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Efeitos da Fotobiomodulação e do Exercício Aeróbico na Sobrevivência Celular e nos Mecanismos de Isquemia e Reperfusão: Estudos na Insuficiência Cardíaca Associada ao Diabetes Experimental e em Cardiomiócitos Isolados

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Orientador: Prof. Dr. Pedro Dal Lago

Coorientador: Dr. Patrick Türck

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RESUMO EXPANDIDO

Introdução: As doenças cardiovasculares, como a insuficiência cardíaca (IC) e a doença arterial coronariana (DAC), estão entre as principais causas de morbidade e mortalidade global. A IC, tem um processo fisiopatológico resultante de estressores celulares como hipóxia e interrupção da síntese proteica, afeta cerca de 2% da população nos países ocidentais e é exacerbada pelo *diabetes mellitus* tipo 2 (DM2) através do aumento do estresse oxidativo e disfunção mitocondrial. Essas condições induzem alterações celulares adversas, como atrofia de miócitos e autofagia deficiente. A fotobiomodulação (FBM) com luz não térmica de lasers ou LEDs minimiza a inflamação e melhora a defesa antioxidante, crucial para o reparo celular. As intervenções de revascularização para DAC, embora necessárias, carregam riscos significativos de lesão por reperfusão, marcada por dano celular agudo. A FBM tem potencial para mitigar esses efeitos, melhorando a função mitocondrial e a produção de ATP. Paralelamente, o exercício aeróbico (EA) melhora as funções cardíacas e músculo esqueléticas, modulando vias como Akt/mTOR, cruciais para a síntese proteica e hipertrofia muscular, além de promover a renovação celular. A combinação de FBM e EA promete melhorar os resultados das intervenções na reabilitação cardiopulmonar, com pesquisas contínuas sendo vitais para desenvolver tratamentos inovadores e não invasivos que maximizem esses benefícios. **Objetivos:** Investigar os efeitos terapêuticos isolados e combinados da fotobiomodulação e do exercício aeróbico sobre os mecanismos de estresse oxidativo, autofagia e apoptose em miócitos e cardiomiócitos, bem como suas implicações na melhora da função cardíaca e muscular em modelos de isquemia cardíaca, insuficiência cardíaca associada com diabetes mellitus tipo 2. **Metodologia do Artigo 1:** Foram utilizados dezoito ratos machos distribuídos em quatro grupos: Controle (CT), IC+DM (modelo de doença), Exercício+IC+DM (EX+IC+DM), e EX+IC+DM+FBM. DM2 foi induzido por dieta hiperlipídica (todo o experimento) e por estreptozotocina (0,25 ml/kg, i.p.), e a IC por ligadura coronária. Uma semana após a indução, iniciou-se um protocolo de oito semanas de FBM e EA. Avaliamos por western blot a expressão de proteínas relacionadas à apoptose (BAX, CASPASE-3, CASPASE-9, Anexina-V, P-ASK) e proteínas relacionadas à autofagia (mTOR, BECLIN-1, P62, LC3-I, LC3- II, Nrf2, P-AKT). **Resultados do Artigo 1:** Não houve diferença significativa na expressão de apoptose e na maioria das proteínas de autofagia entre os grupos. No entanto, o grupo

EX+IC+DM+PBM apresentou um aumento no Nrf2 (p=0,04), p-AKT (p=0,03) e LC3-I (p=0,005) em comparação ao grupo IC+DM, indicando melhora na sinalização de sobrevivência celular. **Metodologia do Artigo 2:** Cardiomiócitos H9C2 foram induzidos à hipóxia por 24h com 300 µM de CoCl2, seguida de 16h de reoxigenação em meio normóxico. As células foram tratadas com Laser GaAlAs (850nm, 1J/cm²) pós-hipóxia. Os grupos incluíram células em condições normóxicas (C), hipóxia e reperfusão sem tratamento (HR), e hipóxia e reperfusão com FBM (HR+FBM). A viabilidade celular e a expressão proteica foram avaliadas via MTT e *Western blot*, respectivamente. **Resultados do Artigo 2:** A FBM atenuou as alterações induzidas pela hipóxia/reoxigenação, aumentando significativamente a expressão de proteínas Nrf2, HSP70, mTOR, LC3II, LC3II/I, Caspase-9 e reduzindo a expressão de PGC-1α, SOD2, xantina oxidase, Beclin-1, LC3I e Bax, indicando uma redução no estresse oxidativo e modulação na autofagia e apoptose. **Conclusão:** Os estudos confirmam que tanto a FBM quanto a combinação de EA com FBM atuam como intervenções terapêuticas eficazes para melhorar a sobrevivência e funcionalidade celular em condições de doenças cardíacas e diabetes. As sinergias observadas entre estas modalidades sugerem a possibilidade de uma nova direção para tratamentos não farmacológicos, contribuindo para o manejo de doenças cardiovasculares como IC, DAC e diabetes.

Palavras-chave: Fotobiomodulação; Hipóxia/Reoxigenação; Diabetes Mellitus; Insuficiência Cardíaca; Exercício Aeróbico.

EXPANDED ABSTRACT

Introduction: Cardiovascular diseases such as heart failure (HF) and coronary artery disease (CAD) are among the leading causes of mortality and morbidity worldwide. HF, has a pathophysiological process cellular stress including hypoxia and interruptions in protein synthesis, affects 2% of the population in eastern countries and is exacerbated by type 2 diabetes mellitus (DM2) through increased oxidative stress and mitochondrial dysfunction. These conditions induce adverse cellular changes, such as myocyte atrophy and deficient autophagy. Photobiomodulation (PBM) with non-thermal lasers or LEDs minimizes inflammation and improves antioxidant defense, which is crucial for cellular repair. Revascularization interventions for CAD, although necessary, carry significant risks of reperfusion injury, marked by acute cellular damage. PBM has the potential to mitigate these effects by improving mitochondrial function and ATP production. In parallel, aerobic exercise (AE) improves cardiac and skeletal muscle functions by modulating pathways such as Akt/mTOR, which are crucial for protein synthesis and muscular hypertrophy, in addition to promoting cellular renewal. The combination of PBM and AE promises to improve intervention results in cardiopulmonary rehabilitation, although continuous research remains vital to develop innovative and non-invasive treatments that maximize these benefits. **Objectives:** Investigate the isolated and combined therapeutic effects of photobiomodulation and aerobic exercise on the mechanisms of oxidative stress, autophagy and apoptosis in myocytes and cardiomyocytes, as well as their implications for improving cardiac and muscular function in models of cardiac ischemia, heart failure associated with diabetes mellitus type 2. **Methods - Article 1:** Eighteen male rats were assigned to one of four groups: Control (CT), HF+DM (disease model), Exercise+HF+DM (EX+HF+DM), and EX+HF+DM+Photobiomodulation (EX+HF+DM+PBM). DM2 was induced by a high-fat diet (the entire experiment) and streptozotocin (0.25 ml/kg, i.p.) was administered. HF was induced via coronary ligation. One week post-induction, an eight-week protocol of aerobic exercise and PBM began. We evaluated by western blot the expression of apoptosis-related proteins (BAX, CASPASE-3, CASPASE-9, Anexin-V, P-ASK) and autophagy-related proteins (mTOR, BECLIN-1, P62, LC3-I, LC3-II, Nrf2, P-AKT). **Results - Article 1:** There was no significant difference in the expression of apoptosis and most autophagy proteins among the groups. However, the EX+HF+DM+PBM group showed a increase in Nrf2 ($p=0.04$), p-AKT ($p=0.03$), and LC3-I ($p=0.005$) compared to

the HF+DM group, indicating enhanced cell survival signaling. **Methods - Article 2:** H9C2 cardiomyocytes were exposed to hypoxia with concentrations of 300 μM CoCl2 for 24 hours, followed by 16 hours of reoxygenation in a normoxic medium. Treatment was conducted using a GaAlAs Laser (850nm, 1J/cm²) after hypoxia. The groups included cells in normoxic conditions (C), hypoxia and reperfusion without treatment (HR), and hypoxia and reperfusion with PBM (HR+PBM). Cell viability was analyzed using MTT, and Western Blot assessed protein expression. **Results - Article 2:** PBM attenuated changes induced by hypoxia/reperfusion by significantly increasing the expression of Nrf2, HSP70, mTOR, LC3II, LC3II/I, Caspase-9 and by reducing the expression of PGC-1α, SOD2, xanthine oxidase, Beclin-1, LC3I, and Bax, indicating a reduction in oxidative stress and modulation of autophagy and apoptosis. **Conclusion:** The studies confirm that both PBM alone and the combination of PBM and AE are effective therapeutic interventions to improve cellular survival and function in conditions of heart disease and diabetes. The synergies observed with these modalities suggest the possibility of a new direction in non-pharmacological treatments, contributing to the management of cardiovascular diseases such as HF, CAD, and diabetes.

Keywords: Photobiomodulation; Hypoxia/Reoxygenation; Diabetes Mellitus; Heart Failure; Physical Exercise.

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LISTA DE ABREVIATURAS E SIGLAS

ATP - Adenosina trifosfato

Ca2+ - Cálcio

COX - Citocromo c oxidase

CRVM - Cirurgia de revascularização miocárdica

DAC - Doença arterial coronariana

DATASUS - Departamento de Informática do Sistema Único de Saúde

DIC - Doença isquêmica coronariana

DM2 - Diabetes mellitus tipo 2

DNA - Ácido desoxirribonucleico

EROs - Espécies reativas de oxigênio

FBM - Fotobiomodulação

H2O2 - Peróxido de hidrogênio

IAM - Infarto agudo do miocárdio

IC - Insuficiência cardíaca

ICP - Intervenção coronária percutânea

IL-6 - Interleucina 6

IL-10 - Interleucina 10

LED - Diodo emissor de luz

mTOR - Alvo da rapamicina em mamíferos

ON - Óxido nítrico

O_2 - Oxigênio molecular

pH - Potencial hidrogeniônico

SCASSST - Síndrome coronariana aguda com supradesnivelamento de segmento ST

SUS - Sistema Único de Saúde

TNF alfa - Fator de necrose tumoral alfa

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PREFÁCIO

Linha de pesquisa no Programa de Pós-Graduação em Ciências da Reabilitação: Fundamentos da Reabilitação Cardiovascular, Pulmonar e Metabólica.

Orientador: Prof. Dr. Pedro Dal Lago, Prof. Titular do Departamento de Fisioterapia da Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) e Coordenador do Grupo de Pesquisa em Interação e Reabilitação Cardiopulmonar.

Coorientador: Prof. Dr. Patrick Türck, Prof. do curso de medicina da Universidade do Vale do Rio dos Sinos (UNISINOS), e Pós-doc no Laboratório de Fisiologia Cardiovascular na Universidade Federal do Rio Grande do Sul (UFRGS).

Parcerias nacionais que envolvem a presente Tese de Doutorado

Profa. Dra. Márcia Rosângela Wink, Profa. Titular do Departamento de Ciências Básicas da Saúde - Bioquímica da Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) e Coordenadora do Laboratório de Biologia Celular.

Profa. Dra. Aparecida Maria Catai, Profa. Titular do Departamento de Fisioterapia da Universidade Federal de São Carlos (UFSCar) e Coordenadora do Laboratório de Fisioterapia Cardiovascular. Durante o doutorado tive o prazer de poder visitar as instalações do laboratório orientado pela Profa. Dra. Aparecida, onde observei os experimentos em humanos em análise da função ventilatória, cardíaca e do balanço simpatovagal. Além disso, participei como ouvinte da disciplina de Tópicos de Fisioterapia Cardiovascular e apresentei resultados parciais dos artigos realizados no período do doutorado para o laboratório durante reuniões do grupo de pesquisa. Isso ocorreu devido a parceria entra UFSCar e UFCSPA a partir da bolsa fornecida pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) número 141194/2020-0 - Chamada Pública Nº 01/2019 - APOIO À FORMAÇÃO DE DOUTORES EM ÁREAS ESTRATÉGICAS.

Prof. Dr. Alex Sander da Rosa Araujo, Professor Associado no Departamento de Fisiologia - Instituto de Ciências Básicas da Saúde (ICBS) da Universidade Federal do Rio Grande do Sul (UFRGS) e Coordenador do Programa de Pós-Graduação em Ciências Biológicas: Fisiologia.

Prof. Dr. Alexandre Luz de Castro, Professor adjunto da Universidade Federal do Rio Grande do Sul (UFRGS) e orientador do Programa de Pós-Graduação em Ciências Biológicas: Fisiologia da UFRGS.

Prof. Dr. Guido Lenz, professor titular do Departamento de Biofísica da UFRGS, membro do CA em Ciências Biológicas da FAPERGS e sócio fundador da Fusiona Biotecnologia Ltda.

Originalidade

Esta tese de doutorado apresenta dois estudos inovadores que exploram modificações em quadros de altas taxas de mortalidade global: a insuficiência cardíaca associada ao *diabetes mellitus* tipo 2 e a hipóxia/reoxigenação em cardiomiócitos, e um estudo complementar focado no efeito da FBM na proliferação celular.

O primeiro estudo é um artigo original que avaliou os efeitos combinados da fotobiomodulação e do exercício físico nas vias de sinalização envolvidas na sobrevivência celular, apoptose e autofagia no músculo esquelético de ratos com insuficiência cardíaca e *diabetes mellitus* do tipo 2. Essa investigação oferece conhecimentos valiosos sobre como essas intervenções podem ser integradas para melhorar resultados clínicos em pacientes com múltiplas condições crônicas.

O segundo estudo detalha uma análise rigorosa dos impactos da fotobiomodulação sobre estresse oxidativo, apoptose e autofagia em cardiomiócitos isolados submetidos a hipóxia/reoxigenação. Este trabalho contribui para o entendimento de como a fotobiomodulação pode mitigar danos celulares em situações de estresse agudo, como as observadas em eventos isquêmicos cardíacos.

Por fim, o terceiro estudo, apresentado no Apêndice 1, é uma comunicação breve que explora os efeitos da fotobiomodulação na proliferação de células de glioblastoma envelhecidas. Este visa identificar intensidades ótimas de tratamento que possam reverter alterações senescentes em células cancerígenas, abrindo caminho para novas abordagens terapêuticas no tratamento de cânceres altamente agressivos.

Cada estudo nesta tese não apenas avança no conhecimento científico em suas respectivas áreas, mas também sugere possíveis e potenciais aplicações clínicas que podem influenciar futuras pesquisas e tratamentos. Através desta pesquisa, contribuições significativas foram feitas para a compreensão e o manejo de condições patológicas complexas, destacando a interdisciplinaridade e a inovação no coração desta tese de doutorado.

Contribuição dos resultados da pesquisa para o avanço científico

Os estudos realizados nesta Tese de Doutorado oferecem contribuições valiosas para o avanço científico à medida que abordam lacunas importantes existentes na literatura na compreensão de doenças cardiovasculares associadas ao diabetes e aprimoram métodos de tratamento em modelos experimentais dessas doenças.

A tese explora o impacto da insuficiência cardíaca associada ao *diabetes mellitus* tipo 2 na função do músculo esquelético. Esta condição frequentemente acelera a degeneração do miócito, levando à morte celular e ao declínio funcional. Através da investigação dos efeitos da fotobiomodulação associada ao exercício aeróbico, os resultados desta tese demonstram novas vias para mitigar esses danos, oferecendo perspectivas promissoras para o tratamento clínico não farmacológico dessas interações patológicas complexas.

Nos casos de pacientes submetidos à revascularização miocárdica, a literatura já discute os efeitos adversos do processo de reperfusão cardíaca, especialmente em termos da sobrecarga de oxigênio que ativa espécies reativas de oxigênio, induzindo caminhos de autofagia e apoptose celular. Nesse contexto, este estudo contribui significativamente ao elucidar os efeitos potencialmente terapêuticos da fotobiomodulação no reparo do tecido cardíaco após lesões induzidas por hipóxia e reoxigenação. Esta descoberta pode

fornecer bases para intervenções menos invasivas em situações pós-isquêmicas, preenchendo uma lacuna crítica na literatura atual.

Em resumo, os resultados apresentados nesta tese ampliam a compreensão dos mecanismos fisiopatológicos de doenças cardiovasculares e diabetes e introduzem abordagens terapêuticas não farmacológicas inovadoras com potencial para melhorar significativamente a qualidade de vida dos pacientes. Tais contribuições são fundamentais para o desenvolvimento futuro de estratégias de tratamento mais eficazes e personalizadas.

Relevância social

A relevância social da tese é um aspecto fundamental que sublinha seu impacto prático e o potencial para avançar no conhecimento tanto na prática clínica quanto na qualidade de vida dos pacientes. Esta tese de doutorado foca em explorar e ampliar o entendimento sobre a aplicação da FBM e do EA no tratamento de duas condições com alta prevalência global: a IC associada a DM2 e a hipóxia/reoxigenação cardíaca na doença isquêmica. Investigando os efeitos da FBM e do EA nas vias de sobrevivência e morte celular, este estudo contribui de forma significativa para um possível refinamento das estratégias de intervenção clínica, introduzindo uma nova ferramenta terapêutica baseada em evidências que pode melhorar substancialmente o manejo dessas doenças crônicas.

Nesse sentido, os resultados observados podem oferecer implicações práticas, capacitando profissionais de saúde, membros da equipe multidisciplinar, na reabilitação cardiopulmonar, a intervir de forma mais eficaz e assertiva. A implementação da FBM combinada ao EA, ou, seus usos isolados, pode ter um potencial de melhorar a capacidade funcional, reduzir a frequência de exacerbações, diminuir as taxas de hospitalização e, por consequência, reduzir a mortalidade associada a essas condições crônicas. Esses benefícios diretos na vida dos pacientes enfatizam a importância social e o impacto dos resultados desta tese.

Adicionalmente, esta tese lança luz sobre as intensidades ótimas de FBM, abrindo caminho para futuras investigações clínicas destinadas a expandir o uso dessa tecnologia para uma gama mais ampla de condições patológicas. Ao

estabelecer parâmetros claros de tratamento, esta tese serve como um recurso valioso para pesquisas subsequentes, encorajando a incorporação da FBM em uma variedade mais ampla de contextos clínicos e contribuindo para a fundamentação de práticas clínicas mais robustas e baseadas em evidências.

Portanto, a relevância social deste trabalho transcende as fronteiras acadêmicas e avança na esfera da saúde pública, promovendo uma abordagem inovadora que tem o potencial de melhorar a reabilitação de doenças cardiovasculares e diabetes, desafios persistentes para os profissionais da saúde em todo o mundo.

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Participação de Bancas de TCC

BAHR, A.C.; FABRIS, V.C.; DADALT, M.L.R. Participação em banca de Antônio Silvestre dos Santos e Lucas Molena. Risco Cardiovascular Associado À Depressão em Mulheres Atendidas em uma Unidade Básica De Saúde No Meio-

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1. CONTEXTUALIZAÇÃO

1.1. *DIABETES MELLITUS* E DOENÇAS CARDIOVASCULARES

A diabetes mellitus tipo 2 (DM2) e as doenças cardiovasculares são condições crônicas, prevalentes, que frequentemente coexistem, influenciando a fisiopatologia, o manejo e os desfechos clínicos dos pacientes. A interação entre doença arterial coronariana (DAC) e DM2 é complexa e multifacetada, abrangendo vários aspectos da epidemiologia, fisiopatologia e manejo clínico.

