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EFEITOS DA FOTOBIOMODULAÇÃO POR DIODOS EMISSORES DE LUZ-LED EM CÉLULAS TUMORAIS

Santa Maria, RS
2020

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Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Reabilitação Funcional, da Universidade Federal de Santa Maria (UFSM, RS), como requisito para obtenção do título de **Mestre em Reabilitação Funcional**.

Orientador: Profa. Dra. Hedioneia Maria FolettoPivetta
Co-orientador: Prof. Dr. Alencar Kolinski Machado

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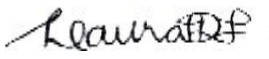

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RESUMO

EFEITOS DA FOTOBIOMODULAÇÃO POR DIODOS EMISSORES DE LUZ-LED EM CÉLULAS TUMORAIS

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ORIENTADORA: Profa. Dra. Hedioneia Maria Folleto Pivetta
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Introdução: A Fotobiomodulação é recurso amplamente utilizado no tratamento de diversas condições clínicas e nas mais diversas áreas. No entanto, na oncologia mamária sua aplicação ainda é bastante limitada por diversas razões, especialmente pela ausência de familiaridade com a terapia e pela incerteza quanto aos mecanismos de ação celular, molecular e tecidual. **Objetivo:** Verificar os efeitos da fotobiomodulação por diodos emissores de luz (LED) azul e vermelho em células de linhagem tumoral de mama. **Metodologia:** Projeto de caráter experimental *in vitro* em que células tumorais da linhagem MCF7 de câncer de mama serão expostas à fotobiomodulação por LED, com dois comprimentos de onda distintos (658 ηm e 470 ηm) e serão acompanhadas por 1 (um), 2 (dois) ou 3 (três) dias, para assim verificar o potencial modulatório da irradiação. Após o período de irradiação, as células foram analisadas quanto aos parâmetros relacionados a viabilidade e proliferação celular, produção indireta de óxido nítrico (ON) e espécies reativas de oxigênio (EROs). **Resultados:** Não houve aumento da proliferação celular em nenhum dos comprimentos de onda. Três doses cumulativas ocasionaram redução da viabilidade celular. A luz azul promoveu redução na produção de ON e EROs. Para a luz vermelha uma única irradiação a 6 J/cm² foi capaz de promover aumento nas taxas de ON e duas doses cumulativas a 19J/cm² incrementou a formação de EROs. **Conclusão:** Nossos achados demostraram que a irradiação com a luz azul e a vermelha para 6 J/cm² e 19 J/cm² não promoveram aumento da proliferação celular, ao contrário, três doses cumulativas induziram ao aumento da mortalidade celular.

Palavras-chave: Fotobiomodulação. Neoplasias da mama. Proliferação de células.

ABSTRACT

EFFECTS OF PHOTOBIOMODULATION BY LED LIGHT-EMITTING DIODES IN TUMOR CELLS

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Introdutction: Photobiomodulation is a widely used resource in the treatment of various clinical conditions and most diverse areas. However, its application in breast oncology is still quite limited for several reasons especially due to the lack of familiatity with the therapy and the uncertainty regarding the mechanisms of cellular, molecular and tissue action. **Objective**: To verify the effects of photobiomodulation by blue and red LED light-emitting diodes on the breast tumor lineage cells. **Methods:** In vitro experimental design in which breast cancer MCF7 strain tumor cells will be exposed to LED photobiomodulation with two distinct wavelengths (658 ηm and 470 ηm) and be accompanied by 1 (one), 2 (two) or 3 (three) days to verify the modulatory potential of irradiation. After the irradiation period, the cells were analyzed for parameters related to cell viability, proliferation, indirect production of nitric oxide (NO) and reactive oxygen species (ROS). Results: There was no increase in cell proliferation at any of the wavelengths. Three cumulative doses reduced cell viability. The blue light promotes a reduction in the production the NO and ROS. For red light, a single irradiation at 6J/cm² was able to promote an increase in NO rates and two cumulative doses at 19J/cm² increased the formation of ROS. Conclusion: Our findings showed that irradiation with blue and red light to 6J/cm² and 19J/cm² did not promote an increase in cell proliferation, on the contrary, three cumulative doses induced an increase in cell mortality.

Keywords: Photobiomodulation. Breast Neoplasms. Cell proliferation.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ATP – Adenosina trifosfato (ATP)
CM – Câncer de mama
DCF – Diclorodiidrofluoresceína
DCFH – Diclorofluoresceína
DCFH-DA – Diclorofluoresceínadiacetato
dsDNA – DNA dupla-fita
EROs – Espécies reativas de oxigênio
FBM – Fotobiomodulação
HER2 – Fator de crescimento humano epidérmico receptor-2
LASER – Light Amplification by Stimulated Emission of Radiation
LED – Light Emitting Diode
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ON – Óxido Nítrico
TFBM – Terapia por fotobiomodulação
UFN – Universidade Franciscana
UFSM – Universidade Federal de Santa Maria

SUMÁRIO

1 INTRODUÇÃO:

A fotobiomodulação (FBM) é o nome dado à ação da luz nos tecidos biológicos capaz de tratar e estimular múltiplos processos fisiológicos e reparar danos causados por lesões e doenças (ROBIJNS et al., 2018; HAMBLIN; NELSON; STRAHAN, 2018).

Durante muito tempo a terapia por FBM ficou conhecida como "terapia a laser de baixa intensidade", porque os primeiros dispositivos utilizados foram o Laser de rubi (694 ηm) e o Laser de He-Ne (633 ηm), no entanto, após decisão consensual, a terminologia foi substituída pelo termo atual, pois o termo "baixo nível" era muito subjetivo e porque outras fontes além dos lasers podem ser igualmente utilizadas (HAMBLIN, 2017).

A terapia por FBM utiliza fontes de luz monocromática, coerentes ou não, e de baixa intensidade, como os Lasers (*Light Amplification by Stimulated Emission of Radiation*) e os LEDs (*Light Emitting Diode)* que são absorvidas a nível celular pelos cromóforos endógenos resultando em reações atérmicas, eventos fotofísicos e fotoquímicos em várias escalas biológicas (ROBIJNS et al., 2018; DE FREITAS; HAMBLIN, 2016; KIRO; HAMBLIN; ABRAHAMSE, 2017).

Acredita-se que os efeitos modulatórios da FBM estão intimamente ligados ao comprimento de onda empregado e variam desde a apoptose à proliferação celular. O efeito inibitório tem sido associado ao estresse oxidativo ocasionado pela produção de espécies reativas de oxigênio (EROs), enquanto que o efeito proliferativo está relacionado ao aumento da produção de adenosina trifosfato (ATP) (KIRO; HAMBLIN; ABRAHAMSE, 2017).

A FBM é recurso amplamente utilizado no tratamento de diversas condições clínicas e nas mais diversas áreas (HEISKANEN; HAMBLIN, 2018). No entanto, na oncologia mamária sua aplicação ainda é bastante limitada (ROBIJNS et al., 2017). Isto ocorre por diversas razões, especialmente pela ausência de familiaridade com a terapia (ROBIJNS et al., 2017) e pela incerteza quanto aos mecanismos de ação celular, molecular e tecidual (DE FREITAS; HAMBLIN, 2016).

Além disso, a preocupação com a segurança de pacientes oncológicos é recorrente no meio científico (ZECHA et al., 2016). Estudos experimentais (SPERANDIO et al., 2013; MONTEIRO et al., 2011; HENRIQUES et al., 2014) e uma recente revisão de literatura (SONIS et al., 2016) evidenciaram um aumento na proliferação celular e um incremento da agressividade tumoral após a irradiação com a FBM. Contudo, outras pesquisas demonstram que em tecidos tumorais, a utilização da FBM não ocasionou qualquer efeito mensurável no crescimento do tumor e não propiciou efeito mutagênico, podendo ser utilizado de maneira

segura (KUFFLER, 2016; KLEINPENNING, 2010; MYAKISHEV-REMPEL et al., 2012; KHAN; TANG; ARANY, 2015). Recentemente estudos demonstraram que a terapia com a FBM foi capaz de modular o crescimento tumoral e exercer efeitos anticancerígenos em pacientes oncológicos, sendo considerada uma terapia promissora no controle do câncer (SANTANA-BLANK et al., 2016; HAMBLIN; NELSON; STRAHAN, 2018), além disso, também foi associada a um melhor prognóstico e melhor sobrevida global nesses pacientes (ANTUNES et al., 2017).

Considerando os resultados conflitantes encontrados na literatura científica, a ausência de revisões sistemáticas com metanálises sobre a segurança da FBM em pacientes oncológicos, e ainda, a possibilidade do uso terapêutico desse recurso, suscita-se a seguinte questão norteadora: Quais os efeitos da FBM por LED azul e vermelho em células tumorais da mama?

1.2 OBJETIVOS:

1.2.1 Objetivo geral:

Verificar os efeitos da fotobiomodulação por diodos emissores de luz-LED azul e vermelho em células de linhagem tumoral de mama.

1.2.2 Objetivos específicos:

- Investigar a ação do LED azul e vermelho na viabilidade de células tumorais de mama;
- Investigar os efeitos do LED azul e vermelho na proliferação celular de células tumorais de mama;
- Determinara ação do LED azul e vermelho quanto ao metabolismo oxidativo de células tumorais de mama.

1.3 JUSTIFICATIVA

A fisioterapia é indispensável no processo de reabilitação dos pacientes oncológicos, através do planejamento e da execução de ações que visem a reabilitação física, seja prevenindo complicações ou promovendo a melhora funcional e, por conseguinte melhorando a qualidade de vida desses pacientes.

Atualmente há inúmeros estudos que apoiam a utilização da FBM com o uso do LED na prevenção e tratamento de diversas toxicidades relacionadas ao tratamento oncológico, no entanto, ainda não existe um consenso universal que sustente sua ampla utilização nessa população, ao contrário, ainda há informações contraditórias e perguntas que permanecem sem respostas concretas nessa área do conhecimento, especialmente quanto à segurança desse recurso.

A utilização do LED na oncologia mamária tem apresentado resultados consistentes, especialmente na cicatrização de deiscência de suturas e na prevenção e tratamento da radiodermite, no entanto, a irradiação sobre a área da mama pode expor células tumorais remanescentes aos efeitos bioestimulatórios desse dispositivo. Diante dessa possibilidade e considerando a controvérsia dos dados relativos à segurança desse recurso surgiu a motivação para a realização dessa pesquisa.

Adicionalmente, a área da fisioterapia carece de estudos em que se analisam os processos e os mecanismos de ação dos recursos terapêuticos em modelos *in vitro*, devido ao número reduzido desses estudos em periódicos nacionais (TOLVES et al., 2016), portanto, pesquisas dessa natureza podem contribuir para sanar importantes lacunas do conhecimento na profissão.

