UNIVERSIDADE ESTADUAL DE MONTES CLAROS

Angeliny Tamiarana Lima Tabosa

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Área de Concentração: Mecanismos e Aspectos Clínicos das Doenças

Orientador: Prof. Dr. André Luiz Sena Guimarães

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BANCA (TITULARES)

Prof. Dr. André Luiz Sena Guimarães ORIENTADOR (participação à distância por videoconferência)

Prof.ª Dr.ª Lucyana Conceição Farias COORIENTADOR (participação à distância por videoconferência)

Prof. Dr. Gefter Thiago Batista Corrêa (participação à distância por videoconferência)

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“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vesce obstáculos, no mínimo fará coisas admiráveis. “

José de Alencar

RESUMO

O carcinoma de células escamosas bucal (CCEB) é o tipo mais comum de neoplasia maligna oral (90%), sendo a principal causa de morbidade e mortalidade em pacientes com câncer de cabeça e pescoço. A radioterapia é atualmente o tratamento adjuvante padrão, no entanto, é acompanhado por várias complicações resultantes do dano dos tecidos radiossensíveis localizados perto do tumor. Por isso, a fotobiomodulação (PBM) tem atraído a atenção em vários campos clínicos, com uma nova geração de LEDs, para a reparação dos tecidos lesados. Assim, o presente estudo teve como objetivo investigar a resposta celular do carcinoma de células escamosas oral com pré-exposição à fototerapia de baixa intensidade antes da radioterapia, além de uma revisão da literatura que avaliou dados que investigaram a aplicação da PBM como um radiosensibilizante de células cancerígenas. Para o trabalho experimental, as linhagens de células SCC9, Cal-27, A431 e HaCaT foram submetidas à fototerapia de baixa potência e radioterapia. As células foram tratadas com uma densidade de energia única (300 J/cm^2) de um diodo emissor de luz (660 nm) antes da radiação ionizante em diferentes doses (0, 2, 4 e 6 Gy). Após 24 h, as análises de migração, proliferação, ensaio clonogênico, morte celular e espécies reativas de oxigênio (ROS) foram realizadas para avaliar a resposta celular. No trabalho de revisão foi feita uma pesquisa nos bancos de dados MEDLINE/PubMed e google acadêmico, usando os termos “PBM, low level light therapy, cell cancer, cell tumors, radiosentitizer, and ionizing radiation”. Os resultados experimentais mostraram que as linhagens celulares pré-expostas ao PBM na dosagem analisada foram radiossensíveis. O tratamento reduziu significativamente a proliferação celular e a sobrevivência das células clonogênicas. Os ensaios de migração e morte celular também revelaram resultados positivos, com o grupo de tratamento apresentando menor taxa de migração e maior morte celular do que o grupo controle. Além disso, o PBM efetivamente aumentou os níveis intracelulares de ROS. Dessa forma, conclui-se com o trabalho experimental que a fototerapia a 300 J/cm^2 é uma modalidade radiossensibilizante promissora para reduzir a dose de radiação e evitar os efeitos colaterais intoleráveis da radioterapia para CCEB de cabeça e pescoço, aumentando assim a probabilidade de sucesso do tratamento. Os resultados obtidos com a revisão mostraram que somente cinco estudos avaliaram a PBM como um possível agente radiosensibilizante de células cancerígenas, sendo que foram avaliadas nesses estudos as linhagens celulares HeLa, HeLa Kyoto e A431, e em quase todos os estudos, a pré-exposição da PBM levou a um efeito positivo no combate das células. Portanto, novos estudos devem ser realizados para avaliar a

PBM como uma técnica que melhora os resultados dos tratamentos oncológicos, aumentando a erradicação do tumor e reduzindo os efeitos adversos da radiação.

Palavras-chave: Câncer de cabeça e pescoço, fotobiomodulação, terapia de luz de baixa intensidade, LED, radiação ionizante, radiossensibilização

ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common type of oral malignancy (90%), being the main cause of morbidity and mortality in patients with head and neck cancer. Radiation therapy is currently the standard adjuvant treatment; however, it is accompanied by several complications resulting from damage to radiosensitive tissues located close to the tumor. Therefore, PBM has attracted attention in various clinical fields with a new generation of LEDs for the repair of injured tissues. Thus, the present study aimed to investigate the cellularresponse of oral squamous cell carcinoma with pre-exposure to low-level phototherapy before radiotherapy, in addition to a literature review that evaluated data that investigated the application of PBM as a radiosensitizer of cancer cells. For the experimental study, cell lines SCC9, Cal-27, A431 and HaCaT were submitted to low-level phototherapy and radiotherapy. Cells were treated with a single energy density (300 J/cm^2) of a light emitting diode (660 nm) before ionizing radiation at different doses (0, 2, 4 and 6 Gy). After 24 h, analyzes of migration, proliferation, clonogenic assay, cell death and reactive oxygen species (ROS) were performed to assess the cellular response. In the review study, a search was carried out in the MEDLINE/PubMed and academic google databases, using the terms “PBM, low level light therapy, cell cancer, cell tumors, radiosentitizer, and ionizing radiation”. The experimental results showed that the cell lines pre-exposed to PBM at the analyzed dosage were radiosensitive. The treatment significantly reduced cell proliferation and survival of clonogenic cells. The migration and cell death assays also revealed positive results, with the treatment group having a lower migration rate and greater cell death than the control group. Furthermore,PBM effectively increased intracellular levels of ROS. Thus, it is concluded with the experimental study that phototherapy at 300 J/cm^2 is a promising radiosensitizing modality to reduce the radiation dose and avoid the intolerable side effects of radiotherapy for head and neck CCEB, thus increasing the probability of treatment success. The results obtained with thereview showed that only five studies evaluated PBM as a possible radiosensitizing agent for cancer cells, and in these studies the cell lines HeLa, HeLa Kyoto and A431 were evaluated, and in almost all studies, pre-exposure of PBM led to a positive effect in fighting the cells. Therefore, further studies should be carried out to evaluate PBM as a technique that improves the results of cancer treatments, increasing tumor eradication and reducing the adverse effects of radiation.

Keywords: Head and neck cancer, photobiomodulation, low-level light therapy, LED, ionizing radiation, radiosensitization

SUMÁRIO

1 INTRODUÇÃO

1.1 Carcinoma de células escamosas bucal

O carcinoma de células escamosas bucal (CCEB) é o tipo mais comum de neoplasia maligna oral (90%). Em geral, ele se desenvolve a partir da progressão de uma hiperplasia epitelial, passando para um carcinoma *in situ* e depois para a forma invasora. É a principal causa de morbidade e mortalidade em pacientes com câncer de cabeça e pescoço e, por isso, é considerado um crítico problema de saúde pública [1, 2]. Ele representa cerca de 2,1% dos cânceres em todo o mundo, sendo que as mais altas taxas de mortalidade (77%) acontecem nos países menos desenvolvidos [2, 3]. As taxas de incidência são mais altas nos homens do que em mulheres com uma grande fração de casos tipicamente diagnosticados em estádios tardios e mais de 50% dos pacientes eventualmente desenvolvem recidiva local ou metástase, geralmente com anos após a conclusão do tratamento. As taxas de sobrevida de cinco anos são superiores a 80% em pacientes com CCEB localizado, no entanto, esta taxa diminui para 40% quando os linfonodos estão acometidos e a reduz para 20% nos pacientes com metástase à distância [4, 5].

O desenvolvimento de CCEB envolve danos às células epiteliais orais devido ao acúmulo de múltiplas mutações genéticas nas células influenciadas pela predisposição genética e por potenciais agentes cancerígenos como o tabaco, o álcool, inflamação e infecções virais, pricipalmente o HPV [5]. A exposição crônica aos carcinógenos podem danificar genes individuais, bem como grandes porções do material genético, incluindo cromossomos. A acumulação de tais alterações causa o desenvolvimento de lesões pré-malignas e posterior carcinoma invasivo. Estas alterações genéticas incluem ativação de mutações ou amplificações de oncogenes que promovem a sobrevivência e proliferação celular, bem como a inativação de genes supressores de tumores envolvidos na inibição da proliferação celular. A partir dessas alterações nos oncogenes e nos genes supressores de tumores, as células adquirem crescimento autônomo e auto-suficiente e escapam dos sinais inibitórios de crescimento, resultando no crescimento incontrolável do tumor [6].

As células tumorais escapam da morte celular programada e se reproduzem infinitamente através do processo de imortalização por alongamento dos telômeros. O carcinoma oral de células escamosas, como a maioria dos tumores, é capaz de criar um suprimento de sangue estimulando a proliferação de células endoteliais e formação de novos vasos sanguíneos. Durante a carcinogênese oral, há uma interrupção seletiva deste

processo, de tal forma que os fatores pro-angiogênicos predominam [6]. Esta angiogénese é uma parte essencial na formação de tumores sólidos, pois a subsequente progressão da CCEB inclui invasão de tecido e metástase, sendo que a invasão do tecido normal requer que moléculas de adesão celular, tais como integrina e caderinas, sejam perdidas, para permitir que as células cancerosas deixem o seu local primário [7].

Os tumores malignos de cabeça e pescoço são classificados pelo Sistema de estadiamento tumor-nódulo-metástase conhecido pela sigla TNM. Os tumores que possuem a mesma combinação TNM são pensados para ter comportamento semelhante e essa estratificação auxilia médicos e pesquisadores no planejamento, prognóstico e avaliação uniforme dos resultados do tratamento. É importante ressaltar que o sistema de estadiamento TNM descreve a extensão anatômica do tumor primário, bem como o envolvimento de linfonodos regionais e metástases à distância. Na prática atual, a informação obtida do exame clínico e radiológico é utilizada para atribuir um estágio clínico (cTNM), que é então utilizado para estratificar os pacientes para a seleção da terapia e para relatar os resultados do tratamento. Se o paciente for submetido à ressecção cirúrgica, o estágio patológico (pTNM) derivado do exame histopatológico do tumor ou linfonodos regionais é útil na seleção de terapia adjuvante pós-operatória e na estimativa do prognóstico [9].

As opções de tratamento do câncer bucal incluem cirurgia, quimioterapia (QT), radioterapia (RT) e terapias sistêmicas adjuvantes. Elas podem causar muitos distúrbios locais como defeitos dos tecidos moles e possivelmente no osso e pele. A terapia de pacientes que sofrem de carcinoma oral de células escamosas visa à reabilitação funcional e estética da deglutição, mastigação, fala e aparência facial, com o intuito de maximizar a qualidade de vida [8, 9].

1.2 Radioterapia

Um dos tratamentos utilizados para a CCEB é a radioterapia, tendo a radiação ionizante como o seu agente terapêutico. O DNA é o principal alvo da radiação e a quebra das cadeias simples e duplas podem gerar danos irreversíveis. As rupturas de dupla cadeia de DNA são as lesões mais prejudiciais associadas à radiação ionizante e quando o dano do DNA não é corrigido corretamente pode ocasionar aberrações cromossômicas, perda de material genético, perda da capacidade reprodutiva e morte celular [10].

A radiação interage com as células e tecidos por meio de mecanismos diretos e indiretos. No mecanismo direto, a energia da radiação é absorvida pelo meio e induz a ejeção de elétrons que interagem diretamente com os componentes celulares, principalmente com o DNA, o que leva a formação de alterações estruturais e funcionais. Enquanto que no mecanismo indireto, a energia da radiação interage com moléculas de água, produzindo radicais livres como a hidroxila (OH). Esses radicais livres quebram as ligações químicas do DNA em busca de estabilidade eletrônica (Figura 1) [11].

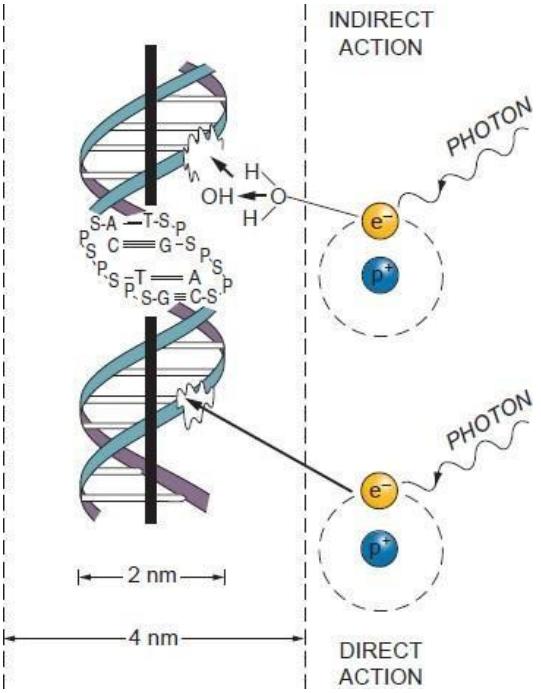

Figura 1: Ação direta e indireta da radiação. Na ação direta um elétron secundário resultante da absorção de um fóton de raio X interage com o DNA para produzir o dano ao DNA. Na ação indireta, o elétron secundário interage com uma molécula de água para produzir um radiacal hidroxilo (OH), que porsua vez produz o dano ao DNA.
Fonte: Hall EJ, Giaccia AJ. Radiobiology for the Radiologist: Lippincott Williams & Wilkins; 2012.

