Effect of nutritional stress and serum starvation on the optical absorbance of normal and malignant epithelial cell lines

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August 29, 2024

Abstract

Photobiomodulation has the potential to modulate cellular responses in various pathological conditions by affecting different signaling pathways. This study aimed to investigate the optical absorbance spectra of normal, dysplastic, and malignant epithelial cell lines under normal and nutritional stress conditions. HaCAT (keratinocyte), DOK (oral dysplastic), and oral squamous cell carcinoma (OSCC) cell lines (CA1, Luc4, SCC9) were evaluated regarding their optical absorbance after culture with 0-10% fetal bovine serum. Absorbance measurements indicated that HaCAT under serum starvation exhibited higher absorbance at blue (430nm) and near-infrared (906nm) wavelengths. DOK showed absorption at 440 nm and 945 nm. OSCC cells showed absorption peaks at blue (400-428nm) and near-infrared. These findings highlight the importance of tailoring PBM parameters to individual needs to achieve optimal absorption and effectiveness. Moreover, the higher absorption peaks in the blue region support further studies to elucidate the potential use of blue light in oral dysplastic lesions and OSCC.

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Abstract

Photobiomodulation has the potential to modulate cellular responses in various pathological conditions by affecting different signaling pathways. This study aimed to investigate the optical absorbance spectra of normal, dysplastic, and malignant epithelial cell lines under normal and nutritional stress conditions. HaCAT (keratinocyte), DOK (oral dysplastic), and oral squamous cell carcinoma (OSCC) cell lines (CA1, Luc4, SCC9) were evaluated regarding their optical absorbance after culture with 0-10% fetal bovine serum. Absorbance measurements indicated that HaCAT under serum starvation exhibited higher absorbance at blue (430nm) and near-infrared (906nm) wavelengths. DOK showed absorption at 440 nm and 945 nm. OSCC cells showed absorption peaks at blue (400-428nm) and near-infrared. These findings highlight the importance of tailoring PBM parameters to individual needs to achieve optimal absorption and effectiveness. Moreover, the higher absorption peaks in the blue region support further studies to elucidate the potential use of blue light in oral dysplastic lesions and OSCC.

Key-words: Photobiomodulation, oral squamous cell carcinoma, oral dysplastic cells, optical absorbance.

INTRODUCTION

Photobiomodulation (PBM) is capable to modulate the behavior and cellular response in different pathological conditions, acting through different signaling pathways and target transcription factors that ultimately activate effector cellular molecules [1]. Because of the absorption of light by cellular and/or tissue chromophores, distinct biological processes are triggered promoting cellular proliferation, differentiation, tissue repair, modulation of inflammation and analgesia [2]. This therapy uses light sources (lasers and light-emitting diodes) mainly in red (600 to 700 nm) and near-infrared (770 to 1200 nm) wavelengths [3]–[5].

Both, the absorption and scattering of light in tissue are wavelength dependent and the effectiveness of PBM on the target tissue is not only dependent on the parameters such as light source, wavelength, energy density, light pulse, and exposition time, but also on the cell type, oxygen availability and metabolic state [6]. After entering the cell, photons are readily absorbed by cellular chromophores located in the mitochondria or in the cell membrane and its energy is converted inside the cell in ATP, which enhances cellular metabolism and proliferation. In this context, it is important to consider the levels of ATP and the metabolic state in PBM, especially when performed in hypoxic, starving or otherwise stressed cells [7].

In vitro studies have demonstrated that under nutritional stress or serum starvation, normal cells are more responsible to PBM. Tagliani et al., [8] showed that PBM contributes to the maintenance of cellular membrane integrity of mesenchymal stem cells from human exfoliated deciduous (SHED) and increases cellular viability and proliferation under cultured with 1% FBS. Similar results were also observed with SHEDS and human fibroblasts cultivated with 5% FBS after PBM, with comparable results to cells cultured with 10% or 15% FBS [9]. However, the underlying mechanisms by which PBM achieve these effects in cells under nutritional stress are largely unknown. It is possible that cellular and molecular changes that occur in this condition will alter the absorption of light, leading to an increase in the biological effects of PBM. In fact, inhibitory or stimulatory effects on the function of photoreceptors after PBM, which integrate the respiratory chain, depend upon external factors such laser parameters and amount of available energy [7].