A DM2 continua a aumentar globalmente. Em 2017, cerca de 60 milhões de europeus foram acometidos pela doença, e na China e Índia estima-se que 10% da população possui a doença. Em 2045, esses números podem chegar a 600 milhões de pessoas em todo o mundo (COSENTINO et al., 2019). Atualmente, mais de 460 milhões de pessoas são afetadas pelo DM2, que corresponde à maioria dos pacientes diabéticos (KANALEY et al., 2022). Essas estatísticas são alarmantes devido às múltiplas comorbidades e alta mortalidade prematura por doenças cardiovasculares e/ou renais (LIM et al., 2022; MA et al., 2022).

Enquanto doença, a DM2 é uma condição crônica, caracterizada por hiperglicemia e hiperinsulinemia, afetando diretamente o metabolismo do músculo cardíaco e esquelético. A disfunção mitocondrial nos cardiomiócitos pode levar à limitação na produção de energia, aumento das espécies reativas de oxigênio (EROs), redução das proteínas de sobrevivência celular e indução de apoptose (GARCÍA-PEÑA; ABEL; PEREIRA, 2024; HO, et al. 2022).

A inflamação sistémica pode causar a DM2, essa pode resultar em disfunção do tecido adiposo e resistência à insulina, produzindo mais citocinas inflamatórias, como TNF-α e IL-6 (SHARIF et al., 2021). Esta inflamação, juntamente com a disfunção mitocondrial, propicia a formação de placas ateroscleróticas (GARCÍA-PEÑA; ABEL; PEREIRA, 2024). Essa placa ateroscletótica associada a DM2 pode ocasionar a doença cardíaca isquemica, doença arterial periférica e insuficiência cardíaca (IC) (MA et al., 2022).

A IC é uma das principais complicações derivadas da DM2 e da DAC, está relacionada ao aumento no índice de mortalidade e morbidade no mundo, com uma prevalência global estimada de 64 milhões de pessoas (SAVARESE et al.,

2023). No Brasil, a prevalência é de aproximadamente 2 milhões de pessoas (CESTARI et al., 2021). As doenças cardiovasculares são a principal causa de mortalidade no Brasil e no mundo, com a DAC sendo responsável por mais de 9 milhões de mortes globais em 2021 e mais de 170 mil mortes no Brasil em 2019 (VADUGANATHAN et al., 2022; OLIVEIRA et al., 2022).

Em pacientes que tiveram infarto agudo do miocárdio (IAM), a perfusão deve ser reestabelecida rapidamente para evitar danos celulares aos cardiomiócitos. O tratamento inclui trombolíticos, nitratos, betabloqueadores e estratégias invasivas como intervenção coronária percutânea (ICP) e cirurgia de revascularização miocárdica (NICOLAU et al., 2021). Embora a reperfusão seja essencial, pode causar danos adicionais às células cardíacas devido aos paradoxos do oxigênio, cálcio e pH (NERI et al., 2017; NAITO et al., 2020).

Estresses como lesão isquêmica desencadeiam uma reprogramação genética dos cardiomiócitos, resultando em disfunção contrátil e remodelamento ventricular que pode levar à IC (MORCIANO et al., 2022). A síndrome cardiometabólica, composta por obesidade, resistência insulínica, dislipidemia, hipertensão e estado pró-inflamatório, contribui para o desenvolvimento de doença cardiovascular e DM2 (AHAMAD; SHAPIRO, 2021).

Devido às modificações bioquímicas, moleculares e estruturais, a associação entre IC e DM2 pode levar a um quadro exacerbado de atrofia muscular, correlacionada com alta mortalidade (WOOD et al., 2021). Intervenções como exercício aeróbico e fotobiomodulação podem melhorar a capacidade funcional e tolerância ao exercício, atuando nas vias bioquímicas e moleculares que envolvem a sobrevivência e morte celular.

1.2. VIAS DE SOBREVIVENCIA E MORTE CELULAR

O estresse causado no tecido cardíaco devido a privação de oxigênio ou nutrientes após o processo de isquemia é caracterizado por hipercontratura por sobrecarga de Ca^{2+} dos cardiomiócitos. Várias formas de morte celular no coração são iniciadas a partir da ativação das vias de sinalização intracelular, incluindo proteínas redox sensíveis, autofagia e apoptose. Esses processos são fundamentais na resposta ao dano isquêmico, contribuindo para a disfunção e morte das células cardíacas (WANG, et al. 2020; HAIKEL DRIDI, et al. 2023).

1.2.1 MODULAÇÃO DO ESTRESSE OXIDATIVO

O termo EROs é utilizado para incluir não apenas radicais livres de oxigênio (O_2), mas também alguns não radicais derivados de oxigênio capazes de gerar radical livre, como o peróxido de hidrogênio (H_2O_2) (SHIELDS, et al. 2021). A maior parte do oxigênio consumido é reduzido a dióxido de carbono e água nas mitocôndrias como resultado da geração de energia. Entretanto, aproximadamente 1-2% do oxigênio presente na mitocôndria não é reduzido completamente e acaba resultando na formação de EROs (DHAR, et al. 2022).

As EROs podem ser produzidas por fontes endógenas ou exógenas. As principais fontes endógenas são: mitocôndria (metabolismo oxidativo), peroxissomos, citocromo P-450 e fagocitose. Independente da fonte, elas acabam danificando a fluidez e seletividade das membranas celulares, além de danificar funções nucleares, por oxidar fosfolipídios e proteínas da membrana (SHIELDS, et al. 2021).

A produção contínua de EROs ativa um mecanismo de defesa antioxidante contrarregulatório na tentativa de manter o sistema estável para que os danos estruturais sejam mínimos (SIES, et al. 2020). Os antioxidantes estão presentes tanto no meio intracelular como no meio extracelular, sendo classificados em enzimáticos (por exemplo, glutationa peroxidase, catalase e superóxido dismutase) e não enzimáticos (por exemplo, vitamina A, vitamina E) (SALEHI, et al. 2018).

O desequilíbrio no estado redox, ou seja, o estresse oxidativo, é caracterizado pela diminuição dos níveis de enzimas antioxidantes, uma vasta produção de EROs, ou uma combinação de ambas as condições (PIZZINO, et al. 2017). Para manter uma condição normal de vida aeróbia, é preciso ter um equilíbrio entre os antioxidantes e pró-oxidantes (SHARIFI-RAD, et al. 2020). Em estado de equilíbrio, os radicais livres formam-se para manter as condições fisiológicas (PHANIENDRA, et al. 2015). Por outro lado, um desequilíbrio associado ao aumento de pró-oxidantes desencadeia o processo de estresse oxidativo, podendo ocasionar danos as estruturas celulares, aos lipídios, proteínas e DNA (ENGWA, et al. 2018).

O estresse oxidativo está associado ao desenvolvimento de diversas doenças cardíacas (KIBEL, et al. 2020). A diminuição do fluxo coronariano, ocasionado pela isquemia, dificulta a função contrátil das células que agora estão hipoperfundidas, favorecendo a formação de EROs que possuem papel chave sobre a indução do estresse oxidativo, participando não apenas dos papéis patológicos nas doenças cardiovasculares, mas também na função fisiológica, regulando a sobrevivência e a morte dos cardiomiócitos (ZHOU, et al. 2018, BUGGER; PFEIL, 2020).

No musculo esquelético, a DM2 e a IC podem ocasionar disfunção mitocondrial, que por consequência leva à produção excessiva de EROs, com efeitos adversos na constituição estrutural das fibrilas. Além disso, a resistência à insulina e a inflamação promovida pelas EROs podem ainda exacerbar a atrofia do musculo esquelético (WOOD, et al. 2021).

1.2.2 MODULAÇÃO DA AUTOFAGIA

A autofagia está envolvida na formação de energia para atender as demandas metabólicas nos miócitos. A redução da autofagia leva a morte dos cardiomiócitos, enquanto intervenções que aumentam a autofagia preservam a função e prolongam a vida útil em vários organismos. Entretanto, em condições excessivas e prolongadas, pode haver a destruição de proteínas e organelas essenciais, resultando assim em morte celular (LAMPERT; GUSTAFSSON, 2018).

A inibição da proteína mTOR, e consequentemente, ativador do processo de autofagia, resulta no aumento da hipertrofia cardíaca e o declínio da função cardíaca em camundongos (LAMPERT; GUSTAFSSON, 2018). O complexo mTOR é uma serina/treonina quinase responsável pelo controle do crescimento celular regulando processos como biogênese de ribossomos, síntese protéica, metabolismo mitocondrial e autofagia (ZOU, et al. 2020). A via da mTOR desempenha função reguladora chave na fisiologia e patologia cardiovascular. O complexo mTOR, regula a síntese de proteínas, o crescimento e a proliferação celular, a autofagia, o metabolismo celular e as respostas ao estresse (SCIARRETTA, et al. 2018; SCIARRETTA, et al. 2014; SILVA, 2015). Vários estudos evidenciam que a mTOR está intimamente envolvida no processo de

modulação da hipertrofia compensatória à sobrecarga hemodinâmica e outros processos decorrentes de determinadas patologias (AMEMIYA et al. 2023; SCIARRETTA, et al. 2018; XU; BRINK, et al. 2016).

Na DM2, o processo inflamatório gerado a partir das EROs pode estimular a via de autofagia, promovendo uma redução acentuada do músculo esquelético. Entretanto, quando se observa na IC, os dados ainda são inconsistentes quanto a ativação dessa via (WOOD, et al. 2021). O estudo de CALLAHAN e TOTH, (2013) evidenciou não haver diferença nas rotas de autofagia em biopsia muscular de pacientes com IC. Já Jiang, et al. (2023) descreveram que a autofagia está presente na IC, no início tem um papel adaptativo, podendo modular e proteger os cardiomiócitos, mas, a longo prazo, a autofagia pode remover excessivamente as células lesadas.

1.2.3 MODULAÇÃO DA APOPTOSE

A apoptose celular pode ocorrer em situações fisiológicas e condições patológicas (OBENG, et al. 2020). A apoptose dos cardiomiócitos é caracterizada por uma série de eventos morfofisiológicos, como o encolhimento da célula e do núcleo, a fragmentação de corpos celulares contendo organelas celulares e fragmentos nucleares, e a fagocitose rápida ou degradação das células vizinhas (HEUSCH, 2020; LODRINI, et al. 2021).

A apoptose celular pode ser regulada e ativada de duas formas diferentes pelas vias de sinalização, entretanto, resultam na ativação das caspases (família de proteases) (OBENG, et al. 2020). A via extrínseca é ativada pela interação entre os ligantes e os receptores de membrana, enquanto a via intrínseca, é ativada a partir da mitocôndria ou do retículo sarcoplasmático, tendo a via intrínseca maior responsabilidade de transmitir os estímulos para a ativação da apoptose celular. Esta via está intimamente ligada com a ativação por estresse oxidativo, dano oxidativo ao DNA, membranas e proteínas, toxinas, inadequação de nutrientes, e fatores de sobrevivência (SHANMUGAM; SETHI, 2022).

Em casos de dano a membrana mitocondrial, a proteína pró-apoptótica Bax pode ocasionar a liberação do Citocromo C. Este é um fator apoptótico, que quando liberado ativam as caspases, e consequentemente ocorre a apoptose (ASSALY, et al. 2012). As caspases-2, 8, 9 e 10, são inicialmente ativadas,

resultando posteriormente na ativação das caspases efetoras do processo de morte, que são as caspases-3, 6 e 7 (SUN, 2024).

A doença isquêmica cardíaca é caracterizada principalmente por disfunção mitocondrial, levando os cardiomiócitos à exacerbação da apoptose, principalmente da região peri-infarto. A alta taxa de apoptose a curto prazo pós-IAM, se correlaciona com remodelamento ventricular mal-adaptado, resultado em IC. Assim, a apoptose desempenha um papel no tamanho do infarto, na extensão da remodelação do ventrículo esquerdo e no desenvolvimento de insuficiência cardíaca após IAM (TERINGOVA; TOUSEK, 2017).

As alterações metabólicas geradas pela DM2 podem ocasionar modificações na estrutura dos miócitos. Nos cardiomiócitos pode levar a hipertrofia cardíaca, alterações na estrutura ventricular, e disfunção diastólica e sistólica. Enquanto a atrofia do musculo esquelético acontece duas vezes mais rápido que em indivíduos saudáveis. Essas alterações ocorrem principalmente pela ativação do processo de apoptose, mecanismo ativado pela grande produção de EROs, e pela redução na capacidade antioxidante (REN, et al. 2020; LOPEZ-PEDROSA, et al. 2024).

Apesar da importância do processo de apoptose nas alterações celulares, existem poucos agentes terapêuticos para doenças cardíacas capazes de atuar sobre o processo de morte programada em cardiomiócitos e miócitos. Neste contexto, diversos trabalhos demonstram um papel positivo da terapia de fotobiomodulação (FBM) como agente terapêutico nos distúrbios cardiovasculares.

1.3 FOTOBIOMODULAÇÃO E EXERCÍCIO AERÓBICO

A FBM é a utilização de doses baixa de luz como forma de tratamento. É caracterizada pelo seu uso na forma de luz, como lasers ou LEDs. O comprimento de onda varia de luz vermelha (600-700 nanômetros [nm]) ao infravermelho (700-1000 nm), e a densidade de energia representa a forma terapêutica, essa energia é medida em Joules (J/cm^2) (SYED, et al. 2023; KLAUSNER, et al. 2021). Além disso, é uma ferramenta simples, de alta segurança e de alto impacto biológico, com efeitos fotofísicos e fotoquímicos não ionizantes e atérmicos (SYED, et al. 2023; DOMPE, et al. 2020).

Os resultados da FBM estão associados à sua absorção nas células através de fotorreceptores, facilitando o transporte de cálcio e a respiração mitocondrial, o que resulta em reparo e regeneração tecidual mais acelerado (DE MATOS, et al. 2021). Além disso, provoca redução do processo inflamatório e alívio do quadro álgico (DOMPE, et al. 2020). Pode também aumentar a expressão de citocinas anti-inflamatórias e diminuir as pró-inflamatórias, principalmente por estimular as células imunológicas no local da lesão (DE MATOS, et al. 2021).

A eficácia do FBM no tecido alvo depende dos parâmetros utilizados, como fonte de luz, comprimento de onda, densidade de energia, estrutura do pulso de luz e duração da aplicação do laser (DOMPE, et al. 2020). Essas respostas terapêuticas da FBM foram amplamente atribuídas a três principais vias fotorresponsivas: homeostase de energia mitocondrial, receptores ou transportadores de membrana celular e ativação do fator de crescimento extracelular (TGF-β1) (SYED, et al. 2023).

A principal via que destaca os efeitos da FBM é a homeostase mitocondrial. As mitocôndrias contêm cromóforos que absorvem fótons da FBM, sendo a enzima Citocromo C oxidase o principal componente celular que absorve a luz vermelha. Localizada na unidade IV da cadeia respiratória mitocondrial, essa absorção resulta na atividade de diversas moléculas como óxido nítrico (NO), ATP, íons cálcio (Ca^{2+}), EROs e outras moléculas sinalizadoras. A FBM estimula os elétrons nos cromóforos, promovendo a glicólise e a produção de ATP. Além disso, ativa vários fatores de transcrição e incentiva a produção de espécies reativas de oxigênio (DOMPE, et al. 2020).

Outra modalidade terapêutica para DM2 e IC, bastante discutida na literatura é o exercício aeróbico. A recomendação de exercício físico é de pelo menos 150 minutos, em intensidade moderada, ou 75 minutos de intensidade vigorosa para a redução de fatores de risco para DCV, destacando-se a obesidade e diabetes mellitus (PIERCY, et al.2018). É descrito na literatura que o exercício físico tem a capacidade de aumentar a produção de EROs através dos mecanismos envolvidos com a fosfolipase A2 (PLA2), da nicotinamida adenina dinucleotídeo fosfato (NADPH) oxidase e da xantina oxidase (XO). Entretanto, essas modificações também propiciam elevação nos níveis de antioxidantes. Ainda é discutido na literatura, mas há evidencias que ocorre

maior produção de antioxidantes no EA, quando comparado ao exercício anaeróbico (AMMAR, et al. 2020).

As EROs produzidas a partir de um quadro patológico como na DAC, IC, DM2 podem levar o tecido cardíaco e musculoesquelético a apoptose celular. O EA pode regular positivamente o a expressão de Bcl-2 e a proteína de choque térmico - HSP70 (um potente inibidor da apoptose), enquanto regula negativamente a expressão de Bax no músculo esquelético e cardíaco, podendo assim reduzir o mecanismo de apoptose provocado pelo aumento de EROs (REZAEE, et al. 2021).

O EA ainda pode modular a função autonômica, melhorando o desequilíbrio no balanço simpatovagal. Além disso, provoca alterações induzidas pela sensibilidade barorreflexa, consequentemente pode melhorar a pressão arterial (LEE, et al. 2022).

A partir do exposto, podemos perceber que a associação do EA e da FBM poderia ter um papel extremamente importante e significativo nas vias envolvidas com o estresse oxidativo, autofagia e apoptose nos mióticos e cardiomiócitos na isquemia cardíaca, IC e DM2.

1.4 FBM E EA NA ISQUEMIA CARDÍACA, IC e NO DIABETES

Dentre as alternativas terapêuticas para melhorar as consequências promovidas pela isquemia cardíaca, podemos destacar a utilização do laser de baixa potência em que foi verificado seu potencial efeito benéfico em relação à inflamação e à formação de EROs no período pós-IAM em modelo animal (HENTSCHKE, et al. 2013; YANG, et al. 2013). Estudos em modelos animais mostraram que a aplicação de laser após reperfusão de IAM foi capaz de reduzir a expansão da isquemia no tecido cardíaco, o que resultou em uma menor dilatação ventricular em ratos (MANCHINI, et al. 2014; MANCHINI, et al. 2017) Além disso, estudos concluíram que o tratamento com laser (1 J/cm²) logo após o infarto pode causar redução da área infartada, atenuação da disfunção sistólica, diminuição de células inflamatórias, reforçando a participação protetora do laser no miocárdio em coração de ratos três dias pós-IAM. Em ratos submetidos à IAM sem reperfusão posterior, a FBM foi capaz de promover aumento da multiplicação celular e angiogênese, além de redução da fibrose no

músculo cardíaco (GAO, et al. 2022). Revisões sistemáticas mostraram melhora em redução de colágeno, redução de espessamento ventricular e aumento de células-tronco mesenquimais (LIEBERT, et al. 2017; CARLOS, et al. 2016).

Além de contribuir para a redução da extensão de músculo cardíaco infartado (MANCHINI, et al. 2014; MANCHINI, et al. 2017; GAO, et al. 2022), a FBM também se mostrou eficaz em modelos animais na atenuação da disfunção ventricular esquerda pós-IAM, bem como demonstrou ser capaz de aumentar a geração de NO pelas células epiteliais dos vasos cardíacos (MANCHINI, et al. 2014; ZHANG, et al. 2021). Esses efeitos revelam o potencial da FBM de (i) reduzir o estresse físico sobre as células cardíacas, (ii) ter os efeitos celulares traduzidos em efeitos clínicos, e (iii) aumentar a oferta de O_2 para o tecido cardíaco por meio da dilatação arteriolar causada pelo NO. A FBM se mostrou capaz de reduzir o estresse oxidativo da desregulação metabólica gerada pelo DM2 através da absorção do laser pela enzima citocromo C oxidase, o que permite o aumento da oxigenação e redução da hipóxia, produção de EROs e do aumento da produção de moléculas antioxidantes (TONETTO, et al. 2023).

Dentre esses aspectos da lesão de isquêmica/reperfusão, a proteção da membrana mitocondrial se estabelece como um dos principais parâmetros para a redução da lesão celular. Já se sabe que a aplicação de FBM é capaz de regular a citocromo C oxidase e também se acredita que seja capaz de melhorar a capacidade de respiração mitocondrial, bem como de sinalização mitocondrial retrógrada, o que favorece a proteção contra a morte celular. Além disso, a FBM também atua em diferentes vias de sinalização celular que atuam principalmente modificando EROs e produção de NO, importante molécula reguladora da resposta ao estresse e da homeostase do cálcio (LIEBERT, et al. 2017).