A radioterapia tem desempenhado um papel importante no controle do crescimento tumoral em muitos pacientes com câncer. Em doentes com carcinoma oral de células escamosas, a radioterapia é atualmente o tratamento adjuvante padrão [12]. No entanto, o tratamento de radioterapia (RT) para tumores de cabeça e pescoço é acompanhado por várias complicações resultantes do dano dos tecidos radiossensíveis localizados perto do tumor [13, 14]. As complicações orais agudas incluem mucosite oral, xerostomia e disgeusia, enquanto as complicações orais crônicas são caracterizadas

por persistência de hiposalivação e xerostomia, trismo, cárie dentária, doença periodontal progressiva e o risco aumentado de osteorradionecrose [15, 16].

Muitos estudos têm como objetivo minimizar os danos causados pela radioterapia sem aumentar as características de radiorresistência nas células cancerígenas e evitar tumores secundários. Além disso, existem estudos sobre métodos de radiossensibilização de células cancerígenas e radioproteção de células normais. Uma das estratégias de radiossensibilização que vem sendo bastante estudada é a fototerapia de baixa potência, atualmente conhecida como fotobiomodulação (PBM) [17].

1.3 Fototerapia de baixa intensidade

A fototerapia de baixa intensidade (LLLT) é uma opção de tratamento nãoinvasiva empregada para a cicatrização de feridas, redução da inflamação e edema, e parao alívio da dor [17]. Recentemente é denominada terapia de fotobiomodulação (PBM) e ganhou o seu lugar na medicina geral há mais de 40 anos. A PBM é aplicada em uma variedade de domínios médicos como a dermatologia, fisioterapia, neurologia e odontologia, sendo que o seu uso em ambientes oncológicos tornou-se objeto de estudo atualmente [17, 18].

As fontes de luz não ionizantes utilizadas na PBM referem-se a várias energias luminosas, tais como o laser e o diodo emissor de luz (LED) no espectro visível e infravermelho próximo (NIR) (600-1000 nm) [19]. As células alvo da terapia à luz têm um limite de sobrevivência. A terapia utilizando luz de alta intensidade (HLLT) é aplicada durante cirurgias para cortes, ablação de tecidos e coagulação, causando danos às células alvo por exceder o limiar de sobrevivência das mesmas. Em contraste, a PBM mantém a reação celular abaixo deste limiar modulando a atividade das células alvo, e, por isso, é empregado como uma modalidade terapêutica [17, 18].

Embora os mecanismos biológicos subjacentes à terapêutica da PBM não foram completamente elucidados, o efeito da terapia sobre o metabolismo de células benignas tem sido amplamente estudado, principalmente na tentativa de entender melhor o seu mecanismo de ação [20, 21]. Ela contribui para a cicatrização de feridas, reparação de ossos e regeneção muscular e neural, além de sua importância para os avanços na engenharia de tecidos usando células-tronco [22, 23]. No entanto, em células malignas, devido a instabilidade genômica, a proliferação induzida pelo laser de baixa intensidade pode aumentar o número de células geneticamente alteradas com alta atividade

proliferativa, acelerando indiretamente o ganho adicional de mutações durante o processo natural de carcinogênese [24].

Os dispositivos da terapia de PBM da nova geração possuem um conjunto de feixes de diodos emissores de luz, fornecendo uma exposição de campos maiores, ou um único ponto do laser. Os feixes led monocromáticos de alta qualidade têm as mesmas propriedades dos lasers, utilizando o mesmo comprimento de onda, mas sua luz é menos coerente. As especificações de led precisam ser cuidadosamente combinadas com a terapia usando lasers ao considerar matrizes de leds [25].

Apesar das variações na instrumentação e parâmetros de dosagem, desde a sua introdução em 1967, a PBM demonstrou melhorar a reparação de feridas e a regeneração de tecidos devido à influência em diferentes fases de resolução das lesões, incluindo a fase inflamatória, na qual as células imunes migram para o local da lesão tecidual; a fase proliferativa, que inclui a estimulação de fibroblastos, macrófagos, bem como outros componentes de reparação; e a fase de remodelação, consistindo em deposição de colágeno e reconstrução da matriz extracelular no local da ferida [26].

O mecanismo básico subjacente à PBM ainda não está completamente elucidado e pode variar entre diferentes tipos de células e condições teciduais. Esse mecanismo é baseado na absorção de luz por cromóforos endógenos induzindo eventos não térmicos, fotofísicos e fotoquímicos em várias escalas biológicas levando a mudanças fisiológicas.

É necessário mencionar que tanto a luz visível (600-750 nm) quanto a luz no infravermelho próximo (750-1000 nm) possuem o mesmo mecanismo básico de trabalho. No entanto, os seus alvos principais e as reações causadas nas células são diferentes [27].

A luz visível visa principalmente a enzima citocromo c oxidase (CCO) localizada na membrana mitocondrial, causando uma reação fotoquímica primária. A energia absorvida leva à ativação da cadeia respiratória mitocondrial, o que resulta em uma aceleração das reações de transferência de elétrons levando a um aumento da produção de ATP. Por outro lado, a luz NIR induz uma reação fotofísica primária que tem como alvo a membrana celular, levando à ativação das bombas Na^+/K^+, ATPase e Ca^{2+}. Comoreação secundária, a produção de ATP mitocondrial na célula é aumentada. O ATP regula a produção de cAMP, que é um segundo mensageiro. Além disso, em ambas as condições,o potencial da membrana mitocondrial é alterado, resultando numa maior actividade dos anti-transportadores Na^+/ H^+ e Ca^{2+}/Na^+ e de todos os transportadores ATP para íons, tais como as bombas Na^+/ K^+, ATPase e Ca^{2+}. Isto leva eventualmente a um aumento do nível intracelular de Ca^{2+}, que é um importante mensageiro secundário, juntamente com o

cAMP [28, 29]. Estes processos irão aumentar a atividade celular e melhorar as vias de transmissão do sinal intracelular, o que leva à síntese de DNA e RNA, enzimas e proteínas, resultando em uma maior proliferação celular, ativação celular e reparação de células lesadas [29].

Além disso, a libertação e produção de óxido nítrico (NO) nas mitocôndrias parecem ser reguladas positivamente pela PBM. Existem duas vias possíveis ligadas à libertação de NO por essa terapia. Em primeiro lugar, é possível que a PBM impede a ligação do NO ao CCO. O óxido nítrico desregula a respiração celular pela ligação ao CCO. O LLLT atua impedindo este processo através da dissociando do NO e do CCO, o que resulta no aumento da produção de ATP. Em segundo lugar, a PBM pode causar um aumento na atividade de nitrito redutase de CCO (uma redução de um eletrón de nitrito forma NO), o que leva a um aumento na produção de NO. Ele causa vasodilatação que aumenta a disponibilidade de oxigênio para as células expostas e também permite maior tráfego de células imunes no tecido [30].

Por fim, a PBM também é capaz de aumentar a produção de espécies reativas de oxigênio (ROS) nas mitocôndrias. A ROS está envolvida na via de sinalização redox entre as mitocôndrias e os núcleos. Nos núcleos, ROS ativará vários fatores de transcrição, o que levará a um aumento na regulação de vários genes estimuladores e protetores. Isto resultará na produção de várias proteínas que acionam efeitos tais como um aumento na proliferação e migração celular, uma modulação nos níveis de citocinas, factores de crescimento e mediadores inflamatórios e um aumento na oxigenação dos tecidos [27].

Evidências sugerem que a PBM aumenta o crescimento de células neoplásicas como resultado da expressão alterada de proteínas relacionadas à regulação do ciclo celular, apoptose, adesão e migração celular, degradação da matriz extracelular e angiogênese. Portanto, o uso não intencional dessa terapia durante o desenvolvimento e progressão de um processo neoplásico pode favorecer atividades biológicas que são determinantes para a tumorigênese, tais como proliferação celular e migração. A identificação de alterações nessas atividades celulares pode restringir o uso de PBM em qualquer situação clínica com potencial de transformação maligna ou quando o tumor está localizado próximo ao campo de irradiação [24].

2. OBJETIVOS

2.1 Objetivo Geral

- Investigar a resposta celular *in vitro* do carcinoma de células escamosas bucal submetido à radioterapia e pré-exposto à fototerapia de baixa intensidade.

2.2 Objetivos Específicos

- Definir a densidade de energia (J/cm^2) a ser trabalhada através de uma curva dose-resposta;
- Comparar a resposta celular do carcinoma de células escamosas bucal tratadas com LED e Laser de baixa intensidade;
- Avaliar se a associação de PBM mais radioterapia causam uma maior taxa de morte celular comparados ao tratamento apenas com radioterapia;
- Revisar a literatura para avaliar os dados disponíveis que investigaram a aplicação do PBM como radiossensibilizador de células cancerígenas.

3. PRODUTOS

3.1 Artigo 1: Application of photobiomodulation in the radiosensitivity of cancer cells: A Review

Application of photobiomodulation in the radiosensitivity of cancer cells: A Review

Angeliny Tamiarana Lima Tabosa[1], Victor Hugo Dantas Guimarães[1], Sérgio Henrique Sousa Santos[2], Alfredo Maurício Batista de Paula [1], Lucyana Conceição Farias [1] and André Luiz Sena Guimarães[1*]

[1] Department of Dentistry, Universidade Estadual de Montes Claros (Unimontes), Montes Claros, Minas Gerais, Brazil.
[2] Institute of Agricultural Sciences, Universidade Federal de Minas Gerais (UFMG), Montes Claros, Minas Gerais, Brazil.

*Corresponding author:
André Luiz Sena Guimarães Universidade Estadual de Montes Claros. Hospital Universitário Clemente Faria. Laboratório de Pesquisa em Saúde, 562 Av. Cula Mangabeira Santo Expedito. Montes Claros, MG. Brazil Zip code: 39401-001 E-mail: andreluizguimaraes@gmail.com

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Abstract

Purpose: Radiotherapy in cancer treatment is associated with several adverse effects to patients. One of the radiosensitizing strategies that has been extensively studied is low-power light therapy, currently known as photobiomodulation (PBM). Thus, the aim of this study is to review the literature to assess the available data that investigated the application of PBM as a radiosensitizer of cells cancer.

Methods: A search of literature was conducted in MEDLINE/PubMed and Google Scholar databases, using search terms "PBM, low level light therapy, cell cancer, cell tumors, radiosentitizer, and ionizing radiation". The literature was reviewed and analysed, and all relevant articles, with English language, were included and narratively discussed.

Results: Only five studies were found that evaluated PBM as a possible radiosensitizing agent. The tumor cell lines used were HeLa, HeLa Kyoto and A431, and in almost all studies, pre-exposure of cells to PBM led to a positive effect in combating these cells.

Conclusion: PBM should be explored as an inexpensive, non-invasive technique that improves treatment outcomes by enhancing tumor eradication and reducing the adverse effects of radiation. However, further studies are needed to assess the benefits caused by PBM-RT.

Keywords: photobiomodulation, low-level light therapy, ionizing radiation, radiosensitization

Introdution

One of the essential characteristics of tumor cells is their infinite and uncontrolled proliferation [1]. It is expected that from 2015 to 2025 there will be an increase of 420 million new cases, which demonstrates that this disease is a major public health problem worldwide [2]. Tumor formation occurs due to mutations in oncogenes and tumor suppressor genes, and these mutations regulate the expression and activity of metabolic enzymes such as c-MYC and TP53, which activates glutamine uptake and regulates lipid metabolism in cancer cells, respectively [3, 4]. Cancer treatment options include surgery, chemotherapy (CT), radiation therapy (RT), and adjuvant systemic therapies [5].

Among the types of treatment, radiotherapy is used in more than 50% of patients, either with the aim of cure or palliative. Radiation therapy has ionizing radiation as a therapeutic agent that will kill tumor tissue but also affect healthy tissue [6]. Despite obtaining great results with radiotherapy, many studies are looking for radiosensitizers to increase the toxic effect on cancer cells, and reduce the side effects on healthy tissue [7, 8]. Radiosensitizers are chemical and pharmaceutical agents that have the ability to accelerate DNA damage and increase the production of free radicals. In recent years, several strategies have been exploited to develop radiosensitizers that are highly effective and have low toxicity [9].

One of the radiosensitizing strategies that has been extensively studied is low-power light therapy, currently known as photobiomodulation (PBM) [10, 11]. PBM is a therapy used to restore and stimulate physiological processes with light in the red and infrared wavelengths [12]. This low-power light causes intracellular chemical and physical reactions causing the emitted photons to reach the inner mitochondrial membrane and act on cytochrome c oxidation, increasing the production of ATP, reactive oxygen species (ROS) and the release of nitric oxide (NO)[13]. Therefore, the PBM improves the vital capacity of cells, induces growth factor production, and enhances the motility and viability of the irradiated cells [14].