In cancer, the effects of PBM on cell proliferation and differentiation have been investigated *in vitro* using malignant cell lines, with controversial results across different cell lines and PBM parameters [10]–[14]. Some studies indicate that PBM can promote malignant behavior due to its positive effect in stimulatory cell signaling pathways that increase cell proliferation [15]. On the other hand, we and others have demonstrated that the parameters of PBM as wavelength range, fluence, time of irradiation, and multiple exposures had no effect or showed negative dose-response relationships [16], [17]. However, there is no information in the

literature regarding the absorption spectrum of malignant cell lines, which could influence the choice of the best wavelength to achieve the desire effect in cancer cells. In addition, it is important to consider the Warburg effect in cancer cells, in which the mitochondria metabolism is switched to carry out aerobic glycolysis and thus, the effects of PBM in normal and malignant cells will be different and will require different PBM parameters [18].

Thus, the present study aimed to measure the optical absorbance spectrum of a keratinocyte cell line, oral dysplastic cell line and oral squamous cell carcinoma cell lines (OSCC) cultured with normal and nutritional stress conditions.

MATERIAL AND METHODS

Cell culture

HaCaT cells spontaneously immortalized human keratinocyte line [19] were cultured in regular Dulbecco's Modified Eagle's Medium (DMEM) (ThermoFisher) supplemented with 10% heat-inactivated fetal bovine serum (FBS), glutamine (2 mM), 1% penicillin/streptomicin (Thermofisher).

The dysplastic oral keratinocyte cell line DOK (ATCC) was grown in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 5ug/mL hydrocortisone (Sigma-Aldrivh).

The OSCC cell lines SCC9 (ATCC), CA1 and Luc4 (kindly given by Prof. Ian Mackenzie, Barts, and the London School of Medicine and Dentistry, UK) were grown in DMEM/F12 medium (ThermoFisher, USA) supplemented with 10% FBS and 1% antibiotic/antimycotic (Vitrocell, Brazil). The culture medium used for SCC9 was supplemented with 400 ng/ml hydrocortisone (Sigma-Aldrich, USA) and the CA1 and Luc4 medium were supplemented with RM^{+} [20]. All cell lines were maintained at 37degC atmosphere containing 5% CO_2 and 95% humidity.

Experimental groups

The cell lines were seeded at a density of 7 x 10^3cells/cm^2 and cultured for 24h with the regular medium supplemented with 10% FBS (control), 5% FBS (nutritional stress) and in the absence of FBS (0% FBS, serum starvation).

Analysis of optical absorbance

After 24h of treatment, the cells were detached, centrifuged (453 xg for 5 min), and counted. A total of 5x10^4 cells from each cell lines treated with different concentrations of FBS were resuspended in 1.5 mL of PBS and placed in a disposable plastic cuvette. The analysis of absorbance was performed in a spectrophotometer (Ocean Optics, USB-2000 model, Florida, USA) with the aid of the SpectraSuite software after calibration and setting the equipment with PBS (blank). The results were analyzed using the OriginPro program (version 2017 SR2, Massachusetts, USA). Mean absorbance was calculated from triplicate readings of each group and the data were normalized for plotting (first-order derivative) and smoothed with the method of Savitzky-Golay. The absorbance spectrum considered was 400 to 1000 nm. In the graphics, regions where the increase in absorbance was directly proportional to the concentration of FBS, that is, from 0% to 10% or inversely proportional, from 10% to 0% of FBS, were highlighted in light gray.

Three independent experiments in triplicate were performed for each cell line.

RESULTS

Analysis of optical absorbance in normal and dysplastic epithelial cell lines

HaCAT cells were able to absorb light over almost the entire spectrum (Figure 1A), with higher absorption at blue, 430 nm and another peak absorption in the near-infrared region, at 906 nm. The highest overall absorbance was observed in cells cultivated under serum starvation (0% FBS), followed by the cells cultivated with 5% FBS, when compared to cells cultivated with 10% FBS.