Além disso, por se tratar de um recurso não farmacológico e de baixo custo, os resultados desse estudo podem fornecer amparo científico para realização de futuras pesquisas com seres humanos e ampliar as possibilidades terapêuticas da fisioterapia.

Diante disso, faz-se necessário compreender os efeitos que a FBM por LED azul e vermelho exercem em células tumorais da mama.

2 REFERENCIAL TEÓRICO

2.1 CÂNCER DE MAMA

O câncer de mama (CM) é a neoplasia maligna mais comum entre as mulheres, representando aproximadamente 29,5% de todos os tumores malignos diagnosticados nessa população. Para o triênio 2020-2022 estima-se o surgimento de 66.280 novos casos ao ano de CM no Brasil, e destes, 4.050 são esperados para o estado do Rio Grande do Sul (INCA, 2019). Logo, esta doença tem se caracterizado como um importante problema de saúde pública mundial, demandando atenção por parte de pesquisas epidemiológicos, observacionais e experimentais.

O avanço das técnicas de diagnóstico e a implementação de tratamentos cada vez mais eficazes têm contribuído com a melhora do prognóstico e o aumento das taxas de sobrevida observada ao longo dos anos, diante disso, é extremamente necessário o gerenciamento adequado dos efeitos colaterais e das sequelas oriundas do tratamento oncológico para propiciar uma qualidade de vida adequada aos sobreviventes (BODAI; TUSO, 2015).

O tratamento cirúrgico, as terapias adjuvantes e neoadjuvantes empregadas no tratamento do CM podem gerar uma gama de comprometimentos físicos que podem impactar negativamente a funcionalidade e a qualidade de vida das pacientes (MARTINS et al., 2017). Nos últimos anos, vários avanços foram obtidos a fim de aumentar a especificidade do tratamento às células cancerígenas e limitar os efeitos tóxicos aos tecidos adjacentes, no entanto, vários efeitos colaterais debilitantes ainda persistem, tais como: dor, mucosite oral, linfedema, radiodemite, neuropatia periférica, entre outros (PAGLIONI et al., 2019; ROBIJNS et al., 2018; RUPEL, et al., 2018; RUNOWICZ, et al., 2016).

Com base nas evidências atuais, a FBM por LED pode se tornar um recurso promissor para prevenir e gerenciar uma gama de efeitos colaterais ocasionados pelo tratamento oncológico (HAMBLIN; NELSON; STRAHAN, 2018), no entanto, a heterogeneidade de resultados observados quanto à segurança desse recurso na população oncológica suscita cautela quanto a sua utilização (SONIS et al., 2016).

2.2 CÉLULAS MCF-7

A linhagem celular MCF-7 foi inicialmente isolada a partir de um derrame pleural metastático de uma mulher de 69 anos oriundo de um carcinoma mamário (COMSA; CÎMPEAN; RAICA, 2015).

Essa linhagem possui receptores positivos tanto para estrógeno quanto para a progesterona e pertence ao subtipo molecular luminal A (COMSA; CÎMPEAN; RAICA, 2015). De acordo com a literatura, esse subtipo representa aproximadamente 60% dos casos de carcinoma mamário e está relacionado ao melhor prognóstico em relação aos demais (SERRA et al., 2014; CIRQUEIRA et al., 2011).

Ainda de acordo com o perfil imuno-histoquímico, o subtipo luminal A possui um baixo índice de proliferação celular (Ki-67 inferior a 14%) e a superexpressão/amplificação do fator de crescimento humano epidérmico receptor-2 (HER2) negativo (SERRA et al., 2014; CIRQUEIRA et al., 2011). O tempo médio de duplicação dessas células é de aproximadamente 29 horas (Banco de Células do Rio de Janeiro, 2019).

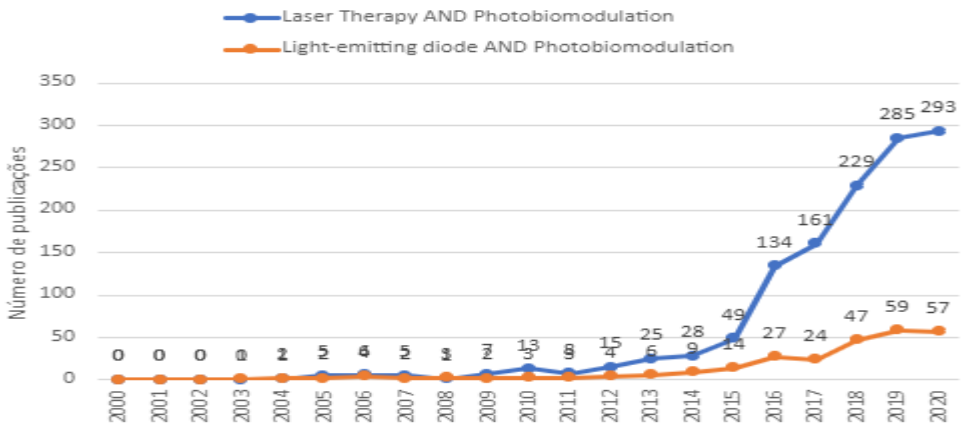
Atualmente, há um considerável apelo para reduzir ou eliminar o uso de animais em pesquisas laboratoriais e por isso estudos com cultivo celular pode ser um caminho a seguir. As células MCF-7 são bem caracterizadas e amplamente utilizadas nos estudos científicos há mais de quarenta anos, e isso, permite aos pesquisadores utilizar esta linhagem celular para ampliar os conhecimentos sobre a patogênese do CM e sobre os protocolos de tratamento através de testes *in vitro* confiáveis (COMSA; CÎMPEAN; RAICA, 2015).

2.3 FOTOBIOMODULAÇÃO

A utilização da FBM completou o seu 50º ano de utilização. A FBM foi descoberta em 1967 por Endre Mester na *Semmelweis* na *Medical University* na Hungria (MESTER et al., 1967). Mester estava tentando reproduzir um experimento realizado por Paul McGuff, em Boston, EUA, que utilizou o laser de Rubi recém descoberto para curar tumores malignos em ratos (HAMBLIN, 2016). Entretanto, apesar de não conseguir curar nenhum tumor com o feixe de laser de baixa intensidade, Mester observou um aumento na taxa de crescimento de pelos e a melhora na cicatrização das lesões ocasionadas pela implantação cirúrgica dos tumores. Este foi, portanto, o primeiro indício de que o laser de baixa intensidade poderia ter aplicações benéficas na medicina (HAMBLIN, 2016).

Conforme o gráfico abaixo (figura 1) pode-se dizer que houve um aumento considerável no número de publicações de artigos científicos envolvendo o laser e o LED na base de dados *National Library of Medicine* (PubMed), o que denota o crescente interesse da comunidade científica sobre a temática ao longo dos anos. Além disso, há ainda 166 ensaios clínicos cadastrados com o termo *"photobiomodulation"* no banco de dados do ClinicalTrials.gov.

Figura 1 – Imagem ilustrativa referente aos artigos científicos disponíveis no PubMed sobre a FBM

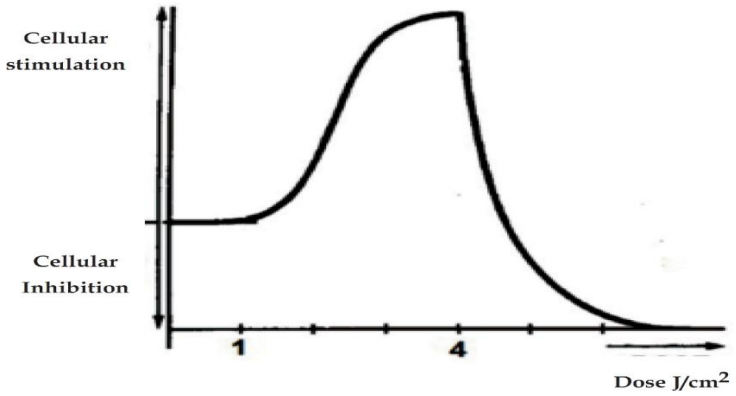


Fonte: https://www.ncbi.nlm.nih.gov/pubmed/, consulta em 05 de dezembro de 2020

Embora as pesquisas envolvendo a FBM tenham avançado nas últimas décadas e gerado inúmeros resultados controversos, a sua relevância biológica é indiscutível. No entanto, para induzir a atividade biológica nos tecidos irradiados, a luz precisa ser absorvida pelos fotorreceptores e ser capaz de excitá-los, para na sequência, afetar alvos secundários dentro da célula, e finalmente ser capaz de converter o sinal de luz em uma resposta molecular (LIEBMANN; BORN; KOLB-BACHOFEN, 2010).

Acredita-se que a FBM exerça um efeito bifásico sobre os tecidos irradiados. A primeira fase é imediata e resulta da irradiação direta dos componentes celulares, enquanto que a segunda é uma resposta mais tardia que ocorre horas ou dias após a irradiação (PINHEIRO et al., 2019). Inicialmente, os efeitos são decorrentes da ativação dos cromóforos endógenos, absorção da luz pela água intercelular e vários mediadores: fatores de crescimento (TGF-β1), citocinas anti e pró-inflamatórias e pequenas moléculas, tais como ATP e ERO. Esses mediadores modulam a proliferação celular, a diferenciação, a angiogênese e a apoptose (PINHEIRO et al., 2019; ELAD et al., 2018). Paralelamente a isso, os efeitos modulatórios da FMB estão de acordo com a Curva de Arndt-Schultz em que o nível da atividade biológica depende da força do estímulo (Figura 2). Essa lei postula que pequenas doses de irradiação ocasionam estímulos fracos que aceleram ligeiramente a atividade vital, doses um pouco mais elevadas aceleram a atividade celular até atingir um pico, enquanto que estímulos bem mais fortes (doses muito elevadas) suprimem a atividade vital, ocasionando uma resposta inibitória ou negativa na célula (CALDERHEAD; VASILY, 2016; DE FREITAS; HAMBLIN, 2016).

Figura 2 - Curva de Arndt-Schultz



Fonte: Chaves e colaboradores (2014)

A primeira fonte de luz utilizada para este fim foi o Laser e o fenômeno físico para a formação dos feixes de laser é a amplificação óptica baseada na emissão estimulada de fótons que ocorre a partir da excitação de elétrons, originando então uma luz monocromática, coerente e colimada (HEINSKANEN; HAMBLIN, 2018).

