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ORIGINAL ARTICLE

Photobiomodulation is associated with a decrease in cell viability and migration in oral squamous cell carcinoma

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Abstract

The treatment of squamous cell carcinoma (SCC) involves surgery, chemotherapy, and/or radiotherapy, which can cause mucositis (inflammation of the oral mucosa that causes considerable pain and can compromise the continuity of oncological treatment). Photobiomodulation (PBM) has been successfully used in the treatment of mucositis, but doubts arise regarding the use of laser for areas in which tumor cells may remain. In this study, the effect of PBM on the viability, mitochondrial activity, proliferation, apoptosis, and migration of cells derived from oral SCC was evaluated. SCC9 cells were irradiated with laser (660 and 780 nm, using 11 dosimetric parameters) and submitted to mitochondrial and caspase 3 activity tests after 1 and 3 days. Based on the results, cell viability (neutral red assay), proliferation (BrdU assay), and migration (scratch-wound assay) were evaluated using only the dosimetric parameters recommended for mucositis. Non-irradiated cells served as the control. The experiments were performed in triplicate. The 11 parameters diminished mitochondrial activity and induced tumor cell apoptosis. Using the parameters recommended for mucositis, irradiation with 780 nm (70 mW, 4 J/cm^2) proved to be the safest and led to a reduction in cell viability, the induction of apoptosis, and a reduction in the migration capacity of the tumor cells.

Keywords Oral squamous carcinoma cells · Photobiomodulation · Low-level laser therapy · Mucositis · Mouth neoplasms

Introduction

Squamous cell carcinoma (SCC) accounts for 95% of all tumors of the oral cavity [1, 2] and is the fifth most common type of tumor worldwide [3]. Treatment normally involves surgery, radiotherapy, and/or chemotherapy, which can lead to the development of oral mucositis [2, 4–10]. This condition is characterized by ulcerations of the mucosa that cause

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considerable pain [2, 8, 11], dysphagia, dehydration, and weight loss, which can compromise the continuity of oncological treatment and affect the prognosis [8, 12]. Treatment for oral mucositis includes cryoprotectants, anti-inflammatory agents, antibiotics, lubricants, antimicrobial agents, vitamin E, and photobiomodulation (PBM) [6, 7, 9, 10].

PBM has analgesic, anti-inflammatory, and healing effects [2, 9, 10, 13]. However, some researchers state that PBM should be avoided or used with considerable caution in patients with head and neck cancer due to the possibility of direct and indirect effects on tumor cells that may have remained in the site of the primary tumor after treatment [2, 8, 11, 14–17]. Indeed, PBM may be capable of enhancing cell proliferation, altering the cell cycle and causing apoptosis in different cell lines [8, 11, 18–20]. These effects vary depending on the dosimetric parameters and type of cell evaluated [11, 21, 22].

To contribute knowledge with regard to the influence of this treatment modality on cells derived from oral SCC, the aim of the present study was to evaluate the effects of PBM on cell viability, mitochondrial activity, proliferation, apoptosis, and migration using the dosimetric parameters recommended for the treatment of mucositis.

Materials and methods

Cell culture

SCC9 cell line (ATCC) was cultivated in DMEM/F-12 medium (Sigma-Aldrich Andover, Hampshire, UK) supplemented with fetal bovine serum 10% (Gibco, Paisley, UK), 400 ng/ml of hydrocortisone (Sigma-Aldrich, Andover, Hampshire, UK), 100 IU/ml of penicillin (Gibco), 2.5 μg/ml of streptomycin (Gibco), and 2.5 μg/ml of amphotericin B (Gibco). The cells were cultivated at 37 °C in a 5% CO_2 atmosphere at 70 to 80% confluence. The cell layer was enzymatically released (0.05% trypsin in 0.25% EDTA solution), and the cell suspension was used to perform the experiments.