Thus, the aim of this study is to review the literature to assess the available data that investigated the application of PBM as a radiosensitizer of cancer cells.

Methods

A search of literature was conducted in MEDLINE/PubMed and Google Scholar databases, using search terms “PBM, low level light therapy, cell cancer, cell tumors,

radiosentitizer, and ionizing radiation". The literature was reviewed and analysed, and all relevant articles, with English language, were included and narratively discussed.

Results

A few studies were found that evaluated the use of PBM as a potent radiosensitizer in cancer cells, and their findings are summarized in Table 1. The studies were performed by pre-exposing cancer cells to PBM at red and infrared wavelengths, with a rate that ranged from 632.8 to 780nm. The types of cancer cell lines used in the studies were HeLa [8, 15, 16], HeLa Kyoto [17], and A431 [18].

In one of the studies with HeLa cell line was used the BTL-5000 laser in 685 or 830 nm at 1 or 5 J/cm^2, and after the X-ray ionizing radiation with the device Siemens Primus linear accelerator at dosages of 2, 4 and 6 Gy [8]. The second study with this cell line, the PBM source was a He-Ne laser in 632.8nm at 100 J/m^2, and for ionizing radiation, ^{197}Cs y-irradiation was used with doses from 0.2 to 10 Gy [15]. The other study that involved HeLa cells, was used a continuous wave 685nm laser at fluences of 0, 5, 10,and 20 J/cm^2, and after the cells were exposed to 6 MeV X-ray photons at 2, 4, and 6 Gy[16]. In the study that used the HeLa kyoto cell line, was used LED light at a wavelenghtof 640nm in diferente energy densities (0, 3, 30 and 300 mJ/cm^2), and the radiation sourcewas gamma photons from a "Terabalt 80" Cobalt^{60} unit at doses 2, 4, and 6Gy [17].

Regarding the study involving the A431, cells were illuminated using a LED array system at 780 nm and total energy density of 5 J/cm^2. After were used the XRAD 225x X-ray generator with a dose rate of 4.0 Gy/min. This same study performed in vivo experimentation where the Xenographic tumor induction was performed with the same cell line of the in vitro experiments. A single point illumination was performed with 5 J using a GaAlAs diode laser system at 780 nm, which delivered the desired energy in 10 s. The ionizing radiation was performed using the same system at a dose rate of 1.7 Gy/min [18].

The findings involving the first study with HeLa cells show that the LLLT in 685nm decreased survival fraction at 5 J/cm^2 energy density, while did not significantly effect the ionizing radiation survival curves of HeLa cell pre-exposure to LLLI at 830nm. This study demonstrate a possible radiosensitizing effect for 685 nm LLLI in HeLa cancer cells [8].

In the second study, there was an increase a viability of cells preirradiated with He-Ne laser, and according to the authors the most importante factor for the manifestation

of this effect was the time between the two types of irradiation. The effect was pronounced when laser irradiation was performed 60 min or 180 min before the exposureof the cells to y-radiation. Futhermore, the colony size distribution changed when the interval between the laser and y-exposures was 60 min. The changes were most pronounced for doses of 2.5 and 5 Gy with a decrease in percentage abortive colonies andan increase in the percentage survivors with delayed growth. It means that the laser radiation stimulated the proliferation of some subpopulations even when the survival of the population did not change [15].

Another study with HeLa cells pre-exposed to 20 J/cm^2 showed that PBM did not significantly influence the proliferation of HeLa cell and enhanced inhibition of colony formation following ionizing radiation. The authors demonstrate that this therapy enhaced radiosensitivity due to increased oxidative stress, DNA damage, and radiation-induced apoptosis and autophagy [16].

The study with HeLa kyoto cells showed that there was a statistically significant decrease in a number of viable tumor cells for the samples that were exposed to low-intensity red light prior to gamma irradiation and a statistically significant increase in a number of viable tumor cells for the samples that were exposed to low-intensity red light after gamma-irradiation [17].

The results of the study using the A431 cell line showed that although there was no increase in cell proliferation in vitro, there was an increase in G2/M fraction by 27% 24h after illumination, resulting in an enhancement of 30% in radiation effect in the clonogenic assay. In addition, in vivo studies the median survival of the PBM-RT group increased by 4 days when compared to radiation alone. An analysis showed that PBM increases tumor necrosis due to radiation, and histological analysis showed that illumination increased cell differentiation and angiogenesis in the group treated with low-level light therapy alone, which may play a role in the synergetic effect of PBM and radiation [18].

Discussion

Radiation therapy is known to cause several side effects to healthy cells located close to the tumor, due this, studies have attempted to develop modalities for increase radiosensitivity of cancer cells and radioprotection of normal cells [19, 20], being the PBM one of the target therapies of these studies [10, 11].

PBM is a therapy widely used to prevent and treat numerous side effects, such as mucositis and dermatitis, which occur as a result of a variety of different types of cancer treatments [21, 22]. There are several studies in the literature that show the effects of PBM on different types of cells [23-25], and this effects are caused due to its influence on the stimulation or inhibition of various metabolic pathways [26].

There are several study in the literature that evaluated cellular responses to treatment with low-level light alone or associated with other therapies, such as ionizing radiation. But it is important to emphasize that in most of these studies, PBM is performed after ionizing radiation, and only a few studies are presented devoted to the stimulating effect of PBM on the cells previously exposed to ionizing radiation, as shown in the results. Thus, the purpose of this review was to examine the results of studies that investigated the application of PBM as a radiosensitizer of cancer cells, and for this assessment, cancer cells must be exposed to PBM before IR.

It can be seen that the second studie with HeLa cells had an opposite result to the others, since increased a viability of cells and the percentage of surviving colonies and the other decreasing it. This result may be due to the difference in wavelength, one being at 632.8 nm and the others at 685 nm. It is important to note that the optical spectral range is from 650 to 950 nm, this means that wavelengths below 650 nm are strongly absorbed mainly by hemoglobin and over 950 is strongly absorbed by water, which correspondingly may cause overheating of tissues [14]. With the spectral range of the optical window at low energy, the penetration of light is maximized through the mucosa without overheating, reaching the inner mitochondrial membrane, and resulting in photochemical effects [27]. Therefore, probably in the second study, the wavelength used was not adequate. While the results of the others studies corresponded, which suggests that 685 nm PBM at 5 and 20 J/cm^2, respectively, could possibly be a promising radiosensitizing agent in cervical cancer.

In the study with HeLa Kyoto cell line, the authors investigated the cellular response in groups which PBM was applied before IR and groups which it was applied after IR, and the results were opposite, that is, when cells were exposed to PBM before IR there was a decrease in viable tumor cells, and when PBM was applied after IR there was an increase in the number of viable cells. According to the authors the study demonstrated the absence of the adaptive (radioprotective) effect of the PBM on the HeLa cells, but revealed a stimulating effect of some PBM modes in relation to cells that had previously been damaged by ionizing radiation [17].

The study with the A431 cell line, the authors investigated the safety of photobiomodulation therapy in tumors and its potential as a radiosensitizer when combined with radiotherapy, and the results were promising, since the combined treatment (PMB+IR) induced cell death and loss of integrity, which cause a clearreduction in the number of colonies, and in the in vivo study there was an increase in tumor necrosis.

There are authors who claim that PBM may be contraindicated in cancer patients, because this therapy, when irradiated close to the tumor mass, can increase the growth and aggressiveness of these cancer cells [28-30]. Djavid et al. show that a preliminary PBM led to a decrease in the number of viable HeLa cells after subsequent gamma irradiation compared with the control. Similar results were obtained when using PBM with a energy density of 20 J/cm^2. The number of viable cells decreased, in particular, due to an increase in the number of apoptotic cells, as well as an increase in the level of autophagy. Thus, the use of LLLT before the radiation therapy for the prevention and correction of mucositis in the studied modes appears to be safe in relation to the possible stimulation of a malignant tumor growth that falls into the laser radiation exposure zone [16]. Althought, Sperandio et al. demonstrated in their study that LLLT can modify oral dysplastic cells (DOK) and oral cancer cells (SCC9 and SCC25) due this therapy modified the expression of proteins related to progression and invasion in all the cell lines, and could aggravate oral cancer cellular behavior, increasing the expression of pAkt, pS6 and Cyclin D1 proteins and producing an aggressive Hsp90 isoform [29].

Cytochrome c oxidase is considered an important photoacceptor and the photoexcitation caused by PBM leads to changes in the redox properties of the respiratory chain components after excitation with the increase in ATP levels, release of nitric oxide (NO) from the catalytic center of cytochrome c oxidase, increase in oxygen levels which leads to singlet oxygen formation, transient local heating of absorber chromophores and increased production of reactive oxygen species (ROS). This cascade of reactions causes a change in cellular homeostasis [31]. Therefore, a high intracellular concentration of ROS caused by PBM, can induce proapoptotic effects makes the cell radiosensitive, that is, when suffering an injury with ionizing radiation, mechanisms will be activated that will lead to its death in a faster way (Figure 1).

The PBM follows the rules of the “biphasic dose–response” curve [32]. This principle states that there are optimal parameters (energy density or power density) that provide a benefit to the irradiated tissues, but if these parameters are significantly

exceeded, the irradiation could lead to harmful effects [14]. Thus, the definition of these parameters is very important for that all the advantages that phototherapy can provide can be properly used.

The results shown are promising, indicating that PBM should be explored as an inexpensive, non-invasive technique that improves treatment outcomes by enhancing tumor eradication and reducing the adverse effects of radiation, However, further studies are needed, using different methods, PBM protocols, cell lines and animal models, since there are still very few studies in the literature that evaluated PBM as a potent radiosensitizing agent.

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Figure Legend

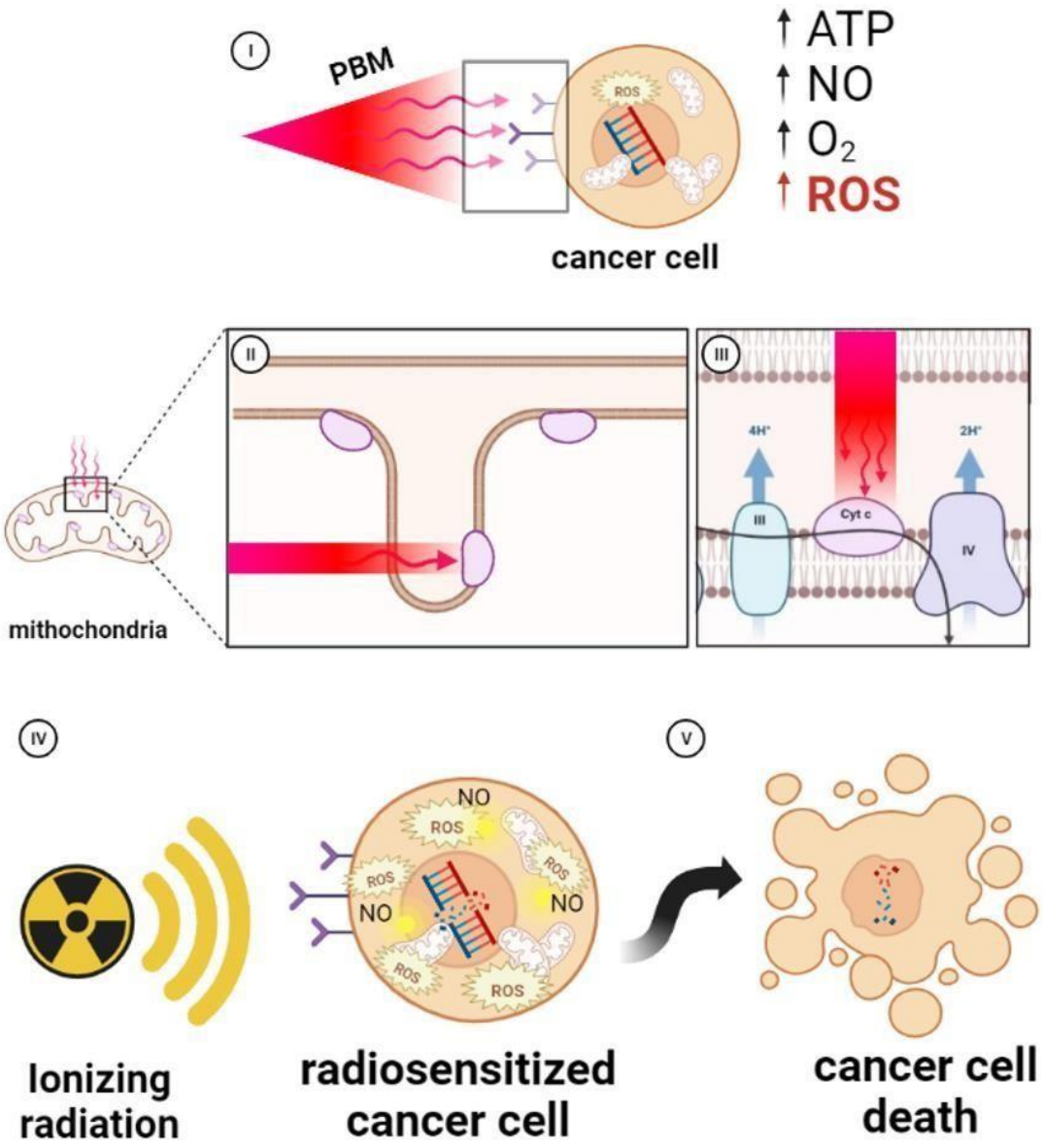

Figure 1. Schematic drawing that explains a possible mechanism for radiosensitization of tumor cells to PBM. (I) Light is incident on the cell that has membrane photoacceptors. (II) Photons of light reach the mitochondria and reach the main target, cytochrome c oxidase (III). A cascade of reactions takes place leading to an increase in the levels of ATP, NO, oxygen, mainly in its singlet form and, consequently, an increase in the levels of ROS. This increase in oxidative stress makes the cell radiosensitized, so when it receives a dose of ionizing radiation (IV), it will activate mechanisms that will lead to death (V).