Os benefícios da FBM com os Lasers vêm sendo reportados desde a década de 60, entretanto, um outro dispositivo – O LED – começou a ganhar destaque a partir de 1990 (CHAVES et al., 2014). Desde então, diversos estudos foram conduzidos com o objetivo de comparar a ação do laser e do LED e concluíram que os benefícios terapêuticos das duas fontes de luz são equivalentes, já que o mecanismo de FBM está relacionado ao comprimento de onda e a dose de energia entregue ao tecido (CHAVES et al., 2014; LANGELLA et al., 2018; HEINSKANEN; HAMBLIN, 2018). Desta forma, o uso terapêutico da luz LED vem ascendendo, porque além de apresentar os mesmos benefícios que o Laser, os equipamentos de LED são capazes de irradiar áreas maiores, com baixo custo e são considerados seguros para o uso domiciliar (NISHIOKA et al., 2012; HEINSKANEN; HAMBLIN, 2018; CALDERHEAD; VASILY, 2016), talvez por isso, alguns autores afirmam que o LED é um dispositivo muito promissor (HEINSKANEN; HAMBLIN, 2018).

2.3.1 FBM por Diodos Emissores de Luz (LED)

A terapia por FBM com a utilização dos dispositivos de LEDs é um fenômeno relativamente novo. Trata-se de um dispositivo constituído por material semicondutor que converte corrente elétrica em emissão de luz através do fenômeno conhecido como eletroluminescência (HEINSKANEN; HAMBLIN, 2018; CALDERHEAD; VASILY, 2016).

Internamente, o LED é formado por junções P-N (P-positivas, N-negativas) que, quando polarizadas, os elétrons atravessam barreira de potencial e se recombinam com

orifícios dentro do dispositivo, sempre no sentido da região N para a região P e para cada recombinação, um fóton é emitido (HEINSKANEN; HAMBLIN, 2018; CHAVES et al., 2014).

A luz de LED vermelha abrange o comprimento de onda de 620-770 ηm e pode atingir a 6 milímetros (mm) de penetração nos tecidos irradiados, já a luz LED azul apresenta uma faixa de comprimento de onda mais estreita que varia de 400-480 ηm com penetração tecidual de aproximadamente 1 mm (ZHANG; WU, 2018; LI et al., 2016).

De acordo com alguns estudos encontrados na literatura, os comprimentos de onda que resultam na luz vermelha, agem estimulando os tecidos biológicos, favorecendo o aumento de síntese de ATP celular. A teoria mais aceita para explicar esse fenômeno é baseada na fotodissociação do óxido nítrico inibitório, já que o óxido nítrico compete com o oxigênio durante a produção de ATP (HAMBLIN, 2017; WANG et al., 2016).

Em contrapartida, a luz azul tem apresentado efeito inibitório em diversos tecidos biológicos e tem demonstrado ser capaz de modular o processo inflamatório (TAFLINSKI et al., 2014; LIEBMANN; BORN; KOLB-BACHOFEN, 2010; OHARA et al., 2002). Evidências apoiam a capacidade da luz azul em exercer ação antibactericida, antiproliferativa, além de ser capaz de reduzir a liberação de citocinas pró-inflamatórias (LIEBMANN; BORN; KOLB-BACHOFEN, 2010; FISCHER et al., 2013; KIM et al., 2013).

2.3.2 FBM por LED em células tumorais

Vários estudos *in vitro* foram realizados para verificar os efeitos dos LEDs vermelho e azul em células normais, porém em células tumorais os estudos são mais limitados.

Estudo realizado por Ohara e colaboradores (2002) utilizou o LED azul (λ= 470 ηm, 5,7 mW/cm2) e o LED vermelho (λ= 634 ηm, 2,9 mW/cm2) por 20 minutos em linhagem celular de melanoma humano (B16). Os resultados demostraram que houve uma redução do número de células e no tamanho das colônias, em contrapartida o LED vermelho não ocasionou mudanças significativas nessas variáveis.

Matsumoto e colaboradores (2014) evidenciaram que as linhagens de câncer colorretal humano (HT29 e HCT116) foram fortemente afetadas pela irradiação do LED azul (λ= 465 ηm, 30 mW, 10 minutos por 5 dias), observado pela inibição da proliferação celular dessas linhagens, porém quanto ao LED vermelho (λ= 635 ηm) sob as mesmas condições de potência e tempo não houve alteração significativa.

Já Hopkins e colaboradores (2016) realizaram um estudo que envolveu a irradiação do LED azul (λ= 455 ηm) e vermelho (λ= 630 ηm) em linhagens celulares humanas de melanoma (A375), carcinoma epidermóide (A431), carcinoma pulmonar (A549), adenocarcinoma da glândula mamária (MCF7 e MDA-MB-231) e em glioblastoma (U-87). Os autores realizaram uma única irradiação com densidades de energia de 3, 6, 9 e 19 (J cm $^{-2}$) correspondentes à 5, 10, 15 e 30 minutos com o LED azul (λ= 455 ηm) e densidades de 6, 12, 19 e 37 (J cm $^{-2}$) correspondentes aos tempos de 3, 6, 9 e 18 minutos com o LED vermelho (λ= 630ηm). Os resultados demostraram o LED azul na densidade de 19J cm $^{-2}$foi capaz de reduzir a viabilidade celular na comparação com os controles escuros em 20% para a linhagem MDA-MB-231, 30% para a A549 e em 60% para a linhagem.

Dessa forma, considerando que o CM se caracteriza como uma problemática de saúde pública, que os efeitos da FBM ainda não estão claros na literatura, a necessidade de desenvolvimento de métodos terapêuticos alternativos, bem como a carência quanto a estudos utilizando LED vermelho ou azul em linhagem tumoral de câncer de mama, acredita-se que o presente projeto de pesquisa possui mérito e relevância.

3 MATERIAIS E MÉTODOS

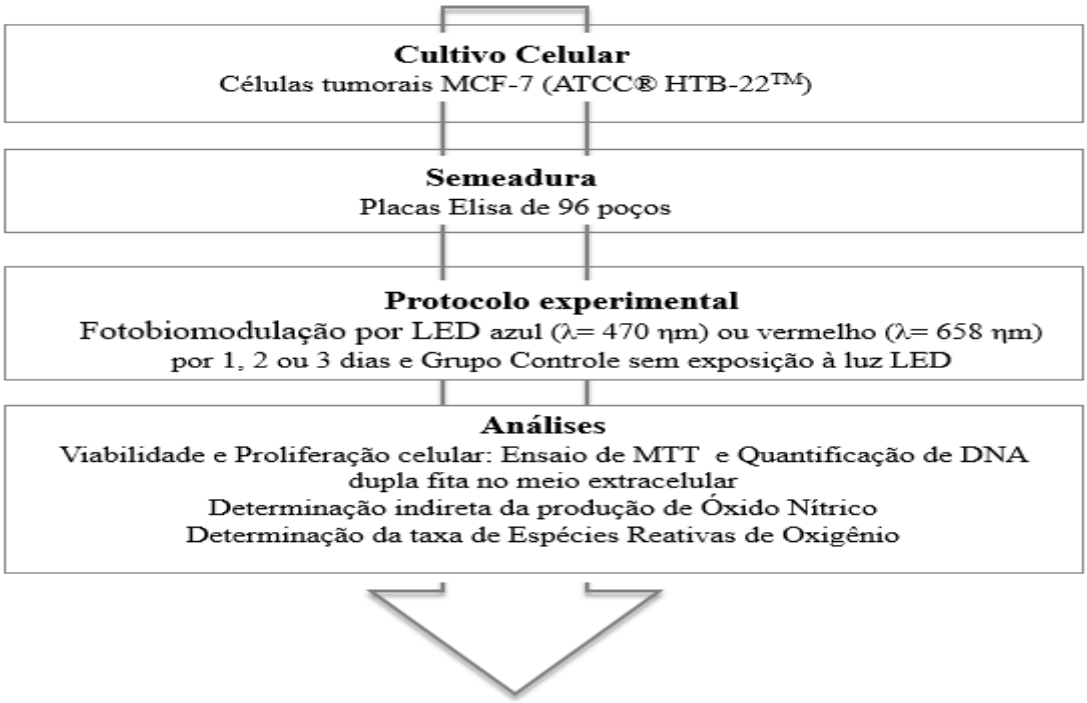
O presente estudo deriva do projeto intitulado “Efeitos da fotobiomodulação por diodos emissores de luz – LED em células tumorais da mama e em células de pele humana envolvidas no processo de reparo tecidual”. Para a realização desse estudo foi solicitado a autorização da instituição responsável pelo laboratório de pesquisa (Anexo A) e posteriormente registrado junto ao Gabinete de Apoio a Projetos do Centro de Ciências da Saúde (CCS) da Universidade Federal de Santa Maria – UFSM (nº 052560) (Anexo B), previamente ao início das coletas de dados.

3.1 DELINEAMENTO EXPERIMENTAL

Projeto de caráter experimental *in vitro* em que células tumorais da linhagem MCF7 de carcinoma mamário foram expostas à fotobiomodulação por LED, com dois comprimentos de onda distintos (658 ηm e 470 ηm), para assim verificar o potencial modulatório da irradiação. As células foram acompanhadas microscopicamente quanto a formação de monocamada em frasco de cultivo celular, sendo que ao atingir confluência entre 90-98% tais células foram repicadas, e após, foram dispostas em placas do tipo Elisa com 96 poços para a

realização dos protocolos de irradiação. Os ensaios incluíram métodos colorimétricos, fluorimétricos e microscópicos de verificação dos parâmetros relacionados a viabilidade e proliferação celular e ao metabolismo oxidativo. A figura 3 apresenta um resumo esquemático do método.

Figura 3: Resumo esquemático do método



Fonte: o próprio autor

3.2 LOCAL E PERÍODO DO ESTUDO

As coletas foram realizadas em colaboração com o Laboratório de Cultivo Celular da Universidade Franciscana – UFN e ocorrerão no mês de dezembro de 2019.

3.3 CULTIVO CELULAR

A linhagem celular MCF-7 (ATCC® HTB-22™), foi obtida a partir do Banco de Células do Rio de Janeiro (Rio de Janeiro, RJ, Brasil). Para o seu cultivo foi utilizado o meio de cultivo celular RPMI 1640 (Sigma-Aldrich, R8758, São Paulo, SP, Brasil) contendo 10% de soro fetal bovino (SFB) (Sigma-Aldrich, F2442, São Paulo, SP, Brasil) e suplementado com 1% de antibióticos penicilina-estreptomicina (100U/mL e 100mg/mL, respectivamente) (Gibco®Thermo Fisher, 15140122, São Paulo, SP, Brasil) e 1% de antifúngico anfotericina B (Gibco®Thermo Fisher, 15290018, São Paulo, SP, Brasil). As células foram mantidas em incubadora de CO_2, com controle da saturação de CO_2 de 5%, temperatura em 37°C e

ambiente umidificado. Após o primeiro frasco de cada um dos cultivos apresentar confluência em torno de 90-98% foi realizado o repique celular. Assim que foi obtido o número de células ideal para a realização dos experimentos e ensaios experimentais, as células foram plaqueadas em placas de 96 poços na concentração de $2,5X10^5$ células/mL. Transcorridas 24h de estabilização celular, o protocolo de irradiação com LED foi iniciado.