Laser irradiation of SCC9 cell line

Viable cells were counted in a Neubauer hemocytometer chamber using a 1:1 proportion of Trypan Blue. The cell suspension was centrifuged at 471.8 G for 2 min at 10 °C. Laser irradiation was performed using the Twin-Laser system (Twin-Laser, MMOptics Ltd., São Carlos, São Paulo, Brazil) directly on the pellet formed at the bottom of the centrifugation tube (TPP, Trasadingen, Schaffhausen, Switzerland) without passing through the culture medium [23]. Centrifugation grouped the cells into a smaller area, enabling the light beam to reach a larger number of cells in a more homogeneous way, as described previously [23–25]. Moreover, the light source could be positioned very close to the target cells, passing only through the thin barrier of the polypropylene tube.

The 11 combinations of dosimetric parameters employed in the present study were among those indicated in recent systematic reviews [7, 10] and also included dosimetric parameters used in previous studies with SCC-derived cells lines [2, 16, 23]. Table 1 shows the dosimetric parameters and the effective average radiant power for each combination of parameters studied taking into account the energy loss during passage through the polypropylene of the Falcon tubes, as described by Silva et al. [26]. With NIR light, the energy intensity that hits the cells is always a little greater. NIR light

Table 1 Parameters used of irradiation: wavelength, output power, effective power, irradiated areas, effective power density, total effective energy density, and irradiation time

Operating mode	Continuous wave										
Beam spot size [cm^2]	0.14										
Aperture diameter [cm]	0.43										
Area irradiated [cm^2]	0.19										
Number of treatment sessions	1 session										
Parameter	*Red laser*					*Infrared laser*					
Center wavelength [nm]	*660*					*780*					
Average radiant power [mW]	*30*		*40*			*30*		*40*			*70*
Effective average radiant power [mW]	22.5		30			23.1		30.8			53.9
Radiant exposure considering target area [J/cm^2]	*1*	*2*	*2*	*4*	*6*	*1*	*2*	*2*	*4*	*6*	*4*
Effective irradiance at aperture [mW/cm^2]	160.7	160.7	214.3	214.3	214.3	165.0	165.0	220.0	220.0	220.0	385.00
Radiant exposure at aperture [J/cm^2]	1.4	2.7	2.7	5.4	8.1	1.4	2.7	2.7	5.4	8.1	5.4
Exposure duration [s]	8.4	16.9	12.7	25.3	38.0	8.2	16.5	12.3	24.7	37.0	14.1
Effective radiant energy [J]	0.19	0.38	0.38	0.76	1.14	0.19	0.38	0.38	0.76	1.14	0.76
Total effective radiant energy [J]	0.19	0.38	0.38	0.76	1.14	0.19	0.38	0.38	0.76	1.14	0.76

normally has greater penetration than the red right because absorption and scattering tend to be smaller at longer wavelengths. The experiments were performed in minimal ambient lighting to avoid the influence of external light. After irradiation, the pellets of the experimental groups were re-suspended and the cells were cultivated and plated for the experiments. The cells in the control group underwent the same procedures but did not receive laser irradiation.

Mitochondrial activity (MTT assay)

Cells were seeded in 96-well microplates at a concentration of 10^4 cells per well (in quadruplicate). After the incubation periods (1 and 3 days), the cells were further incubated for 3 h at 37 °C in the presence of 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The culture medium was removed, and the formazan crystals were solubilized in DMSO. Absorbance of the samples was evaluated at 550 nm in an ELISA microplate reader (Synergy HT, Biotek). The results were expressed as the percentage of the values obtained in the control (non-irradiated SCC9 cells). Three independent experiments were performed.

Apoptosis (caspase 3 activity)

Cells were seeded in 96-well microplates (10^4 cells per well). After 3 days, apoptosis was evaluated using the EnzChek® Caspase-3 Assay kit no. 2 (Molecular Probes, Eugene, OR, USA) following the manufacturer's instructions. Briefly, the cells were washed with PBS and lysed for 30 min. The working solution was added to the wells (including the control). The microplates were incubated for 30 min in the dark at room temperature. Fluorescence was measured at 485 and 528 nm (excitation and emission, respectively) using a microplate reader (Synergy HT, Biotek). The fluorescence data corresponded to caspase 3 activity and were expressed as mean and standard deviation (SD) values. Three experiments were performed in triplicate.

