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Efeitos do exercício físico aeróbio, da terapia por fotobiomodulação e da estimulação elétrica sobre a capacidade funcional, a função hemodinâmica e o controle autonômico de ratos diabéticos com insuficiência cardíaca após infarto do miocárdio.

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Orientador: Dr. Pedro Dal Lago

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Resumo

A doença cardiovascular (DCV) continua sendo a principal causa de morte e incapacidade entre os pacientes com diabetes mellitus (DM) e insuficiência cardíaca (IC). Esta tese foi constituída por dois estudos. **Estudo 1:** O objetivo foi analisar o efeito da estimulação elétrica neuromuscular (NMES) e fotobiomodulação (PBMT) sobre os parâmetros cardiovasculares, função hemodinâmica, sensibilidade do barorreflexo arterial (SBR) e equilíbrio autonômico (ANS) de ratos com insuficiência cardíaca (IC). Ratos Wistar machos (220-290g) foram organizados em cinco grupos: Sham (n=6), Controle-IC (n=5), NMES-IC (n=6), PBMT-IC (n=6) e NMES + PBMT-IC (n=6). O infarto do miocárdio (IM) foi induzido pela ligadura da artéria coronária esquerda. Os animais foram submetidos a um protocolo de NMES e PBMT de oito semanas. Análise estatística com o General Linear Model (GLM) seguido do teste post hoc de Bonferroni. Os ratos do grupo NMES-IC mostraram uma área de MI maior do que o grupo Controle-IC (P=0,003), PBMT-IC (P=0,002) e NMES + PBMT-IC (P=0,012). NMES-IC e NMES + PBMT-IC apresentaram congestão pulmonar maior (P=0,004 e P=0,02) e pressão sistólica mais baixa (P=0,019 e P=0,002) do que o grupo Sham. NMES + PBMT-IC apresentou pressão arterial média menor (P=0,02) do que o grupo Sham. O Controle-IC apresentou frequência cardíaca maior do que o NMES-IC e o NMES + PBMT-IC (P=0,017 e P=0,013). Não houve diferença nas variáveis BRS e ANS entre os grupos. Em conclusão, o NMES de oito semanas isolado ou associado ao protocolo PBMT reduziu a frequência cardíaca basal, pressão arterial sistólica e média, sem influência na sensibilidade barorreflexa e controle autonômico, e nenhum efeito do PBMT foi observado em ratos com IC. **Estudo 2:** Objetivo: analisar os efeitos do exercício aeróbio (EA) e da terapia de fotobiomodulação (PBMT) isoladamente ou combinada na capacidade funcional, função hemodinâmica e características morfológicas de ratos com diabetes mellitus tipo 2 (DM-2) e insuficiência cardíaca (IC). Métodos: Ratos Wistar machos (~120g) foram divididos em três grupos: ratos com DM-2 e IC que não receberam nenhuma intervenção (DMIC-Controle; n=4), ratos com DM-2 e IC que receberam treinamento de EA (DMIC-EA; n=6), e ratos com DM-2 e IC que receberam treinamento EA e PBMT (DMIC-EA + PBMT; n=6). O DM-2 foi induzido por uma dieta rica em gordura associada à injeção de estreptozotocina (STZ), e a cirurgia de infarto do miocárdio (IM) foi usada para induzir IC. Os animais foram submetidos aos protocolos EA e PBMT (8 semanas). Resultados: Comparando os valores do grupo apenas no período pós-protocolo, tanto o grupo EA quanto o grupo EA + PBMT aumentaram o tempo, a distância e a velocidade máxima do que o grupo Controle ($p<0,001$) no teste ergométrico. Pela avaliação do delta (Δ) percentual, apenas o grupo EA + PBMT melhorou o tempo (p=0,016), distância (p=0,019) e velocidade máxima (p=0,011) em relação ao grupo Controle. O grupo EA apresentou menor espessura da parede posterior do ventrículo esquerdo na diástole (EPVEd) do que o grupo controle (p=0,02). EA e PBMT não foram capazes de alterar as características morfológicas. EA e PBMT podem controlar o ganho de massa corporal, mas não influenciam o índice LEE e o controle glicêmico. Conclusões: Um protocolo de 8 semanas de EA combinado com PBMT foi capaz de potencializar os efeitos do EA isolado no desempenho no teste ergométrico de ratos com DM-2 e IC.

Palavras-chave: Insuficiência Cardíaca, Diabetes Mellitus, Infarto do Miocárdio, Estimulação elétrica, Fototerapia, Exercício.

Abstract

Cardiovascular disease (CVD) remains the leading cause of death and disability among patients with diabetes mellitus (DM) and heart failure (HF). **Study 1:** The aim was to analyze the effect of neuromuscular electrical stimulation (NMES) and photobiomodulation (PBMT) on cardiovascular parameters, hemodynamic function, arterial baroreflex sensitivity (BRS), and autonomic balance (ANS) of rats with heart failure (HF). Male Wistar rats (220-290g) were organized into five groups: Sham (n=6), Control-HF (n=5), NMES-HF (n=6), PBMT-HF (n=6), and NMES+PBMT-HF (n=6). Myocardial infarction (MI) was induced by left coronary artery ligation. Animals were subjected to an eight-week NMES and PBMT protocol. Statistical analysis with the General Linear Model (GLM) followed by the Bonferroni post hoc test. Rats of NMES-HF group showed a higher MI area than Control-HF group (P=0.003), PBMT-HF (P=0.002), and NMES+PBMT-HF (P=0.012). NMES-HF and NMES+PBMT-HF showed higher pulmonary congestion (P=0.004 and P=0.02), and lower systolic pressure (P=0.019 and P=0.002) than Sham group. NMES+PBMT-HF showed lower mean arterial pressure (P=0.02) than Sham group. Control-HF showed a higher heart rate than NMES-HF and NMES+PBMT-HF (P=0.017 and P=0.013). There was no difference in the BRS and ANS variables between groups. In conclusion, eight-week NMES isolated or associated with PBMT protocol reduced basal heart rate, systolic and mean arterial pressure, without influence on baroreflex sensibility and autonomic control, and no effect of PBMT was seen in rats with HF. **Study 2:** Purpose: to analyze the effects of aerobic exercise (AE) and photobiomodulation therapy (PBMT) alone or combined on functional capacity, hemodynamic function, and morphological characteristics of type 2 diabetes mellitus (DM-2) and heart failure (HF) rats. Methods: Male Wistar rats (~120g) were divided into three groups: rats with DM-2 and HF that did not received any intervention (DMHF-Control; n=4), rats with DM-2 and HF that received AE training (DMHF-AE; n=6), and rats with DM-2 and HF that received AE training and PBMT (DMHF-AE+PBMT; n=6). DM-2 was induced by a high-fat diet associated with streptozotocin (STZ) injection, and myocardial infarction (MI) surgery was used to induce HF. The animals were subjected to the AE and PBMT protocols (8 weeks). Results: Comparing group values only in the post-protocol period, both the AE and the AE+PBMT groups increased the time, distance, and maximal speed than the Control group (p<0.001) in the exercise test. From the delta (Δ) percentage evaluation, only the AE+PBMT group improved the time (p=0.016), distance (p=0.019), and maximal speed (p=0.011) compared to the Control group. AE group had a lower left ventricular posterior wall in diastole (LVPWd) than Control group (p=0.02). AE and PBMT were not able to change the morphological characteristics. AE and PBMT can control the mass body gain, but not influence the LEE index, and the glycemic control. Conclusions: An 8-week protocol of AE combined with PBMT was able to potentiate the effects of isolated AE on performance in the exercise test of rats with DM-2 and HF.

keywords: Heart Failure, Diabetes Mellitus, Myocardial Infarction, Electric Stimulation, Phototherapy, Exercise.