O estudo realizado por Yang et al. (2013), verificou redução na área de infarto e no acúmulo de EROs utilizando laser de baixa potência em ratos 7 dias após a indução do IAM. O estudo de Oron et al. 2001, mostrou que os ratos tratados com as doses de 2,5 e 20 mW/cm^2 apresentaram redução na área de infarto de até 62%14 dias após o IAM. Já o estudo de Hentschke et al. (2013), em que trataram os ratos com IC, a partir da aplicação de laser durante 10 dias consecutivos, com intensidade de tratamento de 3 e 21 J/cm^2 no gastrocnêmio dos animais, concluíram que o tratamento é capaz de causar a diminuição de

TNF alfa/IL 10, IL6, IL6/IL10 no grupo 21 J/cm^2, sendo assim mostrando efeitos anti-inflamatórios musculares sistêmicos e esqueléticos em ratos com IC.

Exercício físico regular, particularmente o EA, é uma das principais formas de prevenção primária e secundária de doenças cardiovasculares (ALHUMAID et al., 2022). O EA está relacionado com um risco reduzido de mortalidade, de IC, menor incidência de doença coronariana e IAM, além de aliviar as consequências de arritmias e auxiliar no tratamento da hipertensão arterial sistêmica (MANNAKKARA; FINOCCHIARO, 2023). Ademais, a atividade física isolada melhora a sensibilidade à insulina e melhora o metabolismo dos ácidos graxos (RATAJCZAK et al., 2024).

O exercício físico aeróbico se mostrou eficaz em pacientes com IC com melhora da função ventricular e circulação coronariana (STØLEN et al., 2019), além de melhora da capacidade funcional, capacidade de exercício e qualidade de vida (COPS et al., 2019). Em pacientes com DM2, o exercício aeróbico melhora a glicose plasmática, função endotelial, reduz adiposidade visceral, aumenta massa magra e aumenta a disponibilidade do transportador GLUT-4, o que resulta em melhora do controle glicêmico (PARADA FLORES et al., 2023; BENNASAR-VENY et al., 2023).

Desse modo, é possível determinarmos diversos resultados benéficos da FBM, e da FBM associada ao exercício aeróbico. Entretanto, a literatura é escassa quando se analisa as vias para tais efeitos positivos. Neste contexto, entender os efeitos da FBM nos mecanismos de proliferação, estresse oxidativo, senescência e morte celular de miócitos de ratos com IC e DM2 e de cardiomiócitos expostos a hipóxia/reoxigenação, é extremamente relevante e inovador.

A interação entre DAC e DM2 representa um desafio significativo devido à fisiopatologia entrelaçada e aos riscos agravados. As modificações metabólicas propiciam o tecido cardíaco e esquelético a prejuízos bioquímicos e funcionais. Dessa forma, exigem uma abordagem de tratamento que contemple tanto os aspectos musculares quanto metabólicos. Compreender e gerir esta associação é crucial para melhorar os resultados e a qualidade de vida dessa população de pacientes. Pesquisas sobre tratamentos personalizados que envolvam exercício aeróbico e a fotobiomodulação e seus efeitos sobre as alterações celulares e bioquímicas tanto no músculo periférico quanto nos

cardiomiócitos são essenciais para intervenções terapêuticas mais apropriadas e benéficas para esta população.

2. OBJETIVO

2.1 GERAL

Investigar se a fotobiomodulação isolada ou combinada com exercício aeróbico possui efeitos terapêuticos relacionados às vias celulares de mecanismos de estresse oxidativo, autofagia e apoptose em miócitos e cardiomiócitos, com melhora da função cardíaca e muscular em modelos de isquemia cardíaca, insuficiência cardíaca associada com diabetes mellitus tipo 2.

2.2 ESPECÍFICOS

Artigo 1: Verificar se a influência do exercício aeróbico e sua associação com a fotobiomodulação na sobrevivência celular, autofagia e vias de sinalização de apoptose no músculo esquelético de ratos com IC e DM.

Artigo 2: Verificar se os efeitos da fotobiomodulação no estresse oxidativo, autofagia e apoptose de cardiomiócitos isolados expostos a hipóxia/reoxigenação.

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3 ARTIGO 1

Photobiomodulation and Physical Exercise modulate of cell survival proteins in the Skeletal Muscle of Rats with Heart Failure and Diabetes Mellitus

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ABSTRACT

Introduction: Heart failure (HF) and type 2 diabetes mellitus (DM2) are global health problems that often lead to muscle atrophy. These conditions are associated with increased autophagy and apoptosis in the muscle cells, resulting in decreased muscle mass. Physical exercise associated with photobiomodulation seems promising to attenuate the skeletal muscle changes caused by HF and DM2, due to its direct effects on mitochondria, which may result in an increase in antioxidant capacity. **Objective:** To verify the influence of physical exercise and the association with photobiomodulation on autophagy, apoptosis, and cell survival signaling pathways in myocytes from rats with HF and DM2. **Materials and methods:** Eighteen male rats were assigned to one of four groups: Control (CT), HF+DM (disease model), Exercise+HF+DM (EX+HF+DM), and

EX+HF+DM+Photobiomodulation (EX+HF+DM+PBM). DM2 was induced by a high-fat diet (the entire experiment) and streptozotocin (0.25 ml/kg, i.p.) was administered. HF was induced via coronary ligation. One week post-induction, an eight-week protocol of aerobic exercise (5x in week, 50-70% VO2max) and PBM began. We evaluated by western blot the expression of apoptosis-related proteins (BAX, CASPASE-3, CASPASE-9, Anexin-V, P-ASK) and autophagy-related proteins (mTOR, BECLIN-1, P62, LC3-I, LC3-II, Nrf2, P-AKT). **Results:** The EX+HF+DM+PBM group showed a substantial increase in Nrf2, p-AKT, and LC3-I levels compared to the HF+DM group. **Conclusion:** These findings suggest that physical exercise combined with PBM can upregulate proteins that promote myocyte survival in rats with HF and DM2.

Keywords: Autophagy; Apoptosis; Physical Exercise; Photobiomodulation; Heart failure; type II diabetes mellitus.

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1. Introduction

Heart failure (HF) affects approximately 2% of the population in Western countries.[1] It often leads to myocyte atrophy through mechanisms such as hypoxia and disrupted protein synthesis via the Akt/mTOR pathway.[1,2,3] The compounding effects of type 2 diabetes mellitus (DM2) exacerbate cellular stress by increasing reactive oxygen species, causing mitochondrial dysfunction, and reducing Nrf2 expression.[4,5] DM2-related altered insulin signaling also affects the autophagic process in skeletal muscles.[6] Although autophagy can protect against oxidative stress, prolonged activation may lead to cell death.[7,8]

Physical exercise improves heart and respiratory functions, modulates the mTOR pathway, and transiently activates autophagy, which increases protein balance and muscle hypertrophy.[9,10] This modulation is particularly beneficial in managing DM2 and HF.[11,12] Aerobic physical activity intensifies autophagy quickly, with a return to baseline within three hours[13], the increase in the autophagic reaction is beneficial due to its role in removing damaged mitochondria, thus being able to prevent the process of apoptosis.[14] Likewise, Photobiomodulation (PBM) also seems to have an important role in modulating this pathway.[15]

The PBM utilizes non-thermal light from low-power lasers or LEDs (1–500 mW) to reduce inflammation and protect against oxidative stress, as well as to preserve muscle morphology, including conditions such as acute muscle injury.[16,17] In addition, PBM can

protect against oxidative stress by increasing Nrf2 expression.[18,19] It has also been noted to decrease interleukin-1 (IL-1) expression and reduce beclin-1 mRNA expression in different experimental models, indicating its effect on autophagy.[20,21]

The interaction between exercise and PBM may offer synergistic enhancements in cellular repair and survival, particularly in HF and DM2. However, the combined effect on autophagy, apoptosis, and cell survival pathways has yet to be thoroughly investigated. Preliminary data suggest a beneficial interaction; however, rigorous studies are needed to confirm this. Hence, the main objective of our study was to bridge this gap by examining the influence of aerobic exercise combined with PBM on the signaling pathways governing cell survival, autophagy, and apoptosis in the skeletal muscles of rats afflicted with HF and DM2, thereby providing a stepping stone for potential clinical applications.

The hypothesis raised for this work is that the association of aerobic physical exercise and photobiomodulation can have beneficial effects in modulating pathways involved in cell survival, autophagy and apoptosis. To this end, we used wistar rats induced with HF and DM2 that practiced physical exercise and PBM five times a week. After this protocol, skeletal muscle was collected and the expression of proteins involved in cell survival, autophagy and apoptosis was applied.

2. Materials and Methods

2.1 Experimental Design

Male Wistar rats (70 days old, 200–230 g) were used in this study. The study followed the animal care guidelines in the NRC Guide for the Care and Use of Laboratory Animals (8th edition). All experimental procedures were performed according to the ethical principles established by the Committee for the Ethical Use of Animals (CEUA) of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Rio Grande do Sul, Brazil (approval number: 655/19).

To calculate the sample size, we used as a primary outcome the activity of glutathione peroxidase in the gastrocnemius of diabetic rats subjected to fatigue with high-intensity exercise and treated with PBM and GPX activity increased in the group treated with PBM.[22] We used α=0.05, effect size of 1.11 and analysis power of 95%, we estimated a total of 18 animals. The software used for the calculation was G*Power version 3.1.9.2 for Windows (University of Düsseldorf, Düsseldorf, Germany). The animals were conditioned at a controlled temperature of 22 °C with a 12-hour light/dark

cycle and kept in appropriate boxes with a capacity for five animals, lined with shavings, which were changed every two days. The animals received water and food *ad libitum*.

The animals were randomly divided into four groups: control-operated (CT, n =4), heart failure + diabetes and sedentary (HF + DM, n = 4), heart failure + diabetes who performed aerobic exercise (EX+HF+DM, n =5), heart failure + diabetes who performed aerobic exercise and photobiomodulation (EX+HF+DM+PBM, n=5) (Figure 1).

Concerning the heart failure animals, only rats in which an ischemic area in the heart was observed were maintained in the study. The animals were also manually drawn to divide the groups.

2.2 DM induction

To induce DM in the HF+DM, EX+HF+DM, and EX+HF+DM+PBM groups, a high-fat diet was performed for four weeks (lard (57.3%), carbohydrates (26.5%) and proteins (17.2%). To meet the standard (except starch and lard), quantities will badjusted to: 13.7% albumin (Naturovos®), 7.4% amino acids (Aminomix®) and 1.1% dried bone and oyster meal). After that, the animals were submitted to an intraperitoneal injection of streptozotocin (STZ) (0.25 ml/kg, i.p.) (STZ, Sigma, St. Louis, MO).[23] A high-fat diet was maintained for all the experimental protocols. The animals that presented fasting glycemia <7.8 mmol/L (<140.4 mg/dL) received another dose of STZ (30 ml/kg, i.p.).[24] Animals in the control group received the same volume of saline solution and standard chow. The glucose tolerance test (GTT) was performed to characterize the DM model. Briefly, 40% glucose (2.0 g/kg body weight) was administered intraperitoneally (IP). Blood samples were then taken from a needle prick at the tip of the rat tail at 0, 30, 60, 90, and 120 min, and the area under the curve (AUC) calculation was used to assess glucose intolerance. This evaluation was performed before the physical exercise protocol.[25,26]

2.3 HF Induction

Fifteen days after the induction of DM, HF was induced in rats through myocardial infarction surgery. In this experimental model, the rats were anesthetized (ketamine 90 mg/kg/ip and isoflurane 2% inhaled (Isoforine, 100 ml; Cristália, Brazil)) and submitted to left anterior descending coronary artery ligation as previously described.[27,28] Control-operated animals underwent all surgical steps except for coronary occlusion. Post-surgical analgesia was performed with tramadol (12.5 mg/kg,

subcutaneously) (Brainfarma Indústria Química e Farmacêutica SA, Anápolis, GO, Brazil) and dipyrone (200 mg/kg, orally) (Brainfarma Indústria Química e Farmacêutica SA, Anápolis, GO, Brazil), twice a day, for two days.

2.4 Aerobic Capacity Assessment and Aerobic Exercise Protocol

Four weeks after the infarction surgery, EX+HF+DM and EX+HF+DM+PBM animals were subjected to aerobic capacity evaluation and pre-adaptation training (10 min/day, 10 to 30 m/min, 5x a week) on a treadmill. (AVS Projetos, São Carlos, SP, Brazil). The test was performed at the beginning of each week to maintain exercise intensity.[29,30,31] Five weeks after infarction surgery, the animals underwent aerobic physical training on a rodent treadmill. The training protocol was performed for one hour at moderate intensity (50–70% VO_2 max) for eight weeks, five days a week, with a gradual increase in velocity.[31]

2.5 Photobiomodulation Protocol

Treatment with PBM started with the aerobic exercise protocol and lasted for eight weeks. A GaAIAs-850 nm laser (ISO: 13485, model 2779, Chattanooga Group - Intelect® Mobile Laser, Austin, Texas, USA) was used, and the applied parameters are listed in Table 1. A power meter (ThorLABSPM16-121) was used to verify the energy density and the laser output power. The animals received a laser application to the right and left gastrocnemius muscles.[17]

2.6 Non-invasive hemodynamic evaluation through echocardiography

To evaluate HF development, rats were anesthetized with isoflurane (2%) by inhalation and then placed in the left lateral decubitus position (45° angle). A commercially available echocardiograph (GE Vivid I; GE Medical Systems, Israel) with an 8-13 MHz electronic transducer was used. Thus, to calculate ejection fraction (%), the following calculation was performed: [(left ventricular end-diastolic volume – left ventricular end-diastolic volume) – left ventricular end-diastolic volume] × 100).[28,32]

2.7 Infarction Area Measurement

The infarction size was determined through a histological study adapted from a previously described method.[30] Briefly, measurements were taken in mid-ventricular

portions (5-6 mm from the apex), ten µm sections were cut, stained using Masson's trichrome method, and analyzed using the ImajeJ 1.47 software.[17]

2.8 Euthanasia and Tissue Collection

Animals were euthanized by decapitation. The gastrocnemius muscles were removed, weighed, frozen in liquid nitrogen, and stored at -80°C. Subsequently, protein expression assays were performed in the left gastrocnemius muscle. In order to verify the degree of skeletal muscle trophism we used the gastrocnemius/body weight index between groups. The heart was separated into the left and right ventricles, and Image J 1.47 software was used to determine the size of the infarction area.[33]

2.9 Tissue Preparation and Protein Expression Analysis

The gastrocnemius muscle was weighed and homogenized for 40 s with Ultra-Turrax (OMNI Tissue Homogenizer, OMNI International, USA) in the presence of 1.15% KCl (5 mL/g tissue) and 100 mmol/L of phenyl methyl sulfonyl fluoride. The samples were centrifuged (20 min at 10,000 × g at four °C), and the supernatant was collected and stored at -80°C until analysis.[34] A western blot technique was used to analyze protein immunocontent and perform single blinding. Electrophoresis was performed on 10% bis-acrylamide gels, and 30 µg of protein was loaded in a vertical gel system (Bio-Rad). Proteins were transferred to a PVDF membrane (Immobilon-P Transfer Membrane, Millipore) in a mini-transblot electrophoretic tank or Trans-Blot SD Semi-Dry Transfer Cell. Membranes were blocked in 5% nonfat milk prepared in TBS-T (Tris buffer saline — 20 mmol/L Tris, 137 mmol/L NaCl, pH 7.4, with 0.1% Tween-20). Membranes were probed overnight with the primary antibodies Nrf2 (1:1000 - Santa Cruz Biotechnology, Inc.), P-AKT 1:500 -Abcam, USA), Annexin V (1:500 - Santa Cruz Biotechnology, Inc.), CASPASE 3 (1:500 - Abcam, USA), CASPASE 9 (1:500 - Santa Cruz Biotechnology, Inc.), P-ASK (1:1000 - Santa Cruz Biotechnology, Inc.), BAX (1:1000 - Abcam, USA), LC3 I e II (1:1000 - Santa Cruz Biotechnology, Inc.), BECLIN-1 (1:1000 - Santa Cruz Biotechnology, Inc.), p62/SQSTM1 (1:500 - Santa Cruz Biotechnology, Inc.), and an mTOR (1:1000 - Abcam, USA), prepared in 5% TBS-T milk. Bound primary antibodies were detected using anti-rabbit or anti-mouse conjugated secondary horseradish peroxidase antibodies prepared in 5% milk TBS-T (1:1000–1:5000) incubated for two hours at room temperature. Membranes were developed using a chemiluminescent reagent, and fluorescent images were captured using a ChemiDoc MP system (Bio-Rad).

Data were expressed as mean optical densities, as quantified using Quantity One software. Ponceau was used as a loading control for normalization.[35]

2.10 Statistical analysis

Data normality was tested using the Shapiro-Wilk test. Comparison between groups was performed using one-way ANOVA followed by Bonferroni's post-test. Results are expressed as mean ± standard deviation. Differences were considered significant at P <0.05. GraphPad Instat 8.01 for Windows (GraphPad Software Inc, La Jolla, CA, USA) was used for data analysis.

3. Results

3.1 Sample Description

Animals subjected to the physical exercise protocol showed a reduction in body weight compared with the groups that did not practice physical exercise (P < 0.05). Echocardiographic analysis was performed two days after the surgical procedure to confirm the success of infarction induction. This evaluation showed a homogeneous infarction area among the groups, with an average value of 34.9% (HF+DM, 30.4% ± 8.4%) (EX+HF+DM, 36.76% ± 4.6%) (EX+HF+DM+PBM, 37.7% ± 9.6%) p=0,61 (Figure 2). Mortality of the operated animals was 20% within the first 24 hours after surgery. Regarding the infarction area, 20% did not show a significant area of infarction in the initial echocardiogram and were excluded from the study. These data indicate a 60% success rate for acute myocardial infarction (AMI) induction surgery. Additionally, ejection fraction was evaluated using echocardiography. Animals that developed AMI with an ejection fraction <45% were included in the HF group.

The animals in the HF+DM, EX+HF+DM, and EX+HF+DM+PBM groups successfully developed diabetes based on the results of the GTT results at the end of the induction protocol, these results were prior to the interventions. In the glucose tolerance test, a significant difference was observed for all groups at 120 min after glucose application (HF+DM = Time 0 min: 174.4 ± 93.8, and time 120 min: 235.1 ± 134.5, P = 0.03) (EX+HF+DM = Time 0 min: 247.6 ± 82.8, time 120 min: 357.9 ± 91.7, P = 0.0008) (EX+HF+DM+PBM = Time 0 min: 254.8 ± 137.8, time 120 min: 326.9 ± 138.6, P = 0.02). The animals showed an increase in AUC (HF+DM = 492 ± 225 mg/dl) (EX+HF+DM = 745 ± 193 mg/dl) (EX+HF+DM+PBM = 707 ± 258 mg/dl). The glucose AUC cutoff value for screening glucose intolerance was 290 mg/dl.[28] The gastrocnemius

index corrected for body weight showed no significant difference between the groups (CT = 4.11 ± 0.72) (HF+DM = 4.01 ± 0.86) (EX+HF+DM = 4.13 ± 0.55) (EX+HF+DM+PBM = 4.36 ± 0.67).