Choice of dosimetric parameters

For the assays described in the "Cell viability (neutral red)," "Cell proliferation (incorporation of BrdU)," and "Migration (scratch-wound assay)" sections, only the parameters that used an energy density of 4 J/cm^2 were chosen, since recent reviews on treatment for mucositis indicate that the parameters that lead to the best therapeutic results and are believed to be the safest are those that involve red (633 to 685 nm) or infrared (780 to 830 nm) laser with an output power of 10 to 150 mW and energy density between 2 and 6 J/cm^2 [7, 10].

Cell viability (neutral red)

Irradiated and non-irradiated (control) cells were seeded in 96-well microplates at a concentration of 10^4 cells per well. After periods of 1 and 3 days, each well received 200 μL of neutral red solution (40 μg/ml). After incubation for 3 h at 37 °C, the solution was discarded and $CaCl_2$ in formaldehyde was added to each well, followed by further incubation for 2 min at room temperature. A 1% acetic acid solution was then added to each well. The microplate was shaken for 5 min until complete homogenization of the dye. Absorbance was measured at 540 nm in a microplate reader (SpectraMax i3 Imaging Cytometer, Molecular Devices, California, USA). Three experiments were performed in quadruplicate.

Cell proliferation (incorporation of BrdU)

Irradiated and non-irradiated (control) cells were plated on eight-well slides at a concentration of 2.12×10^4 cells/well (to reproduce the same cell density (10^4) used in the 96-well microplate assays) for 1 and 3 days. After these periods, the slides were washed with PBS. BrdU (dilution: 1:100) was added to the medium for 2 h. The cells were then washed and fixed in 70% ethanol for 15 min at 4 °C. The incorporation of BrdU in cells in proliferation was identified using immunocytochemistry with the BrdU Staining kit (Invitrogen, Camarillo, CA, USA) following the manufacturer's instructions. The BrdU incorporation index (expressed as the percentage of BrdU-positive cells) was determined by counting 1000 SCC9 cells with the ImageJ software program (National Institutes of Health, Bethesda, Maryland, USA). Two experiments were performed in duplicate.

Migration (scratch-wound assay)

Irradiated and non-irradiated (control) cells were seeded in six-well plates at a concentration of 2×10^5 cells per well (in duplicate). After 3 days, when the cells had reached confluence, the medium was aspirated and a scratch wound was made in the shape of a cross with the aid of a 10-μL pipette. The cells were washed with PBS and fresh medium was added. Photographs were taken with a camera (Axio Cam HRc-Zeiss, Carl Zeiss, Oberköchen, Germany) until the wound was completely closed. The area was measured using the NIS-Elements D 3.1 software (Nikon, Tokyo, Japan) in a phase-contrast-inverted microscope (Zeiss® Axiovert 25, Carl Zeiss, Oberköchen, Germany). Photographs were taken every 12 h for the first 48 h and then at 24-h intervals until the wound in all groups had closed completely. The area measured in the irradiated groups was compared to the control, and the results were expressed as mean and SD of two independent experiments performed in triplicate.

Statistical analysis

The data was evaluated based on mean and SD values. Analysis of variance (ANOVA) was employed for the comparisons using the GraphPad InStat 3 software program. Statistical significance was determined using Tukey's test, with the level of significance set at 5% ($p \leq 0.05$).

Results

PBM diminished mitochondrial enzymatic activity of SCC9 cells

SCC9 cells irradiated with 660- or 780-nm laser demonstrated a significant decrease in mitochondrial activity with all dosimetric parameters 1 and 3 days after irradiation compared to the control cells (Fig. 1a, b). The exception was the cells irradiated with 660 nm, 30 mW, and 2 J/cm^2 1 day after laser therapy, in which the reduction in mitochondrial activity did not differ significantly from the control.