3.4 PROTOCOLO DE IRRADIAÇÃO COM LED

Antes de iniciar os experimentos, a intensidade luminosa dispensada pelo equipamento LED foi aferida por um luxímetro digital, afim de verificar se a intensidade real de energia recebida pelas células está de acordo com os parâmetros do estudo.

Para uso exclusivo nesse estudo, foi utilizado o equipamento de fotobiomodulação Endophoton - KLD - Brasil, número de série FB4LSD35, validado e autorizado pela ANVISA para uso medicinal. De acordo com as características do equipamento, a área efetiva de irradiação é de 5,3 cm².

O aplicador LED azul (λ=470ηm) contém doze pontos de LED com 0,03 W de potência cada, totalizando 0,36 W de potência por área efetiva de irradiação. Já o LED vermelho (λ=658 ηm) contém doze pontos com 0,04 W de potência cada, totalizando 0,48 W de potência por área efetiva de irradiação.

As doses diárias, tanto para a luz azul quanto para a vermelha foi de 6 J/cm² e 19 J/cm² e tal dosagem está de acordo com o estudo de Hopkins e colaboradores (2016).

As fórmulas abaixo foram utilizadas para definição do tempo de irradiação por ponto:

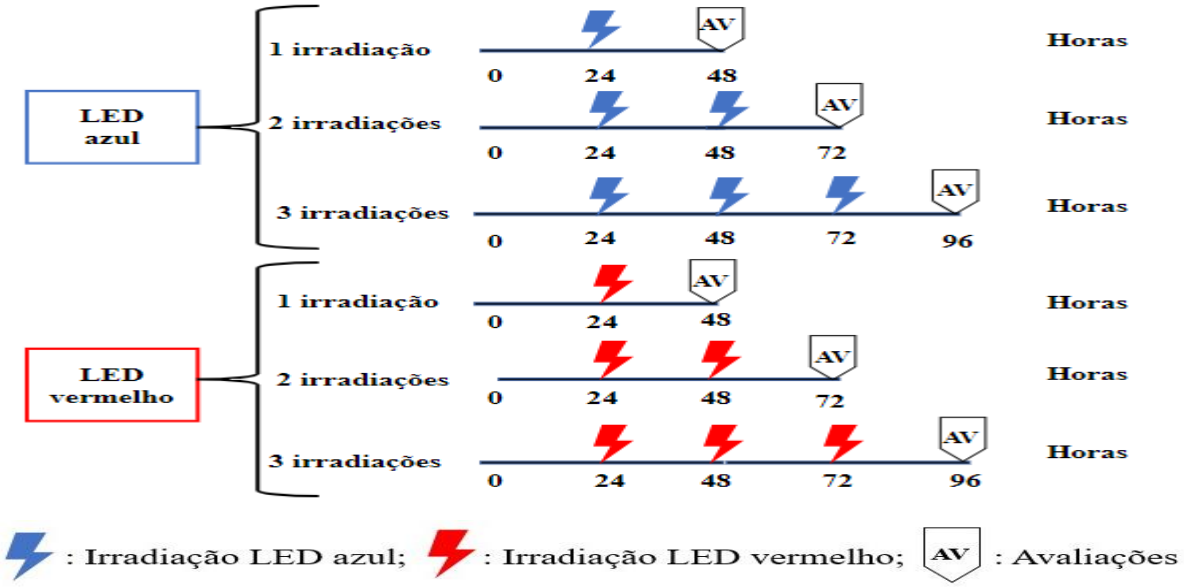
$$\text{Energia} = \text{Potência} \times \text{Tempo} \quad (1)$$

$$\text{Dose} = \text{Energia}/\ \text{Área} \quad (2)$$

O dispositivo de irradiação foi utilizado no modo contínuo e as irradiações foram realizadas no fluxo laminar, com a luz do fluxo apagada e a caneta de LED posicionada em contato com a placa, de forma que a luz fosse irradiada de forma perpendicular. Durante as irradiações, as placas ficaram sem as tampas e sobre um papel opaco para evitar a reflexão da luz. Um único pesquisador foi responsável pela irradiação das células.

As células foram dispostas em 6 placas (3 placas para o LED azul e 3 placas para o LED vermelho) e cada placa com o seu respectivo controle negativo. As irradiações foram realizadas por um, dois ou três dias consecutivos, com intervalo de 24 horas entre elas. As análises experimentais ocorreram 24 horas após a última irradiação. O protocolo de irradiação está esquematizado na figura 4.

Figura 4: Protocolo de irradiação com os LEDs



Fonte: o próprio autor

3.5 TESTES EXPERIMENTAIS

3.5.1 Avaliação da viabilidade e proliferação celular

3.5.1.1 Ensaio do MTT

Transcorridas as irradiações, as células foram avaliados quanto a viabilidade e proliferação celular através do ensaio colorimétrico de MTT (do inglês *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*) (Sigma-Aldrich, M2128, São Paulo, SP, Brasil), de acordo com as instruções descritas por Kang e colaboradores (2010). Este é um ensaio onde o reagente MTT é internalizado pelas células e em seguida metabolizado por enzimas mitocondriais de células viáveis, havendo a formação de cristais de formazan intracelulares. Tais cristais são então solubilizados com a adição de dimetilsulfóxido (DMSO) e a absorbância é determinada a 570 ηm em equipamento leitor de placas. Nesse caso, quanto maior for a absorbância, maior será o número de células viáveis.

3.5.1.2 Quantificações de DNA dupla fita no meio extracelular

Além do ensaio de MTT, a quantificação de DNA dupla fita (dsDNA) no meio extracelular é um parâmetro que contribui com a avaliação de índice de mortalidade celular.

Para esta determinação foi utilizado o reagente DNA PicoGreen® (Quant-It™ PicoGreen™ dsDNA Assay Kit, Thermo Fisher, São Paulo, SP, Brasil). Esse reagente é ultrassensível e possui alta afinidade com o dsDNA que permite quantificar concentrações mínimas desse DNA ao se intercalar com tal molécula e emitir fluorescência. O método adotado baseia-se nas instruções de Cadoná e colaboradores (2014), sendo a intensidade de fluorescência obtida proporcional ao índice de mortalidade celular. A fluorescência foi quantificada através de leitor de placas a 480 ηm de excitação e 520 ηm de emissão.

3.5.2 Avaliação do metabolismo oxidativo

3.5.2.1 Determinação indireta da produção de óxido nítrico

A linhagem celular, após exposição à luz, foi avaliada quanto a taxa de produção de óxido nítrico através do método colorimétrico indireto descrito por Choi e colaboradores (2012). Este ensaio baseia-se no uso do reagente de *Griess* e determinação dos metabólicos nitrato e nitrito, sendo a intensidade da coloração produzida quantificada em leitora de placas a 540 ηm.

3.5.2.2 Determinação da taxa total de espécies reativas de oxigênio

As células foram avaliadas quanto a taxa total de espécies reativas de oxigênio através do ensaio da 2´7´ diclorofluoresceínadiacetato (DCFH-DA) (Sigma-Aldrich, D6883, São Paulo, SP, Brasil), conforme instruções descritas por Costa e colaboradores (2012). Este ensaio fluorimétrico se baseia na internalização do reagente DCFH-DA, seguido da metabolização do mesmo a diclorofluoresceína (DCFH) através de enzimas intracelulares. Este metabólito ao interagir com EROs se transforma em diclorodiidrofluoresceína (DCF), a qual é capaz de emitir fluorescência. Logo, quanto maior a fluorescência emitida, maior a taxa total de EROs. A intensidade de fluorescência será quantificada em leitor de placas a 488 ηm de excitação e 525 ηm de emissão.

3.6 ANÁLISE ESTATÍSTICA

Inicialmente os dados obtidos foram tabulados no programa *Microsoft Excel*, versão 2010. Após, os dados foram convertidos em porcentagem na comparação ao controle negativo. Posteriormente, a análise estatística foi desenvolvida através do programa estatístico

Graph Pad Prism, versão 7.2, por meio de análise de variância (ANOVA), de uma via, seguida do *post hoc* de *Tukey*. Os resultados foram considerados significativos com $p<0,05$.

RESULTADOS:

Os resultados referentes dessa dissertação estão apresentados sob a forma de um manuscrito a seguir. O manuscrito intitulado “Análise de segurança da terapia por fotobiomodulação por diodos emissores de luz – LED e sua potencial aplicação nas toxicidades relacionadas ao tratamento oncológico da mama” será submetido a revista Lasers in Medical Science.

4 ARTIGO

Analysis of photobiomodulation therapy with light-emitting diodes – LED safety and its potential application on the toxicities of breast cancer treatment.

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ABSTRACT

It is known that antitumor therapies can lead to numerous adverse effects. Thus, there have been several alternative proposals to soothe the undesired effects. An example is the use of photobiomodulation therapy (PBMT). However, there is still controversy over the effects induced by PBMT on tumor cells. Hence, the aim of this study was to evaluate the modulatory effect of PBMT with light-emitting diodes (LED) on a tumor line of breast adenocarcinoma. For that purpose, MCF-7 breast tumor cells were irradiated with blue (470 nm) or red (658 nm) light at energy densities of 6 J/cm² and 19 J/cm², with a single or consecutive exposures. Experimental analyses were performed to verify cell viability and

proliferation, release of dsDNA to the extracellular space, nitric oxide (NO) production, and reactive oxygen species (ROS) production. The exposures caused a reduction of cell viability and/or proliferation, and there was no increase of mitosis on either of the wavelengths tested. The blue light caused a decrease in NO and ROS production. A single irradiation of red light at 6 J/cm² was capable of increasing NO levels, and two cumulative doses at 19 J/cm² increased ROS production. In this study, PBMT with blue and red LED at energy densities of 6 J/cm² and 19 J/cm² did not cause an increase in cell proliferation but reduced cell viability and division capacity of breast adenocarcinoma cells.

Keywords: Photobiomodulation. Breast Neoplasms. Cell proliferation.

INTRODUCTION

Oncologic treatment often involves the combination of local and systemic therapies [1]. Although several advances have been made aiming to increase the specificity of the treatment to tumor cells and limit the toxic effects to adjacent tissues, many side effects still persist, which include pain, lymphedema, radiodermatitis, wound healing delay, peripheral neuropathy, among others. [2-4]. Thus, the search for new therapeutic tools that can support the management of these effects is a topic of great relevance.