The dysplastic cell line DOK exhibited higher absorption also at blue, 440 nm, with the second highest peak around 945 nm, also in the near infrared region. Others peaks of absorption were noticed, but only from 635 to 670 nm (red region) and 840 to 955 nm (near infrared) the absorbances were proportional to the concentration, with the cells cultivated with 10% FBS showing higher absorbance than those cultivated with 5% FBS or in starvation condition. In some regions as 450 to 565 nm, 584 to 635 nm and 703 to 805 nm, cells cultivated under starvation showed higher absorbance than cells with 5% FBS, although the highest absorbance was with 10% FBS (Figure 1B).

Taken together, these results demonstrate that normal epithelial cells have higher absorption in the analyzed spectral range than dysplastic cells, especially under serum starvation.

Figure 1: Optical absorbance spectra (400 to 1000 nm) of HaCAT (A and DOK (B) cell lines after 24h of culture in the presence of 10% FBS, 5% FBS and in serum starvation (0% FBS). Graph represents mean of absorbance of three independent experiments. Regions where the increase in absorbance was directly proportional to the concentration of FBS, that is, from 0% to 10% or inversely proportional, from 10% to 0% of FBS, were highlighted in light gray.

Analysis of optical absorbance in malignant epithelial cell lines

The OSCC cell line CA1 showed three high absorbance peaks in the visible range. In blue, at 400 and 428 nm, and in yellow, at 556 nm. However, only in the intervals from 428 to 457 there was a greater absorption with 10% FBS, followed by 5% FBS and 0% FBS, as well as between 497 to 584 nm and 681 to 695 nm. Another three peaks appear in the infrared region of the spectrum, at 904 nm, around 945 nm and 974 nm. However, only two regions, 871 to 917 nm and 963 to 982 nm showed absorbance related to the FBS concentration, being inversely proportional to concentrations with 0% FBS, followed by 5% FBS and then 10% FBS. In most of the region between 608 and 846 nm (red and near infrared) cells with 5% FBS exhibited higher overall absorbance than cells cultivated with 10% FBS or 0% FBS, with the lowest absorbance observed in the serum starvation condition in the red region (608 to 708 nm) (Figure 2A).

LUC4 cells showed the highest absorption peak in the blue region of the spectrum, around 428 nm, followed by the peak at 976 nm (infrared). Two other peaks stand out, at 553 nm (yellow) and 744 nm (near infrared). Almost in the whole spectral range analyzed there was a higher absorbance for the concentration of 10% of FBS, followed by 5% FBS, with the lowest absorbance for 0% FBS. The exception is in the regions 445 to 500 nm, 752 to 760 nm, 902 to 922 nm and 965 to 991 nm, with the highest absorption using 5% FBS followed by absorbance of 10% FBS (Figure 2B).

In contrast to the results observed for CA1 and Luc4 cell lines, SCC9 cells showed the highest absorption peak in the red region, around 690 nm and the second highest peak at 818 nm. Cells cultivated in serum starvation exhibited higher optical absorbance in wavelengths up to 673 nm, at 719 nm and 783 nm when compared to those cells cultivated with 5% FBS or 10% FBS, which showed similar results. Interestingly, a switch in the optical absorbance was seen at 700 nm, in which cells cultivated in serum starvation showed lower absorption in relation to 10% FBS or 5% FBs. Moreover, cells with 10% FBS demonstrated greater absorbance than 0% FBS and 5% FBS, in this sequence, at wavelengths 751 to 772 nm, 791 to 836 nm, 844 to 882 nm, 895 to 947 nm and above 958 nm (Figure 2C).

Figure 2 : Optical absorbance spectra (400 to 1000 nm) of CA1 (A), LUC4 (|B) and SCC9 (C) cell lines after 24h of culture in the presence of 10% FBS, 5% FBS and in serum starvation (0% FBS). Graph represents mean of absorbance of three independent experiments. Regions where the increase in absorbance was directly proportional to the concentration of FBS, that is, from 0% to 10% or inversely proportional, from 10% to 0% of FBS, were highlighted in light gray.

DISCUSSION

PBM can modulate cellular behavior and tissue response in pathological conditions, however its effectiveness depends on factors like wavelength, energy density, exposure time, cell type, oxygen availability, and metabolic state [2]. Studies show that the effect of PBM is better in cells with nutritional deficit, however

there is a lack of studies that have studied in depth the light absorption spectrum of different cell types under different nutritional conditions, especially regarding PBM and cancer cells. This could impact the selection of the optimal wavelength to achieve the desired effect on cancer cells and may be one of the reasons for the contradictory results found in the literature.