3.2 Autophagy Proteins

Animals subjected to the HF and DM protocol followed by aerobic exercise and photobiomodulation were euthanized. The gastrocnemius muscle was collected for western blot analysis of LC3I, LC3II, LC3II/LC3I ratio, BECLIN-1, p62 /SQSTM1, and mTOR, which are proteins associated with the autophagy pathway. The results are shown in Figure 3.

Concerning autophagy protein expression, EX+HF+DM and EX+HF+DM+PBM animals showed an increase in LC3I compared to CT (P = 0.02 and 0.009, respectively). In addition, the EX+HF+DM+PBM group showed significantly increased protein content compared to the CT group (P = 0.02). The other proteins showed no statistically significant differences between the groups.

3.3 Cell Survival Proteins

The animals subjected to the HF and DM protocol, and later, aerobic exercise and/or photobiomodulation, were euthanized, and the gastrocnemius muscle was collected to analyze cell survival protein expression. The results are shown in Figure 4.

The animals in the EX+HF+DM+PBM group showed a significant increase in Nrf2 protein and p-Akt levels compared to the HF+DM group (P = 0.04 and 0.03).

3.4 Apoptosis Proteins

To evaluate apoptosis, animals subjected to the HF and DM protocol followed by aerobic exercise and/or photobiomodulation were euthanized. The gastrocnemius muscle was collected to analyze annexin V, p-ASK, BAX, caspase-3, and caspase-9 levels. The results are shown in Figure 5.

4. Discussion

The efficiency of our surgical model for HF induction was validated by echocardiographic analyses, which showed a decrease in systolic function characterized by an ejection fraction of < 45%. Similarly, the DM induction model was confirmed using glucose tolerance testing. Significant alterations were found in the expression of LC3I

and cell survival proteins, P-AKT and Nrf2, underscoring the complex interplay of autophagy and survival pathways. However, no significant differences were observed in apoptosis-related proteins.

The role of the Akt/mTOR pathway in autophagy regulation is well documented, [36,37] however, contrary to expectations, mTOR expression remained unchanged across all groups. This suggests that the HF+DM model did not significantly activate the autophagy pathway, possibly accounting for the limited impact of physical exercise and photobiomodulation on the assessed autophagy proteins, except for LC3I expression. Evidence shows that physical exercise can modulate autophagic and apoptotic processes and improve cell survival in skeletal muscles.[38,39,40] However, few studies have analyzed the association between physical exercise and photobiomodulation in animals with HF and DM. We observed that eight weeks of physical exercise and photobiomodulation application increased LC3I expression, a protein related to the autophagy process. To LC3II expression, no change was observed in this protein content in the treated groups compared to that in the HF+DM group, similar to the findings of Brandt et al.,[41]and Brandt et al.,[42] Autophagosomes can undergo modifications by increasing the expression of LC3 protein by converting LC3I to LC3II through lipidation. Therefore, the LC3II/I ratio can determine the state of autophagy.[37,38] Evaluation of the LC3II/I ratio revealed no statistical differences among the groups. Similar to our results, other authors have reported increased LC3I expression without modifying LC3II expression and the LC3II/I ratio. In addition to LC3I and LC3II, other autophagy proteins were evaluated.

Beclin-1 is the first protein related to the initiation of autophagy and is one of the most essential proteins in the formation of autophagosomes.[36] Although Beclin-1 and p62/SQSTM1 levels did not vary, suggesting that the autophagy pathway was not modulated, it is essential to consider that factors such as the model used, exercise duration, and exercise intensity may significantly affect these outcomes.[43,44,45] This is supported by the work of Seiler et al., which indicated that autophagy markers do not change in the skeletal muscle in an HF context, challenging the assumed link between autophagy and muscle atrophy in HF.[46] Interestingly, while exercise alone did not elevate Nrf2 expression, its combination with photobiomodulation did, aligning with the literature positing Nrf2's vital role in antioxidant defense, especially in response to exercise-induced ROS.[47,48] However, our study did not corroborate the acute redox imbalance reported by Fasipe, Li, and Laher[49] following physical exercise. In the context of HF and DM2, where muscle atrophy is not universally observed, our findings could

prompt a re-evaluation of the implicated pathways, suggesting that alternative mechanisms may contribute to muscle preservation in these conditions.[49] This transcription factor activation mitigates oxidative stress by increasing the expression of many antioxidant genes while mediating additional beneficial effects through other cytoprotective mechanisms.[50] Although there was a significant difference between the group that practiced exercise associated with photobiomodulation and the controls, the group that practiced only exercise did not demonstrate an increase in Nrf2 expression. These results highlight the positive influence of photobiomodulation associated with training on cytoprotective protein expression. Yadav et al.,[51] evaluated the effects of photobiomodulation for 10 min daily for seven days in an animal burn model. They assessed its impact on oxidative stress, endogenous antioxidant capacity, and redox homeostasis.[51] The authors concluded that photobiomodulation reduced oxidative stress, increased superoxide dismutase (SOD) and catalase (CAT) activity, and maintenance of redox homeostasis.

Similarly, Wei et al.,[50] reported that high levels of ROS increase the release of Nrf2, which upregulates the expression of antioxidant enzymes such as SOD, CAT, and glutathione S-transferase (GST). Nrf2 acts through heterodimeric reactions with cis-regulatory elements in the gene promoter regions that encode defense enzymes.[50] Based on these previous studies, we hypothesized that the association between photobiomodulation and exercise could increase ROS levels, leading to Nrf2 activation. This activation likely prevents oxidative stress and cellular injury. This differs from what Fasipe et al.,[51] found, who demonstrated that acute physical exercise could generate redox imbalance.[51] However, we did not find articles in the literature describing the role of photobiomodulation in the Nrf2 signaling pathway in the skeletal muscle.

Interestingly, many patients with HF and DM2 do not develop muscle atrophy. HF occurs in 20-40% of patients; in DM2, the rate is 15%.[52, 53, 54] Under these conditions, muscle atrophy is marked by metabolic abnormalities, myocyte apoptosis, and hormonal shifts that favor catabolism over anabolism.[55,56,57] Furthermore, muscle mass loss leads to inhibition of the pro-hypertrophic PI3K-AKT-mTOR pathway, known for its role in protein synthesis, thus potentially affecting protein degradation.[56,57] Proteasome activity and autophagy are pivotal in the pathophysiology of muscle atrophy in both DM2 and HF.[52,53,58] Inflammation and mitochondrial dysfunction have also been implicated in DM2-related muscle atrophy.[59] Collectively, DM and HF can expedite the onset of cachexia and muscle wasting associated with heightened factors that mediate cell

apoptosis.[54] However, our study did not find any variance in the expression of apoptosis-related proteins, indicating potential alternative pathways or mechanisms.

Wang et al.,[60] investigated whether levosimendan could improve skeletal muscle contractility and atrophy in an animal model of HF.[60] Similar to our study, the authors found that the isolated model of HF did not show significant changes in the apoptosis pathway, indicating that under remote HF conditions, the skeletal muscle does not undergo substantial apoptosis. We believe that, as seen in the studies performed by Seiler et al.,[46] and Wang et al.,[60] the HF model was insufficient to influence autophagy- and apoptosis-related protein expression in skeletal muscle.[46,60]

5. Conclusion

In this study, we observed that physical exercise combined with photobiomodulation increased Nrf2 levels in the gastrocnemius muscle of animals with HF and DM, indicating the protective effect of this combined treatment. These findings are significant given the current gap in effective therapies for mitigating the muscle damage associated with concurrent DM and HF. Despite these promising results, our investigation revealed that HF and DM animal models did not exhibit altered expression of proteins related to apoptosis and autophagy. Additionally, neither exercise alone nor exercise in conjunction with photobiomodulation affected the expression of these proteins.

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Credit authorship contribution statement

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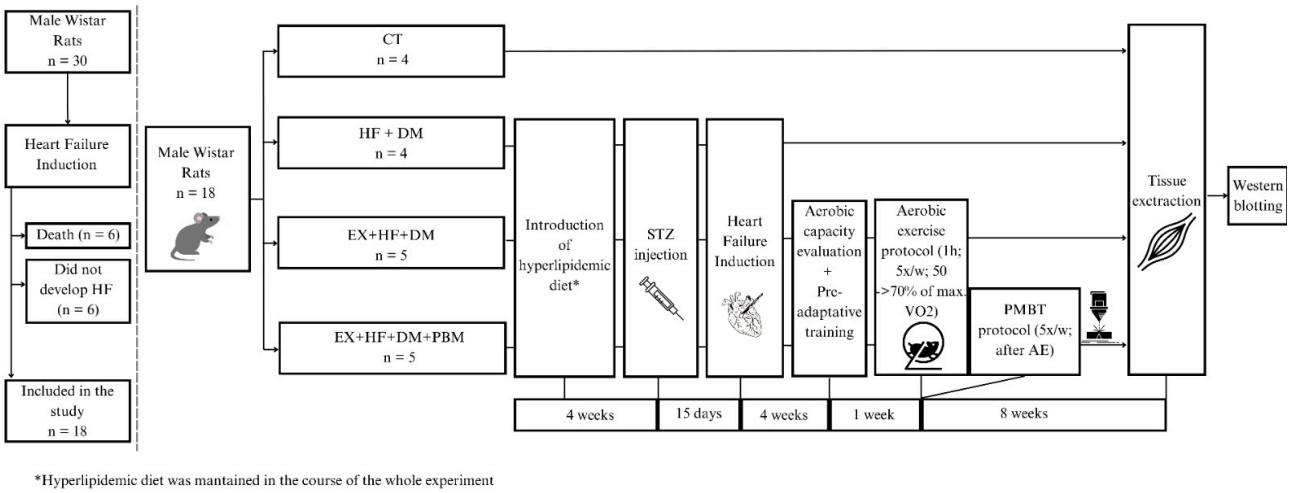


Figure 1. Experimental protocol.

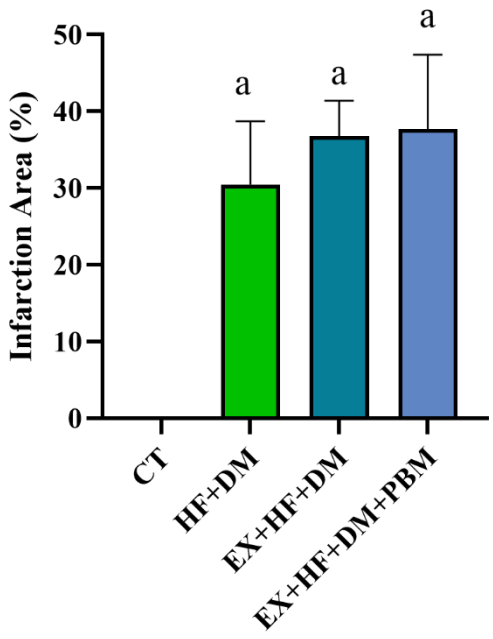


Figure 2: Evolution of the infarct area by echocardiography in rats with HF and DM before physical exercise and photobiomodulation protocol. One-way ANOVA followed by the Bonferroni post-test. [a] Different from the HF+DM group n = 4 for CT and HF+DM-groups and n= 5 for EX+HF+DM and EX+HF+DM+PBM groups.

Fig.3

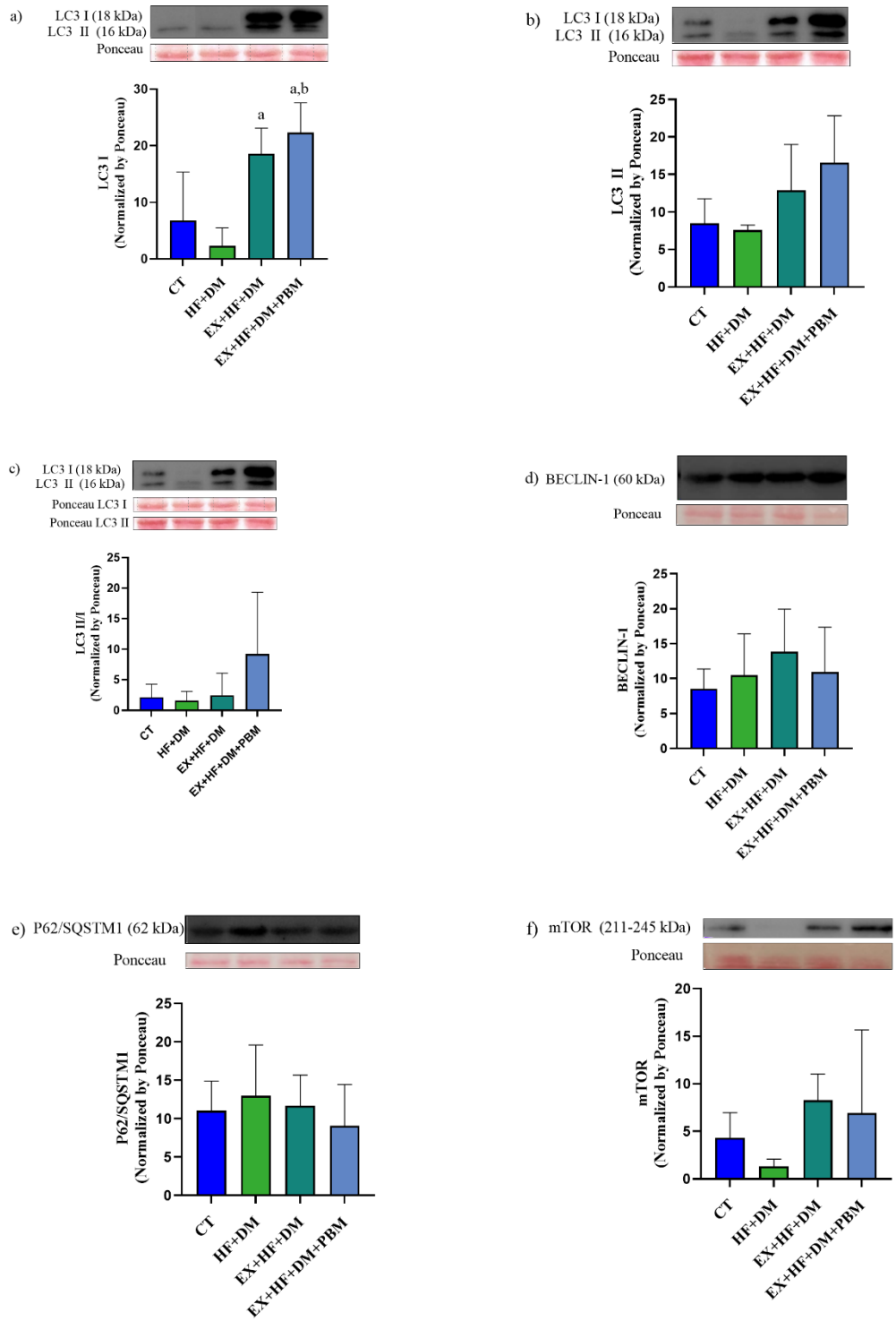


Figure 3: Evaluation of autophagy proteins in the gastrocnemius of rats with HF and DM after a physical exercise protocol and photobiomodulation. Western Blot analyzed protein content. Results were expressed as mean ± standard deviation. One-way ANOVA followed by the Bonferroni post-test. [a] Different from the HF+DM group, [b] Different from the CT group. n = 4 for CT and HF+DM- groups and n= 5 for EX+HF+DM and EX+HF+DM+PBM groups.

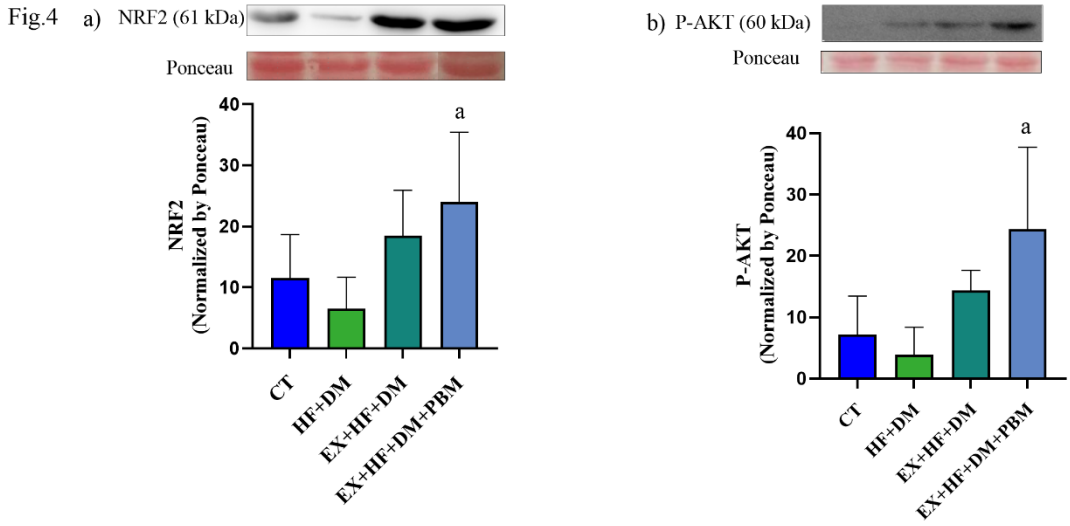


Figure 4: Evaluation of cell survival protein expression in the gastrocnemius of rats with HF and DM after physical exercise and photobiomodulation. Western Blot analyzed Nrf2 and p-AKT protein content. Results were expressed as mean ± standard deviation. One-way ANOVA followed by Bonferroni post-test. [a] Different from the HF+DM group. n = 4 for CT and HF+DM groups and n= 5 for EX+HF+DM and EX+HF+DM+PBM groups.

Fig.5

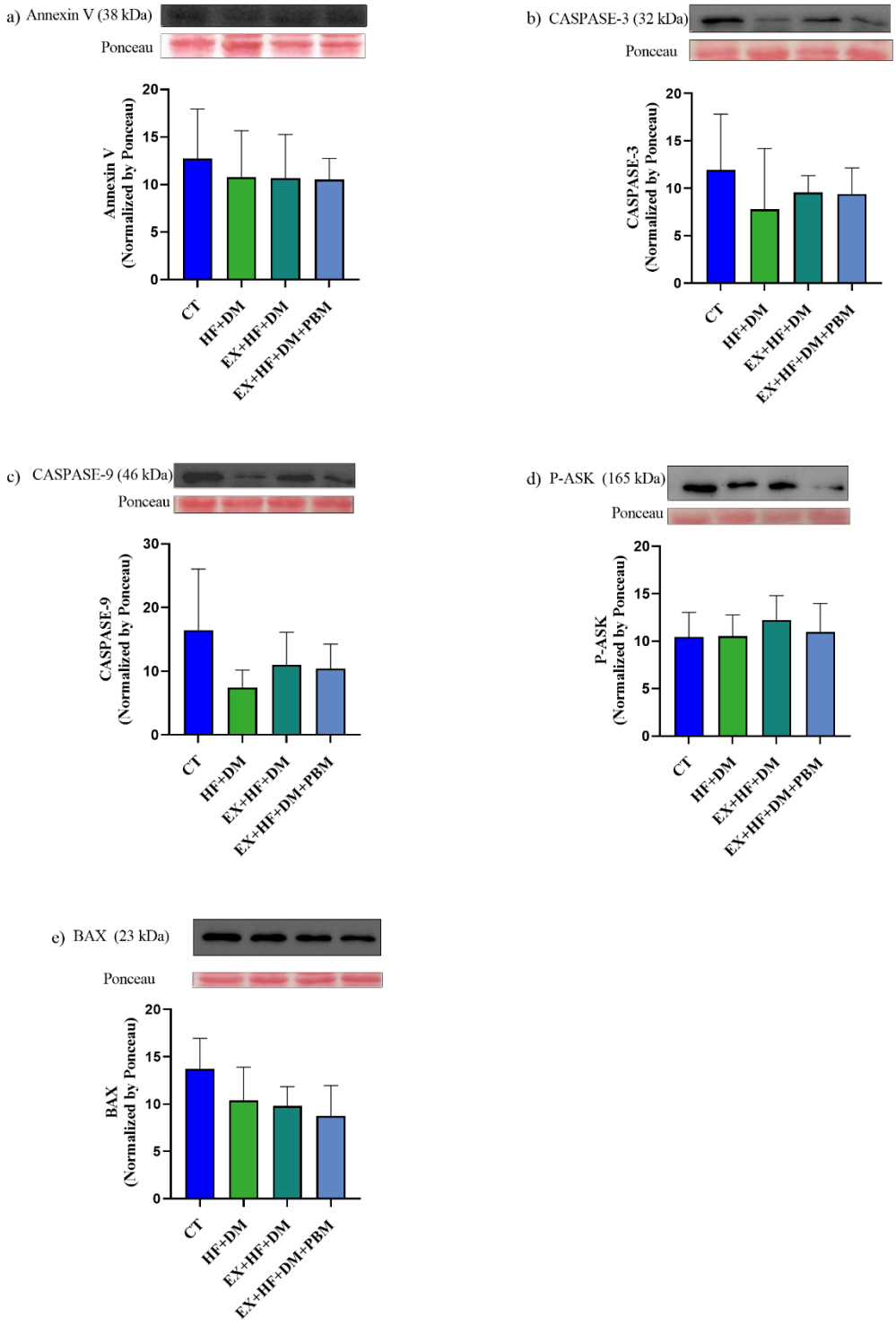


Figure 5: Evaluation of apoptosis in the gastrocnemius of rats with HF and DM after a physical exercise protocol and photobiomodulation. Western Blot analyzed Annexin V, p-ASK, Bax, caspase-3, and caspase-9. Results were expressed as mean ± standard deviation. One-way ANOVA followed by Bonferroni post-test. n = 4 for CT and HF+DM groups and n= 5 for EX+HF+DM and EX+HF+DM+PBM groups.