SUMÁRIO

1 TRAJETÓRIA ACADÊMICA DO CANDIDATO

Este trabalho é uma continuação das atividades que venho desenvolvendo no laboratório de fisiologia experimental desde o início do ano de 2012. Todos os integrantes, alunos de iniciação científica, mestrado, doutorado e pós-doutorado, que desenvolvem suas pesquisas nesse laboratório, estão vinculadas ao Grupo de Pesquisa e Interação Cardiopulmonar (GPIC), coordenado pelo Professor Pedro Dal Lago.

Historicamente, nosso grupo vem pesquisando vários aspectos relacionados à influência de diversas modalidades terapêuticas não-farmacológicas (exercício aeróbio, exercício de força, treinamento muscular respiratório, eletroestimulação, fototerapia, suplementação alimentar) sobre a Insuficiência Cardíaca (IC). O modelo experimental de ratos com IC, através da cirurgia de infarto agudo do miocárdio (IAM), em que é feito o bloqueio da artéria coronária descendente anterior esquerda, já está bem estabelecido na literatura e é a base das nossas pesquisas.

Durante o mestrado, de 2012 a 2014, tive a oportunidade de aprender diversos métodos de análise e avaliação (teste cardiopulmonar de exercício, análise hemodinâmica e barorreflexa, e análise ecoradiografica em ratos), bem como aperfeiçoar as técnicas cirúrgicas (cirurgia de infarto e canulações arteriovenosas).

No período final do mestrado, através de leituras e estudos, fiquei intrigado com as questões relacionadas às complicações cardiovasculares causadas pelo diabetes mellitus (DM). Entendendo um pouco mais sobre os benefícios do exercício aeróbio, eletroestimulação e fototerapia, fui instigado a iniciar as pesquisas associando o modelo de IC com o DM.

A partir disso, iniciei a pesquisa por protocolos de indução do DM a fim de encontrar algum que fosse possível de desenvolver no nosso laboratório. Foram várias conversas com o Professor Pedro e com o colega Giuseppe Potrick Steffani no intuito de definirmos o melhor protocolo.

Bom, como todo novo método que é desenvolvido no laboratório, sabíamos de algumas dificuldades que poderíamos encontrar, mesmo assim,

resolvemos topar o desafio. As atividades foram iniciadas e, através desse projeto, fomos contemplados com um apoio financeiro proveniente do Programa Pesquisador Gaúcho da FAPERGS, que possibilitou o aprimoramento tecnológico do laboratório. Até o momento, conseguimos alguns resultados interessantes. Porém, ainda há muito a fazer, as atividades continuam e já temos outros alunos interessados em seguir o modelo.

2 REVISÃO DA LITERATURA

2.1 DADOS EPIDEMIOLÓGICOS

A doença cardiovascular (DCV) é a principal causa de morte no mundo, responsável por 17,3 milhões de mortes por ano, que correspondeu a 31,5% de todas as mortes no ano de 2013 (SACKS et al., 2017). Aproximadamente 808.000 pessoas morreram de doenças cardíacas, acidente vascular cerebral e outras doenças cardiovasculares nos Estados Unidos em 2014, o que corresponde a cerca de 1 em cada 3 mortes. Os custos diretos e indiretos anuais dessas mortes totalizam mais de US$ 316,1 bilhões, incluindo gastos com saúde e perda de produtividade (BENJAMIN et al., 2017).

Nos Estados Unidos, mais de 6 milhões de adultos sofrem de IC e há uma perspectiva de que a prevalência aumente cerca de 50% até 2030, o que está diretamente relacionado ao envelhecimento da população com maiores comorbidades cardiometabólicas (BENJAMIN et al., 2017; PANDEY et al., 2018).

No Brasil, as DCVs são responsáveis por um terço da taxa de mortalidade total e aproximadamente 30% das mortes na faixa etária dos 20 aos 59 anos. A IC é responsável por cerca de 4% do total de internações e 31% das internações por doenças cardiovasculares. O tempo médio de internação é de 5,8 dias, com mortalidade relacionada à infecções hospitalares variando entre 5,6 a 6%, acarretando em um custo superior à R$ 200 milhões (ANDRADE et al., 2013).

Atualmente estamos passando por uma grande crise na qualidade alimentar da população mundial. Dessa forma, surgem várias doenças cardiometabólicas que são influenciadas por fatores de risco relacionados à

dieta, tais como: a doença arterial coronariana (DAC), o acidente vascular cerebral (AVC), o DM, a doença arterial periférica (DAP), a doença renal crônica (DRC), o déficit cognitivo, a IC e a fibrilação atrial. De modo geral, as doenças crônicas causarão um prejuízo estimado em US$ 17,3 trilhões até 2030, somando gastos em saúde, redução da produtividade e perda de capital (MOZAFFARIAN, 2016).

Nas últimas duas décadas, vários dados clínicos e epidemiológicos comprovaram que, além do infarto do miocárdio e de outros eventos cardiovasculares relacionados à aterosclerose, a IC é um dos principais fatores que contribuem para o aumento da morbidade e mortalidade cardiovascular em pacientes com diabetes (LEHRKE; MARX, 2017).

A DCV continua sendo a principal causa de morte e incapacidade entre os pacientes com diabetes mellitus. A presença de DM duplica o risco de mortalidade, independentemente da presença ou ausência de doença cardiovascular (BECKMAN et al., 2013). O DM exacerba os mecanismos subjacentes à aterosclerose e à IC. Infelizmente, esses mecanismos não são adequadamente modulados por estratégias terapêuticas que se concentram apenas no controle glicêmico ideal com drogas ou abordagens atualmente disponíveis (LOW WANG et al., 2016).

Nos pacientes com DM, a prevalência de IC é maior do que na população em geral, uma vez que 1% de aumento na hemoglobina glicosada está associado à 8% de aumento no risco de IC. Indivíduos com DM têm um risco 3 a 7 vezes maior de desenvolver doenças cardiovasculares quando comparados aos não diabéticos (CALL et al., 2015). Há mais de 35 anos o *Framingham Heart Study* demonstrou, pela primeira vez, um aumento no risco de IC em pacientes diabéticos e, com o passar dos anos, estas doenças têm sido tratadas separadamente, no entanto, muitas vezes se sobrepõem (KANNEL, 1979).

Pacientes com IC apresentam significativa resistência à insulina, o que aumenta o risco de desenvolver DM tipo 2 em comparação com os indivíduos normais ou portadores de doença arterial coronariana (DAC). Da mesma forma, a resistência à insulina em pacientes com IC, mesmo na ausência evidente de DM, é um preditor independente de pior prognóstico, sugerindo um envolvimento

fisiopatológico da resistência à insulina na progressão da IC (HAMBLIN; SMITH; HILL, 2007). Assim, a relação entre DM e IC é bidirecional, com cada doença aumentando de forma independente o risco para o desenvolvimento da outra.

2.2 Definições, sintomas e fisiopatologia da Insuficiência Cardíaca e do Diabetes Mellitus

Uma das doenças cardiovasculares mais importantes é a IC, em função do aumento na sua prevalência, bem como da alta taxa de mortalidade. Está associada com diversas complicações, tais como: alto índice de hospitalizações, arritmias fatais e morte durante sua progressão (CHOI; PARK; YOUN, 2019).

A IC é definida classicamente como uma síndrome clínica, caracterizada por sintomas como fadiga e dispneia, surgindo a partir de uma desordem cardíaca estrutural e funcional, que compromete a função hemodinâmica do coração (PONIKOWSKI et al., 2017). Atualmente, a IC tem sido descrita como insuficiência cardíaca com fração de ejeção preservada, do inglês "*heart failure with preserved ejection fraction"* (HFpEF) ou insuficiência cardíaca com fração de ejeção reduzida, *"heart failure with reduced ejection fraction"* (HFrEF), de acordo com o grau de comprometimento funcional do ventrículo esquerdo (LEHRKE; MARX, 2017).