Table 1. Summary of some studies on the effects of exposure to PBM on tumor cells subsequently treated with ionizing radiation.

Title	Tumor cell	Device	Wavelenght (nm)	Effect	Reference
Analysis of Radiomodulatory Effect of Low-Level Laser Irradiation by Clonogenic Survival Assay	HeLa	Laser BTL-5000	685 e 830 nm	Inhibit clonogenic growth of HeLa at 685nm, and did not significantly effect the ionizing radiation survival curves of HeLa cell pre-exposure to LLLI at 830nm.	[8]
Irradiation with He-Ne laser can influence the cytotoxic response of HeLa cells to ionizing radiation	HeLa	He-Ne laser (Spectra Physics model)	632.8 nm	Increased a viability of cells, decreased in percentage abortive colonies and increased the percentage survivors with delayed growth.	[15]
Photobiomodulation leads to enhanced radiosensitivity through induction of apoptosis and autophagy in human cervical cancer cells	HeLa	Laser (BTL-5000, Prague, Czech Republic)	685 nm	Enhanced inhibition of colony formation following ionizing radiation, increased oxidative stress, DNA damage, and induced apoptosis and autophagy.	[16]
Effects of photobiomodulation in relation to HeLa Kyoto tumor cells exposed to ionizing radiation	HeLa Kyoto	LED light (IPT RAS, Russia)	640 nm	Decreased a number of viable tumor cells (for the samples that were exposed tolow-intensity red light prior to gamma irradiation), and increased a number of viable tumor cells (for the samples that were exposed to low-intensity red light after gamma-irradiation).	[17]
Tumor radiosensitization by photobiomodulation	A431	LED array system (Biotable®, MM OpticsLtda.,Brazil)	780nm	There was no induction in the proliferation rate, increased the G2/M fraction, enhanced of 30% in radiation effect in the clonogenic assay (In vitro). Increased tumor necrosis (In vivo)	[18]

3.2 Artigo 2: Effect Effect of low-level light therapy before radiotherapy in oral squamous cell carcinoma: An *in vitro* study

Effect of low-level light therapy before radiotherapy in oral squamous cell carcinoma: An *in vitro* study

Angeliny Tamiarana Lima Tabosa[1], Marcela Gonçalves Souza[1], Sabrina Ferreira de Jesus[1], Danielle Ferreira Rocha[1], Lorena dos Reis Pereira Queiroz[1], Eloá Mangabeira Santos[1], Victor Hugo Dantas Guimarães[1] Luciano Alves de Araújo Andrade[3], Sérgio Henrique Sousa Santos[2], Alfredo Maurício Batista de Paula [1], Paulo Eduardo Narcizo de Souza[4], Lucyana Conceição Farias [1] and André Luiz Sena Guimarães[1*]

[1] Department of Dentistry, Universidade Estadual de Montes Claros (Unimontes), Montes Claros, Minas Gerais, Brazil.
[2] Institute of Agricultural Sciences, Universidade Federal de Minas Gerais (UFMG), Montes Claros, Minas Gerais, Brazil.
[3] Dilson Godinho Hospital, Montes Claros, Minas Gerais, Brazil.
[4] Institute of Physics of the University of Brasilia, University of Brasilia, Federal District, Brazil.

*Corresponding author:
André Luiz Sena Guimarães Universidade Estadual de Montes Claros. Hospital Universitário Clemente Faria. Laboratório de Pesquisa em Saúde, 562 Av. Cula Mangabeira Santo Expedito. Montes Claros, MG. Brazil Zip code: 39401-001 E-mail: andreluizguimaraes@gmail.com

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Abstract

Purpose: Radiation therapy for head and neck squamous cell carcinoma (HNSCC) is associated with several complications. Although photobiomodulation (PBM) has radioprotective effects in normal tissue, it could also enhance the growth of neoplastic cells. Thus, the present study aimed to investigate the cellular response of oral squamous cell carcinoma with pre-exposure to low-level phototherapy before radiotherapy.

Methods: SCC9, Cal-27, A431, and HaCaT cell lines were subjected to low-level light therapy and radiotherapy. The cells were treated with a single energy density (300 J/cm^2) of a light-emitting diode (660 nm) prior to ionizing radiation at different doses (0, 2, 4, and 6 Gy). After 24 h, wound scratch, proliferation, clonogenic cell survival, cell death, and reactive oxygen species (ROS) analyses were performed to evaluate cell response.

Results: The cell lines pre-exposed to PBM at the analyzed dosage were radiosensitive. The treatment significantly reduced cell proliferation and clonogenic cell survival. Migration and cell death assays also revealed positive results, with the treatment group showing lower rate of migration and higher cell death than did the control group. Moreover, PBM effectively increased the intracellular levels of ROS.

Conclusion: PBM at 300 J/cm^2 is a promising radiosensitizing modality to reduce the radiation dose and avoid the intolerable side effects of radiotherapy for HNSCC, thus increasing the probability of successful treatment. However, further studies are needed to support and confirm the results.

Keywords: head and neck cancer, photobiomodulation, low-level light therapy, LED, ionizing radiation, radiosensitization

List of abbreviations

A431	Epidermoid carcinoma cell line
AO	Acridine orange
Cal-27	OSCC cell line
DEMEM/Ham's F-12	Dulbecco's modified Eagle's medium/nutrient medium
EB	Ethidium bromide
HaCaT	Skin keratinocyte cell line
HNSCC	Head and neck squamous cell carcinoma
IR	Ionizing radiation
LED	Light-emitting diode
LLLT	Low-level light therapy
OSCC	Oral squamous cell carcinoma
PBM	Photobiomodulation
ROS	Reactive oxygen species
RT	Radiotherapy
SCC9	OSCC cell line

Introduction

Oral squamous cell carcinoma (OSCC) is a heterogeneous, aggressive, genetically complex cancer. It accounts for approximately 90% of all head and neck cancers and is the main cause of morbidity and mortality in this type of malignancy [1-3]. OSCC accounts for approximately 2.1% of all cancers worldwide, with the highest mortality rate (77%) reported in developing countries [4]. In 2020, 264,211 new cases of oral cavity cancer and 125,022 deaths were recorded. Men are also more affected by this disease [5]. The development of OSCC is generally associated with intrinsic factors, such as genetic predisposition, tobacco consumption, alcohol abuse, inflammation, and viral infections [6, 7]. Premalignant lesions and subsequent invasive carcinoma are caused by chronic exposure to carcinogens that damage individual genes or large portions of the genome [8].

Oral cancer treatment options include surgery, chemotherapy, radiation therapy, and adjuvant systemic therapies. In current practice, information obtained from the clinical and radiological examination is used to assign a clinical-stage, which is then used to stratify patients for therapy selection and to report treatment outcomes [9]. Among the surgical approach options, high-power laser equipment is very effective for tissue dissection and provides several adjunctive advantages, such as clear evidence of the surgical plans, precise cut, and better control of intraoperative bleeding [10]. The diode laser diode demonstrated good surgical capabilities and is undoubtedly the most widely used for surgical excision of proliferative lesions of the oral cavity. It minimizes or avoids thermal and morphostructural damage, and it has recently been reported to reduce the migration of metastatic cells during surgery [11, 12]. Furthermore, if the patient undergoes surgical resection, the pathologic stage derived from the histopathologic examination of the tumor or regional lymph nodes helps select postoperative adjuvant therapy and estimate prognosis [9].

Radiotherapy is an important treatment for OSCC, with ionizing radiation (IR) as a therapeutic strategy that controls tumor growth. Radiation mainly targets the DNA, breaking of single and double chains and generating irreversible damage such as chromosomal aberrations, loss of genetic material, loss of reproductive capacity, and cell death [13]. Radiotherapy is currently the standard treatment modality for OSCC, but it is also associated with several complications resulting from damage to radiosensitive tissues located near the tumor [14, 15]. Many studies have aimed to minimize the damage caused

by radiotherapy without enhancing radioresistance characteristics in cancer cells and avoiding secondary tumors. In addition, there have been studies on methods for radiosensitization of cancer cells while protecting normal cells [16-19].

Low-level light therapy (LLLT) could protect cells against cytotoxic agents such as IR [19]. LLLT, also known as photobiomodulation (PBM), is a noninvasive treatment for wound healing, inflammation, edema reduction, and pain relief [20, 21]. The non-ionizing light sources used in the treatment involve light energies (e.g., lasers and light-emitting diodes (LEDs) in the visible and near-infrared spectrum (600-1000 nm) [22]. LLLT stimulates the respiratory chain in the mitochondria, increasing the production of adenosine triphosphate (ATP) and, consequently, the synthesis of DNA, RNA, and proteins [14]. The field cancerization hypothesis explains the development of multiple primary tumors and locally recurrent cancers, with carcinogen exposure affecting several cells, including healthy tissue cells, at different sites in the field. Therefore, the presence of a field containing genetically altered cells is a risk factor for the growth of secondary cancers [23, 24].

Although many clinical reports support the radioprotective effects of PBM in normal tissue [20, 25, 26], evidence suggests that this therapy also enhances the growth of neoplastic cells due to a biphasic dose-response pattern [27]. Thus, the present study aimed to investigate the cellular response of OSCC with pre-exposure to low-level phototherapy before radiotherapy.

METHODS

Cell lines and culture conditions

The tumorigenic cell lines originating from human tongue squamous cell carcinomas used in this study were human OSCC cell lines SCC9 (Code 0196) and Cal-27 kindly donated by Dra. Lídia Maria de Andrade (Nanobiomedical Research Group, Department of Physics, ICEx/Federal University of Minas Gerais, UFMG). For comparative analysis, we used HaCaT human normal keratinocytes (donated by Dra Lídia Maria de Andrade) and an epidermoid carcinoma cell line A431 (Code 0032). All cell lines were maintained in Dulbecco's modified Eagle's medium (F12, GIBCO, Billings, MT, USA) supplemented with 10% fetal bovine serum (GIBCO, Billings, MT, USA), 400 ng/mL hydrocortisone, and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% CO_2. During subculture, cells were

grown in 175 cm^2 culture flasks and detached by trypsinization. The experiments were performed when the cells reached 70-80% confluency.

Comparison of laser and LED

Initially, an experiment was conducted to compare cellular responses using two different PBM devices. For this, groups were irradiated with a 660-nm red laser with a power of 100 mW/cm^2 for 90 s, spot size 3 mm^2, at the distance of 0.5 cm, and an energy density of 300 J/cm^2 (GaA1As and InGaAlP dual diode LASER, LASER duo —MMOptics®, MMOptics Ltda., São Carlos, São Paulo, Brazil, RRID: SCR_015955). Other groups were irradiated with a visible red (660 nm) LED device (prototype number 02 produced by the Institute of Physics at the University of Brasília, Brazil) in a continuous mode witha power of 102.2 mW/cm^2 for 49 min, a distance of 7 cm, and an energy density of 300.37J/cm^2. Notably, the choice of LLLT parameters was based on previous studies [28] and adose-response curve, which evaluated cellular response to different energy densities.

Grouping and cell treatment

After choosing the PBM device, the experimental groups were defined, and five groups were created: one control group (not subjected to any treatment) and four treatment groups that received LLLT (300 J/cm^2) irradiated with 0, 2, 4, and 6 Gy. The plated cellswere stimulated with the LED in partial darkness without the influence of other light sources and after irradiation with different doses (0, 2, 4, and 6 Gy) using the Elekta Synergy linear accelerator (Atlanta, GA) with a field-source distance of 97.5 cm. The Effect on cell lines was evaluated in all assays after 24 h, as the study intended to evaluatethe immediate cellular Effect.

Cell proliferation assay

For the treatment groups, the cells were seeded at a density of 8×10^4 cell/well in a 12-well culture plate. Control cells were grown in an untreated medium. The cells were collected 24 h later by trypsinization and evaluated with a 0.4% trypan blue exclusion test using an automated cell counter (IIFL Countess^{TM} Invitrogen). A single sample measurement using this counter yielded the following data: total cell concentration, viable and dead cell concentrations, and cell size.