Table 1: Protocol table of laser radiation

Model	Laser GaAIAs (850nm)
Number of laser diodes	3
Pulse frequency	Continuous output
Output power (mW)	100/diode
Spot size (cm2)	0.012
Power density (W/cm2)	8.33
Protocol regime	Immediately after aerobic training protocol; bilaterally in hind limbs
Number of points	3
Irradiation sites per animal	12
Total energy per point (J)	0.735
Total energy per animal (J)	8.82J
Dose per treatment (J/cm2)	61.25 J/cm²
Time per point (s)	7.3s
Time total (s)	21.9s
Application mode	Spot held stationary in skin contact at 90° angle with slight pressure
Frequency treatment	1x day for five days/week
Protocol duration (weeks)	8

Table 2. Result of the expression of proteins involved in autophagy.

Parameters	CT	HF+DM	EX+HF+DM	EX+HF+DM+PBM	p
LC3 I	6.8 ± 6.6	7.2 ± 10.2	15.2 ± 8.5[a]	13.6 ± 12.5[a,b]	0.005
LC3 II	9.7 ± 3.6	6.2 ± 2.8	12.9 ± 6.1	14.4 ± 7.3	0.16
LC3II/I ratio	2.1 ± 2.1	1.6 ± 1.5	2.5 ± 3.6	9.2 ± 10.1	0.22
BECLIN-1	8.6 ± 2.8	10.5 ± 5.9	13.8 ± 6.1	10.9 ± 6.4	0.58
P62/SQSTM1	11.1 ± 3.9	13.0 ± 6.6	11.7 ± 4.0	9.1 ± 5.3	0.70
mTOR	19.5 ± 30.5	13.0 ± 23.4	7.0 ± 3.7	6.9 ± 8.7	0.71

NOTE: Result of the expression of proteins involved in autophagy [a] $p<0.05$ compared to CT, [b] $p<0.05$ compared to CT. The results were expressed as mean ± standard deviation.

Table 3. Result of the expression of proteins involved in cell survival.

Parameters	CT	HF+DM	EX+HF+DM	EX+HF+DM+PBM	p
Nrf2	11.5 ± 7.1	6.5 ± 5.2	18.5 ± 7.4	24.0 ± 11.4[a]	0.04
P-AKT	7.2 ± 6.3	6.8 ± 6.8	14.8 ± 3.3	20.5 ± 13.4[a]	0.03

NOTE: Result of the expression of proteins involved in cell survival [a] $p<0.05$ compared to CT, [b] $p<0.05$ compared to CT. The results were expressed as mean ± standard deviation.

Table 4. Result of the expression of proteins involved in process apoptosis.

Parameters	CT	HF+DM	EX+HF+DM	EX+HF+DM+PBM	p
Anexin V	12.7 ± 5.2	10.8 ± 4.9	10.7 ± 4.6	10.5 ± 2.2	0.85
CASPASE-3	11.0 ± 5.9	7.8 ± 6.4	9.6 ± 1.8	9.4 ± 2.7	0.62
CASPASE-9	16.5 ± 9.6	7.4 ± 2.7	11.0 ± 5.1	10.5 ± 3.8	0.20
P-ASK	10.4 ± 2.6	10.5 ± 2.2	12.2 ± 2.6	11.0 ± 2.9	0.71
BAX	13.7 ± 3.2	10.4 ± 3.6	9.8 ± 2.0	8.8 ± 3.2	0.13

NOTE: The result of the expression of proteins involved in the process of apoptosis. The results were expressed as mean ± standard deviation.

4 ARTIGO 2

Photobiomodulation Improves Cell Survival and Death Parameters in Cardiomyocytes Exposed to Hypoxia/Reoxygenation

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Data availability and material: data generated or assessed during the study is available to the corresponding author through reasonable solicitation.

Code availability: N/A

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ABSTRACT

Introduction: Cardiovascular diseases are the leading cause of morbidity and mortality worldwide. The ischemic heart disease is one of the most harmful conditions to cellular structure and function. After reperfusion treatment, a spectrum of adverse effects becomes evident, encompassing altered cell viability, heightened oxidative stress, activated autophagy, and increased apoptosis. Photobiomodulation (PBM) has been utilized in experimental models of cardiac hypoxia to enhance mitochondrial response and ameliorate the biochemical changes in the injured tissue. However, the effects of PBM on cultured cardiomyocytes subjected to hypoxia/reoxygenation are not yet well established. **Method:** H9C2 cardiomyocytes were exposed to hypoxia with concentrations of 300 μM $CoCl_2$ for 24 hours, followed by 16 hours of reoxygenation through incubation in a normoxic medium. Treatment was conducted using GaAlAs Laser (850nm) after hypoxia at an intensity of 1J/cm^2. Cells were divided into three groups - Group CT: cells maintained under normoxic conditions; Group HR: cells maintained in hypoxia and reoxygenation conditions without treatment; Group HR+PBM: cells maintained in hypoxia and reoxygenation conditions that underwent PBM treatment. Cell viability was analyzed using MTT, and western blot assessed protein expression. One-way ANOVA with the Tukey post hoc test was used for data analysis. Differences were significant when $p<0.05$. **Results:** PBM at intensities of 1J/cm^2 mitigated the alterations exposed by hypoxia/reoxygenation in cell survival. Additionally, significantly increased the expression of proteins Nrf2, HSP70, mTOR, LC3II, LC3II/I, Caspase-9, and reduced the expression of PGC-1α, SOD2, xanthine oxidase, Beclin-1, LC3I, and Bax. **Conclusion:** PBM at intensities of 1J/cm^2 reverses the changes related to oxidative stress, mitochondrial biogenesis, autophagy, and apoptosis caused by hypoxia and reoxygenation in a culture of cardiomyocytes.

Keywords: Photobiomodulation, hypoxia/reoxygenation, H9C2, oxidative stress, autophagy.

1. INTRODUCTION

Coronary diseases are the leading causes of death and disability [1,2]. In 2020, they affected approximately 244 million individuals worldwide, and it is estimated that by 2030, ischemic coronary disease will globally affect 1,845/100,000 individuals [3]. Myocardial revascularization is the most recommended treatment for ischemic coronary artery disease [4]. From 2003 to 2016, twelve million, sixty-two thousand, and eighty-one surgeries were performed in the United States [5], and almost 400,000 surgical procedures are conducted worldwide each year [6].

Although revascularization can effectively manage ischemic emergencies, it is imperative to address the subsequent risk of reperfusion injury [7]. Ischemia/reperfusion injury or hypoxia/reoxygenation (H/R) in the myocardium is initiated in the early minutes of reoxygenation, and, therefore, this period represents a valuable "therapeutic window" for myocardial protection [8,9]. Thus, the primary strategies to minimize the harmful effects of H/R are implemented in the early minutes of reoxygenation [10,11].

Simulating this process in culture models of H9C2 cardiomyocyte cell line [12] is possible. The hypoxia model involves reducing the O_2 concentration through an anaerobic incubator (95% N^2 and 5% CO_2) or by chemically inducing hypoxia with cobalt chloride ($CoCl_2$), followed by reoxygenation in an aerobic medium [13,14].

In hypoxia, blood flow is interrupted, limiting adenosine triphosphate (ATP) formation due to mitochondrial changes in the electron transport chain and the failure of ionic pumps, initiating anaerobic metabolic pathways [15,16]. Consequently, there is a decrease in antioxidant defenses, pH, and ion exchange. These changes make ATP-dependent transporters nonviable, potentially leading to mitochondrial dysfunction and cell death [1,17].

When revascularization is performed, cellular reoxygenation occurs. The amount of oxygen supplied induces a marked formation of reactive oxygen species (ROS), exceeding the capacity of the free radical elimination system [1,11]. As a consequence of increased ROS mainly through the canteen oxidase pathway, there is a decrease in membrane fluidity, increased calcium permeability, and changes in mitochondrial function, resulting in cardiomyocyte death through multiple mechanisms, including the activation of apoptosis and modifications in the autophagy process [1,7].

Under cellular stress conditions like hypoxia the autophagy pathway begins, the mTOR protein becomes inhibited, leading to the activation of Beclin-1 and the initiation of autophagosome formation. The autophagosome expands through the action of autophagy-related proteins (ATG), such as ATG7 and ATG3, facilitating the conversion of LC3I to LC3II. LC3II is integral to autophagosome closure and the recruitment of adapter proteins like p62/SQSTM1. The subsequent fusion with lysosomes signifies the transition to an autolysosome [18,19].

The therapeutic alternatives to improve cellular damage caused by H/R injury include photobiomodulation (PBM), which has a beneficial effect on tissue repair [20], antioxidant production [21], mitochondrial protection [22], and cellular survival in ischemic cardiac tissue [23]. It is known that these effects are related to the ability of PBM to produce ATP by stimulating the activity of cytochrome c oxidation and consequently increasing mitochondrial function, thus improving cellular metabolism [24]. The current literature lacks a comprehensive analysis of the pathways involved in cell survival and death in cardiomyocyte cultures subjected to H/R and treated with PBM. This study aims to elucidate the effects of PBM on these cellular pathways.

2. MATERIALS AND METHODS

2.1 Cell culture

H9c2(2-1) cardiomyoblast cell line was obtained from the Rio de Janeiro Cell Bank (BCRJ). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin and maintained at 37◦C, 5% CO_2, and 95% humidity. The medium was changed every second day. Cells were passaged when they reached 80% confluence.

2.2 Hypoxia/Reoxygenation (H/R)

The H9c2(2-1) cells were divided into three experimental groups: Group CT, cells maintained under normoxic conditions throughout the protocol described below; Group HR, cells maintained in hypoxia and reoxygenation conditions without treatment; and Group HR+PBM, cells maintained in hypoxia and reoxygenation conditions that underwent treatment with PBM.

To mimic the hypoxic conditions, the cells of the HR and HR+PBM groups were treated with $CoCl_2$ (300 μM; Sigma-Aldrich; Merck KGaA) in DMEM without glucose with 1% FBS and 1% penicillin/streptomycin media, for 24h. After that, the cells were washed with phosphate-buffered saline (PBS), and reoxygenation was performed after incubation in a normoxic medium for 16h (DMEM, 10% FBS, and 1% penicillin-streptomycin solution) in an incubator with 5% CO_2 at 37°C for 16h [25,26]. Cells from the CT group were also incubated in a normoxic medium and manipulated with a medium change in all processes to which we subjected cells from HR and HR+PBM groups.

2.3 Stage 1

Photobiomodulation is intensity and power-dependent. This research stage was designed to determine the intensity of treatment application based on cell viability results in cardiomyocytes exposed to H/R.

2.3.1 PBM Protocol

Irradiation was performed shortly after hypoxia using the GaAlAs Laser (850nm) (ISO: 13485, model 2779, Chattanooga Group - Intelect® Mobile Laser, Austin, Texas, USA) using the pen directly in the wells of the 96-well plate. At a continuous pulse frequency, with different energy intensities, thus, the power density varied from 8W/cm² to 12W/cm², and application time ranging from 0,15s to 250s, depending on the energy intensity. To identify the best intensity, we used the MTT technique, and the wells of the plates were divided into different groups by energy intensity: HR +PBM1= 1J/cm²; HR +PBM2,2= 2,2J/cm²; HR +PBM3= 3J/cm²; HR +PBM9= 9J/cm² and HR +PBM21= 21J/cm² [27,28,29,30,31,32] (Fig. 1). Intensity correction was performed for the culture plate [33]. The application occurred in the lower region of the plate using a point pen, with a distance of 10mm between the cells and the laser. A power meter (ThorLABSPM16-121 - New Jersey, United States) was used to verify energy density and laser power output. The intensity that yielded the best cellular viability result was chosen for the treatment protocol.

2.3.2 MTT

Cardiomyocyte cell viability was assessed by the MTT technique (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [34], adapted for this culture model. Group CT was maintained in a normoxic environment, while groups HR and HR+PBM were submitted to the hypoxia/reoxygenation and treatment protocol, as described above. Cardiomyocytes were seeded in 96-well plates at a density of 3x103 cells/well. After 24 hours, they were incubated with MTT for three hours. The crystals were dissolved in dimethyl sulfoxide (DMSO), and the amount of formazan (presumably directly proportional to the number of viable cells) was measured by the SpectraMax Microplate Reader (Molecular Devices Corporation, USA) at 570 nm.

2.4 Stage 2

After determining the most effective intensity for cell viability, we then examined the impact of photobiomodulation at 1J/cm^2 on the pathways related to oxidative stress, autophagy, and cellular apoptosis in cardiomyocytes H/R exposed.

2.4.1 PBM Protocol

Irradiation was performed shortly after hypoxia using the GaAlAs Laser (850nm) (ISO: 13485, model 2779, Chattanooga Group - Intelect® Mobile Laser, Austin, Texas, USA) in culture bottles, at a continuous pulse frequency, with energy intensities of 1J/cm^2 (HR+PBM), power density ranging from 8W/cm² to 12W/cm², and application time ranging from 150s. Intensity correction was performed for the culture plate [33]. The application occurred in the lower region of the plate using a cluster with multiple points (each point emitted the described energy), with a distance of 10mm between cells and the laser.

2.4.2 Cell preparation and protein analysis

The cells were washed twice with PBS in RIPA buffer (Beyotime, Shanghai, China) [35]. Proteins were quantified using the method described by Lowry et al. (1951) [36], which utilizes a bovine albumin solution at 1 mg/mL concentration as standard. The measurement was carried out on a spectrophotometer at 625 nm, and the results were expressed in mg/mL.

2.4.3 Western Blot

Western blot was used to analyze protein immunocontent. Electrophoresis was performed in 10% bis-acrylamide gels, loading 50 µg of proteins in a vertical gel system (Bio-Rad). Proteins were transferred to a PVDF membrane (Immobilon-P Transfer Membrane, Millipore) in a mini-transblot electrophoretic tank or Trans-Blot SD Semi-Dry Transfer Cell. Membranes were blocked in 5% nonfat milk prepared in TBS-T (Tris buffer saline - 20 mmol/L Tris, 137 mmol/L NaCl, pH 7.4, with 0.1% Tween-20). Membranes were probed overnight with the primary antibodies PGC-1α (1:500 - Santa Cruz Biotechnology, Inc.), Nrf2 (1:1000 - Santa Cruz Biotechnology, Inc.), SOD2 (1:1000 - Santa Cruz Biotechnology, Inc.), xanthine oxidase (1:500 - Santa Cruz Biotechnology, Inc.), mTOR (1:500 - Abcam, USA), Beclin-1 (1:500 - Santa Cruz Biotechnology, Inc.), LC3 I e II (1:1000 - Santa Cruz Biotechnology, Inc.), caspase-9 (1:1000 - Santa Cruz Biotechnology, Inc.), Bax (1:1000 - Santa Cruz Biotechnology, Inc.) and HSP70 (1:1000 - Santa Cruz Biotechnology, Inc.) prepared in 5% TBS-T milk. Bound primary antibodies were detected using anti-rabbit or anti-mouse conjugated to secondary horseradish peroxidase antibodies prepared in 5% milk TBS-T (1:5000) incubated for two hours at room temperature. Membranes were developed using a chemiluminescent reagent, and fluorescent images were captured using a ChemiDoc MP system (Bio-Rad). Data were expressed as mean optical densities as quantified using Quantity One software. Alpha-actinin and beta-actin were used as the loading control for normalization [37].

2.5 Statistical analysis

The normality of data was tested with the Shapiro-Wilk test. Different groups were compared using one-way ANOVA with the Tukey post hoc test. The square of partial Eta (n^2) was derived from a general linear way to assess effect size. Effects were classified as low (0.01-0.06), moderate (0.06 – 0.14), and high (>0.14). MTT results were calculated from the mean ± standard deviation, but expressed on the graph as a percentage. Pearson correlation test was used to determine the correlation between autophagic flow (LC3II/I - LC3II divided by the amount of LC3I) and Caspase-9. Results were expressed as the mean ± standard deviation. The differences were statistically significant when the assessment showed $p<0.05$. GraphPad Instat 9.05 for (GraphPad® Software Inc, La Jolla, CA, USA) was used as a tool for data analysis.

3. RESULTS

3.1 Stage 1

3.1.1 Cell Viability

Cell viability was significantly lower in the HR, HR+PBM2.2, HR+PBM3, HR+PBM6, and HR+PBM9 groups (27%, 26%, 27%, 28%, and 24% $n^2= 0.53$, respectively) compared to the CT group. The other groups did not significantly differ (Fig 2 and 3). Based on the cell viability data, the intensity of 1J/cm^2 was selected for subsequent experiments.

3.2 Stage 2

3.2.1 Oxidative Stress

H/R can alter oxidative stress, with xanthine oxidase being an essential protein that produces reactive oxygen species (ROS). This protein was increased in the HR group compared to the CT and HR+PBM groups ($p=0.002$ e $p=0.001$, $n^2=0.64$, Fig. 4a). Thus, we demonstrate the positive impact of PBM on modulating this pathway. As a compensatory response to H/R injury, an elevation in PGC-1α, a protein associated with mitochondrial biogenesis, was noted, leading to a notable was increased in the HR group compared to the CT and HR+PBM groups ($p= 0.01$ e $p= 0.0003$, $n^2= 0.67$, Fig. 4b). Increased Nrf2 expression protects cardiomyocytes by stimulating the production of antioxidant enzymes and protecting against ROS damage. Therefore, in our study, we observed a significant reduction in Nrf2 in the HR group compared to the CT and HR+PBM groups ($p = 0.04$ e $p = 0.05$, $n^2 = 0.4$, Fig. 4c). Even with the increase in Nrf2, we observed that SOD2, the enzyme responsible for reducing ROS, increased in the HR group compared to the CT and HR+PBM groups ($p=0.003$ e $p=0.03$, $n^2=0.52$, Fig. 4d).