Antes mesmo de aparecerem os primeiros sintomas, algumas alterações já estão presentes. A principal delas é a hiperatividade do sistema nervoso simpático (SNS) associada à redução na atividade parassimpática, o que caracteriza o estado de excitação neuro-humoral, diretamente relacionado à disfunção dos barorreceptores, principais responsáveis pela modulação da frequência cardíaca (FC) e da pressão arterial (PA) momento a momento (BESNIER et al., 2017). Inicialmente, a excitação neuro-humoral pode ser considerada um mecanismo compensatório, a fim de manter a homeostase a curto prazo. Porém, cronicamente, torna-se prejudicial e acelera a progressão da IC, levando ao pior prognóstico (MAY et al., 2013).

As alterações estruturais e funcionais cardíacas reduzem as respostas inotrópicas e cronotrópicas, aumentam as pressões intracardíacas, acarretando disfunções sistólica e diastólica, com aumento da pós-carga. Como resultado, há

um comprometimento na capacidade do coração em responder de forma adequada em situações de estresse, tanto fisiológicas (exercício físico) quanto patológicas (infarto do miocárdio) (DHARMARAJAN; RICH, 2017).

Após serem instalados, esses distúrbios são responsáveis por severas alterações em órgãos vitais, incluindo coração (disfunção hemodinâmica), pulmão (congestão pulmonar que compromete a relação ventilação/perfusão, prejudicando as trocas gasosas) e sistema muscular esquelético, desencadeando os principais sintomas da síndrome da IC, tais como: fadiga, dispneia e intolerância ao exercício (PIEPOLI et al., 2010).

Nos portadores de IC e diabetes, pode-se encontrar um quadro fisiopatológico diferente daqueles sem DM, pois há um comprometimento na liberação de cálcio (Ca^{+}) intracelular, no metabolismo de lipídios no miocárdio e na função endotelial (SARMA et al., 2013). O DM tipo 2 apresenta a resistência à insulina e a hiperglicemia como principais alterações metabólicas, responsáveis por várias respostas celulares adaptativas e prejudiciais, que desencadeiam mudanças específicas na estrutura e na função do miocárdio, levando ao surgimento da cardiomiopatia diabética (CMD) (DEI CAS et al., 2015).

A cardiomiopatia diabética foi descrita pela primeira vez em 1972 como insuficiência cardíaca crônica em pacientes diabéticos e, a partir disso, tem sido diagnosticada como insuficiência ventricular esquerda, mesmo na ausência de aterosclerose e hipertensão (RUBLER et al., 1972). É uma das principais complicações cardiovasculares que acomete aproximadamente 60% dos pacientes diabéticos bem controlados. Frequentemente ocorre quando há um comprometimento da função sistólica associada à disfunção diastólica (LITWIN, 2013).

A estrutura cardíaca dos pacientes diabéticos passa por um processo de remodelamento concêntrico do ventrículo esquerdo, que pode estar associado à alterações no metabolismo energético do miocárdio, bem como à redução na pressão sistólica (LEVELT et al., 2016a, 2016b). A hipertrofia cardíaca diabética está relacionada ao aumento na deposição de triglicerídeos no miocárdio e consequente aumento no volume extracelular, importante indicador de

deposição de colágeno e fibrose (FALCÃO-PIRES et al., 2011), além de ser um fator preditivo de mortalidade e IC nesta população (WONG et al., 2014).

Acredita-se que a resistência insulínica, causada pela hiperinsulinemia, também está diretamente relacionada à hipertrofia miocárdica (SHIMIZU et al., 2010). Alguns estudos mostram uma correlação positiva entre a perfusão tecidual miocárdica, o fornecimento de oxigênio, a disponibilidade de substrato energético e a função miocárdica em pacientes diabéticos, indicando o dano microcirculatório como um dos principais fatores desencadeadores da cardiomiopatia diabética (LEHRKE; MARX, 2017; LEVELT et al., 2016c).

O aumento circulante nas concentrações de glicose e ácidos graxos livres leva ao inapropriado acúmulo de lipídeos em outros tecidos, aquém do tecido adiposo, incluindo o coração (ERTUNC; HOTAMISLIGIL, 2016). A ocorrência da resistência insulínica leva à limitação no fornecimento de glicose para os cardiomiócitos, desencadeando o aumento na oxidação de ácidos graxos, que é uma característica do coração diabético (RIJZEWIJK et al., 2010). Isso gera um desequilíbrio na capacidade funcional da fosforilação oxidativa, sobretudo pela excessiva oferta calórica em decorrência da redução na demanda energética suficiente (LIESA; SHIRIHAI, 2013; MONTAIGNE et al., 2014).

O estado de desequilíbrio metabólico oxidativo, causado por uma demanda energética insuficiente associada à um ambiente energeticamente saturado, reduz a necessidade de ATP, gerando um acúmulo de elétrons ao longo da cadeia respiratória (FINK et al., 2016; LIESA; SHIRIHAI, 2013). Como consequência, os elétrons são liberados e passam a reagir com o oxigênio molecular, formando espécies reativas de oxigênio em excesso, o que caracteriza o estresse oxidativo (LIESA; SHIRIHAI, 2013). O estresse oxidativo é uma complicação central no diabetes, no entanto, as estratégias destinadas a evitar esse processo, podem ser capazes de reverter a disfunção cardíaca metabolicamente induzida (SHAH; BROWNLEE, 2016; SVERDLOV et al., 2016).

Importa destacar que o desenvolvimento da IC causa um grande impacto no metabolismo cardíaco, responsável pela alteração no tipo de substrato utilizado pelos cardiomiócitos, passando a utilizar mais os ácidos graxos para a oxidação de glicose, oposto do que ocorre na situação diabética (NEUBAUER,

2007). A partir de então, há uma redução no nível de ácidos graxos celulares, com supressão do mecanismo de oxidação dos ácidos graxos (CHOKSHI et al., 2012). Consequentemente, o coração insuficiente passa a depender mais da oxidação de glicose. Nessa situação, a resistência insulínica tem o potencial de limitar o suprimento energético e prejudicar ainda mais a função cardíaca (LEHRKE; MARX, 2017).

Além disso, a IC promove a resistência insulínica sistêmica, que está associada ao aumento do tônus simpático e das perturbações nas vias metabólicas causadas pelo estresse (DOEHNER; FRENNEAUX; ANKER, 2014). A partir dessas alterações metabólicas, alguns autores passaram a considerar a IC como “um motor sem combustível” (NEUBAUER, 2007), enquanto a resistência insulínica se mostra um preditor independente de mau prognóstico em pacientes com IC (DOEHNER et al., 2005).

Em pacientes diabéticos que são acometidos por infarto do miocárdio, há o comprometimento da função autonômica decorrente das complicações do próprio infarto, porém, sobretudo, por neuropatia autonômica cardíaca pré-existente. A combinação do diabetes com a IC após o infarto do miocárdio requer ações preventivas, isso porque, pacientes diabéticos podem ter uma diminuição da capacidade de remodelamento do ventrículo esquerdo após o infarto, levando assim ao desenvolvimento de IC em volumes ventriculares menores do que os pacientes não diabéticos com áreas de infarto semelhantes (BARTHEL et al., 2011; SOLOMON et al., 2002).

2.2.1 Modelo animal de insuficiência cardíaca e diabetes mellitus

Os modelos animais de IC e diabetes vêm sendo extensivamente utilizados na literatura científica, a fim de mimetizar as alterações causadas por estas patologias em ambiente clínico, sobretudo para que possam ser realizados procedimentos invasivos impossíveis de serem implementados em humanos, bem como para testar novas estratégias de tratamento farmacológicas e não-farmacológicas.