Wound scratch assay

The cell lines were plated at a density of 1×10^5 in 12-well plates and incubated at 37°C and 5% CO2 for 24 h. Cell migration was monitored using a wound scratch assay as described previously [2, 29]. Briefly, a scratch was made with a sterile pipette tip in the confluent cell layer; the cells were then washed twice in PBS and then added to serum-free medium. The wells were photographed at the beginning of the experiment and after 24 h. Images were obtained with a camera SC30 (Olympus, Center Valley, PA, USA) in an IX81 inverted microscope (Olympus, Center Valley, PA, USA). Images were analyzed using ImageJ software [30]. To calculate the wound healing ratio, the initial area (in pixels) was divided by the final cell-free area (in pixels). The experiments were performed in triplicate.

Cell death/viability assay

Apoptotic cells were detected by simultaneous staining with both acridine orange (AO, Sigma, St. Louis, MO, USA) and ethidium bromide (EB, Sigma, St. Louis, MO, USA). The cells were seeded at a density of 1×10^5 cells/well and incubated at 37°C and 5% CO_2 for 24 h. After treatment, the cells were incubated in a mixed solution containing 100 μg/mL AO and 100 μg/ml EB (1:1) in a dark room for 5 min. The cells were then examined using fluorescence light microscopy FSX100 (Olympus, Center Valley, PA, USA). Intense EB (Ex360-370, Em420-460, filter DM400) staining indicated cell death, while extreme AO (Ex460-4 95, Em510-550, filter DM505) indicated live cells. The automatic count and threshold were determined in the merged image using ImageJ software [30].

Clonogenic assay

The clonogenic assay was performed as previously described [31]. Briefly, the cell lines were seeded in 12-well plates and exposed to the experimental treatments. After irradiation, cells were incubated at 37°C in a humidified incubator containing 5% CO_2 for 24 h. The cells were then trypsinized and plated again in 6-well plates at a density of 5×10^2 cells. The cells were maintained in the growth medium for 15 days to form colonies. Subsequently, colonies (clusters containing ≥50 cells) were fixed with 70% ethanol, stained with 2% Giemsa, and quantified using ImageJ software [30]. Non- irradiated controls were used to normalize the surviving fractions of the experimental groups.

Reactive oxygen species assay

Intracellular quantification of reactive oxygen species (ROS) generated by the treatment was performed using 2',7'-dichloro-fluorescin diacetate (H_2DCFDA; Invitrogen, Carlsbad, CA). The cells were seeded at a density of 4×10^4 into 12-well plates, and after 24 h of experimental treatment, they were incubated with 10 μM H_2DCFDA for 30 min in the dark at 37°C with 5% CO_2. The medium containing DCFDA was removed, washed twice with PBS, and immediately photographed under a fluorescence microscope (Olympus, Center Valley, PA, USA). Quantification was performed using ImageJ software [30].

Statistical analysis

All statistical analyses were performed using SPSS software (version 20.0) and GraphPad Prism (version 5.0). The Shapiro-Wilk test was performed to evaluate the data distribution. Data were analyzed according to the normal distribution using ANOVA one-way variance test. The half-maximal inhibitory concentration (IC50) was calculated using GraphPad Prism version 5.00. A p value of <0.05 was considered statistically significant.

RESULTS

Both laser and LED PBMs induced the same cellular response

The dose-response curve showed that at an energy density of 300 J/cm², there was a delay in cell death. This indicates that the energy density delivered did not induce cell death, but rather induced radiosensitization of the cells (SI 1A). In addition, IC50 was calculated using the AO/EB cell death assay (SI 1 B). Initially, a comparative study was carried out to evaluate the cellular response in two PBM devices, both at the same wavelength (660 nm) and energy density (300 J/cm^2). The results of the cell death analysis showed that there was no significant difference between the groups irradiated with laser and LED. It was also evaluated whether treatment with laser plus IR and with LED plus IR will induce a higher apoptosis rate than treatment with IR alone. The results showed that both laser plus IR and with LED plus IR were associated with a significantly higher rate of cell death than IR alone. Comparison between laser+IR and LED+IR showed similar benefits (SI 2). Based on these results, the LED device was selected for further experiments as this equipment operates on a larger surface area (SI 3).

PBM increased IR efficacy by reducing proliferative activity of SCC9, Cal-27, and A431 but protected normal keratinocytes at low radiations doses

The results of the proliferation assay of SCC9, Cal-27, A431, and HaCaT cell lines measured 24 h after treatment with PBM associated with IR are shown in Figure 1. The number of viable cells in SCC9, Cal-27, and A431 was lower in the combined treatment group than in the control and LED groups. Interestingly, in normal keratinocytes, there was a significant difference only in the group irradiated with the highest dose of radiation. This result shows that the associated treatment exerted an adjuvant effect on IR at lower doses of 2 Gy and 4 Gy, promoting the death of neoplastic cells and protecting normal HaCaT cells.

PBM reduced cell migration and induced cell death

The treatment effect on cell migration showed a lower migration percentage in LED+IR group than in the control and LED alone groups in all cell lines (Fig. 2). The AO/EB cell death assay on SCC9, Cal-27, and HaCaT cell lines showed that in all cell lines, the number of cell deaths was higher in PBM+IR (Fig 3). There was no significant difference between the groups exposed to the associated treatment, even with an increase in IR dosages, only in the group irradiated with 2 Gy in the Cal-27 cell line. This indicates that cells pre-exposed to PBM are radiosensitized.

PBM reduced cell survival fraction after X-ray IR in clonogenic survival assay

In the clonogenic formation analysis, the cells in the PBM group showed lower colony-forming ability than did those in the control and LED+0 Gy groups. For the cell lines Cal-27 and HaCaT, there were no significant difference in the groups treated with 4 Gy and 6 Gy. For the A431 cell line also did not significantly differ according to the IR dose in LED+IR group (2, 4, and 6 Gy). However, the SCC9 cell line showed decreasing clone formation with increasing IR dose. These results indicated that PBM at 300 J/cm^2 led to radiosensitization of the cells and enhanced the cytotoxic response to IR (Fig 4).

PBM induced intracellular ROS production

Fluorescent microscopy showed that the PBM+IR groups showed a significantly higher intracellular ROS production in SCC9, Cal-27, and HaCaT cells at all radiation doses than did the control and LED+0 Gy groups (Fig 5). However, the fluorescence intensity of ROS production in the HaCaT cell line was lower than SCC9 and Cal-27 cell lines,

indicating a protective effect of PBM on normal cells. Finally, there was no significant difference among the groups treated with LED+IR, even with an increase in IR dose, consistent with the other analyses. This supports that PBM leads to radiosensitivity in the cells.

DISCUSSION

Radiotherapy is the standard adjuvant treatment for patients with OSCC, and it is also associated with several complications resulting from damage to radiosensitive tissues near the tumor [14, 15]. Due to this, many studies have aimed to develop therapies that minimize the damage caused by radiotherapy without enhancing radioresistance characteristics in cancer cells, in addition to protecting normal cells located close to the tumor [16-18]. The present study proposes to investigate the cellular response of oral squamous cell carcinoma submitted to radiotherapy and pre-exposed to low-level phototherapy. Our findings suggested that PBM at a 300 J/cm^2 can be a promising radiosensitizing agent in a HNSCC and also to protect normal cells.

LLLT has attracted attention in many clinical fields, with a new generation of LEDs that can irradiate large targets. This therapy activates or inhibits physiological, biochemical, and metabolic processes [32]. PBM is mainly used for pain control, accelerated wound healing, inflammation, tissue regeneration, and edema. The current study showed that both laser and LED PBM achieve similar cellular response with or without IR. This indicates the superiority of LED over laser as it can be used for a larger surface area. A study has reported this advantage of LLLT with an LED system over a laser source. LED-based systems cover large planar arrays, irradiating a larger body area hands-free than the point-by-point application of a laser system. In addition, many different cell types can be simultaneously targeted [22] .

PBM can modulate several cellular responses. Studies have shown that light therapy can protect cells against ionizing radiation [21, 33]. Furthermore, LLLT has a biphasic dose-response pattern at low fluences, and PBM leads to cellular biostimulation. Meanwhile, it has an inhibitory effect at higher energy densities, interfering with the cell cycle and inhibiting cell proliferation [34, 35]. These findings support our results. The dose-response curve showed that a higher energy density resulted in decreased cell death, that is, it had an inhibitory effect on cell death. In addition, a study suggests that larger fluences could increase cell radiosensitization in a paradoxical manner [19].

In the current study, PBM increased the benefit of IR by reducing the proliferative activity of SCC9, Cal-27, and A431, and protected normal keratinocytes at low radiation doses. A study that used PBM with IR at an energy density of 20 J/cm^2 found no influence on cell proliferation in HeLa cells [33]. In another study, the proliferation rate of the SCC cell line decreased with 4 Gy IR and PBM at 3 or 6 J/cm^2 from 72h and 120h. Further, HaCaT proliferation decreased only in PBM delivered at 6 J/cm^2 after 120h [36]. Ramos et al. evaluated the Effect of PBM on fibroblasts and human breast cancer cells and found an increase in the rate of fibroblast proliferation when irradiated with PBM at 90 J/cm^2 and 150 J/cm^2 plus 2.5 Gy. Meanwhile, in breast cancer cells irradiated at 2.5 Gy and 10 Gy, none of the PBM doses influenced proliferation of breast control group at 0 Gy [37].

LLLT alters the expression of several proteins responsible for various cellular mechanisms such as cell cycle regulation, migration, and apoptosis [32]. Thus, we analyzed the migration and apoptosis of cell lines 24 h after treatment, and the results showed that PBM with IR reduced cell migration and induced cell death, there was no significant difference between the groups exposed to the associated treatment, even with an increase in IR dosages. This indicates that cells pre-exposed to PBM are radiosensitized. A study that evaluated the Effect of PBM on cancer cells previously treated with IR reported that PBM at 6 J/cm^2 increased the migration of non-irradiated HaCaT and SCC cells. Meanwhile, no effect was observed at 3 J/cm^2, suggesting that the biological effect depends on the PBM dose [36]. Regarding cell death, a study with HeLa cells showed that PBM alone at a 20 J/cm^2 energy density was unable to efficiently induce apoptosis. After 6 Gy X-ray irradiation alone, the apoptotic cell fraction significantly increased, but the combined treatment with PBM and X-ray radiation induced a higher apoptotic cell fraction. The study concluded that PBM and IR could additively increase apoptosis [33]. Therefore, it appears that the rate increase of cell death was due to the additive contribution of PBM rather than any synergistic combination.

The present study showed that PBM reduced the cell survival fraction after X-ray irradiation in all cell lines, and there was no difference between the groups as the IR dose increased. These results were similar to those of a study in which a clonogenic radiation survival assay showed that the application of LLLI at 685 nm and 5 J/cm^2 prior to IR could significantly inhibit the clonogenic growth of HeLa cells. In contrast, the survival curves of NIH 3T3 cells pre-exposed to LLLI at 1 J/cm^2 and 5 J/cm^2 energy densities with 685 nm were the same as those for the control group [19]. Another study reported that the survival fraction of HeLa cells treated with 4 Gy and 6 Gy doses of X-ray radiation was

significantly decreased when pre-treated with PBM at 20 J/cm^2 [33]. Collectively, these results support that PBM enhances the cytotoxic response to IR due to the radiosensitization of cancer cells.

Cells have photoacceptors that absorb photons emitted from lasers or other light sources, causing oxidative stress at the cellular level and generating a burst of intracellular ROS [38]. Low-intensity ROS, produced by lower energy densities of PBM, can stimulate beneficial processes, such as cell proliferation, differentiation, and viability [21]. However, a high intracellular concentration of ROS caused by a high dose of light can induce proapoptotic effects and inhibit proliferation in vitro [39, 40]. Apoptosis is induced by inactivation of the Akt/GSK3beta signaling pathway [34, 40]. Furthermore, apoptosis can be directly initiated from mitochondrial ROS generation following high-energy PBM [38, 41]. Therefore, we performed ROS assay to further confirm the mechanism of cell death. The results showed that PBM was associated with IR-induced intracellular ROS production, as evidenced by the increase in ROS accumulation in SCC9 and Cal-27 cells. Notably, the intensity of ROS in the HaCaT cell line was lower than that in the SCC9 and Cal-27 cell lines, which can be explained by the protective Effect of PBM in normal cells. A study with a HeLa cell line showed that PBM before IR led to an increase in ROS intensity in a dose-dependent manner, and treatment with higher energy densities led to higher intracellular ROS intensity [33].

An important factor is the mechanism by which LLLI exerts its various effects and the choice of appropriate parameters such, wavelength, power density, energydensity, pulse structure, and treatment timing, plays an essential role in the result of the treatment. However, further studies are necessary in order to find an optimum way of applying such therapy, and increase its advantages [42, 43].

In conclusion, PBM at 300 J/cm^2 is a promising radiosensitizing strategy to reduce the radiation dose and avoid the intolerable side effects associated with radiotherapy in HNSCC, thus increasing the probability of successful treatment. The number of studies that evaluate the response of tumor cells exposed to PBM and, later, to ionizing radiation remains limited. Therefore, it is necessary to conduct further studies in vitro, with future in-vivo and clinical investigation to explore the details and elucidate the molecular mechanisms involved in radiosensitization caused by PBM.