Under conditions of nutritional stress induced by serum starvation, certain cell types, particularly normal cells, and dental-origin stem cells, exhibit a more favorable response to PBM [8], [9], [21], [22]. This phenomenon can be attributed to the fact that PBM enhances the activity of cells experiencing compromised growth at the time of irradiation. Conversely, cells in their typical physiological state may not exhibit a significant response to PBM, as there is no underlying stress to alleviate [7]. However, this response does not appear to be uniform across all cell types. To our knowledge, the present study is the first to investigate the absorbance spectrum of normal and malignant epithelial cells under different nutritional conditions.

Considering nutritional stress, HaCAT cell line have the most linear and stable behavior across the analyzed spectral range, with the absorbance being highest for 0% FBS and the lowest absorbance for 10% FBS.*In vitro* studies using HaCAT cell lines have analyzed the effects of different wavelengths on proliferation, viability, and wound healing. Fushimi et al. [23] investigated the impact of red (638 nm), blue (456 nm), and green (518 nm) LEDs. They observed that green light (518 nm) significantly accelerated wound healing, promoting faster cell proliferation and modulating growth factors and cytokines, resulting in more efficient wound recovery *in vitro* and *in vivo* . Blue light (456 nm) had a moderate effect, enhancing cell proliferation and wound closure rates but less effectively than green light. Red light (638 nm) also promoted wound healing but to a lesser extent, enhancing cell proliferation and modulating inflammatory responses.

Similarly, Kim et al. [24] found that in HaCAT cells, green light (525 nm) significantly increased reactive oxygen species (ROS) production, activating focal adhesion kinase (FAK) and promoting cell viability and proliferation. Blue light (470 nm) also increased ROS production but to a lesser extent, resulting in moderate FAK activation and cell viability. Red light (630 nm) had the least effect on ROS production and FAK activation, minimally enhancing cell viability and proliferation compared to green and blue light. Also Basso FG et al., [25] showed effects of PBM (780nm) in HaCAT with positive biostimulator effect, promoting proliferation, viability, and migration, thereby aiding in wound healing and tissue regeneration. Taken together, these studies indicate positive effects of PBM on proliferation, migration, and wound healing at various wavelengths, which can be associated with the absorption of light over almost the entire optical spectrum by HaCAT cells. Although all studies used 10% serum for cell supplementation, no impact on the evaluated outcomes was noticed. However, the conditions with highest light absorption were under nutritional stress and serum starvation, which is more similar to the clinical context when PBM is used, where the ATP levels and the cellular metabolic states are disturbed. Future studies should test the effects of low serum concentration or serum starvation in normal cells submitted to PBM to determine the relation between the levels of light absorption and cell behavior.

For the DOK cell line, we observed higher absorption at blue light (440 nm), with a second peak around 945 nm in the near-infrared region. The literature is scarce regarding the DOK cell line and PBM. Notably, Sperandio FF et al. [26] demonstrated that PBM wavelengths of 660 nm (red) and 780 nm (near-infrared) can increase the aggressiveness of dysplastic cells by activating the Akt/mTOR pathway, with the 780 nm wavelength showing a more pronounced effect compared to 660 nm. Our findings corroborate with this study, as we observed higher absorption near 780 nm than 660 nm in the DOK cell line. In regards to the blue light, future studies should address whether this wavelength will have inhibitory effects in dysplastic oral lesions.