PBM-activated apoptosis mechanism in SCC9 cells

Caspase 3 activity was significantly increased in the cells irradiated with 660 or 780 nm laser with all parameters evaluated (Fig. 2a, b). The cells irradiated with either wavelength using 30 mW and 2 J/cm^2 exhibited the greatest caspase 3 activity ($p < 0.001$).

PBM diminished viability and cell migration of SCC9 cells but does not interfere with cell proliferation

SCC9 cells demonstrated a significant decrease in cell viability 3 days after irradiation with 660 nm (40 mW; 4 J/cm^2) and 780 nm (40 and 70 mW; 4 J/cm^2). In the first day of culture, only cells irradiated with 660 nm laser (40 mW) demonstrated a decrease in cell viability compared to control cells (Fig. 3a).

In the first 36 h of culture, no significant difference in wound closure was found between the cells irradiated with the different PBM parameters and the control cells. In the non-irradiated group (control), wound closure began after 48 h and complete closure occurred after 192 h of cultivation. Cells irradiated with 660 nm laser demonstrated greater wound closure capacity, with complete closure after 120 h. In the cells irradiated with 780 nm laser, the onset of wound closure occurred 72 h after irradiation; cells irradiated with 40 mW and 4 J/cm^2 required a longer time to close (218 h) in comparison to the control. This increase in time was even greater when the power was 70 mW (242 h) ($p < 0.001$) (Fig. 3b).

No differences in the number of BrDU-positive cells were found following the irradiation of SCC9 cells with 660 or 780 nm when compared to control cells 1 and 3 days after irradiation (Fig. 3c).

Discussion

In the present study, a reduction in mitochondrial activity was found in the SCC9 cells irradiated with laser using 11 different dosimetric parameters. However, unlike the other parameters employed, 660 nm laser with 30 mW and 2 J/cm^2 did not lead to a statistically significant reduction in comparison to non-irradiated (control) cells on the MTT test 1 day after irradiation.

Sperandio et al. (2013) [2] also evaluated the viability (MTS assay) of the SCC9 cell line following irradiation with combination of dosimetric parameters for 660 and 780 nm lasers with 40 mW and radiant exposure (fluence) of 2, 3, and 6 J/cm^2. The authors report that the cells irradiated with 780 nm laser using fluences of 2 and 3 J/cm^2 demonstrated an increase in viability after 1 and 2 days in comparison to the control. In the present study, SCC9 cells irradiated with these same parameters demonstrated a reduction in viability compared to the control. This divergence may be associated with

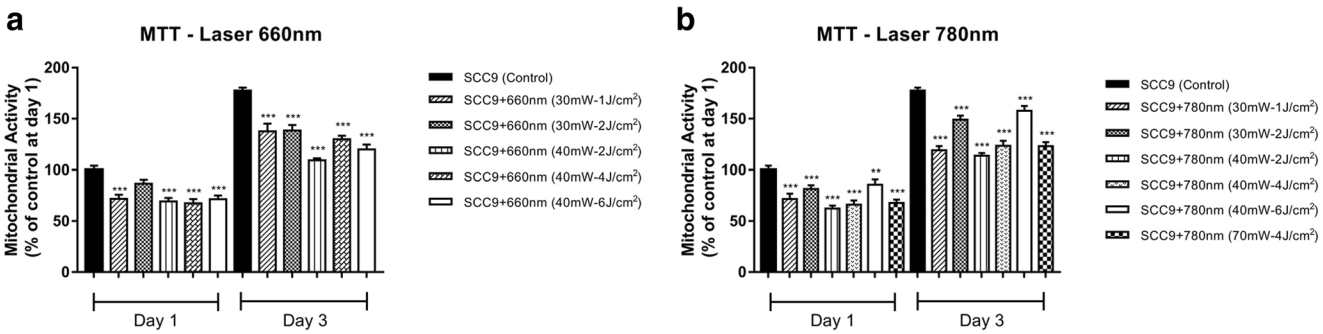

Fig. 1 MTT assay 1 and 3 days after PBM with 660 nm (**a**) and 780 nm (**b**). Results are expressed as mean ± SD of three independent experiments in quadruplicate. ***Significant difference in relation to control ($p < 0.001$)