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Figure Legends

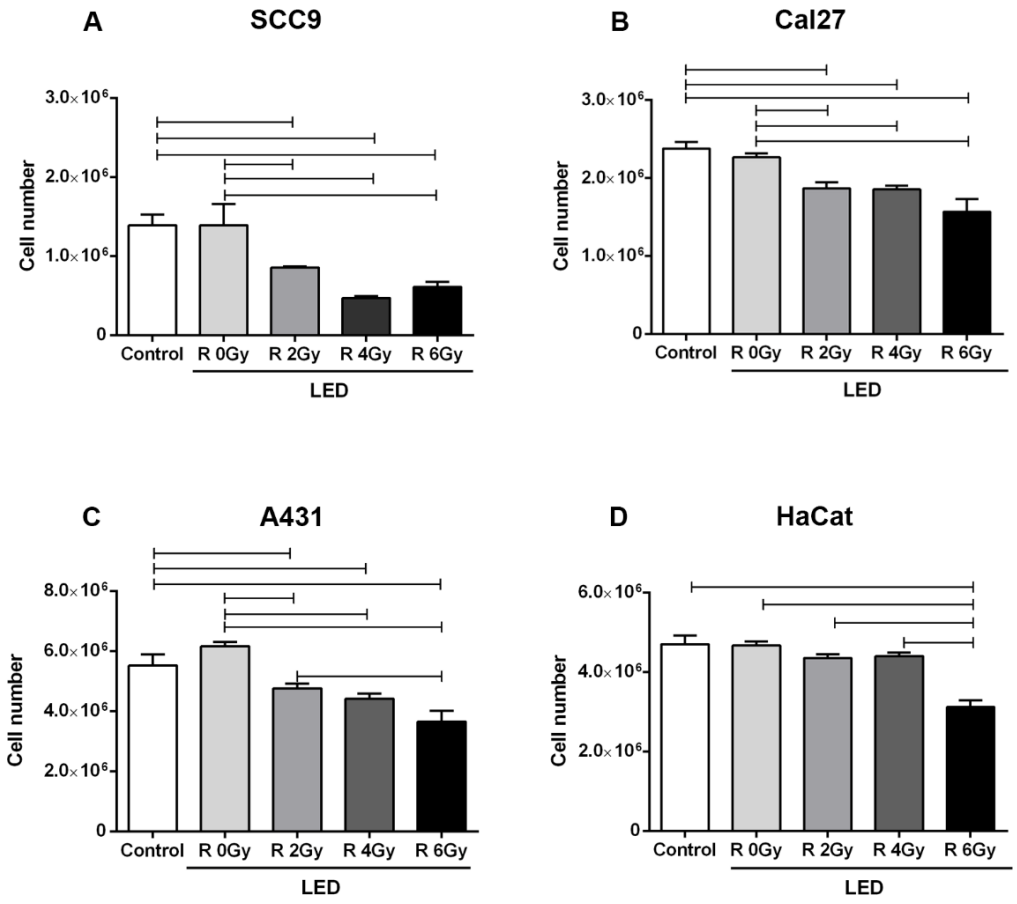


Figure 1. Effect of PBM on cell proliferation
Proliferation rate of (a) SCC9 cells, (b) Cal-27 cells, (c) A431 cells, and (d) HaCaT cells. The horizontal bars represent the significant differences between the groups (control, LED + IR 0 Gy, LED + IR 2 Gy, LED + IR 4 Gy, and LED + IR 6 Gy). LED light source: 300 J/cm^2).

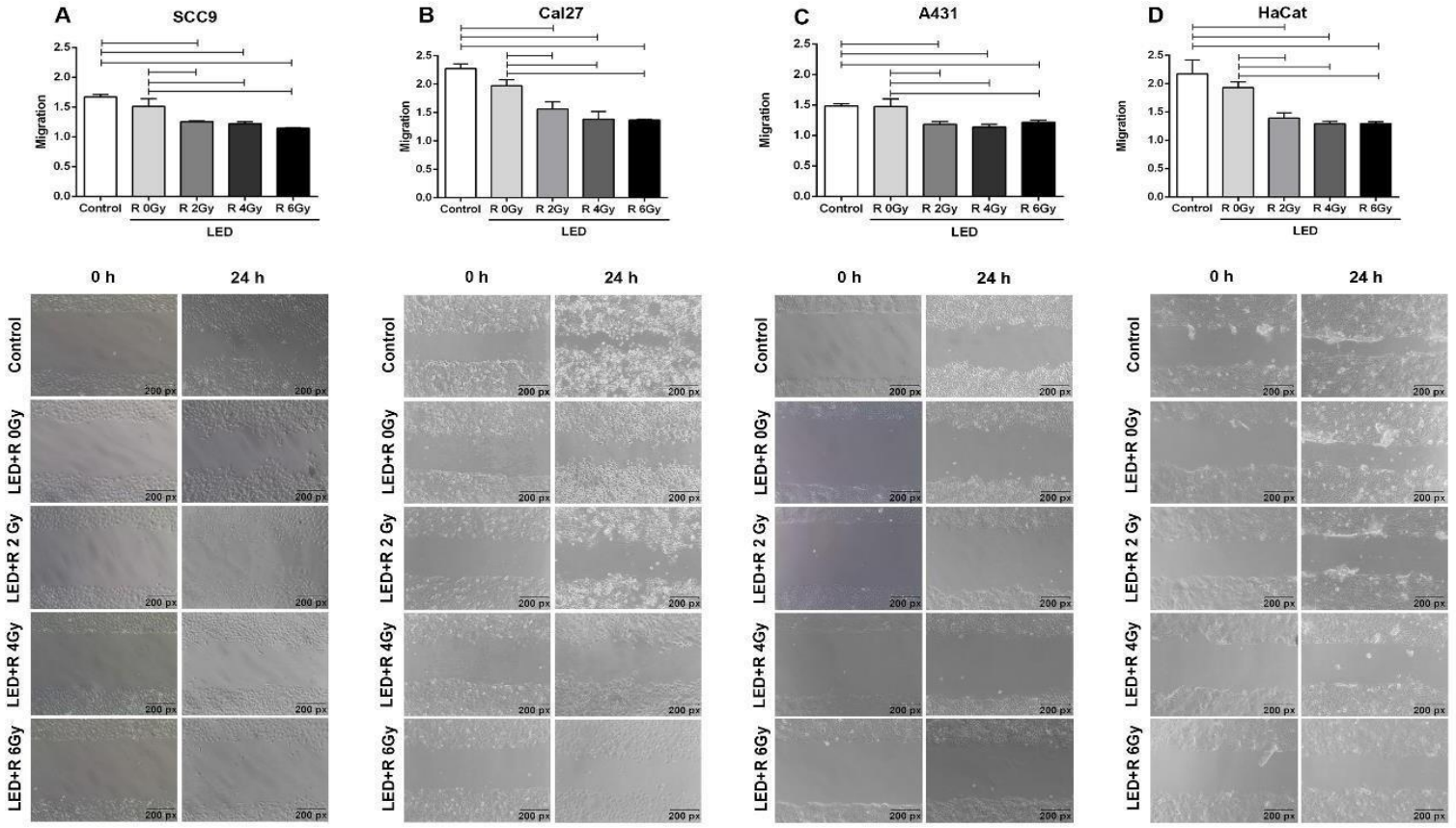


Figure 2. Cell migration rate after treatment with PBM.
Effect on cell migration rate and microscopic representation of a wound before (0 h) and after (24 h) treatment in (a) SCC9 cells, (b) Cal-27 cells, (c**)** A431 cells, and (d) HaCaT cells. The horizontal bars represent significant differences between the groups ($p<0.05$).

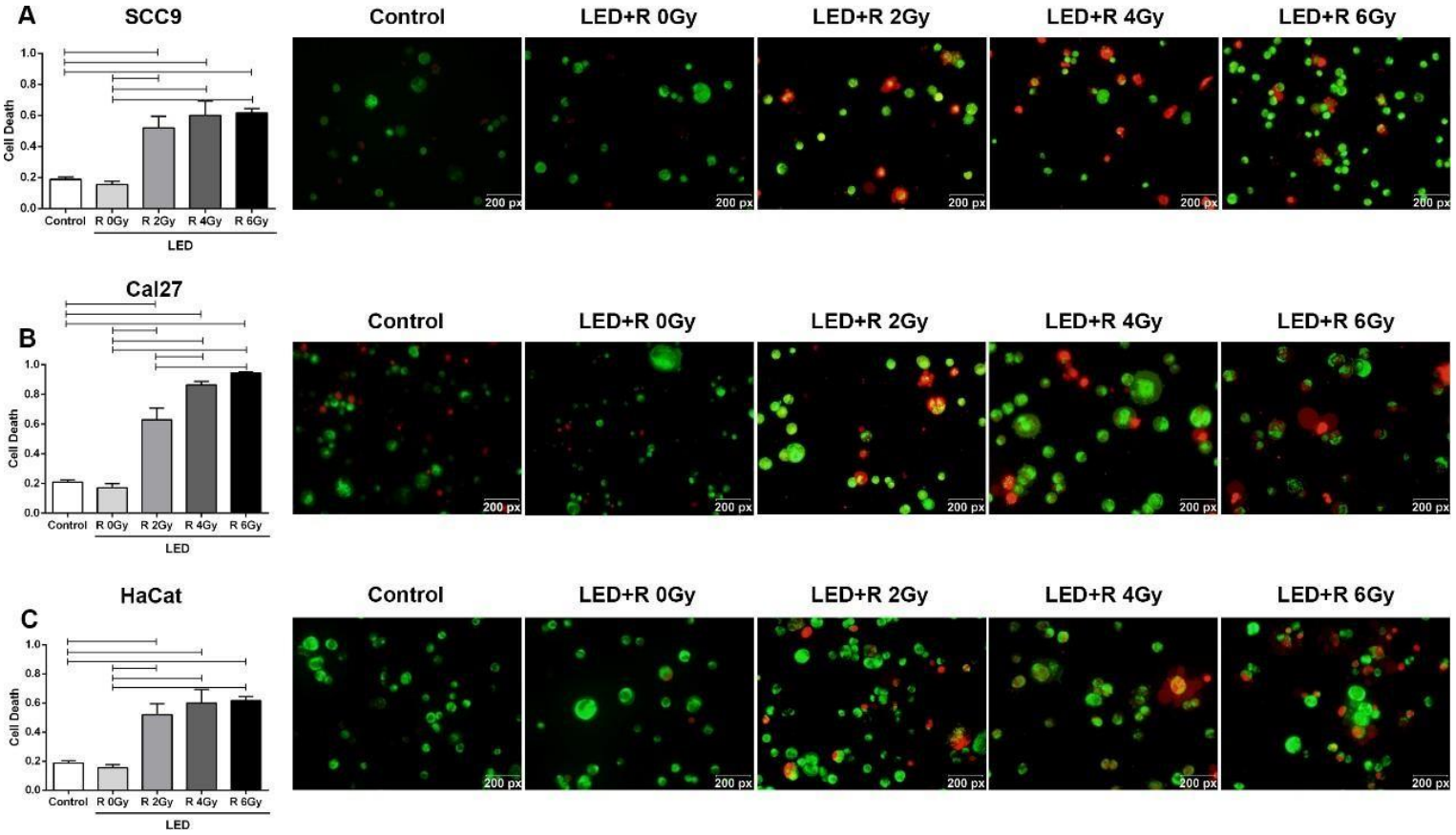


Figure 3. Posttreatment AO/EB double staining of SCC9, Cal-27, and HaCaT cell lines for cell death assessment.
Graph and fluorescence microscopy representation of dead and viable (a) SCC9 cells, (b) Cal-27 cells, and (c**)** HaCaT cells. Viable cells are shown in green, whereas dead cells are shown in red and orange. The horizontal bars represent significant differences between the groups ($p<0.05$).