No modelo de IC, após indução do infarto agudo do miocárdio, ocorre o comprometimento da função sistólica e diastólica, com consequente aumento da

pressão diastólica final do ventrículo esquerdo (PDFVE), tanto em repouso quanto durante o exercício, menor pressão arterial média, menor frequência cardíaca máxima (NUNES et al., 2008), remodelamento cardíaco patológico, caracterizado pela dilatação do VE (TRUEBLOOD et al., 2005), diminuição do débito cardíaco e disfunção endotelial associada ao aumento da resistência vascular periférica, com resultante hipoperfusão periférica (JORGE et al., 2011).

Ao mesmo tempo, surgem alterações autonômicas e neuro-humorais, caracterizadas pela hiperatividade simpática e atenuação da atividade parassimpática, associadas à diminuição da atividade barorreflexa e da variabilidade da frequência cardíaca, que podem ser inicialmente benéficas, mas subsequentemente tornam-se prejudiciais levando à perpetuação da síndrome (JAENISCH et al., 2011; JORGE et al., 2011; PIEPOLI et al., 2010). Juntamente, há uma alteração no perfil inflamatório, com aumento das citocinas pró-inflamatórias circulantes, tais como o fator de necrose tumoral alfa (TNF-α), a interleucina-1 beta (IL-1β), a interleucina-6 (IL-6) e a proteína c reativa (PCR). Em contrapartida, os níveis de mediadores anti-inflamatórios estão diminuídos na IC, o que resulta em um desequilíbrio do sistema imunológico (NUNES et al., 2008).

O somatório dos eventos descritos anteriormente compromete o bom funcionamento de diversos órgãos e sistemas, com consequente aumento do estresse oxidativo no músculo esquelético (HAMBRECHT et al., 1998; TSUTSUI et al., 2001) e diminuição da capacidade funcional, representada pelo consumo máximo de oxigênio ($\dot{V}O_{2máx}$) (JORGE et al., 2011).

Importa destacar que o grau de comprometimento estrutural e hemodinâmico cardíaco, causado pela cirurgia de infarto do miocárdio, está diretamente relacionado à redução da capacidade funcional. Estudo recente, realizado pelo nosso grupo de pesquisa, demonstrou que animais com áreas de infarto grandes (≥ 40%), apresentaram redução nas funções sistólica (fração de ejeção e fração de encurtamento) e diastólica do ventrículo esquerdo (relação E/A), menor $\dot{V}O_{2max}$, consumo de oxigênio de reserva ($\dot{V}O_{2reserva}$), tempo de exaustão, e velocidade máxima atingidos no teste de esforço máximo, quando comparados aos animais controle. Além disso, foi possível perceber que os animais com maiores áreas de infarto reduziram a massa muscular periférica

(gastrocnêmio e sóleo). Portanto, através destes resultados, foi possível confirmar a hipótese de que as variáveis de capacidade funcional ($\dot{V}O_{2max}$) e tolerância ao exercício são dependentes da área de infarto (HENTSCHKE et al., 2017a).

Animais diabéticos, induzidos por estreptozotocina, apresentam alterações semelhantes àquelas encontradas no modelo animal de IC, tais como a diminuição do débito cardíaco e aumento da resistência vascular periférica, menor frequência cardíaca e menor consumo máximo de oxigênio ($\dot{V}O_{2máx}$) (RODRIGUES et al., 2007, 2013). Além disso, há comprometimento do sistema autônomo com redução do tônus vagal e manutenção do tônus simpático, sugerindo a presença de neuropatia vagal (DE ANGELIS et al., 2000), aumento do estresse oxidativo e das citocinas inflamatórias, tais como, proteína C reativa (PCR), fator de necrose tumoral alfa (TNF-α), interleucina 6 (IL-6) e interleucina 1 beta (IL-1β) (TEIXEIRA DE LEMOS et al., 2012).

Entretanto, estudo realizado por (RODRIGUES et al., 2013) demonstrou que quando estas patologias apresentam-se associadas, as funções hemodinâmica e autonômica tornam-se ainda mais prejudicadas, apresentando menor pressão arterial sistólica, menor pressão arterial média, menor débito cardíaco e maior pressão vascular periférica, bem como menor variabilidade da frequência cardíaca e menor tônus vagal quando comparadas aos animais apenas com diabetes ou infarto isolados. Os autores também perceberam que o comprometimento da capacidade cardiorrespiratória é ainda maior, pois animais diabéticos e infartados tiveram uma redução adicional no $\dot{V}O_{2máx}$ quando comparados aos animais diabéticos ou infartados, o que está diretamente associado ao menor débito cardíaco e à maior resistência vascular periférica que comprometeu a oferta de O_2 e remoção do CO_2.

A intolerância ao exercício é uma das principais características observadas tanto em pacientes diabéticos como naqueles com insuficiência cardíaca, tornando-se um fator determinante de mortalidade nesta população (RODRIGUES et al., 2013). Tendo em vista todas as alterações hemodinâmicas, autonômicas, inflamatórias e da musculatura periférica já descritas, tanto no diabetes quanto na IC, é possível que o conjunto de anormalidades intrínseca de

cada uma, pode desempenhar um papel crítico no comprometimento da performance de exercício.

2.2.2 EXERCÍCIO FÍSICO AERÓBIO

Atualmente sabe-se que o exercício físico é capaz de influencia positivamente sobre uma ampla gama de desfechos cardiovasculares e metabólicos, incluindo a sensibilidade insulínica, o perfil lipídico, a reatividade vascular e o condicionamento cardiorrespiratório, beneficiando especialmente os pacientes com DM tipo 2 (KRÄNKEL et al., 2018). Conforme estudos recentes, pacientes com DM tipo 2 que sofreram infarto do miocárdio, ou foram submetidos à uma intervenção coronariana, devem ser encaminhados para um programa de reabilitação cardíaca, onde o exercício físico pode ser iniciado sob supervisão de um profissional habilitado (KEMPS et al., 2019).

A melhora do condicionamento cardiorrespiratório, medida através do $\dot{V}O_{2pico}$ ou do $\dot{V}O_{2max,}$ induzida pelo treinamento físico reflete na melhora da tolerância ao exercício, na qualidade de vida e reduz a morbidade e a mortalidade de pacientes com IC (GILCHRIST et al., 2013). Somado à isso, é um forte e independente preditor de morbidade e mortalidade em pacientes diabéticos, bem como na população em geral (KAVANAGH et al., 2003; MCAULEY et al., 2007; MYERS et al., 2002).

Infelizmente, os potenciais benefícios da reabilitação cardíaca são subutilizados, muitas vezes em função do custo, da falta de disponibilidade e altas taxas de abandono por parte dos pacientes (GOLWALA et al., 2015). Isso torna-se preocupante, pois o exercício é capaz de melhorar significativamente e de forma sustentada a capacidade funcional e a qualidade de vida dos pacientes com IC (BELARDINELLI et al., 2012). Entender melhor os mecanismos base da disfunção muscular induzida pela IC é de extrema importância para desenvolver estratégias terapêuticas mais eficazes e sustentáveis (POOLE et al., 2018).

O exercício físico aeróbio pode ser utilizado como importante ferramenta não-farmacológica, capaz de restaurar a função endotelial (COUTO et al., 2018; HAMBRECHT et al., 2003; KEMI et al., 2013; VARIN et al., 1999), promover a agiongênese (LEOSCO et al., 2008), melhorar a miopatia esquelética

(ANTUNES-CORREA et al., 2014; BRUM et al., 2014; CUNHA et al., 2012) e, consequentemente, melhorar de forma significativa a tolerância ao exercício (HAMBRECHT et al., 1995; PIÑA et al., 2003). Além disso, estudos mostraram que o exercício aeróbio pode, melhorar a capacidade funcional, o perfil inflamatório, a função hemodinâmica (NUNES et al., 2008), o estresse oxidativo e normalizar a disfunção autonômica tanto de ratos diabéticos induzidos por estreptozotocina (DE ANGELIS; IRIGOYEN; MORRIS, 2009) quanto daqueles infartados (JORGE et al., 2011).