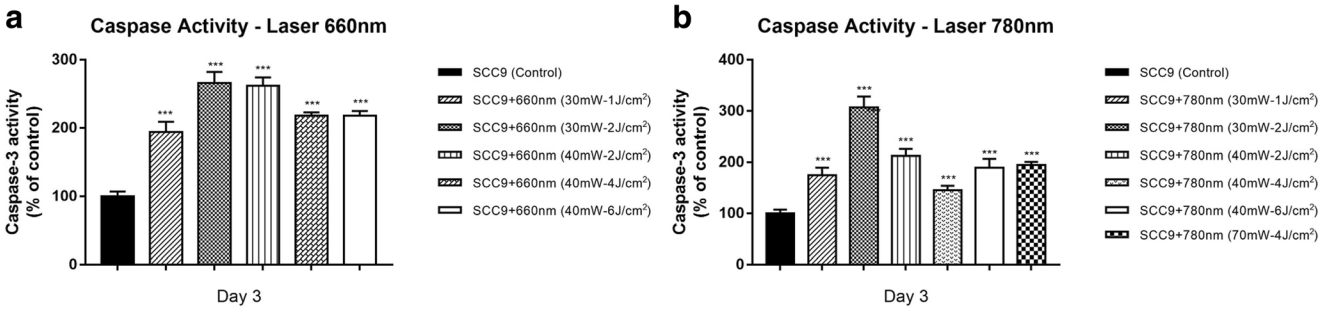

Fig. 2 Caspase activity after 3 days of PBM with 660 nm (**a**) and 780 nm (**b**). Results are expressed as mean ± SD of three independent experiments in triplicate. Significant difference in relation to control. *$p < 0.05$; *$p < 0.01$; ***$p < 0.001$

differences in the irradiation method employed and incubation time.

In another cell line derived from oral squamous cell carcinoma (SCC25), the same authors found an increase in cell viability 2 days after irradiation with 660 nm (2, 3 and 6 J/cm^2) and 780 nm (2 and 3 J/cm^2) [2]. Moreover, Gomes Henrique et al. [15] demonstrated that SCC25 cells irradiated in microplates (immediately and 24 h after plating) at a wavelength of 660 nm, power of 30 mW, and dose of 1 J/cm^2 demonstrated greater proliferation in comparison to non-irradiated cells after 24, 48, and 72 h. Schartinger et al. [8] found a reduction in the viability (MTT assay) of SCC25 cells using 660 nm laser with power of 350 mW for 15 min in three sessions with 24-h intervals [no information on energy density was given], but these parameters are not recommended for the treatment of oral mucositis [7, 10].

With regard to cell death, Sperandio et al. [2] found similar apoptosis rates between irradiated SCC9 cells (660 nm with 2.05 J/cm^2 or 780 nm with 6.15 J/cm^2) and control cells using the TUNEL assay. In the present study, all parameters tested were capable of increasing caspase 3 activity, which is directly associated with the activation of pro-apoptotic genes. This is a reliable test for the evaluation of apoptosis that has advantages over the TUNEL assay, which fails to discriminate among apoptosis, necrosis, and autolytic cell death [27, 28]. The difference regarding the effect of laser irradiation on SCC9 cells