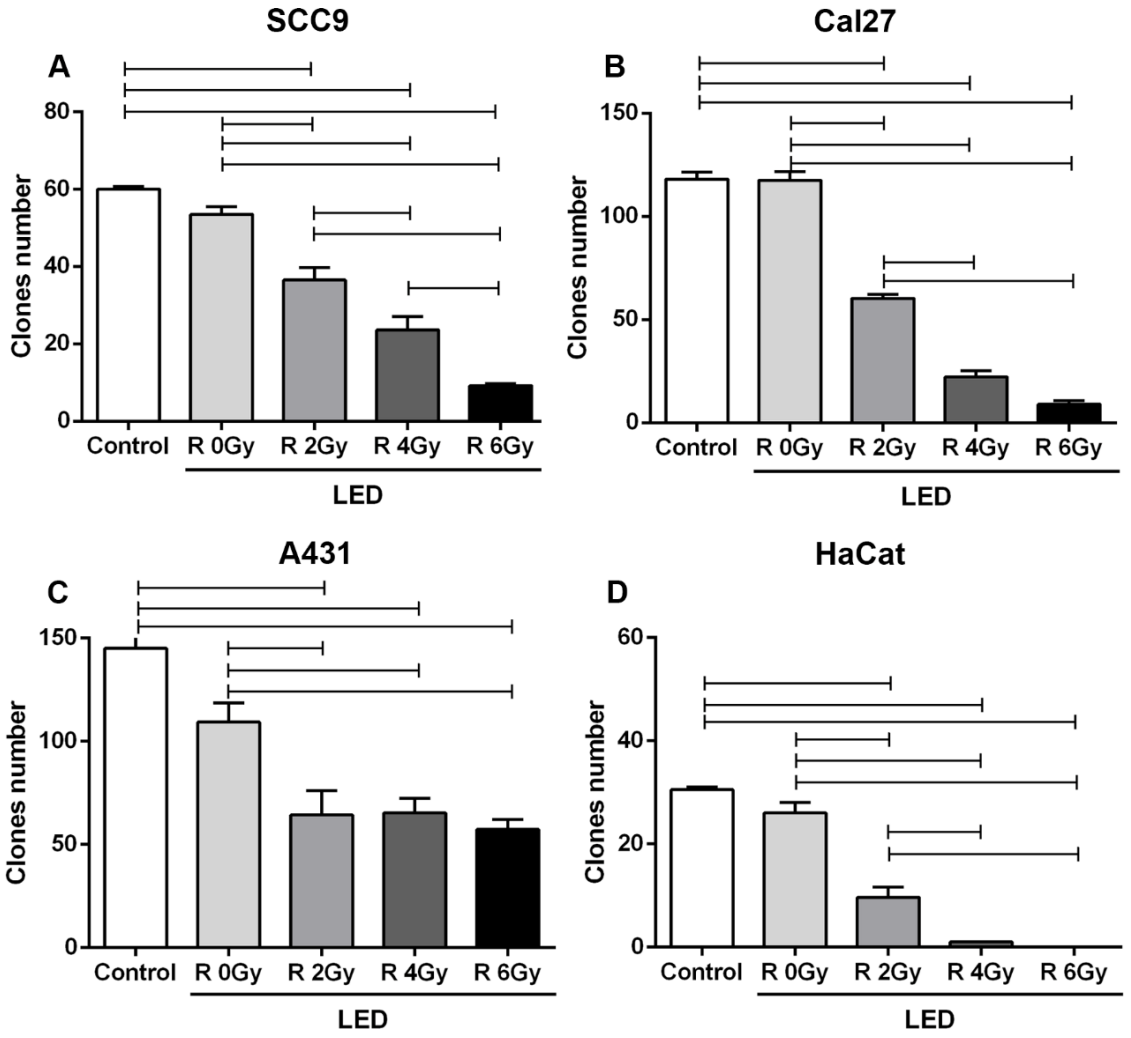


Figure 4. Clonogenic assay of SCC9, Cal-27, A431, and HaCat cells.
Cluster indicating clonogenic formation of (a) SCC9 cells, (b) Cal-27 cells, (c) A431 cells, and (d) HaCaT cells. Statistical significance is determined using one-way ANOVA.The horizontal bars represent the significant difference between the groups ($p<0.05$).

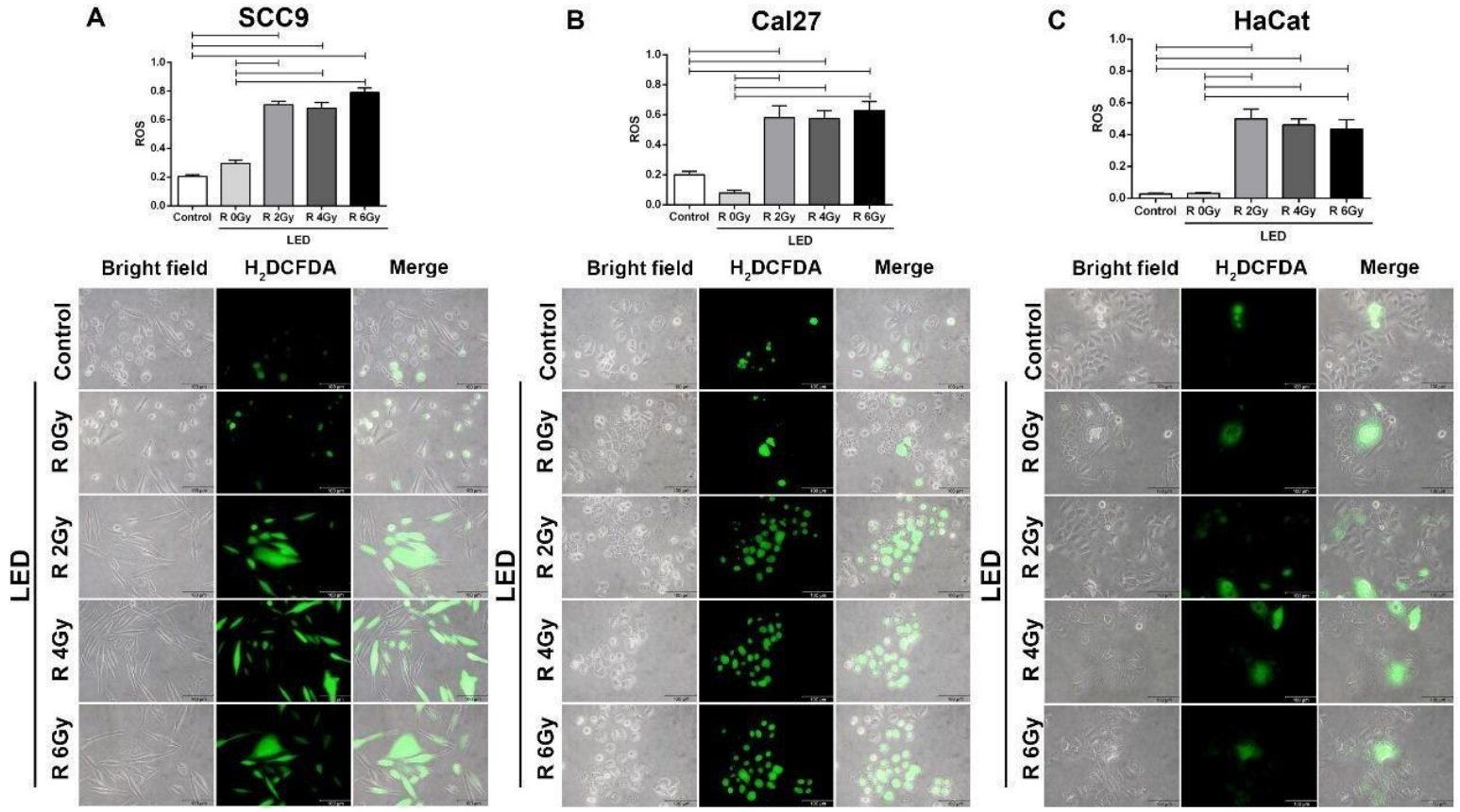
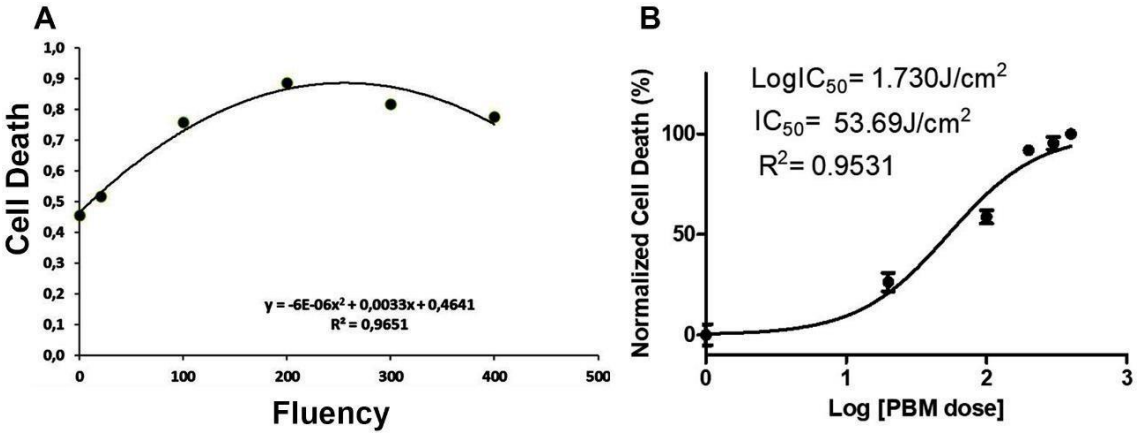
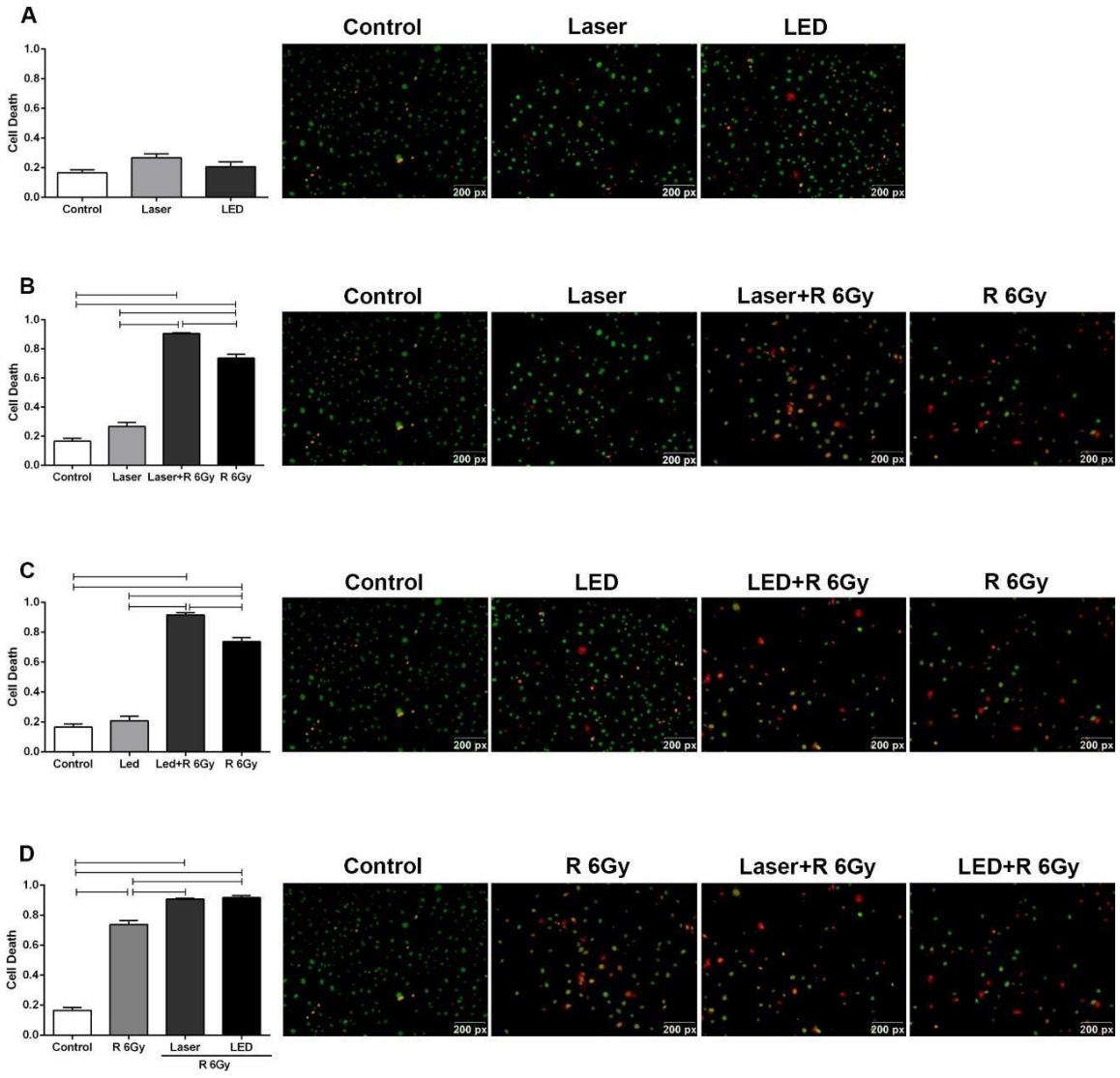


Figure 5. Detection of intracellular ROS in SCC9, Cal-27, and HaCat cells treated with PBM and IR at different doses.
Graph and representative microscopic images of ROS generation in (a) SCC9 cells, (b), Cal-27 cells, and (c) HaCaT cells. The results are shown as the mean percentage of fluorescent hotspots in microscopic fields, considering the total fluorescent cells/cell ratio. Significance is determined using one-way ANOVA ($p<0.05$). The horizontal bars represent significant differences between the groups.