Estudos têm demonstrado que ratos diabéticos e infartados, submetidos ao exercício físico aeróbio, melhoram as funções sistólica e diastólica, provavelmente relacionadas à maior expressão de proteínas associadas à homeostase intracelular de Ca^{+}; normalizam seus parâmetros hemodinâmicos e o fluxo sanguíneo regional, bem como aumentam a expressão proteica e genética do fator de crescimento endotelial vascular *(vascular endothelial growth fator - VEGF)*; e melhoram suas funções autonômicas cardiovasculares. Estes resultados culminaram no aumento da capacidade funcional ($\dot{V}O_{2máx}$) e na queda da mortalidade (RODRIGUES et al., 2012).

2.2.3 Fototerapia ou terapia por fotobiomodulação

A fototerapia ou terapia por fotobiomodulação (PBM) pode ser aplicada através da terapia laser de baixa intensidade (LLLT) ou terapia por diodos emissores de luz (LEDT) (HEISKANEN; HAMBLIN, 2018). O mecanismo de ação que explica os efeitos da fotobiomudulação baseia-se na ativação de fotorreceptores celulares primários, principalmente a proteína citocromo c oxidase (CCO), presente na membrana mitocondrial atuando como unidade IV na cadeia de transporte de elétrons (KARU, 1999; KARU et al., 2005). Os efeitos terapêuticos da fotobiomodulação podem ser, em algumas circunstâncias, inibitórios, mas os efeitos de estimulação são mais comumente encontrados (HEISKANEN; HAMBLIN, 2018). Comprimentos de onda específicos, variando do vermelho ao infravermelho próximo, promovem aumento do potencial da membrana mitocondrial, do consumo de oxigênio e dos níveis de ATP. Evidências preliminares sugerem que alguns comprimentos de onda podem ser

usados para inibir o transporte de elétrons, o que pode ser útil no tratamento de danos por isquemia-reperfusão (SANDERSON et al., 2018).

Ao incidir sobre o tecido, inicialmente ocorre a interação entre os fótons e os fotorreceptores celulares, chamada de “mecanismo primário dos fotorreceptores”, seguida pela ativação de vários mediadores secundários. Essas interações desencadeiam mudanças na expressão gênica, na sinalização celular, no metabolismo celular e na secreção de citocinas (DE FREITAS; HAMBLIN, 2016; PRINDEZE; MOFFATT; SHUPP, 2012).

Embora a maioria dos tratamentos foque apenas na reabilitação de lesões específicas locais, a PBM pode ser capaz de desencadear efeitos sistêmicos relacionados à estimulação mitocondrial. Atualmente, com o envelhecimento da população, o índice de doenças crônicas também tem aumentado. Grande parte dessas doenças está relacionada às disfunções mitocondriais e estresse oxidativo. Portanto, parece adequado pensar que a melhoria da função mitocondrial e das defesas antioxidantes sejam capazes de aliviar os sintomas. Como muitas doenças crônicas compartilham causas metabólicas comuns, métodos sistêmicos de tratamento que alteram o metabolismo podem aliviar doenças de várias partes do corpo (CAMPS; GARCIA-HEREDIA, 2014; HEISKANEN; HAMBLIN, 2018; PIECZENIK; NEUSTADT, 2007).

A partir dos efeitos citados anteriormente, alguns estudos mostraram que a terapia por fotobiomodulação tem demonstrado efeitos benéficos sobre a performance muscular de atletas, envolvendo exercícios de força (BARONI et al., 2010; FERRARESI et al., 2011), resistência a fadiga (DE BRITO VIEIRA et al., 2012; LEAL JUNIOR et al., 2009, 2010), efeitos protetores contra o dano muscular induzido pelo exercício (BARONI et al., 2010; BORSA; LARKIN; TRUE, 2013) e a diminuição da dor muscular após o exercício de indivíduos saudáveis submetidos a um protocolo de exercício excêntrico (ANTONIALLI et al., 2014).

Além disso, a fototerapia pode ser utilizada para prevenir a fadiga muscular, diminuir a sensação de dispneia, aumentando assim o tempo de resistência em pacientes com DPOC (MIRANDA et al., 2014a, 2014b). Recentemente, uma revisão sistemática com meta-análise realizada por (VANIN

et al., 2018) evidenciou que a fototerapia aumenta a performance e acelera a recuperação muscular, principalmente quando aplicada antes do exercício.

Embora os estudos anteriores tenham demonstrado os efeitos benéficos da fototerapia em seres humanos, estudos com ratos saudáveis também têm demonstrado que a fototerapia é eficaz no aumento do desempenho muscular, do metabolismo mitocondrial e ATP, no equilíbrio do estresse oxidativo e na reparação do dano muscular (FERRARESI et al., 2015; HAYWORTH et al., 2010; SANTOS et al., 2014). Além disso, foi possível notar que a fototerapia é capaz de promover melhora no $\dot{V}O_{2basal}$ e no $\dot{V}O_{2max}$, na produção máxima de dióxido de carbono ($\dot{V}CO_{2max}$) e na distância percorrida, apresentando um padrão dose-dependente, pois estes resultados só foram demonstrados com o uso de doses maiores (61,2J/cm^2), mas não em doses menores (8,7J/cm^2) (PERINI et al., 2016).

Esta modalidade terapêutica vem sendo testada pelo nosso grupo de pesquisa há alguns anos de forma experimental, a fim de tentar desvendar seus efeitos em ratos com insuficiência cardíaca. Até agora, foi possível perceber que, a curto prazo, a aplicação da fototerapia pode melhorar o perfil inflamatório em ambos os níveis sistêmico e muscular esquelético (HENTSCHKE et al., 2013); reduzir o estresse oxidativo e o dano ao DNA, quando aplicado em baixas doses (3J/cm^2), porém pode causar danos no DNA em doses mais altas (21J/cm^2) (BIASIBETTI et al., 2014).

Entretanto, os efeitos a longo prazo estão relacionados à melhora da capacidade funcional, através do aumento da distância, do tempo e da velocidade percorridos no teste de esforço máximo (CAPALONGA et al., 2016), bem como pelo aumento do consumo máximo de oxigênio ($\dot{V}O_{2máx}$), consumo de oxigênio de reserva ($\dot{V}O_{2reserva}$) e consumo de oxigênio basal ($\dot{V}O_{2basal}$), quando associado ao treinamento resistido (HENTSCHKE et al., 2017b).

2.2.4 Eletroestimulação

Atualmente, a grande maioria dos programas de reabilitação para pacientes com IC utiliza os exercícios aeróbio e de força como base de treinamento (ARENA et al., 2010). Embora a literatura reforce a importância da

realização do exercício como base de treinamento para essa população, os pacientes que apresentam um grau mais avançado da síndrome, são incapazes de alcançar o débito cardíaco exigido no exercício, tornando-os adequados apenas para aqueles com níveis de IC moderadamente avançado (DOBSAK et al., 2006). Portanto, no intuito de proporcionar maiores benefícios aos pacientes com IC, a estimulação elétrica neuromuscular (EENM) torna-se uma importante forma de terapia coadjuvante, especialmente naqueles com redução mais significativa da capacidade funcional (ARENA et al., 2010).

A EENM consiste em um estimulador que emite estímulos elétricos leves através de eletrodos conectados à pele para produzir uma contração muscular controlada e confortável. Atualmente, a EENM tem apresentado resultados animadores em relação à melhora da capacidade funcional dos pacientes com IC, tais como: distância no teste de caminhada de 6 minutos e $\dot{V}O_{2pico}$, função e força muscular, perfil inflamatório, estresse oxidativo, função endotelial, rigidez arterial e controle autonômico (DOBSAK et al., 2006; DOBŠÁK et al., 2012; KARAVIDAS et al., 2013, 2006).

