SHED viability was 95% over the experimental period, being superior to that observed by Suchanek et al. [20], who isolated and cultivated SHED up to the 19th passage with no stimulation, obtaining an average viability of 90.6% (86.0–94.0%).

Due to the great variability of irradiation parameters in the several studies involving PBMT, it is difficult to compare our results with others [29, 45, 46]. Besides, results from in vitro studies must be interpreted with caution, because they cannot be extrapolated for clinical applications since the interaction of the laser with the target tissue of the organism cannot be reproduced in culture [4, 35, 47]. Therefore, more studies with different irradiation parameters, both in vitro and in vivo, must be conducted to obtain predictable clinical effects after PBMT.

Conclusion

The application of red laser delivering fractionated total energy (two or threee applications of 2.5 J/cm^2) induced higher cell viability at 48 h, while the single irradiation with 2.5 J/cm^2 did not stimulate both the metabolic activity in such period and the proliferation for 72 h. The 5.0 and 7.5 J/cm^2 single doses and the three applications of 2.5 J/cm^2 maintained cell viability and stimulated proliferation of SHED at 72 h.

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Compliance with ethical standards

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

Ethical approval This study was approved by the ethics committee of Bauru School of Dentistry, University of São Paulo, Brazil (protocol no. 88330218.6.0000.5417).

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