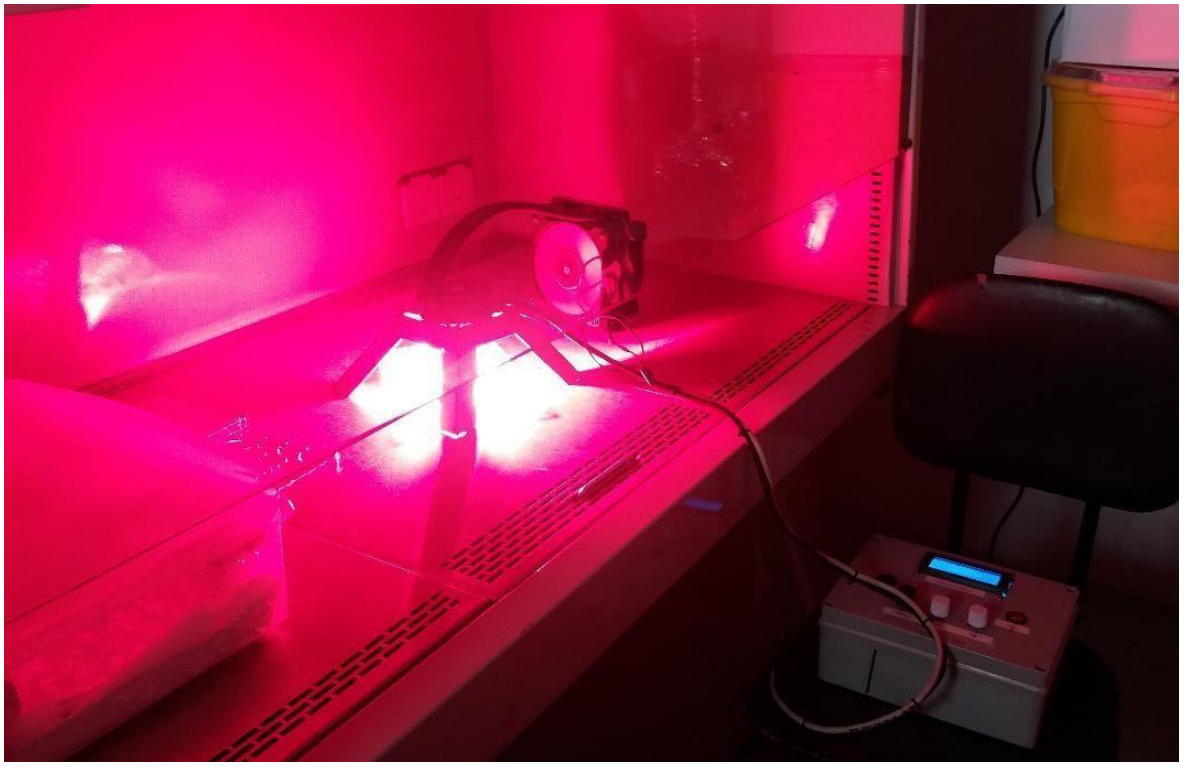
Supplementary Information



Supplementary Information 1 (SI 1). (a) Dose-response curve obtained with the AO/EB cell death assay for SCC9 cells treated with PBM at different energy densities (0, 20, 100, 200, 300, and 400 J/cm^2). (b) IC50 graph calculated from cell death analysis data.



Supplementary Information 2 (SI 2). (a) Graph and fluorescence microscopy representation of cell death analysis (AO/EB) to compare between SCC9 cells irradiated with laser and with LED at a fluence of 300 J/cm^2 (control, laser, LED). (b) Graph and fluorescence microscopy representation of cell death analysis (AO/EB) to evaluate the cellular response of the groups to treatment (control, laser, laser + R 6 Gy, and R 6 Gy). (c) Graph and fluorescence microscopy representation of cell death analysis (AO/EB) to evaluate the cellular response of the groups to treatment (control, LED, LED + R 6 Gy, and R 6 Gy). (d) Graph and fluorescence microscopy representation of cell death analysis (AO/EB) to evaluate the cellular response of the groups to treatment (control, R 6 Gy, laser + R 6 Gy, and LED + R 6 Gy). Light source: 300 J/cm^2.



Supplementary Information 3 (SI 3). Picture of a cell culture plate being irradiated with the LED device (102.2 mW/cm^2, 49 min, 7 cm, 300.37 J/cm^2).

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ANEXOS

ANEXO A- Parecer do Comitê de Ética e Pesquisa

UNIVERSIDADE ESTADUAL DE MONTES CLAROS - UNIMONTES

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito da fototerapia de baixa intensidade associado à radioterapia no carcinoma de células escamosas bucal

Pesquisador: ANGELINY TAMARANA LIMA TABOSA

Área Temática:

Versão: 2

CAAE: 45230821.3.0000.5146

Instituição Proponente: Programa de Pós-Graduação em Ciências da Saúde

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.726.497

Apresentação do Projeto:

As informações descritas nos campos "apresentação do projeto", "objetivo da pesquisa" e "avaliação dos riscos e benefícios" foram retiradas do arquivo PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1715715.pdf de 18/05/2021) e/ou do projeto detalhado (versão de 18/05/2021) que foi anexado à Plataforma.

Estudo experimento laboratorial, que pretende fazer análises fenotípicas e genotípicas de células imortalizadas de carcinoma escamoso bucal e de células saudáveis do tecido epitelial escamoso, que serão coletadas de 10 pacentes e cultivadas em meio próprio. Propõe-se investigar a resposta celular do carcinoma de células escamosas oral submetidas à radioterapia e pré-expostas à fototerapia de baixa potência. Será investigada a hipótese de que as células cancerígenas se tornam mais radiosensíveis quando expostas à fototerapia de baixa potência antes da radiação ionizante. As análises fenotípicas que serão utilizadas são os testes de migração e apoptose. Já as análises genotípicas são PCR em tempo real e Imuno-histoquímica com contagem dos marcadores. Esses testes serão realizados nas linhagens celulares imortalizadas e primárias.

Objetivo da Pesquisa:

Segundo os pesquisadores.

Objetivo Primário:

Investigar a resposta celular do carcinoma de células escamosas bucal e de células saudáveis

Endereço: Av.Dr Rui Braga s/n-Camp Univers Profª Darcy Rib
Bairro: Vila Mauricéia **CEP:** 39.401-089
UF: MG **Município:** MONTES CLAROS
Telefone: (38)3229-8189 **Fax:** (38)3229-8193 **E-mail:** smelocosta@gmail.com

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Continuação do Parecer: 1.726.497

submetidas à radioterapia e pré-expostas à fototerapia de baixa intensidade.

Objetivo Secundário:

Analisar a resposta celular ao tratamento associado através da utilização de diferentes doses da radiação ionizante;

Avaliar a expressão das proteínas AKT1 e TP53 nas diferentes sessões do tratamento;

Verificar correlações entre a análise do RT-PCR nos diferentes grupos.

Avaliação dos Riscos e Benefícios:

Conforme os pesquisadores o projeto envolve os seguintes riscos e benefícios:

Como os participantes da pesquisa (incluindo a respectiva amostra de tecido e dados clínicos), serão provenientes do Biobanco de Materiais Biológicos Humano do Norte do Estaco de Minas Gerais (Biobanco Institucional-UNIMONTES/Registro CONEP:B- 013), os riscos serão aqueles apresentados no TCLE do biobanco, devidamente esclarecidos e aceitos pelo indivíduo doador quando da assinatura do TCLE:- "...Os riscos quanto à cessão do material estão relacionados ao tipo de procedimento realizado pela equipe médica, necessário para diagnóstico e tratamento que deverão ser claramente esclarecidos para você pela equipe (ANEXO II). A coleta de material biológico procedente do Biobanco envolve apenas o excedente material biológico proveniente desse procedimento médico. Visando à confidencialidade das informações, os indivíduos receberão uma codificação numérica e apenas o coordenador do projeto terá a posse da identificação dos nomes dos participantes e sua respectiva codificação.

Benefícios:

A relevância deste estudo fundamenta-se na avaliação de que o FTBI possa ser um agente radiossensibilizante promissor no CCEB com o intuito de diminuir a dose de radiação administrada e evitar os efeitos colaterais intoleráveis associados à radioterapia aumentando, assim, as chances de um tratamento bem-sucedido.

Comentários e Considerações sobre a Pesquisa:

O carcinoma de células escamosas bucal (CCEB) é o tipo mais comum de neoplasia maligna oral (90%). É a principal causa de morbidade e mortalidade em pacientes com câncer de cabeça e pescoço e, por isso, é considerado um crítico problema de saúde pública. Dentre as modalidades básicas de tratamento para o CCEB, a radioterapia desempenha um papel crítico na gestão terapêutica do câncer através da radiação ionizante. O resultado da radioterapia é frequentemente limitado pela radiorresistência das células cancerígenas e efeitos colaterais em células e tecidos saudáveis. A ciência busca formas de melhorar os efeitos terapêuticos da radioterapia, uma dessas tentativas é a proteção seletiva de células normais sem efeitos de radiorresistência em células

Endereço: Av.Dr Rui Braga s/n-Camp Univers Profª Dercy Rib
Bairro: Vila Mauricéia CEP: 39.401-089
UF: MG Município: MONTES CLAROS
Telefone: (38)3229-8180 Fax: (38)3229-8103 E-mail: smelcosta@gmail.com

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Continuação do Parecer: 4.720.497

cancerosas. Dessa forma, esse estudo se justifica e está fundamentado na necessidade de avaliar os efeitos modificadores do FTBI tanto no carcinoma de células escamosas bucal quanto em células normais expostos a radiação ionizante.

Considerações sobre os Termos de apresentação obrigatória:

Todos os documentos de caráter obrigatório foram apresentados e estão adequados: folha de rosto, TCLE (modelo utilizado para coleta de materiais biológicos pelo Biobanco de Materiais Biológicos do Norte de Minas Gerais), projeto detalhado e declaração de aceite do Biobanco para a concessão das amostras e dados clínicos para a execução do projeto.

Recomendações:

1 - Apresentar relatório final da pesquisa, até 30 dias após o término da mesma, por meio da Plataforma Brasil, em "enviar notificação".

2 - O CEP da Unimontes deverá ser informado de todos os efeitos adversos ou fatos relevantes.

3 - Caso a pesquisa seja suspensa ou encerrada antes do previsto, o CEP da Unimontes deverá ser comunicado, estando os motivos expressos no relatório final a ser apresentado.

4 - O TCLE impresso deverá ser obtido em duas vias, uma ficará com o pesquisador e a outra com o participante da pesquisa.

5 - Em conformidade com a Carta Circular nº. 003/2011/CONEP/CNS e Resolução 466/12, faz-se obrigatório a rubrica em todas as páginas do TCLE pelo participante de pesquisa ou responsável legal e pelo pesquisador.

6 - O registro do TCLE pelo participante da pesquisa deverá ser arquivado por cinco anos, conforme orientação da CONEP na Resolução 466/12: "manter os dados da pesquisa em arquivo, físico ou digital, sob sua guarda e responsabilidade, por um período de 5 anos após o término da pesquisa".

Conclusões ou Pendências e Lista de Inadequações:

Não foram identificados óbices éticos nesse estudo.

Considerações Finais a critério do CEP:

O projeto respeita os preceitos éticos da pesquisa envolvendo seres humanos, sendo assim somos favoráveis à aprovação do mesmo.

Endereço: Av.Dr Rui Braga s/n-Camp Univers Profª Darcy Rib
Bairro: Vila Mauricéia CEP: 39.401-089
UF: MG **Município:** MONTES CLAROS
Telefone: (38)3229-8180 **Fax:** (38)3229-8103 **E-mail:** smelocosta@gmail.com

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Plataforma Brasil

Coordenação do Parecer: 4.720.487

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BASICAS_DO_PROJETO_1715715.pdf	18/05/2021 19:47:31		Aceito
Outros	Carta_resposta_CEP.docx	10/05/2021 19:45:43	ANGELINY TAMIARANA LIMA TABOSA	Aceito
Orçamento	Orcamento.docx	18/05/2021 19:38:58	ANGELINY TAMIARANA LIMA TABOSA	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.docx	18/05/2021 19:38:06	ANGELINY TAMIARANA LIMA TABOSA	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_CEP.docx	18/05/2021 19:37:17	ANGELINY TAMIARANA LIMA TABOSA	Aceito
Cronograma	Cronograma.docx	18/05/2021 19:36:19	ANGELINY TAMIARANA LIMA TABOSA	Aceito
Folha de Rosto	Folha_rosto_CEP.pdf	26/03/2021 20:36:37	ANGELINY TAMIARANA LIMA TABOSA	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

MONTES CLAROS, 21 de Maio de 2021

Assinado por:
SIMONE DE MELO COSTA
(Coordenador(a))

Endereço: Av.Dr.Rui Braga s/n-Camp Univers Pro* Darcy Rib
Bairro: Vila Mauricéia **CEP:** 39.401-089
UF: MG **Município:** MONTES CLAROS
Telefone: (38)3229-8100 **Fax:** (38)3220 8109 **E-mail:** emelocosta@gmail.com