3.2.2 Autophagy

The mTOR protein is associated with cell survival, and increased expression is correlated with inhibition of the autophagy process. We observed in our study that the HR group showed a significant decrease in mTOR expression when to the CT and HR+PBM groups (p=0.001 and p=0.009, respectively, n^2=0.6, Fig. 5a). Following the autophagy pathway, decreased mTOR expression induces increased in Beclin-1 expression. In our study, the HR group showed a significant incresead in Beclin-1 expression when compared to the CT and HR+PBM groups (p=0.01 and p=0.008, n^2=0.5, Fig. 5b). The increased in Beclin-1 caused an elevation in LC3I expression in the HR group compared to the CT and HR+PBM groups (p=0.01 and p=0.04, n^2=0.45, Fig. 5c). Contradictorily, there was a decreased in LC3II expression in the HR group compared to the CT and HR+PBM groups (p=0.02 and p=0.04, n^2=0.47, Fig. 5d). We can suggest that H/R injury by cobalt hydrochloride may act on proteins that lipidate LC3I into LC3II. Therefore, we can observe that the HR+PBM group showed no difference with CT maintaining autophagic flux by the LC3II/I ratio (p=0.004 and p=0.03, n^2=0.66, Fig. 5e). While the HR group was different from the HR+PBM and CT groups. It is currently known that there is a relationship between Caspase-9 levels and autophagy. We demonstrated a decreased in caspase-9 expression in the HR group compared to the CT and HR+PBM groups (p=0.003 and p=0.05, n^2=0.058, Fig. 5f). In fact, we observed a positive correlation between caspase-9 and LC3II/I (r=0.74 and p=0.004, Fig. 5g).

3.2.3 Apoptosis

Bax plays a crucial role in the mitochondrial apoptotic pathway. When activated, it induces an inflammatory process in mitochondria, leading to their rupture and subsequent release of proteins, resulting in cell death. In our study, Bax protein expression was significantly taller in the HR group when to the CT and HR+PBM groups (p = 0.02 and p = 0.0004, n^2 = 0.72, Fig. 6a). As a cellular protective measure, the expression of the HSP70 protein increases, thereby inhibiting apoptosis. HSP70 protein expression decreased dramatically in the HR group compared with the CT and HR+PBM groups (p = 0.01, Fig. 6b). We did not observe a significant difference between the CT and HR+PBM groups (p=0.14, n^2=0.39).

4. DISCUSSION

We reveal that PBM treatment significantly improves cellular functions, such as viability, oxidative stress, autophagy, and apoptosis in rat cardiomyocytes (H9c2) during H/R, suggesting it plays a protective regulatory role. These findings may provide a new understanding of cellular adaptation to

stress (H/R) and inform the development of groundbreaking treatments for cardiovascular diseases.

Previous studies suggest that PBM enhances cell viability and mitochondrial efficiency and reduces oxidative stress while improving the processes of cellular autophagy and apoptosis [38,39]. However, there is limited research regarding the effects of PBM on H9c2 cells experiencing H/R. Our study demonstrates that $CoCl_2$-induced hypoxia in H9c2 cells leads to notable changes in survival, mitochondrial generation, oxidative stress management, autophagy, and apoptosis. $CoCl_2$ induces cellular injury by blocking the activity of the Prolyl Hydroxylase Domain (PHD), leading to stabilization of HIF-alpha, inducing a state similar to intracellular hypoxia, also known as hypoxia-reoxygenation injury or ischemia-reperfusion injury [40].

Effects of PBM on cellular viability: The study by Li (2021) [26] demonstrated that H9c2 cells exposed to $CoCl_2$ at doses of 200μM and 400μM experienced decreased cell survival after 24 hours. Similarly, Gómez-Arniz (2020) [41] showed that exposure of neurons to $CoCl_2$ (300μM) for 24 hours reduced cell survival, as indicated by the MTT assay. In our study, we determine the intensity of PBM using the MTT cell survival test results. The analyzed intensities were defined based on studies already published in the literature that focused on the effects of laser on post-cardiac ischemia in cell culture and animal models [27,28,29,30,31,32,42]. We observed that intensities of 1 and 21 J/cm^2 attenuated the changes in cell viability promoted by H/R, indicating that the effect of PBM is intensity-dependent. Corroborating, the study by Hoh & Mitrofanis (2023) [43] reported that the exposure of PBM (910nm) at an intensity of 7.2 J/cm^2 in cultured mouse fibroblast cells (L-929) did not induce cytotoxicity and, at the same time, it did not increase cell viability and proliferation. Contradictorily, the study by Dawoud et al. (2021) [44] found that the application of PBM (627nm) at intensity of 2 J/cm^2 resulted in an increase in cell growth rates and cell viability of stem cells from the dental pulp of human permanent teeth.

Effects of PBM on oxidative stress: In response to reduced cell survival in the H/R model, we observed an increase in PGC-1α. H/R, induced by $CoCl_2$, may have augmented ROS formation and diminished oxygen utilization efficiency in cardiac cell mitochondria [45]. ROS are generated by xanthine oxidase. During hypoxia, xanthine dehydrogenase converts to xanthine oxidase, which, upon reoxygenation, can transfer electrons to oxygen, forming ROS (superoxide and hydrogen peroxide) [1,46].

Griguer et al. 2006 [47] showed that the $CoCl_2$ at 100μM and 200μM for 5 hours in glioma U251-MG cells increased xanthine oxidase expression and ROS levels. Our findings reveal increased xanthine oxidase in H9c2 cells post-H/R, which was reversed with PBM treatment. This reduction in xanthine oxidase by PBM may relate to enhanced mitochondrial functions, such as improved ATP synthesis and respiration [48,49].

Reduced mitochondrial functionality prompts a rise in PGC-1α levels, fostering mitochondrial biogenesis and shielding cells from ROS-induced death via antioxidant enzyme modulation [50]. Yet, excessive PGC-1α in cardiac tissue can increase mitochondria numbers with structural and functional alterations leading to dysfunction [51,52]. Overexpressed PGC-1α also heightens BNIP3 activity, potentially obstructing mitophagy [51,53]. Our study corroborated an increase in PGC-1α in the H/R group. Zhu et al. 2010 [54] and Arany et al. 2008 [55] found increased myocardial PGC-1α expression under hypoxic conditions. We noted decreased PGC-1α expression in the PBM-treated group, likely due to diminished ROS production [56].

The study conducted by Bonvicini et al. (2021) [57] investigated the effects of PBM on cell viability and oxidative stress in human dental pulp fibroblasts subjected to inflammation caused by lipopolysaccharides (LPS). They determined that treatment parameters of 15 J/cm^2 and 30 J/cm^2 were the most effective in decreasing the levels of NO and ROS while also enhancing cell viability.

The downregulation of PGC-1α negatively affects the binding of Nrf2 to the GCLC ARE gene, decreasing SOD2 expression [58], which our study observed. Conversely, the H/R group exhibited increased SOD2, likely a compensatory mechanism to the injury.

The rise in Nrf2 in the HR+PBM group may be tied to sustained autophagy due to p62 expression regulation in response to oxidative stress, with p62 further boosting Nrf2 in a positive feedback loop [59,60].

Effects of PBM on autophagy: The autophagy process is associated with maintaining cellular homeostasis and survival [61]. It also plays a crucial role in preserving cardiovascular function, where its inhibition may lead to cardiomyocyte death and subsequent cardiovascular diseases [62].

$CoCl_2$ has been used to modulate autophagy in H9c2 cells, among others. Chen et al. (2017) [63] reported increased autophagy in mouse myoblasts (C2C12), as evidenced by a higher LC3II/I ratio following CoCl2 treatment. Similarly, Li et al. (2021) [25] observed an increased autophagic flux in H9c2 cells exposed to CoCl2.

Our findings indicate an increase in autophagy in PBM-treated H9c2 cells, shown by an elevation in mTOR proteins, a reduction in Beclin-1, a decrease in LC3I, and an elevation in LC3II. Furthermore, we noted an elevated autophagic flux by the LC3II/I ratio. Therefore, the effects of the H/R model using CoCl2 depend on the duration, dose, and reoxygenation conditions [64].

Our study suggests that reoxygenation-induced mitochondrial alterations inhibit autophagic flux in the H/R group by increasing ROS, which can oxidize enzymes initiating autophagy, promoting cell death and oxidative stress accumulation [58]. Under ischemic/hypoxic conditions, autophagy can inhibit apoptosis, protecting cardiac tissue [61,62].

The interplay between autophagy and caspase-9, which can bind to ATG7 and ATG3 to assist LC3II formation, has been discussed. Decreased caspase-9 correlates with reduced autophagy and increased apoptosis, indicating that caspase-9's apoptotic function is suppressed during interactions with autophagy proteins [65]. Our study found that the PBM elevated caspase-9 expression and autophagic flux, as measured by the LC3II/I ratio, with a positive correlation between these variables.

As mentioned, autophagy is initiated by activating the PI3K/AKT/mTOR signaling pathway. This pathway governs autophagy and regulates apoptosis and cell proliferation [66]. These mechanisms are ATP-dependent [67]. Our results show that PBM positively influences mTOR expression. Liao et al. (2022) have demonstrated that PBM at 660nm induces AKT phosphorylation and enhances the proliferation of primary neural stem cells following hypoxic-ischemic injury [68]. The precise mechanisms by which PBM activates the PI3K/AKT/mTOR pathway remain to be fully elucidated. Nonetheless, it is plausible to propose that the ATP made available by PBM may trigger this pathway, thereby improving autophagic flux.

The study by Zuo et al. (2022) [69] examined the effects of 808nm PBM on autophagy and apoptosis in MC3T3-E1 pre-osteoblasts from mice treated with hydrogen peroxide (H_2O_2). They observed that PBM demonstrated the ability to modulate autophagy and apoptosis and improve osteogenesis at high levels of oxidative stress.

On the other hand, Pevna et al. (2022) [70] investigated the influence of PBM (808nm) and photodynamic therapy associated with chemotherapy on U87 MG human glioblastoma cells. They demonstrated that the effects on autophagy and apoptosis parameters depend on the dose administered but are independent of the technique used, whether PBM or photodynamic therapy. They noted that PBM can preferentially induce autophagy in non-cancerous cells.

The interplay between autophagy and apoptosis is intricate. Autophagy maintains cellular homeostasis by clearing damaged organelles, particularly mitochondria, potentially preventing apoptosis under cellular stress [71,72]. Conversely, apoptosis can suppress autophagy by degrading the proteins vital for its execution [73].

Effects of PBM on apoptosis: Our study observed that the H/R model increased cell death by upregulating the Bax protein while concurrently inhibiting autophagy. PBM treatment mitigated these effects, reducing cell death and restoring normal autophagic flow. Chen et al. 2020 [35] found that exposing mouse digital flexor tendon cultures to 300 μM CoCl2 during a 24-hour hypoxic period followed by 24 hours of reoxygenation increased apoptosis by altering the Bax/Bcl-2 ratio. They also noted that hypoxia alone did not increase apoptosis levels. Supporting these findings, Li et al. (2021) [73] reported that exposure to 800 μM of $CoCl_2$ for 48 hours induced apoptosis in H9c2 cells.

The study by Kim et al. 2023 [74] examined the effects of PBM on geniculate neuronal ganglion cells (GGNs) on cell viability and proliferation. Treatment with 10J PBM significantly improved cell viability in GGNs and reduced apoptosis. The study conducted by Chaudary et al. (2020) [75] investigated the effects of pulsed red LED light at 635 nm on cellular function in cultured mouse myoblasts and fibroblasts exposed to H/R. The study concluded that PBM significantly restored ATP levels during H/R and effectively promoted cell growth, preventing the process of cell apoptosis.

We further explored PBM's potential effects on the expression of the HSP70, known for their anti-apoptotic properties [15]. Our results showed increased HSP70 expression in the group treated with HR+PBM compared to the HR group. HSP70 serves various protective roles, including reducing ROS levels and interacting with the Nrf2 antioxidant system [76]. During cardiac ischemia, HSP70 can act as a cardioprotective agent, mitigating damage from H/R and ROS, thus enhancing cardiomyocyte survival and function [77,78]. Liu et al. (2022) [15] demonstrated that transfection treatments increased cell viability and decreased apoptosis rates in H9c2 cells undergoing H/R. Evangelista et al. (2021) [79] observed increased HSP70 in rats with acute Achilles tendinitis treated with PBM. Similarly, Yaakobi (2001) [80] highlighted that PBM (at 804nm) mechanisms include upregulating HSP70 in rat hearts subjected to ischemia/reperfusion.

We believe that the main limitation of our study was the absence of other techniques to confirm the findings and the lack of results with different intensities. On the other hand, the results of the present study may encourage other groups to use different intensities to minimize or reverse the biochemical changes that occur in hypoxia/reoxygenation injury in cardiomyocytes.

In conclusion, our results showed that PBM therapy, applied at 850nm with an intensity of 1J/cm^2, effectively neutralized the changes caused by H/R. PBM specifically increased the expression of HSP70 and Nrf2, reduced xanthine oxidase levels, maintained physiological levels of autophagy, and reduced apoptosis. These cellular responses contributed to improved cell survival under H/R stress. H9c2 cells subjected to H/R stress showed a notable increase in xanthine oxidase activity. This increase was compensated by SOD2 and PGC-1α levels. Changes induced by oxidative stress reduced autophagy, as evidenced by changes in the LC3II/I ratio, and there was also an increase in apoptotic activity, resulting in impaired cell proliferation. Our primary findings and the underlying mechanisms are succinctly depicted in Figure 7, which provides a visual summary of the results and illustrates the pathways involved.

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Credit authorship contribution statement

Alan Christhian Bahr: Conceptualization, Methodology, Software, Investigation, and Writing- Original draft preparation. **Liliana Ivet Sous Naasani**: Conceptualization and Methodology. **Elizama de Gregório**: Writing- Reviewing and Editing. **Márcia Rosângela Wink:** Conceptualization and Methodology. **Alex Sander da Rosa Araujo**: Writing- Reviewing and Editing. **Patrick Türck**: Methodology, Investigation, Validation and Supervision. **Pedro Dal Lago**: Conceptualization, Validation, and Supervision.

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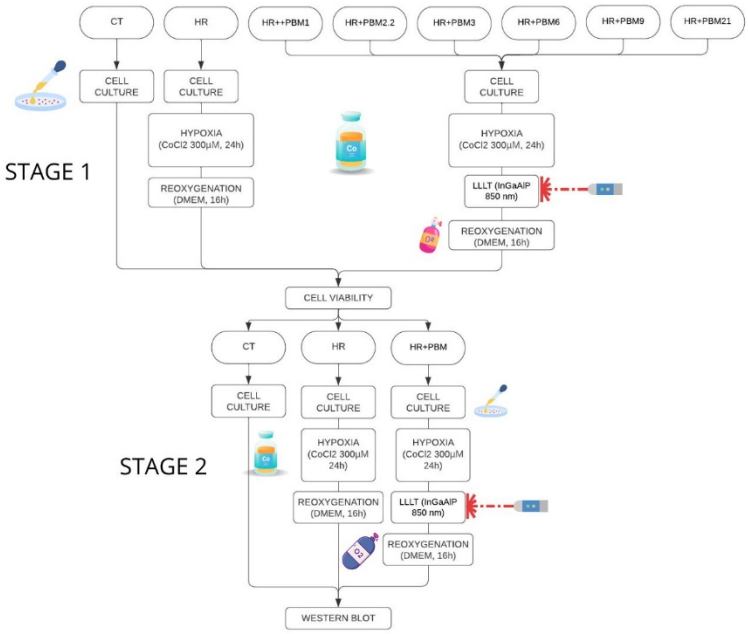


Fig. 1: Experimental design. Stage 1: Irradiation with the GaAlAs Laser (850nm) was carried out immediately after hypoxia directly in the wells of the 96-well plate, to identify the best intensity, using the MTT technique, dividing the wells into different energy intensity groups: 1J/cm², 2.2J/cm², 3J/cm², 9J/cm² and 21J/cm². The intensity that provided the best cellular options was chosen for treatment. Stage 2: Irradiation was carried out in culture bottles, with an energy intensity of 1J/cm², the protein content was analyzed via Western Blot, with detection by chemiluminescence.

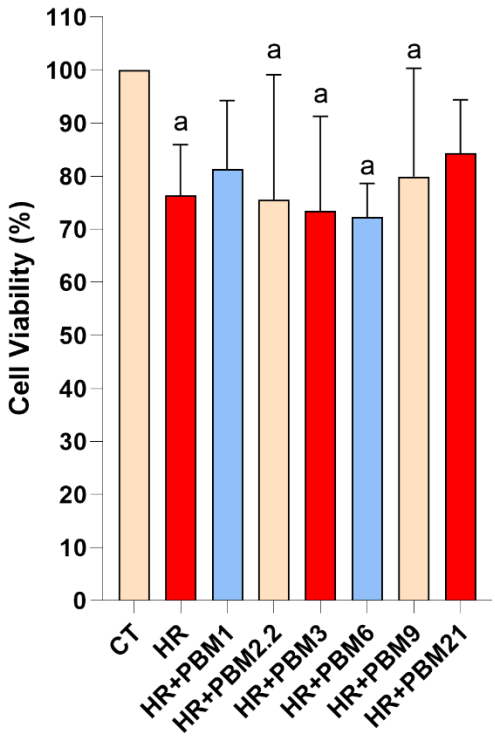


Fig. 2: Evaluation of H9c2 cell survival subjected to H/R and different treatment intensities. Results were expressed in percentage. The Tukey post-test followed

one-way ANOVA. [a] Different from the CT group. n = 3 for CT, HR, HR+PBM1, HR+PBM 2.2, HR+PBM3, HR+PBM6, HR+PBM9 and HR+PBM21 groups.

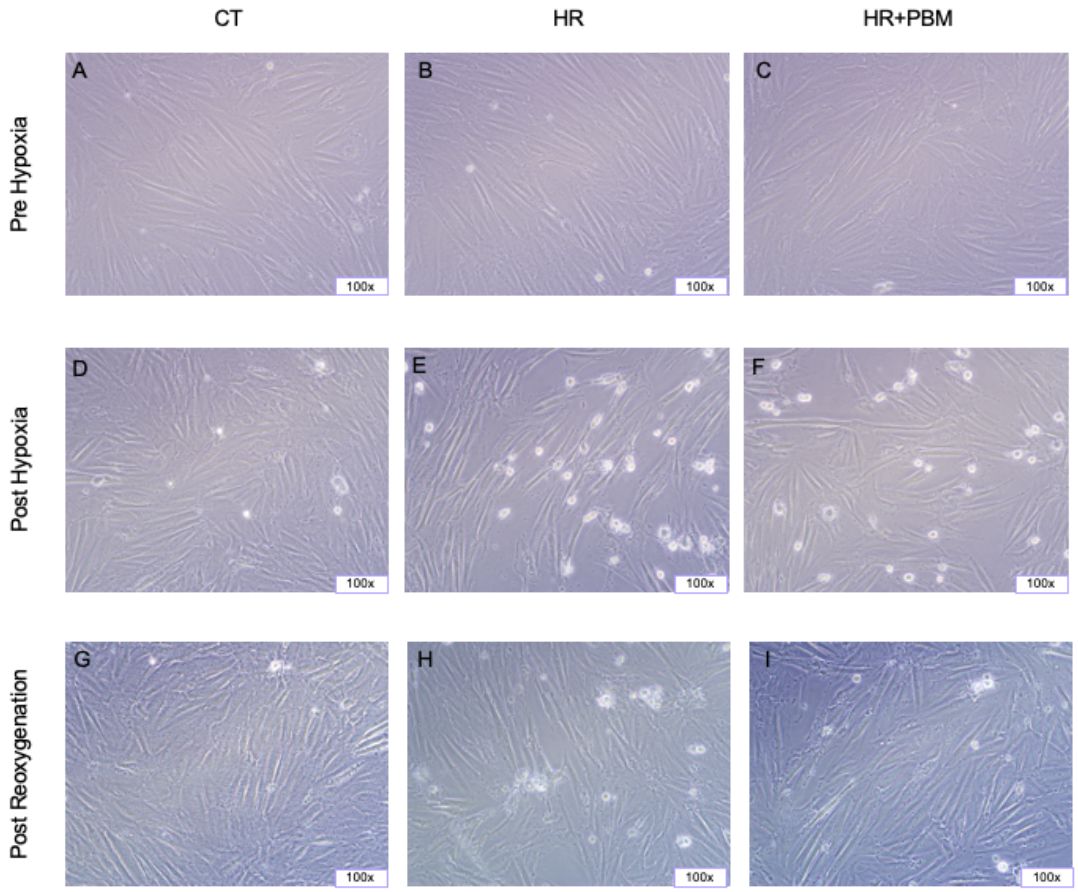


Fig. 3: A-I: Representative phase contrast microscopy of H9c2 cells. Representative phase contrast images were taken pre-hypoxia (A, B, C), post-hypoxia (D, E, F), and 18h after treatment application (G, H, I). The hypoxic condition was using $CoCl_2$ for 24h and reoxygenation for 18h.