In recent years, photobiomodulation therapy (PBMT) has been used for the management of these adverse effects, especially concerning wound healing, with promising results [5]. However, some remaining tumor cells after treatment, especially in breast cancer (BC), can be accidentally exposed to the biostimulating effects of light [6].

In view of the therapeutic potential of PBMT in breast oncology and considering the conflicting results found in the literature regarding the safety of this therapy, this study aimed to evaluate the potential in vitro cell modulation induced by two wavelengths on a line of breast adenocarcinoma (MCF-7).

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

The present study is characterized as an in vitro experimental research conducted through cell culture of MCF-7 breast tumor line. These cells were exposed to two different wavelengths of light-emitting diodes (LED). From the exposures, parameters related to cell modulation in terms of cell viability and proliferation, and rates of oxidative metabolism,

were analyzed through colorimetric and fluorometric assays. All determinations were performed in quadruplicate.

CELL CULTURE

MCF-7 breast adenocarcinoma cell line (ATCC® HTB-22TM) was obtained from the Cell Bank of Rio de Janeiro (Rio de Janeiro, RJ, Brazil). The cells were cultivated in RPMI 1640 medium (Sigma-Aldrich, R8758, São Paulo, SP, Brazil) with 10% fetal bovine serum (FBS) (Sigma-Aldrich, F2442, São Paulo, SP, Brazil), supplemented with 1% penicillin-streptomycin (100U/mL and 100mg/mL, respectively) (Gibco®Thermo Fisher, 1140122, São Paulo, SP, Brazil) and 1% antifungal amphotericin B (Gibco®Thermo Fisher, 15290018, São Paulo, SP, Brazil). The cells were maintained in a humidified incubator with a control saturation of CO_2 at 5% and a temperature of 37°C. After the culture flasks presented 90-98% confluency, periodic transfer was performed. When the number of cells necessary for performing all treatments and experimental assays was reached, cells were placed in 96-well plates at a concentration of $2.5X10^5$ cells/mL. After 24h of cell stabilization, the LED irradiation protocol was initiated.

LED IRRADIATION PROTOCOL

The equipment used in this study was the Endophoton - KLD - Brazil, serial number FB4LSD35, validated and authorized by ANVISA (Brazil, registration 10245239007). Irradiations were performed according to the parameters described in Table 1.

Cells were placed in 6 plates (3 plates for blue LED and 3 plates for red LED), each plate with its negative control (cells in ideal culture conditions and not submitted to the exposures). Irradiations were performed one, two, or three times consecutively, with a 24-hour interval between exposures. Experimental analyses were performed 24 hours after the last irradiation. Daily energy densities, both for blue and red light, were 6 J/cm² and 19 J/cm², according to Hopkins et al. [7].

Irradiations were performed in laminar flow, with the light flow turned off and the LED pen positioned in contact with the plate so that the light would be irradiated perpendicularly. During irradiations, the plates were left without lids and over an opaque paper to avoid light reflection. A single researcher was responsible for irradiating the cells. The arrangement of cells in the 96-well plates in shown in Figure. 1

TAB. 1

FIG. 1

EXPERIMENTAL TESTS

Cell viability and proliferation analysis

Cell viability and proliferation were assessed 24 hours after the irradiations were finished, through MTT colorimetric assay (*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*) (Sigma-Aldrich, M2128, São Paulo, SP, Brazil), following the instructions of Kang et al. (2010) [8]. The absorbance obtained was determined at 570 nm using the Anthos 2010 microplate reader (Biochrom Ltd, United Kingdom).

Quantification of double-stranded DNA (dsDNA) in the extracellular space

Quantification of dsDNA is a parameter that contributes to the evaluation of the cell death index. For that, PicoGreen® DNA reagent (Quant-ItTM PicoGreenTM dsDNA Assay Kit, Thermo Fisher, São Paulo, SP, Brazil) was used, which has a high affinity for dsDNA, and emits fluorescence when intercalating with this molecule. This assay was conducted according to instructions previously published by Cadoná et al. (2014) [9]. The fluorescence intensity was quantified at 480nm of excitation and 520nm of emission using the SynergyTM HTX Multi-mode microplate reader (BioTek, Winooski, Vermont, USA).

Indirect determination of nitric oxide production

The rate of nitric oxide production was evaluated using the indirect colorimetric method described by Choi et al. (2012) [10]. This assay is based on the use of the Griess reagent and the determination of nitrite and nitrate metabolites. Staining intensity was quantified using the Anthos 2010 microplate reader (Biochrom Ltd, United Kingdom) at 540nm.

Determination of the total rate of reactive oxygen species (ROS)

Quantification of the total rate of ROS was measured through 2´7 dichlorofluorescein diacetate (DCFH-DA) assay (Sigma-Aldrich, D6883, São Paulo, SP, Brazil) as described by Costa et al. (2012) [11]. According to this method, the greater the fluorescence emitted, the higher the total rate of ROS. Fluorescence intensity was quantified using the SynergyTM HTX Multi-mode microplate reader (BioTek, Winooski, Vermont, USA) at 488 nm of excitation and 525 nm of emission.

STATISTICAL ANALYSIS

Data obtained was converted in percentage in comparison with negative control. Statistical analysis was performed using a graphic design and statistical analysis software, through one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Results were considered significant when $p<0.05$.

RESULTS

This study evaluated the effects of PBMT with LED on cell viability, proliferation, and death index, as well as on oxidative metabolism parameters, in a tumor line of breast adenocarcinoma (MCF-7). Analyses were performed 24 hours after irradiations, which consisted of one, two, or three exposures to blue or red light, with a 24-hour interval between them and with two different wavelengths (470 ηm and 658 ηm), with energy densities of 6 J/cm² and 19 J/cm².

Cell viability and proliferation

MCF-7 cells exposed to blue LED light showed reduced cell viability after one exposure, both with 6J and 19J of intensity. Similarly, cells exposed to blue LED for 72h (3 irradiations) also showed a reduction in cell proliferation with both intensities used (Fig. 2A), when compared to negative control. Conversely, tumor cells exposed to red LED showed a reduction in cell proliferation when exposed to 3 consecutive irradiations (72h) with 6 and 19J. However, no cell modulations were observed on the other periods (24 and 48h) tested for red light (Fig. 2B).

FIG. 2

Quantification of double-stranded DNA (dsDNA) in the extracellular space

The results concerning the quantification of dsDNA in the extracellular space demonstrated that after two consecutive irradiations (48h) with blue light at 6 J/cm² and 19 J/cm² there was a significant reduction of the dsDNA released by the irradiated cells when compared to control group ($p<0.05$). In addition, a significant difference was found between the two intensities in the 48h exposure, with a greater reduction observed in the 19 J/cm²

exposure. Furthermore, blue light also led to a reduction in dsDNA release after 3 applications (72h) at 19 J/cm² ($p<0.05$), when compared to control group (Fig. 3A).

Cells exposed to only one radiation (24h) of red light with 6 J/cm² showed a reduction in dsDNA released, both when compared to control and when compared to the 19 J/cm² group. In contrast, 3 consecutive doses with the same wavelength of 19 J/cm² led to an increase of dsDNA in the extracellular space when compared to control group and also when compared to the 6 J/cm² group (Fig. 3B).

FIG. 3

Indirect determination of nitric oxide production

Cumulative doses with blue LED were able to influence nitric oxide production. With this wavelength, two irradiations (48h) at 6 J/cm² were able to reduce nitric oxide production when compared to control group ($p<0.05$) as well as when compared to the 19 J/cm² group. Still regarding blue light, three sequential irradiations (72h) at 19 J/cm² led to a reduction in nitric oxide when compared to control group (Fig. 4A).

However, for the red light, a single exposure (24h) at 6 J/cm² was able to increase nitric oxide production both when compared to control group and when compared to the 19 J/cm² group. Nevertheless, tree irradiations (72h) with this wavelength at 19 J/cm² were able to reduce nitric oxide when compared to control group (Fig. 4B).

FIG. 4

Analysis of reactive oxygen species (ROS) production

The results demonstrated that BPM with blue light induced a reduction in intracellular levels of ROS. A single irradiation (24h) with blue light at 19 J/cm² reduced the total ROS when compared to control group as well as when compared to the 6 J/cm² group. Considering the same wavelength, two irradiations (48h) at 6 J/cm² and 19 J/cm² led to a reduction in ROS when compared to the negative control. The same was observed after three applications (72h),

with a steeper reduction in the 19 J/cm² group (Fig. 5A). Conversely, two irradiations with red light at 19 J/cm² led to an increase in ROS levels when compared to control group (Fig. 5B).

FIG. 5

DISCUSSION

The present study aimed to analyze PBMT safety. For that purpose, the energy densities used were carefully selected in order to be equivalent to the ones used in clinical practice, especially in the treatment of toxicities and aftereffects of BC treatment. [5,12,13].

The effects of BPMT have been extensively studied, but definitive results are yet to be reached. Moreover, most of the studies have used wavelengths between 630 ηm and 840 ηm [5,14]. The great differential and novelty of this study is that it provides data on the in vitro behavior of tumor breast cells irradiated with a shorter wavelength LED (470 ηm) as well as with daily and consecutive emissions.

The results of this study showed a reduction in cell viability and proliferation levels of MCF-7 tumor cells exposed to PBMT when compared to non-irradiated control. This effect was especially observed with blue light, and with one and three applications. Concerning the red light, this effect was only observed with three cumulative applications. It is important to highlight that the energy densities used for both blue and red lights did not cause any measurable increase in cell proliferation levels, which is a relevant aspect to be considered.

Corroborating with our results, a study performed with melanoma cells in vitro (irradiance of 15.6 w/cm²) and in vivo (irradiance of 0.6 w/cm²) demonstrated that PBMT with blue light (450 ηm) was able to reduce tumor growth by inducing apoptosis through mitochondrial pathway at the early stage of the disease [15]. More recently, the same group of researchers found that an in vitro and in vivo protocol using blue LED was able to induce cell apoptosis and autophagy in two lymphoma cell lines, and also increase the survival rate of mice with leukemia [16]. Similarly, another in vitro and in vivo study demonstrated that blue light irradiation inhibited cell migration and invasion of solid tumors on the colon cancer cell line in rats (line CT-26) and on human fibrosarcoma (HT-1080) by suppressing the expression of extracellular matrix degrading metalloproteinases [17]. It is important to note that although

these studies did not show an increase in cell proliferation, irradiation times were much longer than ours.