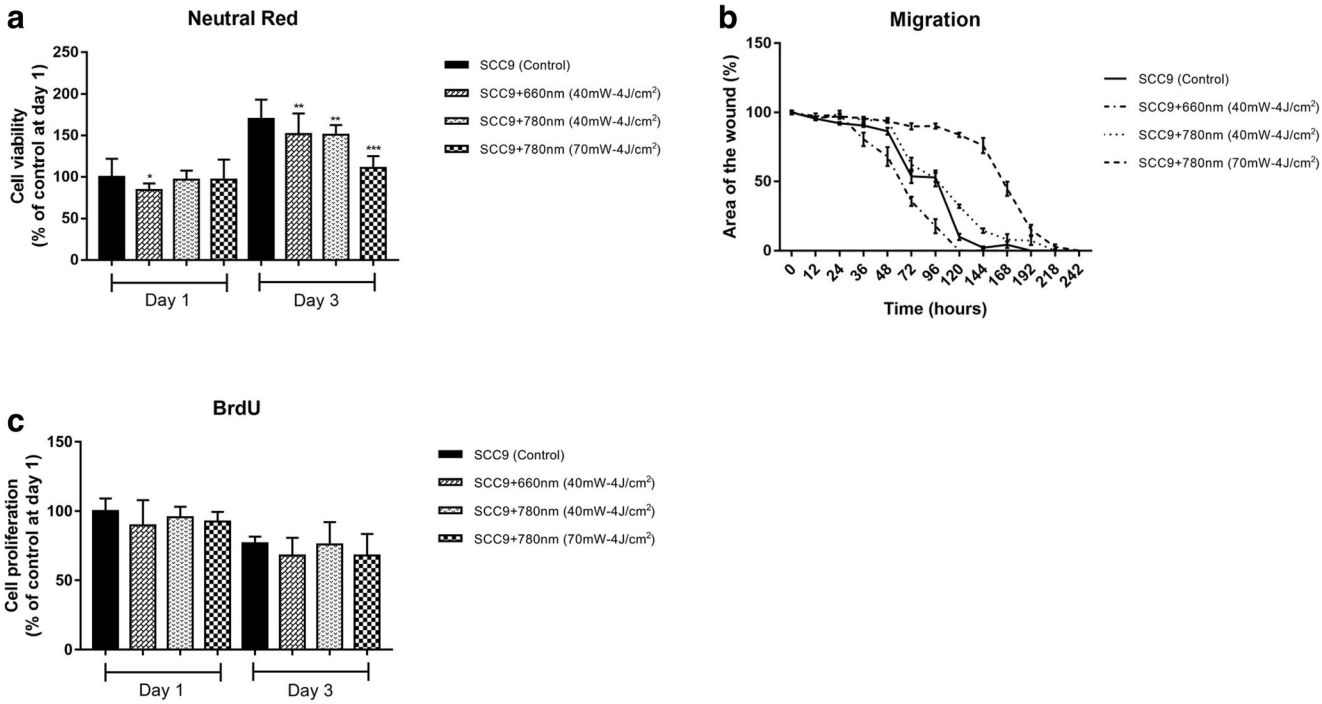

Fig. 3 Neutral red assay after 1 and 3 days of PBM treatment (**a**). Migration of SCC9 cell lines after 240 h of culture (**b**). Results are expressed as mean of percentage remaining of original wound area in each group in different evaluation periods. Percentage of BrdU positive cells after 1 and 3 days of PBM (**c**). Results are expressed as mean ± SD and significant difference in relation to control. *$p < 0.05$; *$p < 0.01$; ***$p < 0.001$

could be explained by the different dosimetric parameters employed in the present investigation as well as the different methods used to evaluate apoptosis.

As experiments that evaluate cell death may demonstrate distinct results, viability tests can also lead to results that are not in agreement. These differences may be related to the type of evaluation used in each test [29], evaluation time, and the intensity/concentration and nature of the agent to be studied [29–31]. In relation to the type of evaluation, the MTT/MTS tests evaluate the enzymatic activity of intracellular dehydrogenases that convert (possibly by the transfer of electrons) water-soluble tetrazolium salt into insoluble purple formazan in the mitochondria [32, 33]. Regarding the nature of the agent to be tested, it is important to consider that the mechanism of action of photobiomodulation is linked to the absorption of light by chromophores present mainly in mitochondria [34]. After photon absorption, a brief burst of reactive oxygen species and increases in the production of ATP and nitric oxide occur and only then is there the activation of different transcription factors that regulate cell viability and proliferation mechanisms [34]. It is also important to consider that light absorption may modify some NADH-linked dehydrogenase reactions of the mitochondria and that tetrazolium salts can be reduced by the presence of superoxides. Therefore, tests such as MTT/MTS may exhibit overestimated values when evaluating the effects of PBM [35, 36]. Thus, it is essential to use more than one test to evaluate cell viability and proliferation [37]. The neutral red test evaluates the uptake of this dye by functional lysosomes [38] and is considered more sensitive than MTT [31].