Fig. 4

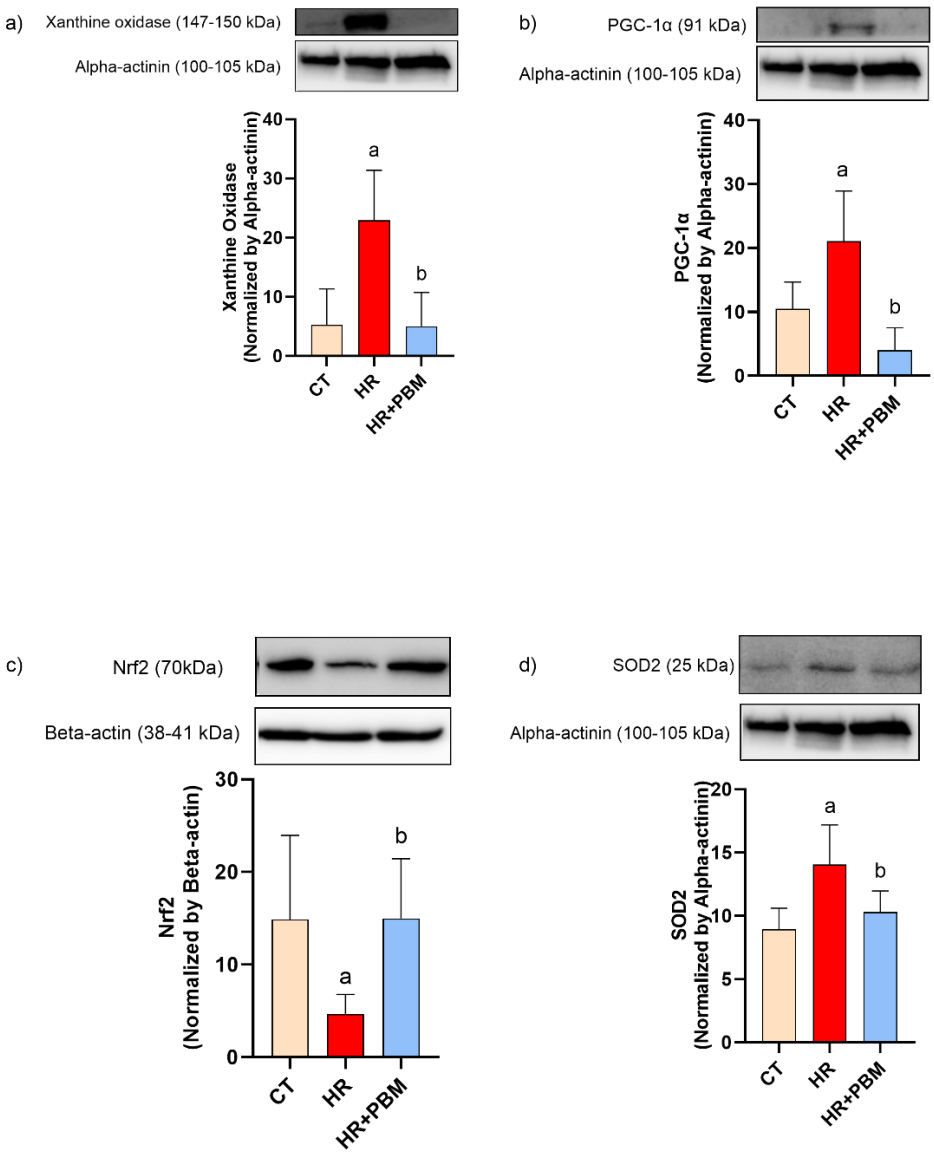


Fig. 4: Evaluation of the expression of proteins related to oxidative stress of H9c2 subjected to H/R and treated with PBM at an intensity of 1J/cm^2. Bands represent one sample per group. Western Blot analyzed a) xanthine oxidase b) PGC-1 α, c) Nrf2 and d) SOD2, and protein content. Results were expressed as mean ± standard deviation. One-way ANOVA followed by Tukey post-test. [a] Different from the CT group. [b] Different from the HR group. n = 6 for CT, HR, and HR+PBM. n = 6 for CT, HR and HR+PBM groups.

Fig. 5

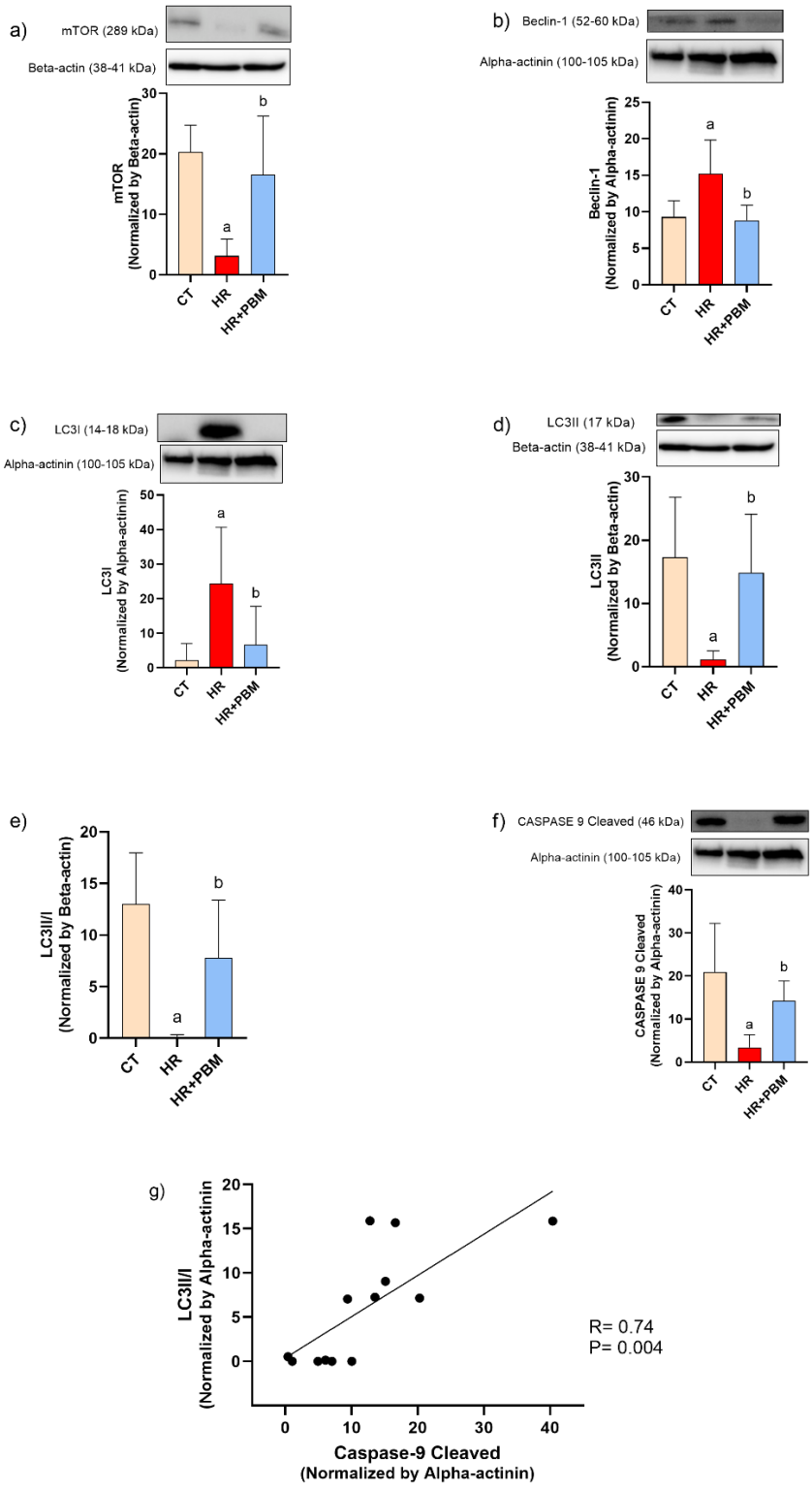


Fig. 5: Evaluation of the expression of proteins related to autophagy of H9c2 subjected to H/R and treated with PBM at an intensity of 1J/cm^2. Bands represent one sample per group. Western Blot analyzed a) mTOR, b) Beclin-1, c) LC3 I, d) LC3 II, e) LC3II/I ratio and f) CASPASE-9 and protein content. Results were expressed as mean ± standard deviation. One-way ANOVA followed by Tukey

post-test. [a] Different from the CT group. [b] Different from the HR group. n = 6 for CT, HR and HR+PBM groups.

Fig. 6

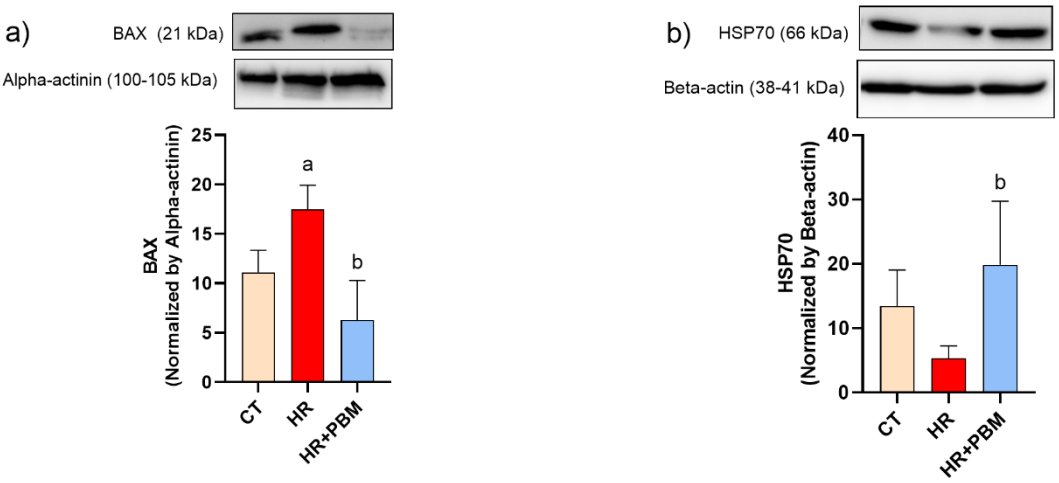


Fig. 6: Evaluation of the expression of proteins related to apoptosis of H9c2 cells to H/R and treated with PBM at an intensity of 1J/cm^2. Bands represent one sample per group. Western Blot analyzed a) Bax and b) HSP70 and protein content. Results were expressed as mean ± standard deviation. One-way ANOVA followed by Tukey post-test. [a] Different from the CT group. [b] Different from the HR group. n = 6 for CT, HR and HR+PBM groups.

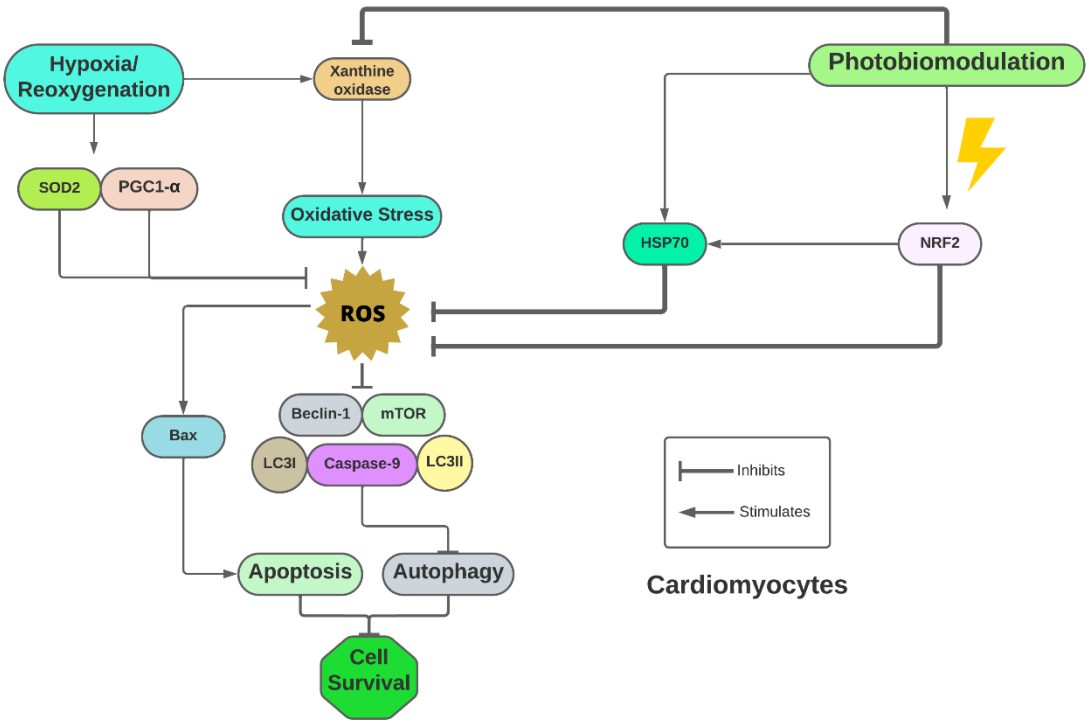


Fig. 7: Summary of the results and the pathways involved. PBM specifically increased the expression of HSP70 and Nrf2, reduced xanthine oxidase levels, maintained physiological levels of autophagy, and reduced apoptosis. These cellular responses contributed to improved cell survival under H/R stress. H9c2 cells subjected to H/R stress showed a notable increase in xanthine oxidase activity. This increase was compensated by SOD2 and PGC-1α levels. Changes induced by oxidative stress reduced autophagy, as evidenced by changes in the LC3II/I ratio, and there was also an increase in apoptotic activity, resulting in impaired cell proliferation.

5 CONCLUSÃO GERAL

Com base nos estudos realizados no desenvolvimento desta tese, podemos concluir que a FBM e o EA oferecem abordagens promissoras e menos invasivas no tratamento de doenças cardíacas, particularmente em condições cardiovasculares complicadas por diabetes mellitus tipo 2. Estas modalidades terapêuticas não apenas melhoram a funcionalidade e sobrevivência celular em condições de estresse como hipóxia e reperfusão, mas também reduzem o estresse oxidativo e modulam processos de autofagia e apoptose. Os estudos destacam a eficácia da FBM em atenuar os efeitos danosos de lesões por reperfusão em cardiomiócitos isolados, além de ressaltar os benefícios do EA na melhoria do tecido cardíaco e musculoesqueléticas através de vias metabólicas essenciais para a hipertrofia muscular e renovação celular. A combinação de FBM e EA tem potencial para melhorar significativamente os resultados em pacientes com doenças cardiovasculares e DM2, oferecendo uma direção inovadora para tratamentos não farmacológicos. Esses achados sugerem que a integração de FBM e EA em protocolos clínicos pode maximizar benefícios terapêuticos, proporcionando um manejo mais efetivo das condições cardíacas e metabólicas. É fundamental a continuidade das pesquisas para otimizar essas intervenções, desenvolvendo tratamentos personalizados que atendam às necessidades específicas dos pacientes, e para explorar mais profundamente as interações sinérgicas entre essas modalidades de tratamento.

6 IMPACTOS DO TRABALHO

Esta tese fornece contribuições significativas tanto para o avanço científico quanto para o impacto social, particularmente na área da reabilitação das doenças cardiovasculares associadas com DM2. Os estudos conduzidos oferecem entendimento profundo sobre os mecanismos pelos quais a FBM e o EA influenciam a funcionalidade e a sobrevivência celular sob condições de hipóxia e reperfusão. A pesquisa explorou a intensidade do laser de 1 J/cm², revelando como este pode ser aplicado para mitigar os efeitos adversos pós-isquêmicos, sugerindo novas abordagens para intervenções menos invasivas.

A combinação da FBM com o EA abre novos caminhos para a reabilitação integrada de doenças cardiovasculares e DM2, destacando-se pelo potencial de personalização para atender às necessidades específicas dos pacientes. Esta integração de modalidades terapêuticas visa modular vias metabólicas essenciais e reduzir o estresse oxidativo, representando um avanço significativo na busca por tratamentos mais eficazes e menos invasivos.

Os resultados experimentais também estabelecem uma base sólida para futuros estudos clínicos, especialmente para pacientes submetidos a revascularização miocárdica. Este paradigma pode ser explorado para avaliar a eficácia da FBM e do EA como coadjuvantes de tratamentos convencionais, com potencial para revolucionar as práticas atuais.

Do ponto de vista social, as abordagens investigadas prometem melhorar substancialmente a qualidade de vida dos pacientes, reduzindo complicações e hospitalizações e melhorando o manejo diário de condições crônicas. Além disso, oferecem a possibilidade de reduzir os custos associados ao tratamento de doenças cardiovasculares e DM2, aliviando o fardo econômico sobre os sistemas de saúde.

A inclusão da FBM durante procedimentos cardíacos pode ser uma inovação que pode transformar os protocolos de reabilitação pós-operatória, promovendo melhores resultados de recuperação. Além disso, a divulgação desses métodos de tratamento fomenta a educação em saúde pública, incentivando estilos de vida mais saudáveis e o reconhecimento de novas tecnologias para tratamento destas condições.

Em resumo, esta tese não só enriquece o corpo de conhecimento científico, mas também apresenta implicações práticas que podem transformar significativamente o tratamento de doenças cardiovasculares e metabólicas, beneficiando a sociedade de maneira ampla e profunda.