Estudos anteriores, realizados em nosso laboratório, mostraram que a EENM foi capaz de reduzir a congestão pulmonar, evitar a atrofia muscular, aumentar a densidade dos vasos sanguíneos e, consequentemente, o fluxo sanguíneo muscular, recuperar os níveis do transportador de glicose 4 (GLUT-4) (DE LEON et al., 2011), reduzir o balanço simpato-vagal sobre o controle da frequência cardíaca através da melhora na ativação dos barorreceptores arteriais e, dessa forma, proporcionar melhor controle autonômico cardiovascular em ratos com IC (LAZZAROTTO RUCATTI et al., 2015). No entanto, esses estudos investigaram apenas os efeitos a curto prazo da EENM no sistema autonômico e nos reflexos periféricos.

3 HIPOTESE DO TRABALHO

A IC é considerada consequência de uma disfunção hemodinâmica central, proveniente do comprometimento circulatório responsável por desencadear diversas alterações nos níveis de oxigenação tanto ao tecido

cardíaco como aos órgãos periféricos. O DM tipo 2 é considerado fator de risco para as doenças cardiovasculares que aumenta a chance de desenvolver infarto do miocárdio.

Conforme descrito no referencial desta tese, o exercício físico já vem sendo utilizado a bastante tempo tanto de maneira preventiva como estratégia terapêutica para a reabilitação de pacientes com doenças cardiovasculares. No entanto, algumas modalidades terapêuticas surgem como possíveis coadjuvantes, a fim de sobrepujar os efeitos do exercício físico isolado. Dentre elas destacam-se a terapia por fotobiomodulação e a eletroestimulação. Ambas atuando sobre a musculatura periférica, no intuito de potencializar a produção e liberação de energia e força muscular, culminando na melhora da capacidade funcional.

Vários estudos do nosso grupo de pesquisa e de outros grupos demonstraram efeitos positivos da terapia por fotobiomodulação sobre o perfil inflamatório, o estresso oxidativo e a capacidade funcional, bem como da eletroestimulação sobre o controle autonômico e o metabolismo de glicose de ratos com IC. A partir disso, a principal hipótese de trabalho desta tese é que a associação do exercício físico aeróbio com a terapia por fotobiomodulação e a eletroestimulação poderá potencializar os efeitos relacionados à melhora da capacidade funcional; da disfunção hemodinâmica; do controle autonômico, além de influenciar nas variáveis morfológicas tanto do músculo cardíaco quanto esquelético de ratos diabéticos com insuficiência cardíaca após o infarto do miocárdio.

Portanto, a realização deste estudo justifica-se pela necessidade de ampliarmos o conhecimento acerca dos reais efeitos do exercício físico aeróbio, da fototerapia e da eletroestimulação para que, posteriormente, possam ser utilizadas com segurança na reabilitação de pacientes acometidos pelo diabetes mellitus tipo 2 e insuficiência cardíaca.

4 OBJETIVOS

4.1 OBJETIVO GERAL

Analisar os efeitos de um protocolo de eletroestimulação, de fototerapia e de exercício físico aeróbio sobre: a capacidade funcional, a função hemodinâmica, o controle autonômico, bem como sobre as alterações morfológicas dos músculos cardíaco e esquelético de ratos diabéticos e IC após serem submetidos à cirurgia de indução do infarto do miocárdio.

4.2 OBJETIVOS ESPECÍFICOS

- Analisar os efeitos da eletroestimulação associada à fototerapia sobre o controle autonômico de ratos com insuficiência cardíaca.
- Analisar os efeitos da eletroestimulação associada à fototerapia sobre a variabilidade da frequência cardíaca de ratos com insuficiência cardíaca.
- Analisar os efeitos da eletroestimulação associada à fototerapia sobre a sensibilidade barorreflexa de ratos com insuficiência cardíaca.
- Analisar se o exercício físico aeróbio e a fototerapia melhoram a capacidade funcional e a performance durante o teste de esforço cardiopulmonar de ratos diabéticos e com insuficiência cardíaca.

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6 ARTIGO 1

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O controle do sistema nervso autonômico (SNA) cardíaco é um processo dinâmico tanto na saúde quanto na doênça. A disfunção do SNA pode ser resultado de um distúrbio primário dos nervos autonômicos ou doenças cardiacas e sistemicas de forma secundária. Como exemplo de distúrbios secundários, podemos citar o infarto do miocárdio (IM), a insuficiência cardíaca (IC) e a cardiomiopatia. De forma agurda, o SNA cardíaco responde para manter a homeostase, promovendo um aumento da atividade simpática e redução da atividade parassimpática, a fim de manter a contratilidade cardíaca e o débito cardíaco após uma lesão. No entanto, à longo prazo, essas alterações tornam-se prejudiciais, gerando uma cascata de modificações na atividade neuro-humoral entre os sistemas cardiovascular, vascular periférico e renal. A partir disso, algumas terapias são utilizadas como coadjuvantes no tratamento e reabilitação de pacientes com IC bem controlados. O exercício aeróbio e a estimulação elétrica têm alguns efeitos benéficos já comprovados sobre o controle do SNA tanto em estudos clínicos com pacientes, como experimentais no modelo animal de IC. No entanto, a terapia por fotobiomodulação ainda carece de estudos que possam comprovar sua eficácia na reabilitação de pacientes que sofrem com doenças crônicas, como a IC. Este artigo descreve os efeitos de um protocolo de oito semanas de intervenção baseada em

estimulação elétrica neuromuscular (NMES) e terapia por fotobiomodulação (PBMT) sobre o controle do SNA em ratos com IC.

Neuromuscular electrical stimulation but not photobiomodulation therapy improves cardiovascular parameters of rats with heart failure

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Abstract:

The aim was to analyze the effect of neuromuscular electrical stimulation (NMES) and photobiomodulation (PBMT) on cardiovascular parameters, hemodynamic function, arterial baroreflex sensitivity (BRS), and autonomic balance (ANS) of rats with heart failure (HF). Male Wistar rats (220-290g) were organized into five groups: Sham (n=6), Control-HF (n=5), NMES-HF (n=6), PBMT-HF (n=6), and NMES+PBMT-HF (n=6). Myocardial infarction (MI) was induced by left coronary artery ligation. Animals were subjected to an eight-week NMES and PBMT protocol. Statistical analysis with the General Linear Model (GLM) followed by the Bonferroni post hoc test. Rats of NMES-HF group showed a higher MI area than Control-HF group (P=0.003), PBMT-HF (P=0.002), and NMES+PBMT-HF (P=0.012). NMES-HF and NMES+PBMT-HF showed higher pulmonary congestion (P=0.004 and P=0.02), and lower systolic pressure (P=0.019 and P=0.002) than Sham group. NMES+PBMT-HF showed lower mean arterial pressure (P=0.02) than Sham group. Control-HF showed a higher heart rate than NMES-HF and NMES+PBMT-HF (P=0.017 and P=0.013). There was no difference in the BRS and ANS variables between groups. In conclusion, eight-week NMES isolated or associated with PBMT protocol reduced basal heart rate, systolic and mean arterial pressure, without influence on baroreflex sensibility and autonomic control, and no effect of PBMT was seen in rats with HF.

Keywords: Heart failure, Electric Stimulation, Photobiomodulation, Myocardial infarction, Autonomic nervous system.

1. Introduction

Heart failure (HF) is a clinical syndrome characterized by symptoms such as dyspnea and fatigue, resulting from a structural or functional cardiac disorder that impairs the hemodynamic function of the heart (Ponikowski et al., 2017; Poole, Hirai, Copp, & Musch, 2012). During the development of HF, some changes arise early, even before the symptoms appear. The main one is the sympathetic nervous system hyperactivity associated with the reduction of parasympathetic activity, which characterizes the state of neurohumoral excitation, directly related to the baroreceptors dysfunction and responsible for modulating heart rate and arterial vasomotor tone (Besnier et al., 2017; Lazzarotto Rucatti et al., 2015). The arterial baroreflex system is one of the most important mechanisms that act on cardiovascular homeostasis, can regulate heart rate (HR) and blood pressure (BP), and therefore, directly influence the autonomic nervous system (Bristow, Honour, Pickering, Sleight, & Smyth, 1969; Eckberg, Drabinsky, & Braunwald, 1971; Quagliotto et al., 2008).