With the selection of dosimetric parameters that were associated with a reduction in mitochondrial activity, an increase in caspase 3 activity and a dose ranging from 2 to 6 J/cm^2, which have demonstrated better therapeutic effects in the treatment of mucositis [7, 10], three combinations were selected (1–660 nm with 40 mW and 4 J/cm^2; 2–780 nm with 40 mW and 4 J/cm^2; 3–780 nm with 70 mW and 4 J/cm^2), all of which leading to a reduction in cellular viability (neutral red test) but not in the proliferation rate of SCC9 cells.

As oral SCC is an aggressive, invasive tumor commonly involving subjacent mandibular and maxillary bones as well as regional lymph nodes [39–45], which are determinant factors of the prognosis [46, 47], it is important to evaluate the effect of irradiation on the invasion capacity of tumor cells. In the present study, cells irradiated with 780 nm laser (40 and 70 mW with 4 J/cm^2) demonstrated lower migration capacity than the non-irradiated control cells, with the least invasiveness found when the cells were irradiated with 70 mW.

To the best of our knowledge, no previous study has evaluated the effect of PBM on the invasion capacity of SCC9 cells. However, Dias Schalch et al. [23] evaluated the effect of PBM using diverse dosimetric parameters on the induction of TRAP activity (necessary enzyme for bone resorption) as well as the expression of PTHrP and IL-11 (cytokines related to invasiveness used to indicate the prognosis of a tumor) on SCC9 cells. Irradiation with 780 nm laser (70 mW and 4 J/cm^2) also demonstrated greater capacity for diminishing the osteoclastogenic potential of the SCC9 cell line, which would indirectly reduce the capacity for migration and invasion. In contrast, Gomes Henriques et al. found an increase in the invasion capacity of SCC25 cells irradiated with 660 nm laser (30 mW and 1 J/cm^2) in a transwell assay after 72 h of culture, but this dosimetric parameter is not within the range indicated for the treatment of oral mucositis.

As any other therapeutic or pharmacological agent, the effects of PBM depend on dosimetry as well as the type of cell and tissue evaluated [11, 21, 22]. The direct relationship between dosimetry and effect is a consensus in the literature [21, 22]. It is also important to remember that the absorption of light by different cellular chromophores guides the effects of its application. Cytochrome-c-oxidase is indicated as the main cellular chromophore absorbing light at the red and NIR wavelengths, but it has been shown that other molecules can absorb light of different wavelengths, which could lead to changes in the effects. For example, flavins and flavoproteins absorb only in green and blue light; porphyrins absorb yellow and red light and opsins can absorb UV, green, and red light [48–51]. Thus, there is a need to evaluate dosimetric parameters used in the treatment of oral mucositis in a deeper, more translational way [7, 9, 10, 52], especially when used on patients with a history of head and neck cancer [2, 8, 11, 14–17].

Considering the limitations of the present study (in vitro model, use of a single cell line and use of a single irradiation, with no normal cells tested), it is fundamental to continue evaluating the effects of PBM on different types of tumor cells as well as the effects of irradiation with the parameters indicated for the treatment of oral mucositis [11]. However, taken together, photobiomodulation with 780 nm and the parameters recommended for mucositis (70 and 40 mW with 4 J/cm^2) demonstrated negative effects on cell viability and migration as well as a positive effect on caspase 3 activity, suggesting that this therapy may contribute to inducing cell death and reducing cell migration in the SCC9 cell line.

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Compliance with ethical standards

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

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