ARTIGO 3

PHOTOBIOMODULATION INDUCES CELLULAR PROLIFERATION AND PRESERVES NUCLEAR SIZE IN GLIOBLASTOMA CELLS

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Abstract

Introduction: Cellular senescence is an irreversible state of cell cycle arrest, thus being characterized by decreased cell proliferation and increased nucleus area, often acting as a tumor suppressor program. Photobiomodulation (PBM) has been used in several conditions to increase the mitochondrial response, promoting nuclear changes and cell proliferation. However, the effects of PBM on cells are still unclear. Materials and methods: We utilized A172 glioblastoma cells transduced with H2B-mCherry by lentivirus to nuclear tagging. Treatment was done with GaAlAs Laser (850nm). Cells were divided by intensity into the following groups: C= Control, L1= 1J/cm², L2= 2.2J/cm², L3= 3J/cm², L9= 9J/cm², L15= 15J/cm², L21= 21J/cm², nuclear evaluation was performed at experimental times (0h, 24h, 48h, and 72h). For data analysis, two-way ANOVA with the Tukey post hoc test was used. Differences were significant when $p<0.05$. Results: PBM on intensities of 1J/cm², 2.2J/cm², 3J/cm², 9J/cm² e 15J/cm² showed a lower increase at the nuclear size compared to time 0h and 72h in the control group. All intensities (1, 2.2, 3, 9, 15, and 21 J/cm²) promoted cellular proliferation after 72 hours, while 15J/cm² presented an accentuated increase compared to groups L1, L2.2, and L3. Conclusion: PBM enhanced cellular proliferation while causing a reduced nuclear increase in glioblastoma cells.

Keywords: Photobiomodulation, nuclear size, proliferation, senescence cellular.

Statements

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Data availability and material: Data generated or assessed during the study is available to the corresponding author through reasonable solicitation.

Code availability: N/A

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INTRODUCTION

Photobiomodulation (PBM) is characterized by the application of non-thermal light (1-500 mW), exploiting light absorption to affect intracellular chemical changes, and it has been widely used in the clinical scenario as low-level laser (light) therapy (LLLT) [1,2,3]. The positive properties of PBM allow its use in the treatment of several physiological disorders. One of the main influences of PBM is on cellular proliferation.

The effects of PBM on cell proliferation are described as the emitted light having the ability to stimulate photon absorption in cytochrome *c* oxidase, stimulating mitochondrial membrane potential, resulting in higher levels of adenosine triphosphate (ATP) synthesis and growth factors [1,3,4]. The release of growth factors, associated with interaction with their cell receptors, induces signaling pathways that transmit signals to the nucleus for gene transcription, promoting increased cell proliferation, viability, and migration [1]. Another effect of PBM is related to the synthesis of DNA and RNA in the cell nucleus from photoreceptor feedback [5]. Thus, there is an interaction between the mitochondria and the nucleus as an effect of PBM, generating structural changes in the cell [6].

One of the changes associated with cell proliferation is aging, characterized by a permanent arrest of the cell cycle that increases inflammation and reduces cell proliferation [7]. Associated with this, abnormalities in nuclear morphology and architecture can be observed in aging, with nuclei enlarging, losing shape, appearing lobulated, and harboring invaginations of the nuclear membrane [8].

From the assumption that there is an inversely proportional connection between proliferation and cellular senescence, PBM could be a therapeutic alternative to prevent cellular aging because of its ability to stimulate proliferation. Thus, the present study aimed to analyze the temporal modifications of cell proliferation and nuclear area in cultured cells under different intensities of PBM.

MATERIALS AND METHODS

A172 glioma cells were cultured in Dulbecco's Modified Eagle's Medium Low Glucose (Gibco #31600-034) supplemented with 10% fetal bovine serum (Laborclin #630111), 1% penicillin/streptomycin (Gibco #15140-122) and 0.1% amphotericin B (Gibco #15290-018), maintained at 37ºC and 5% CO_2 in a humidified incubator. Cells were transduced with H2B-mCherry marker (Addgene #89766) by lentivirus as previously described for nuclear tagging [9,10].

1.5 x 10^4 cells were plated in each well (24 total). For irradiation, an aluminum gallium-indium-phosphorus (GaAlAs) Laser was used (850nm) (ISO: 13485, model 2779, Chattanooga Group - Intelect® Mobile Laser, Austin, Texas, USA) in continuous pulse frequency, with different energy intensities, potency density variated from 8W/cm² to 12W/cm² and application time from 0,15s to 250s, depending on energy intensity. Plate wells were divided into different groups by energy intensity: L1= 1J/cm²; L2= 2.2J/cm²; L3= 3J/cm²; L9= 9J/cm²; L15= 15J/cm² e L21= 21 J/cm² [11,12]. Intensity correction was done for the culture plate [13]. Application happened on a single point, with a distance between cells and laser being 10mm. A potency measurer (Table 1) (ThorLABSPM16-121 - New Jersey, United States) was used to verify energy density and laser potency output. After irradiation, cells were incubated and photographed every 30 minutes with Incucyte® Live-Cell Analysis Systems (Sartorius Stedim Biotech GmbH, Göttingen, Germany). The number of nuclei and the measurement of the nuclear area were performed with the ImageJ® software at times 0, 24, 48, and 72h after the treatments. To determine the growth rate per day, the quantified number of cells at the start and the end of the experiment was applied in the formula PD = ($\log_2$(end) - $\log_2$(start))/time in days [14].

The normality of data was tested with the Shapiro-Wilk test. The comparison between different times after laser application, nuclear area, and cellular proliferation was made using two-way ANOVA with the Tukey post hoc test. The square of partial Eta (n^2) was derived from a general linear way to assess effect size. Effects were classified as low (0.01-0.06), moderate (0.06 – 0.14), and high (>0.14). Results were expressed as the mean ± standard deviation. The differences were statistically significant when the assessment showed $p< 0,05$. GraphPad Instat 9.01 for Windows (GraphPad® Software Inc, La Jolla, CA, USA) was used as a tool for data analysis.

RESULTS

As seen in Figure 1, the control group (C) presented a time-dependent increase in nucleus size, observing the gain at 72h compared to time 0h, 24h, and 48h (44%, 46%, and 30%, respectively). In the same way, the L21 group showed a significant increase in the nucleus size at 72h time, compared to 0h, 24h, and 48h (42%, 43%, and 26%, respectively).

However, unlike what was observed for the Control (C) and the L21 group, L1, L2.2, L3, L9, and L15 groups did not present a significant difference between the nucleus size for 0h and 72h, indicating prevention in the nuclear area increasing.

It is also possible to highlight the significant prevention of increased nucleus size at 24h in groups L2.2, L3, L9, and L15 of 25%, 30%, 28%, and 27%, respectively, compared to 72h. At 48h, groups L1, L2.2, L3, L9, and L15 showed significant prevention of increased nucleus size of 19%, 19%, 25%, 22%, and 18%, respectively, compared to 72h. For these results, an effect size between times of 0.10 and power of 1.0 was found.

Figure 2 shows data on cellular proliferation (72h). It is possible to verify a significant increase in every irradiated group compared to Control (L1=15%, L2.2=16%, L3=16%, L9=23%, L15=35%, and L21=25%). Nevertheless, the L15 group still showed an increase in cellular proliferation compared to L1, L2.2, and L3 (18%, 16%, and 16%, respectively), with an effect size of 0.06. A representative image of the number of cell nuclei in glioblastoma culture subjected to different intensities of PBM at times 0h and 72h is shown in Figure 3.

DISCUSSION

In this study, we investigated the influence of different intensities of PBM on the morphological characteristics of the cell nucleus and the proliferative capacity after 72 hours of application. The increase in nuclear size and its proliferative capacity is associated with both replicative and oncogene-induced senescence [15]. We found in the present study that the groups treated with intensities L1 (1J/cm²), L2.2 (2.2 J/cm²), L3 (3 J/cm²), L9 (9 J/cm²), and L15 (15 J/cm²) prevented the increase in the area of the nuclei of the glioblastoma cells

comparing the timepoints 0h and 72h, indicating a protective effect of low-level laser light. In the control group and L21 (21J/cm²), there was an increase in nuclear area in time points between 0h and 72h. On the other hand, all intensities of laser increased cell proliferation.

During the period of cellular senescence, it is possible to observe that cells can be metabolically active; however, with dysfunctional mitochondria, resulting in increased oxygen consumption, decreased efficiency of oxidative phosphorylation, and increased levels of reactive oxygen species (ROS), resulting in reduced cell multiplication process [16]. On the other hand, PBM can interact with mitochondria photoreceptors, stimulating ATP synthesis and consequently resulting in increased cell proliferation [17].

PBM can stimulate cell proliferation, as observed for mesenchymal and cardiac stem cells and oligodendrocyte precursor cells. The present study found an increase in cell proliferation in all groups treated with PBM [12,18]. The study by Maldaner et al., 2019, demonstrated that PBM presented an essential protective and proliferative action, partially or wholly reversing the negative effects in senescent fibroblasts induced by hydrogen peroxide (H_2O_2) [19]. Yoon et al., 2021, demonstrated that 660nm PBM affects the proliferation of cultured astrocytes and provides evidence of differentiation [20]. An inversely proportional relationship between cell proliferation and senescence has been reported in oncogenic cells [21,22].

Serrano et al., 1997 demonstrated that the ras family of proto-oncogenes expression stimulates senescence in primary cell cultures, a process called oncogene-induced senescence [21]. This senescence leads to an irreversible state of cell cycle arrest that can act as a tumor suppressor program [22]. In this study, we found that the laser decreased the nuclear area, an indicator of cellular senescence, from the evaluation of the morphological parameters and cell proliferation. However, we hypothesize that the evaluated doses could have an oncogenic effect by reducing the senescence process.

The present study has limitations, such as not quantifying the total cell area and not investigating the signaling pathways involved in changes in the nuclear area. It is worth mentioning that the biological effects may be related to the increase in the photo exposure of cells due to the number of photographs. This increase in the incidence of energetic photons on the biological material may

even have caused a certain level of phototoxicity to the cells [23]. However, results of nuclear size and cell proliferation in glioblastomas treated with different laser intensities and at other times are new. They may bring new perspectives on the effects of PBM.

CONCLUSION

We conclude that PBM using low energy intensities (1J, 2.2J, 3J, 9J, and 15J/cm²) prevented the increase in nuclear area in glioblastoma culture cells. In addition, all PBM intensities stimulated cellular proliferation, mainly the 15J/cm² intensity.

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Table 1. Protocol table of laser radiation

Model	Laser GaAlAs (850nm)
Number of laser diodes	1
Pulse frequency	Continuous output
Output power (mW)	100
Spot size (cm^2)	0.012
Power density (W/cm^2)	8 à 12
Protocol regime	Performed in the oven with a distance between the cells and the laser of 10mm
Dose per treatment (J/cm^2)	1 – 2.2 – 3 – 9 – 15 – 21
Time for point	0,15s to 250s
Frequency treatment	1X

Figures

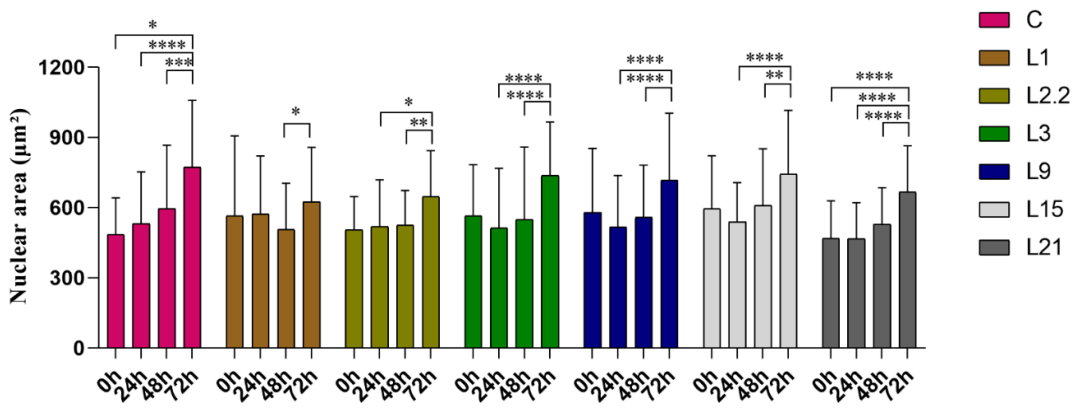


Fig 1. Quantification of the nucleus area (µm²) in a glioblastoma culture treated with different intensities of PBM at different experimental times. Results were expressed as mean ± SD. $^{*}p\leq0.05$, $^{**}p\leq0.01$, $^{***}p\leq0.001$, $^{****}p<0.0001$, compared to their respective group in the experimental times.

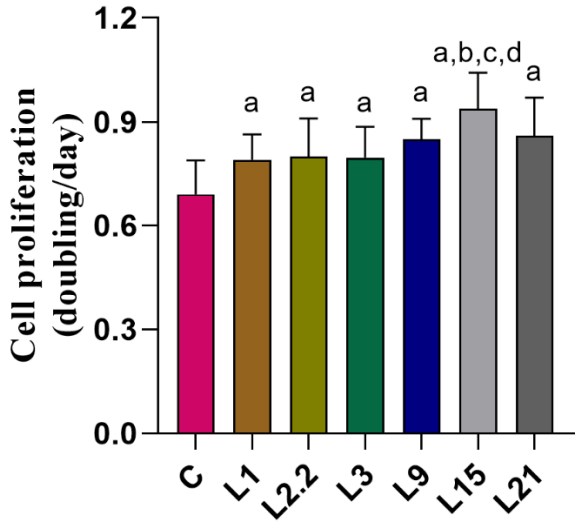


Fig 2. Quantification of the cell proliferation (doubling/day) in glioblastoma cultures treated with different intensities of PBM after three days (0h and 72h). Results were expressed as mean ± SD. ^{a} $p<0.05$ compared to the control. ^{b} $p<0.05$ compared to L1. ^{c} $p<0.05$ compared to L2.2. ^{d} $p<0.05$ compared to L3.

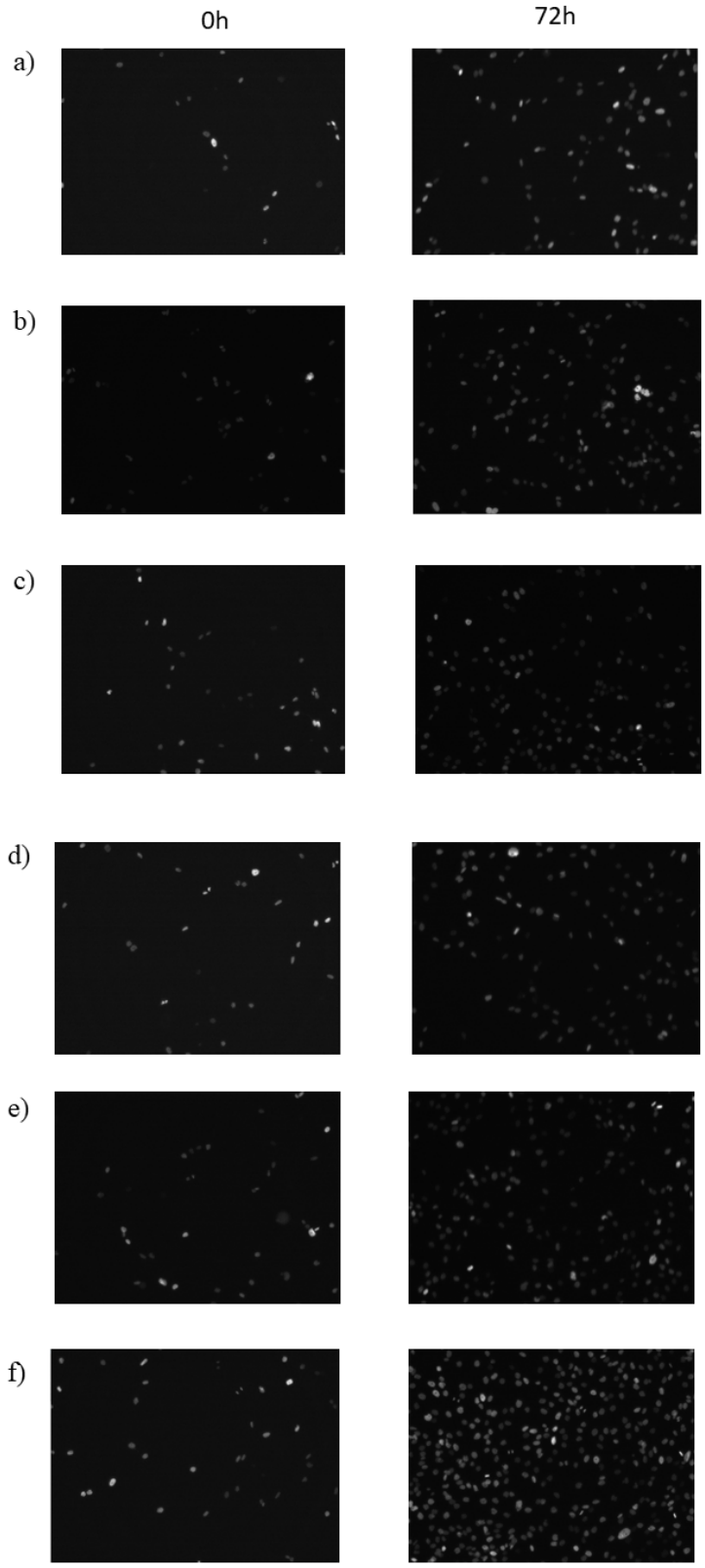


Fig 3. Representative image of the number of cell nuclei in glioblastoma culture subjected to different intensities of PBM at timepoints 0h and 72h. a) Control group. b) L1 group. c) L2.2 group. d) L3 group. e) L9 group. f) L15 group. g) L21 group.

ANEXO A

Aprovação do Comitê de Ética em Utilização Animal

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Projeto 256/19

Parecer: 655/19

Data: 11/09/2019

Título: Efeitos do exercício físico aeróbico associado à fotobiomodulação sobre a função autonômica e hemodinâmica, sensibilidade dos barorreceptores, mecânica ventilatória e marcadores bioquímicos em ratos diabéticos com insuficiência cardíaca

Após reanálise do projeto em reunião realizada no dia 11/09/2019, a Comissão considera que os autores realizaram as correções e ajustes apontados estando o projeto aprovado.

Situação: APROVADO

ANEXO B

Comprovante de Registro na COMPESQ-UFCSPA

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Pró-Reitoria de Pesquisa e Pós-Graduação

Comissão de Pesquisa - COMPESQ

CARTA DE APROVAÇÃO

A Comissão de Pesquisa analisou o projeto:

Número: **485/2023**
Título: **Efeitos da terapia com laser de baixa potência em cultura de cardiomiócitos submetidos à isquemia/reperfusão**
Pesquisador(a) Responsável: **Pedro Dal Lago**
Vigência: **15/03/2019** a **31/12/2023**

Pesquisadores:

Equipe UFCSPA:

- Lucas Kieling
- Pedro Dal Lago
- Alan Christhian Bahr

Equipe Externa: Não possui.

Atestamos que o projeto de pesquisa acima identificado foi registrado na Comissão de Pesquisa da UFCSPA. Salientamos que este registro não autoriza o pesquisador a coletar ou analisar dados oriundos de sujeitos de pesquisa. Salientamos também que este registro não garante a concessão de recursos financeiros por parte da UFCSPA a este projeto de pesquisa.

Porto Alegre, 10 de abril de 2023

RENATA PADILHA GUEDES
Coordenadora Da Comissão De Pesquisa

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Pró-Reitoria de Pesquisa e Pós-Graduação

ATESTADO DE PARTICIPAÇÃO

Atestamos que ALAN CHRISTHIAN BAHR participou do projeto Efeitos da terapia com laser de baixa potência em cultura de cardiomiócitos submetidos à isquemia/reperfusão na qualidade de ALUNO.

Pesquisador(a) Responsável: PEDRO DAL LAGO

Vigência do projeto: de 15/03/2019 até 31/12/2023

Porto Alegre, 03 de junho de 2024

Documento assinado eletronicamente por **Pedro Dal Lago** em 03/06/2024, às 18:58:03, conforme horário oficial de Brasília. A autenticidade deste documento pode ser conferida em: validadorqr/?code=pzDh1fxIST2BdYmG4SQvcA==.