A potential explanation for PBMT not having induced an increase in tumor cell proliferation is related to the Warburg effect, in which tumor cells alter their metabolism in order to perform anaerobic glycolysis instead of oxidative phosphorylation. This happens because in the body, rapid tumor growth overcomes the necessary blood supply, forcing tumor cells to develop tolerance to chronic hypoxia. [12,18]. As consequences of the Warburg effect, tumor and normal cells can behave differently in response to BPMT, since in tumor cells the ATP supply is very limited, and the ATP enhancement given by photobiomudulation can allow tumor cells to respond more efficiently to pro-apoptotic cytotoxic stimuli that are executed more efficiently and are also highly dependent on energy [12].

The presence of dsDNA in the extracellular space is an indicator of cell death by membrane lysis and leakage of nuclear DNA to the extracellular space. In this study, blue light irradiation caused a reduction of dsDNA levels, whereas the red light was only able to cause an increase of dsDNA released with 72h at 19 J/cm², corroborating with data found with red light through the MTT assay.

Thus, it is believed that the blue light could have induced cell death through apoptosis, without the occurence of membrane lysis. It is known that during the process of cell death through apoptosis, occurs chromatin condensation, internucleosomal DNA degradation, and cell shrinkage, and as a general rule there is no release of the material of cytosol to the extracellular space, hence avoiding an inflammatory response, for example [19-21]. Previous studies demonstrated that irradiation with blue LED was able to unleash mitochondrial mediated apoptosis (intrinsic pathway) [15-17]. This pathway triggers caspase-3 activation, which leads to DNA fragmentation and cell death [19].

NO is a gaseous molecule and a short-lived free radical endogenously produced, that acts as a signaling molecule of multiple physiological and pathological processes, including cancer [22,23]. In tumor cells, NO can inhibit or induce tumor progression and metastasis formation, depending on the amount and duration of the exposure to NO and cell sensitivity. Tumor progression and metastasis occur by direct induction of proliferation, migration, and invasion of tumor cells and also indirectly through the expression of angiogenic and lymphangiogenic factors. However, high doses of NO can have cytotoxic effects on the cell through DNA damage, genetic mutation and tumor cell death, or even decreasing the

expression of adhesion molecules, resulting in the regression and inhibition of the tumor and the metastatic process. Nevertheless, genetic mutation and/or transformation can also contribute to the clonal selection of adapted cells and facilitate tumor progression. [24-26].

The results obtained demonstrate that both blue and red LEDs were able to modulate NO levels. However, even with these variations, there was no increase in cell proliferation, which shows that separately these levels did not have an “antitumor” nor a “protumor” effect and that there are other factors to be considered, such as cell type, tumor microenvironment, NO concentration, and cell time of exposure to NO [23,27-28]. Although many variables act upon the inhibitory or stimulatory action of NO, once carcinogenesis begins, NO seems to have a more protumoral rather than antitumor action, since NO concentrations necessary to cause cytotoxic effects on the cell cannot be reached by tumor cells [29]. Moreover, an important factor to be considered is that analyses were performed 24 hours after the irradiations, which could have contributed to the reduction of NO levels in this study, considering it is a short-lived molecule.

It is known that in healthy cells PBMT is able to increase the mitochondrial membrane potential and promote a brief dose-dependent increase in ROS [30,31]. However, in cells previously submitted to oxidative stress, the levels of ROS can be reduced, and although the mechanisms involved in this reduction are yet to be elucidated, this could justify the efficacy of this therapy on inflammatory conditions [3]. Although blue light is known for its phototoxic and antiproliferative effects due to the increase of ROS production [17], our findings, in general, demonstrate a reduction in ROS levels when compared to non-irradiated groups, Overall, there are two plausible explanations for these results: 1) intracellular chromophores, especially cytochrome c oxidase, alter their light absorption spectra depending on their oxidation state [3]; and 2) PBMT can cause a brief increase in ROS levels and 24 hours after irradiations this effect may not remain, since this effect could be related to a recovery of cell homeostasis followed by apoptotic activation.

This study provides experimental evidence that PBMT with blue (470 ηm) and red (658 ηm) LED, with the dosages of 6 J/cm² and 19 J/cm² did not cause an increase in cell proliferation in a breast adenocarcinoma cell line, and at times led to a reduction in cell number and cell proliferation ability.

Regardless of the limitations of an in vitro experiment that cannot accurately simulate a clinic situation in vivo, this study suggests that PBMT with blue and red LED is a

potentially safe treatment option for breast cancer. However, more researches with different cell lines and different PBMT parameters are necessary to support these findings. In conclusion, our data demonstrate that irradiation with blue and red light at 6 J/cm² and 19 J/cm² did not increase cell proliferation, and that three cumulative doses actually induced an increase in cell death.

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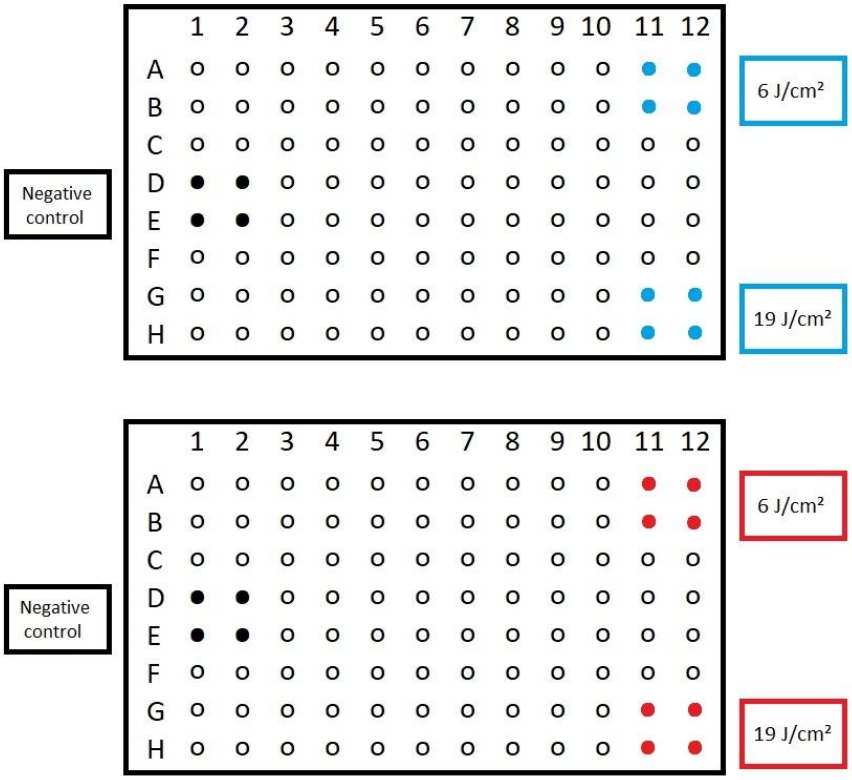
Table 1: PBMT parameters used for blue and red lights.

Wave length (ηm)	Effective radiation area (cm²)	Total emmiting LEDs	Total power (W)	Beam divergence (rad)	Energy density (J/cm²)	Irradiation time (s)	Energy per point (J)
470	5,3	12	0,36 (±20%)	0,52 (±30%)	6	90	2,7
					19	270	8,1
658	5,3	12	0,48 (±20%)	0,52 (±30%)	6	60	2,4
					19	210	8,4

Fig. 1: Disposição das células nas placas irradiadas.



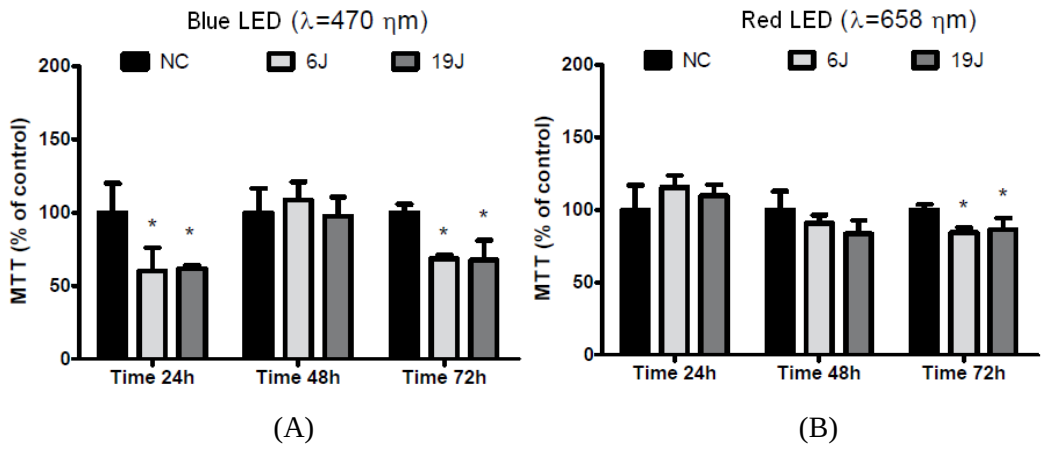


Fig 2: Analysis of cell viability and proliferation through MTT assay, after one (24h), two (48h), or three (72h) exposures to LED (470 nm and 658 nm) and at different energy densities (6 J/cm² and 19 J/cm²). A) Cells exposed to blue LED; B) Cells exposed to red LED. NC: negative control. Statistical analysis was performed using one-way analysis of variance, followed by Tukey's post hoc test. ***** $p<0.05$.