Regarding the OSCC cell lines, the LUC4 cell line showed a spectral absorption response to nutritional stress inversely proportional to the HaCAT, with the highest absorbance for 10% FBS and the lowest for 0% FBS. Regarding both, CA1 and SCC9 cell lines, a variable absorption spectrum was noticed depending on the FBS concentration and wavelength, indicating that the response to PBM may be variable according to the nutritional condition and cancer cell line. In SCC9 cell line, the highest absorbance peak occurred at 690 nm (red region) and the second highest peak was at 818 nm (near infrared). CA1 and LUC4 cell lines also showed

absorption peaks at red and infrared regions lower than SCC9. Studies with PBM in tumor cells have focused more on these regions of the absorbance spectrum as they are used in the treatment of oral mucositis, the main adverse effect of the oncological treatment, mainly in patients with head and neck cancer submitted to radiotherapy. However, there is no consensus regarding whether PBM could stimulate the remaining tumor cells in oral mucosa, possibly leading to tumor progression or recurrence [27]. Research on OSCC cells, especially SCC9 cell line, has indicated that PBM might promote tumor growth in a dose-dependent manner, enhancing cell viability and proliferation, activating Akt/mTOR pathways [26]. Conversely, other studies have reported that PBM can have inhibitory effects, reducing cell viability and *in vitro* cell migration [16]. A systematic review showed that despite conflicting *in vitro* results, according to *in vivo* studies and clinical trials currently available, PBM for oncology patients appears to be safe within the parameters established by international guidelines. Nevertheless, they indicate that it would be irresponsible to overlook the potential influence of PBM on tumor behavior, as it could adversely affect the patient's oncological prognosis [28]. Thus, as OSCC cells absorb photons at red and infrared region, which can modulate cellular behavior, future studies are needed to clarify the safety issues related to the use of PBM in cancer patients.

Interestingly, in both OSCC cell lines CA1 and Luc4 showed higher absorption in wavelengths up to 440 nm (blue region) and between 904 to 976 nm (near infrared). The blue light has recently been investigated as a possible tool for cancer treatment. [29], [30]. Several studies *in vitro* and *in vivo* have already shown inhibitory effects of blue light irradiation alone on specific cancer cells, through the induction of autophagy, apoptosis, necrosis, production of reactive oxygen species and DNA damage, possibly facilitated mainly by the inhibition of the MAPK/MEK/ERK pathway [29], [31]–[37]. Furthermore, the effect of blue light has already been shown to decrease the activity of cancer-associated fibroblasts and cancer-associated macrophages, important therapeutic targets in the tumor microenvironment [38]. In oral cancer cells, especially, studies with blue light irradiation showed a suppressive effect in cellular proliferation and migration, mainly via oxidative stress, DNA damage and a pronounced mitochondrial dysfunction [29], [33]. In fact, the positive anti-tumoral effects of blue light in OSCC might be related with the highest absorption of light at this spectrum region, which was observed in the present study, corroborating to future studies aiming to evaluate the therapeutic use of blue light in oncology.

CONCLUSION

This study revealed significant variations in optical absorbance among normal, dysplastic, and malignant epithelial cells under different nutritional conditions. PBM effects vary based on cell type and metabolic state, with normal cells (HaCaT) showing higher absorption under serum starvation. Dysplastic (DOK) and OSCC cells had distinct absorption peaks, especially in blue and near-infrared regions. These findings highlight the importance of tailoring PBM parameters to individual needs and underscore the need for further research to guarantee both the safety and effectiveness of its application, especially, to elucidate the potential use of blue light in oral dysplastic lesions and in OSCC.

Author Contributions

Juliana Stephan Nobile: Writing – original draft, visualization, validation, methodology, investigation, formal analysis, data curation, conceptualization. **Daniele Heguedusch** : Writing – original draft, methodology, investigation, formal analysis, conceptualization. **Giovanna Lopes Carvalho** : Writing – original draft, methodology, investigation, formal analysis, conceptualization.**Daniela de Fatima Teixeira da Silva:** Writing – original draft, methodology, investigation**. Rebecca Boltes Cecatto:** Writing – original draft, methodology, investigation. **Rodrigo Labat Marcos:** Validation, methodology, investigation, formal analysis**Fabio Daumas Nunes:** Writing- review & editing, investigation, methodology, formal analysis. **Maria Fernanda Setubal Destro Rodrigues:** Writing –review & editing, supervision, resources, project administration, methodology, conceptualization

Funding information

This work was supported by the Sao Paulo Research Foundation (FAPESP, grant number 2018/08540-8). Nobile JS., Heguedusch D., Carvalho GL., were supported with scholarships by the Coordenacao de

Aperfeicoamento de Pessoal de Nivel Superior - Brasil (CAPES) - Finance Code 001.

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