In the early stages of the disease, the sympathetic excitation plays a compensatory role to keep hemodynamic parameters within the normal range. However, it becomes detrimental as the disease progresses, contributing to the aggravation of cardiovascular function and the consequent death of the patient with HF (Gronda et al., 2014).

Currently, is known that NMES, at short-term, has been reducing pulmonary congestion, preventing muscular atrophy, increasing muscle blood vessel density, recovering GLUT-4 protein levels (de Leon et al., 2011), reducing sympathetic-vagal ratio over heart rate, activating arterial baroreceptors, and providing better cardiovascular autonomic control in rats with HF (Lazzarotto Rucatti et al., 2015).

Moreover, PBMT was tested a few years ago by our laboratory group in an innovative way, to elucidate its effects on rats with HF. So far, it has been possible to notice that the short-term application of PBMT can improve inflammatory profile at both levels systemic and skeletal muscle (Vítor S. Hentschke et al., 2013); reduce oxidative stress and DNA damage when applied at low doses (3J/cm^2) but may cause DNA damage at higher doses (21J/cm^2) (Biasibetti et al., 2014). However, the long-term effects are related to the improvement of functional capacity, by increasing the distance, time and speed in the maximal exercise test (Capalonga et al., 2016), as well as by the increase in maximal oxygen uptake ($\dot{V}O_2max$), oxygen uptake of the reserve ($\dot{V}O_2reserve$) and in basal oxygen uptake ($\dot{V}O_2basal$), when associated to resistance training (Vítor Scotta Hentschke, Capalonga, Rossato, Perini, Alves, Stefani, et al., 2017). In healthy rats, it was possible to notice that, when applied in higher doses (61.2J/cm^2), but not in lower does (8.7J/cm^2), PBMT promoted improvement in ($\dot{V}O_2basal$), ($\dot{V}O_2max$), maximum carbon dioxide output ($\dot{V}CO_2max$) and distance covered in a dose-dependent pattern (Perini, Scotta Hentschke, Sonza, & Dal Lago, 2016).

However, there is a gap in the literature that describes the effects of these therapies on autonomic balance, baroreflex activity, and heart rate variability in the long-term. Therefore, this study aimed to test the hypothesis that an eight-week program of NMES and PBMT could improve the cardiovascular parameters, hemodynamic function, baroreflex sensitivity, heart rate variability, and autonomic balance of rats with HF.

2. Material and methods

2.1 Animals

This experimental controlled study was performed on 43 male Wistar rats (220 to 290 g), from the Animal Breeding Unit of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA). The rats were housed three per cage and received food and water ad libitum in an animal room under a 12:12-h light-dark cycle, at 22 ºC. The study's protocol followed the ethical rules established by the Guide for Care and Use of Experimental Animals published by the National Institute of Health (NIH publication 85-23, revised in 1996). The UFCSPA's Ethics Committee on Animal Use approved all procedures (protocol 189/16). For the sample size calculation, we used as the main outcome the maximum gain variable. To detect a minimum difference of 66% (Lazzarotto Rucatti et al., 2015), with an alpha error of 0.05, an effect size of 0.80, and a power of analysis of 95%, we estimated a total of 35 animals. The software used for the calculation was GPower version 3.1.9.2 for Windows (University of Düsseldorf, Düsseldorf, Germany).

2.2 Surgery to induce myocardial infarction (MI)

The rats were weighed and then anesthetized with inhaled isoflurane 2% (Isoforine, 100 ml - Cristália, Brasil) in a bell glass. The surgical procedure started with the trichotomy of the left hemithorax. A small incision was made in the skin at the fourth and fifth ribs, and the pectoral muscles were removed. Briefly, the heart was exposed through a left thoracotomy. A mono-nylon suture 6-0 was threaded around the main left descending coronary artery between 1 and 2 mm distal to the edge of the left atrium, and the left coronary was ligated. Sham-operated animals underwent the same procedure, without tying the suture, and served as controls. The chest wall was closed; the skin was sutured with mono-nylon suture 3-0, and the pneumothorax was drained using continuous aspiration. After the surgery, saline was applied, 1 ml intraperitoneally at room temperature for fluid replacement, as well as a single dose of gentamicin, 0.1 ml intramuscularly for prevention of infection. For the management of post-operative pain, Turbogesic® analgesic (butorphanol) was used, 0.5 mg/kg intraperitoneally 12/12h during the first 24 hours. For recovery, the rats were placed in a heated environment (Capalonga et al., 2016).

2.3 Experimental design

To compare the effects of the interventions, of the 43 animals initially started, only 35 survived the MI. After the eight-week recovery period, they were divided into five experimental groups: Sham control group (Sham, n = 6), HF control group (Control-HF, n = 6), neuromuscular electrical stimulation group (NMES-HF, n = 8), photobiomodulation therapy (PBMT-HF, n = 8) and neuromuscular electrical stimulation plus photobiomodulation therapy (NMES + PBMT-HF, n = 7). After this period, the animals underwent eight weeks of NMES, PBMT, or both protocols. At the end of the protocols, a hemodynamic analysis was performed, followed by euthanasia, in which six rats showed no signs of left ventricular dysfunction, characterized by Left Ventricular End Diastolic Pressure (LVEDP) less than 15mmHg or myocardial infarction area less than 20%, totaling 32.5% loss. Thus, 29 rats were selected for study: Sham control group (Sham, n = 6), HF control group (Control-HF, n = 5), neuromuscular electrical stimulation group (NMES-HF, n = 6), photobiomodulation therapy (PBMT-HF, n = 6), and neuromuscular electrical stimulation plus photobiomodulation therapy (NMES + PBMT-HF, n = 6), as shown in Figure 1.

2.4 Neuromuscular Electrical Stimulation (NMES) protocol

Electrically stimulated groups were subjected to a protocol of neuromuscular electrical stimulation (FES, VIF 995, Quark, Piracicaba, Brazil) for eight weeks, five days per week for 30 minutes per day. The electrical stimulation was applied to the gastrocnemius muscle of the right leg through the surface electrode fixed by a system with a velcro fastener. Animals were placed in a rigid corset, along with the trunk, allowing the movement of fore and hind paws to prevent the removal of the electrodes. This followed previous studies and was considered appropriate after simulation of therapeutic intervention (Durigan et al., 2014). The dose used was enough to generate a visible muscle contraction, starting from four to six mA, ten mA until reaching the end of the protocol, respecting parameters described in Table 1 (Lazzarotto Rucatti et al., 2015). Training progression was ensured by increasing the dose during each session as well as during the entire period of the protocol. This procedure was applied immediately after the PBMT protocol for the group NMES + LEDT-HF.

2.5 Photobiomodulation therapy (PBMT) protocol

The rats were placed on a surgical table and immobilized manually by the researcher. The LEDT (equipment developed at the Institute of Physics at São Carlos of the Universidade de São Paulo in cooperation with researchers from the Laboratory of Electrophysical Agents of the Universidade Federal de São Carlos, SP, Brazil) was applied directly on the right gastrocnemius muscle, with a shaved paw as described above, for eight weeks, five days per week (Capalonga et al., 2016), according to the parameters of Table 1. This procedure was applied immediately before the NMES protocol for the group NMES + LEDT-HF.