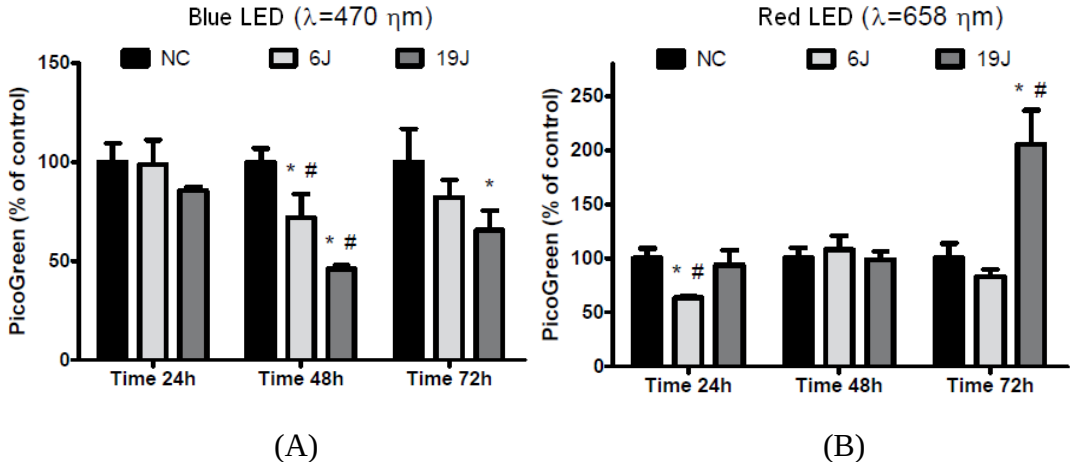
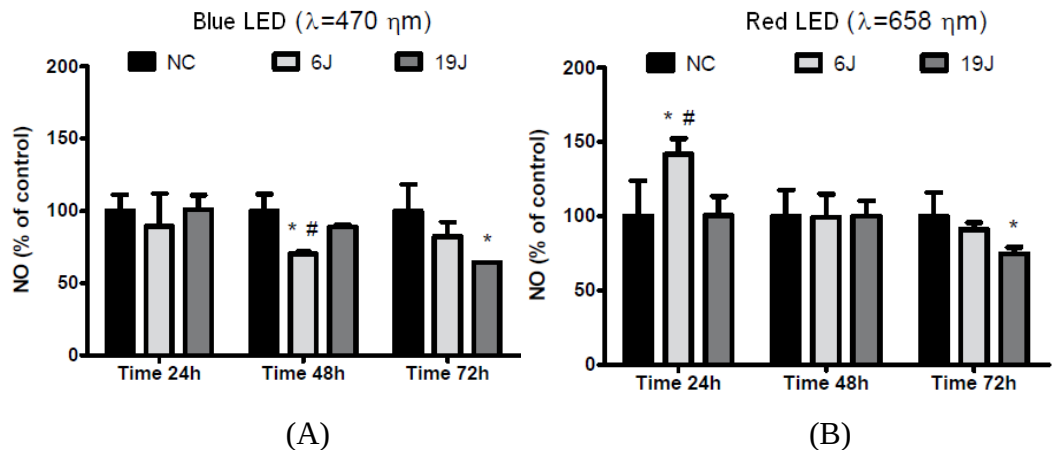
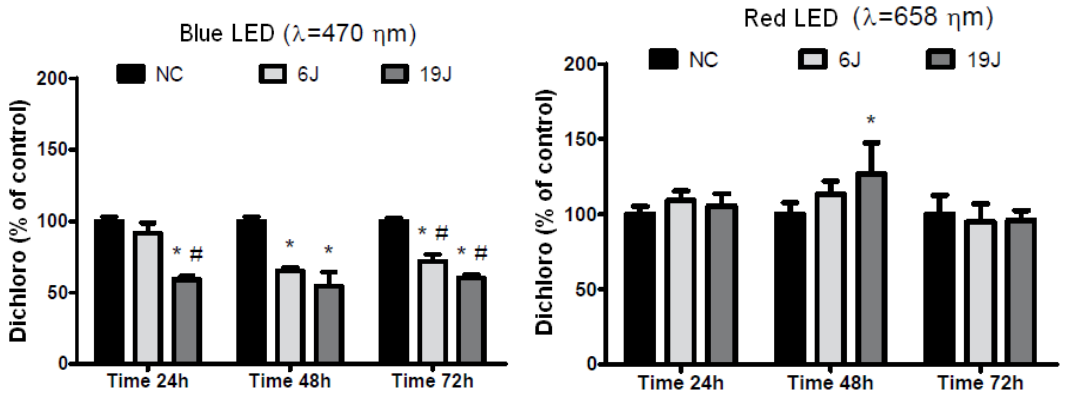


Fig. 3: Quantification of dsDNA in the extracellular space using PicoGreen® fluorescent probe, after one (24h), two (48h), or three (72h) exposures to LED (470 nm and 658 nm) and at different energy densities (6 J/cm² and 19 J/cm²). A) Cells exposed to blue LED; B) Cells exposed to red LED. NC: negative control. Statistical analysis was performed using one-way analysis of variance, followed by Tukey's post hoc test. ***** $p<0.05$ when compared to NC; # $p<0.05$ in the comparison between the irradiated groups at a same incubation period.



(A) (B)

Fig. 4: Determination of nitric oxide production through indirect measurement, after one (24h), two (48h), or three (72h) exposures to LED (470 nm and 658 nm) and at different energy densities (6 J/cm² and 19 J/cm²). A) Cells exposed to blue LED; B) Cells exposed to red LED. NC: negative control. Statistical analysis was performed using one-way analysis of variance, followed by Tukey's post hoc test. ***** $p<0.05$ when compared to NC; # $p<0.05$ in the comparison between the irradiated groups at a same incubation period.



(A) (B)

Fig. 5: Determination of total reactive oxygen species rate after one (24h), two (48h), or three (72h) exposures to LED (470 nm and 658 nm) and at different energy densities (6 J/cm² and 19 J/cm²). A) Cells exposed to blue LED; B) Cells exposed to red LED. NC: negative control. Statistical analysis was performed using one-way analysis of variance, followed by Tukey's post hoc test. ***** $p<0.05$ when compared to NC; # $p<0.05$ in the comparison between the irradiated groups at a same incubation period.

CONCLUSÃO

Em geral, o tratamento oncológico da mama, pode acarretar inúmeras repercussões sobre a funcionalidade e a qualidade de vida dos pacientes, e mesmo após o término do tratamento algumas sequelas podem permanecer.

A fisioterapia como integrante da equipe multiprofissional desempenha um papel singular durante todas fases do tratamento e atua desde a prevenção até a reabilitação das disfunções cinético funcionais desses pacientes. Com vistas às condutas fisioterapêuticas empregadas para tratar as disfunções provenientes da terapia oncológica no CM está a TFBM.

Apesar dos resultados promissores da TFBM no tratamento de diversas toxicidades relacionadas ao tratamento oncológico da mama, ainda há muito receio por parte dos profissionais em utilizar esse recurso pelo medo de estimular a recorrência local do tumor e as metástases.

Por isso, esse estudo se propôs a avaliar a segurança *in vitro* da TFBM em uma linhagem de adenocarcinoma mamário, que possui o subtipo mais prevalente nas mulheres em todo o mundo, como dois comprimentos de onda (470 ηm e 658 ηm) e duas dosagens distintas (6J/cm² e 19J/cm²). A segurança foi avaliada através do perfil modulatório da luz quanto à proliferação, morte celular, produção indireta de óxido nítrico e de produção de espécies reativas de oxigênio.

Como resultado obtidos, observamos que não houve aumento da proliferação celular em nenhum dos comprimentos de onda e em nenhuma das dosagens empregadas. Ao contrário, três doses cumulativas nos dois comprimentos de onda e para as duas doses ocasionaram redução na viabilidade celular na comparação com os grupos controles não expostos à luz. Ainda, pela análise do dsDNA observamos que o principal mecanismo de morte celular não ocorreu por lise das membranas, pois não houve extravasamento do DNA para o meio extracelular, inferindo que a apoptose pode ser o mecanismo envolvido.

Além disso, a luz azul foi capaz de modular negativamente a produção de ON e de EROs. Já para a luz vermelha houve um breve aumento de ON em uma única irradiação em 6J/cm² e duas doses de 19J/cm² promoveu aumento na produção de EROs.

Embora dentro das limitações de uma experimentação *in vitro*, este estudo pode sugerir que a TFBM com LED azul e vermelho para 6J/cm² e 19J/cm² é uma opção de tratamento potencialmente segura na oncologia mamária. No entanto, mais pesquisas com diferentes linhagens celulares e com diferentes parâmetros da TFBM são necessários para apoiar nossos achados.

Apesar da pandemia do Covid-19 ter impactado o andamento dessa pesquisa, pois testes adicionais sobre a análise do perfil modulatório do LED nas fases do ciclo celular não puderam ser realizados pelo fechamento dos laboratórios, posso dizer, que a pesquisa cumpriu o seu papel na produção de novos conhecimentos para a sociedade e agregou um valor inestimável ao crescimento profissional e acadêmico da pesquisadora.

Trabalhar com as pesquisas *in vitro* representou um enorme desafio por diferir completamente das minhas vivências acadêmicas, mas ao mesmo tempo, proporcionou um aprendizado ímpar ao me permitir aprofundar os estudos na oncologia mamária – e que é o principal foco dos meus estudos desde a graduação.

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ANEXO A – AUTORIZAÇÃO DO LOCAL DE PESQUISA

Universidade Franciscana
Pró-Reitora de Pós-Graduação e Pesquisa
Santa Maria – RS, Brasil
Rua dos Andradas, nº1614, prédio 07
Contato: +55 55 3220 1200

Santa Maria, 16 de agosto de 2019.

Destinado a quem interessar

De: Prof. Dr. Marcos Alexandre Alves, Universidade Franciscana

Re: Projeto de Pesquisa da Mestranda Thaís Nogueira de Oliveira Martins

Eu, Marcos Alexandre Alves, Pró-Reitor de Pós-Graduação e Pesquisa da Universidade Franciscana, venho por meio deste declarar às partes interessadas que é de conhecimento da referida Pró-Reitoria do desenvolvimento de estudos in vitro referentes ao projeto de pesquisa intitulado "Fotobiomodulação por diodos emissores de luz-led em células tumorais da mama", referente ao mestrado da aluna Thaís Nogueira de Oliveira Martins, pertencente ao Programa de Pós-Graduação em Reabilitação Funcional e orientada pela Professora Hedioneia Maria Foletto Pivetta da Universidade Federal de Santa Maria.

A realização dos experimentos na Universidade Franciscana caracteriza um trabalho em parceria para fins de produção acadêmica, não havendo qualquer benefício financeiro pelas partes envolvidas. As atividades serão realizadas no Laboratório 011, prédio 04, conjunto I, junto ao Professor Alencar Kolinski Machado. Além disso, os materiais de custeio/consumo serão obtidos pela aluna e professora orientadora.

A comissão de Gestão de Laboratórios da Universidade Franciscana possui ciência do exposto.

Atenciosamente,

Prof. Dr. Marcos Alexandre Alves
Pró-Reitor de Pós-Graduação e Pesquisa

(Representante da Comissão de Gestão de Laboratórios

Universidade Franciscana
Rua dos Andradas, nº 1614
Santa Maria – RS, Brasil
97010-032

ANEXO B – REGISTRO DO PROJETO

UNIVERSIDADE FEDERAL DE SANTA MARIA - UFSM

Data/Hora: 02/09/2019 11:54
Autenticação: 10C3.FF2D.2500.2688.4819.5CD5.50A5.1664
Consulte em http://www.ufsm.br/autenticacao

PROJETO NA ÍNTEGRA

Título: EFEITOS DA FOTOBIOMODULAÇÃOPOR DIODOS EMISSORES DE LUZ-LED EM CÉLULAS TUMORAIS DA MAMA E EM CÉLULAS DE PELE HUMANA ENVOLVIDAS NO PROCESSO DE REPARO TECIDUAL

Número: 052569 **Classificação**: Pesquisa **Registrado em**: 02/09/2019

Situação: Em trâmite para registro **Início**: 30/09/2019 **Término**: 30/09/2021

Avaliação: Avaliado **Última avaliação**:

Fundação: Não necessita contratar fundação **Número na fundação**: *Não se aplica*

Supervisor financeiro: *Não se aplica*

Proteção do conhecimento: Projeto não gera conhecimento passível de proteção

Tipo de evento: Não se aplica **Carga Horária**: *Não se aplica* **Alunos matriculados**: *Não se aplica* **Alunos concluintes**: *Não se aplica*

Palavras-chave: Neoplasia da mama, Fotobiomodulação, Radiodermatite

Resumo: Introdução: O tratamento com a fotobiomodulação por LED é um recurso amplamente utilizado no reparo tecidual com resultados promissores, no entanto, na fisioterapia oncológica, mais precisamente na prevenção de radiodermite, o uso desse recurso ainda é recente e os resultados obtidos precisam ser melhor elucidados. Diante disso, faz-se necessário avaliar e compreender os efeitos que a fotobiomodulação por LED exerce sobre as células tumorais da mama e sobre as células da pele envolvidas no processo de reparo tecidual, no tempo de exposição ao longo do tratamento radioterapêutico.Objetivo:Verificar os efeitos da fotobiomodulação por diodos emissores de luz em células tumorais da mama e em células da pele humana envolvidas no processo de reparo tecidual. Metodologia:Este projeto de pesquisa caracteriza-se como um estudo integrado de caráter experimental in vitro em que células tumorais da mama (MCF7) e normais, queratinócitos da linhagem HaCat e fibroblastos da linhagem HFF-1, serão expostas a diferentes comprimentos de onda: LED vermelho, comprimento de onda 658 nm e LED azul 470nm. As linhagens serão acompanhadas microscopicamente quanto a formação de monocamada em frasco de cultivo celular, sendo que ao atingir confluência entre 90-98% tais células serão repicadas, de modo que metade da suspensão celular seja disposta em novo frasco de cultivo e a outra metade seja destinada às avaliações experimentais. Os ensaios a serem realizados incluem métodos colorimétricos, fluorimétricos e microscópicos de verificação dos parâmetros relacionados a viabilidade e proliferação celular e ao metabolismo oxidativo.

Objetivos: Verificar os efeitos da fotobiomodulação por diodos emissores de luz em células tumorais da mama e em células da pele humana envolvidas no processo de reparo tecidual. Investigar os efeitos do LED vermelho e azul na viabilidade e proliferação celular de fibroblastos e queratinócitos expostos a cada comprimento de onda. Investigar a ação do LED vermelho e azul nas taxas de apoptose celular de fibroblastos e queratinócitos expostos a cada comprimento de onda. Verificar a ação do LED vermelho e azul no metabolismo oxidativo em fibroblastos e queratinócitos expostos a cada comprimento de onda. Comparar os efeitos agudos e cumulativos da fotobiomodulação por LED vermelho e azul em fibroblastos e queratinócitos. Investigar a ação do LED azul e vermelho na viabilidade de células tumorais de mama; Investigar os efeitos do LED azul e vermelho na proliferação celular de células tumorais de mama; Determinara ação do LED azul e vermelho quanto ao metabolismo oxidativo de células tumorais de mama; Avaliar o efeito do LED vermelho e azul na modulação do ciclo celular

ANEXO C – NORMAS PARA A PUBLICAÇÃO DO ARTIGO

Lasers in Medical Science

Journal home > Submission guidelines

Submission guidelines

Contents

- Research involving human participants, their data or biological material
- Informed consent
- Research Data Policy
- After Acceptance
- Open Choice
- English Language Editing

- Open access publishing

Instructions for Authors

Types of papers

- Original Article – limited to 4000 words, 45 references, no more than 5 figures
- Review Article – limited to 5000 words, 50 references, no more than 5 figures
- Brief Report - limited to 2000 words, 25 references, no more than 4 figures - Case Reports will not be accepted!
- Letter to the Editor – up to 600 words

Back to top ↑

Manuscript Submission

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Permissions

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

Online Submission

Please follow the hyperlink "Submit manuscript" and upload all of your manuscript files following the instructions given on the screen.

Source Files

Please ensure you provide all relevant editable source files at every submission and revision. Failing to submit a complete set of editable source files will result in your article not being considered for review. For your manuscript text please always submit in common word processing formats such as .docx or LaTeX.

Back to top ↑

Editorial Procedure

Double-blind peer review

This journal follows a double-blind reviewing procedure. Authors are therefore requested to submit:

A blinded manuscript without any author names and affiliations in the text or on the title page. Self-identifying citations and references in the article text should be avoided.

A separate title page, containing title, all author names, affiliations, and the contact information of the corresponding author. Any acknowledgements, disclosures, or funding information should also be included on this page.

Back to top ↑

Title Page

Title Page

Please make sure your title page contains the following information.

Title

The title should be concise and informative.

Author information

- The name(s) of the author(s)
- The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country
- A clear indication and an active e-mail address of the corresponding author
- If available, the 16-digit ORCID of the author(s)

If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

Abstract

Please provide a structured abstract of 150 to 250 words which should be divided into the following sections:

- Purpose (stating the main purposes and research question)
- Methods
- Results
- Conclusion

For life science journals only (when applicable)

- Trial registration number and date of registration for prospectively registered trials
- Trial registration number and date of registration followed by "retrospectively registered", for retrospectively registered trials

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Statements and Declarations

The following statements should be included under the heading "Statements and Declarations" for inclusion in the published paper. Please note that submissions that do not include relevant declarations will be returned as incomplete.

- **Competing Interests:** Authors are required to disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication. Please refer to "Competing Interests and Funding" below for more information on how to complete this section.

Please see the relevant sections in the submission guidelines for further information as well as various examples of wording. Please revise/customize the sample statements according to your own needs.

Back to top ↑

Text

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.

- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX. We recommend using Springer Nature's LaTeX template.

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

Back to top ↑

Scientific style

Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should be given at first mention.

Units and abbreviations

- Please adhere to internationally agreed standards such as those adopted by the commission of the International Union of Pure and Applied Physics (IUPAP) or defined by the International Organization of Standardization (ISO). Metric SI units should be used throughout except where non-SI units are more common [e.g. litre (l) for volume].
- Abbreviations (not standardized) should be defined at first mention in the abstract and again in the main body of the text and used consistently thereafter.

Drugs

- When drugs are mentioned, the international (generic) name should be used. The proprietary name, chemical composition, and manufacturer should be stated in full in Materials and methods.

Back to top ↑

References

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].

2. This result was later contradicted by Becker and Seligman [5].

3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

The entries in the list should be numbered consecutively.

If available, please always include DOIs as full DOI links in your reference list (e.g. "https://doi.org/abc").

- Journal article
  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of "et al" in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329
- Article by DOI
  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086
- Book
  South J, Blass B (2001) The future of modern genomics. Blackwell, London
- Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document
  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007
- Dissertation
  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

ISSN.org LTWA

If you are unsure, please use the full journal title.

Authors preparing their manuscript in LaTeX can use the bibliography style file sn-basic.bst which is included in the Springer Nature Article Template.

Back to top ↑

Tables

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

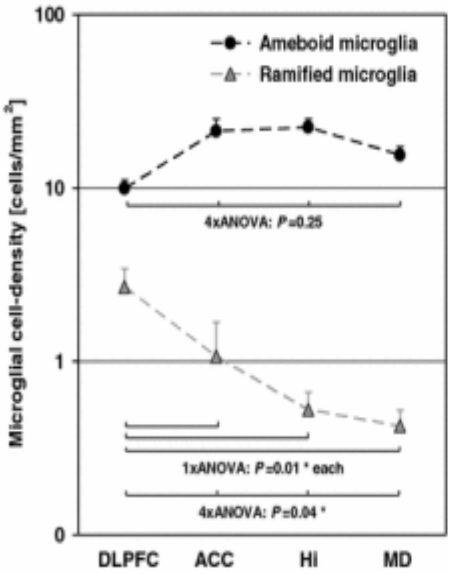
Back to top ↑

Artwork and Illustrations Guidelines

Electronic Figure Submission

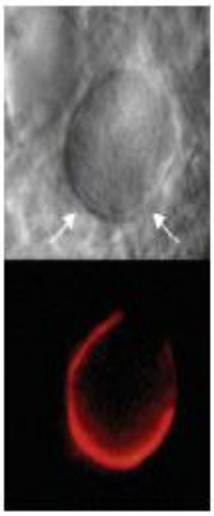
- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Line Art



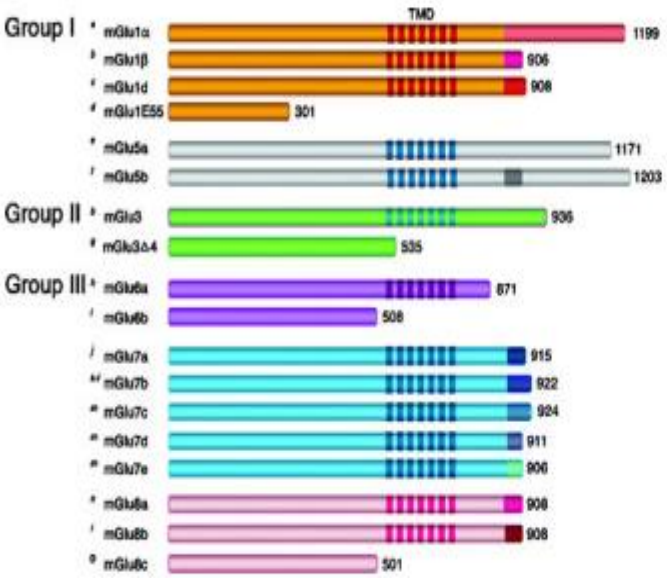
- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art



- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.

Combination Art



- Definition: a combination of halftone and line art, e.g., halftones containing line

drawing, extensive lettering, color diagrams, etc.

- Combination artwork should have a minimum resolution of 600 dpi.

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Back to top ↑

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Back to top ↑

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Back to top ↑

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Back to top ↑

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Back to top ↑

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Back to top ↑

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• This research study was conducted retrospectively from data obtained for clinical purposes. We consulted extensively with the IRB of XYZ who determined that our study did not need ethical approval. An IRB official waiver of ethical approval was granted from the IRB of XYZ.

• This retrospective chart review study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Human Investigation Committee (IRB) of University B approved this study.

Examples of statements to be used when no ethical approval is required/exemption granted:

• This is an observational study. The XYZ Research Ethics Committee has confirmed that no ethical approval is required.

• The data reproduced from Article X utilized human tissue that was procured via our Biobank AB, which provides de-identified samples. This study was reviewed and deemed

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Back to top ↑

Informed consent

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Back to top ↑

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Back to top ↑

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Back to top ↑

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