2.6 Awake measurements of cardiovascular parameters

One week after the protocols, all rats were anesthetized with xylazine (12 mg/kg, i.p.) and ketamine (90 mg/kg, i.p.) for the femoral catheterization. Two catheters filled with saline (0.06 ml) and heparin (0.01 ml) were implanted into the abdominal aorta and inferior vena cava, which were used to measure mean arterial pressure (MAP) and drugs administration, respectively. Conscious rats were studied on a subsequent day after the catheter placement. The arterial catheter was connected to a 40 cm tube attached to a strain-gauge pressure transducer (Miniature Pulse Transducer RP-155, Houston, USA), coupled to a pressure amplifier (General Purpose Amplifier 4 - model 2, Stemtech Inc., Houston, USA), and blood-pressure signals were recorded over a 15 minutes at 1 kHz sampling frequency (Windaq - AT/CODAS, Dataq Instruments Inc., Akron, USA). On a beat-to-beat basis, the measured data were analyzed to quantify the variables of interest (Lazzarotto Rucatti et al., 2015; Quagliotto et al., 2008).

2.7 Baroreflex sensitivity (BRS) and autonomic modulation

On a subsequent day following the catheter placement, MAP and HR were registered for 15 minutes as baseline control. HR changes to test BRS were recorded during peak changes (augmentation or reduction) in MAP due to a single dose of venous injection of phenylephrine (8 µg/ml; Sigma Chemical, St. Louis, USA) or sodium nitroprusside (100 µg/ml; Sigma Chemical), respectively (Lazzarotto Rucatti et al., 2015; Quagliotto et al., 2008). The alterations in MAP were within the 10 to 30 mm Hg range, and the BRS determination was made by fitting the MAP and HR alterations to a sigmoidal logistic equation (Head & McCarty, 1987).

The autonomic modulation of HR was analyzed by spectral analysis. The signals were divided into windows of 300 beats for spectral analysis, with a 50% overlap with the next window. The signal spectral densities were estimated using an autoregressive algorithm by the Yule-Walker method. For the autoregressive algorithm, the filter order was determined for each sample based on the methodology shown in “Heart rate variability signal processing - a quantitative approach as an aid to diagnosis in cardiovascular pathologies.” Windows containing irregular beats were rejected in the analysis. The cut-off frequencies of LF= 0.25-0.75 Hz and HF=0.75-3.0 Hz were chosen according to (Dabiré, Mestivier, Jarnet, Safar, & Chau, 1998). When they presented non-stationary episodes, they were discarded, and a new random selection was performed (Quagliotto, Casali, Dal Lago, & Rasia-Filho, 2015; Rigatto, Casali, Shenoy, Katovich, & Raizada, 2013). The low-frequency spectral components (LF, 0.25 to 0.75 Hz) represents predominantly sympathetic modulation, and the high frequency (HF, 0.75 to 3.00 Hz) represents vagal modulation. The values were expressed in absolute (LFa and HFa; ms2) and normalized (LFnu and HFnu; %) units. This method estimates the frequency and power of each component and indicates the involvement of the heart modulation of sympathetic and parasympathetic systems. The ratio between the LF and HF (LF/HF) components was considered a synthesis of sympathovagal balance.

2.8 Hemodynamic evaluation

After the baroreflex evaluation, animals were anesthetized with xylazine (12 mg/kg, i.p.) and ketamine (90 mg/kg, i.p.) and a small incision was made in the anterior cervical region to access the right carotid artery and introduce a polyethylene catheter (PE-50) for cannulation of the left ventricle (LV) (Jaenisch et al., 2011). The AP was recorded first during for five minutes. Then, the catheter was positioned inside the LV, and the pulse wave was monitored using the typical graphic registration of ventricular pressure and recorded for 5 min. These data were used to determine LV systolic pressure (LVSP), LV maximum change in pressure over time (+dP/dtmax), LV minimum change in pressure over time (-dP/dtmax), and LV end-diastolic pressure (LVEDP).

2.9 Heart hypertrophy, lung, and hepatic congestion

The heart, lungs, and liver were removed and weighed. The right ventricle (RV) and left ventricle (LV) were dissected, separated, and weighed. The heart-to-body mass (H/BM), LV-to-body mass (LV/BM), and RV-to-body mass (RV/BM) were determined and used as an indication of heart hypertrophy. The lungs and liver of each animal were dehydrated (80°C) for 48h and then reweighed to determine the water content. The lung and liver wet-to-dry weight ratios were used to determine the percentage of water in those tissues, as an indication of congestion (Vítor Scotta Hentschke, Capalonga, Rossato, Perini, Alves, Quagliotto, et al., 2017).

2.10 Infarct size

The myocardial infarct scar and the total area of the left ventricle were traced manually on the scanned images and measured automatically by computer (Image Pro-Plus 6.1, Media Cybernetics, Silver Spring, USA) program. The infarct size, expressed as a percentage, was calculated by dividing the sum of infarct areas of all sections by the amount of the areas of the left ventricle (including those without infarct) and multiplied by 100. The analysis was performed in triplicate by three blinded observers (Alves, Nunes, Stefani, & Dal Lago, 2014). All data collection procedures are summarized in Figure 2.

2.11 Statistical analysis

The Shapiro-Wilk test was conducted to evaluate normality for all variables. Statistical differences among groups were identified using the General Linear Model (GLM), followed by the Bonferroni post hoc test. The infarct size was considered a covariate for the analysis of BRS, autonomic modulation and hemodynamic variables, and the infarct size of the Sham group was considered zero. A P value <0.05 was considered statistically significant. To further examine the statistical difference obtained, we performed Cohen's d test to measure the effect size test, followed by a power analysis of the comparison. We considered a reliable power analysis with at least 80.0%. All statistical analyses were performed with IBM SPSS Statistic Data Editor version 22. The images and graphs were created with GraphPad Prism version 7.0

for Windows (San Diego, California, USA). Data are presented as mean and standard deviation (SD).

3. Results

3.1 Mortality, morphological characteristics, pulmonary and hepatic congestion

The rats with HF showed a mortality level of ~20% during and after MI-induced surgery. Table 2 summarizes the data on body mass, heart hypertrophy, infarct size, and pulmonary and hepatic congestion. Sham group animals showed lower wet to dry lung ratio compared with NMES-HF ($P = 0.004$; effect size = 2.60; power analysis = 99%), and NMES + PBMT-HF groups ($P = 0.02$; effect size = 2.36; power analysis = 98%). Importantly, the animals in the NMES-HF group developed larger infarct areas than the Control-HF animals ($P = 0.003$, effect size = 1.92, power analysis= 89%), the PBMT-HF animals ($P = 0.002$; effect size = 2.21; power analysis = 97%), and the NMES + PBMT-HF animals ($P = 0.012$; effect size = 1.81; power analysis = 89%). There was no difference in other morphological variables and hepatic congestion.

3.2 NMES and PBMT did not influence autonomic control

Although the animals in the NMES-HF and NMES + PBMT-HF groups had higher participation of the high-frequency component HF and lower LF/HF ratio values when compared to the Control-HF group, this difference was not significant. The infarct size did not influence ($p > 0.05$) any of the outcomes. Data is presented in Table 3.

3.3 NMES and PBMT on cardiovascular parameters and hemodynamic function

The values of hemodynamic parameters are presented in Table 4. The animals of the NMES-HF and NMES + PBMT-HF groups showed lower systolic arterial pressure (SAP) than Sham group ($P = 0.019$, effect size = 2.21, power analysis = 97%; and $P = 0.002$, effect size = 2.79, power analysis = 99%) respectively. The NMES + PBMT-HF group showed lower mean arterial pressure (MAP) than Sham group ($P = 0.02$, effect size = 2.22, power analysis = 97%). The animals of the Control-HF group demonstrated higher heart rate (HR) than NMES-HF group ($P = 0.017$, effect size = 1.75, power analysis = 84%), NMES + PBMT-HF ($P = 0.013$, effect size = 1.86, power analysis = 88%), and Sham ($P = 0.058$, effect size = 1.73, power analysis = 83%) respectively.

Heart failure groups showed higher LVEDP than Sham group, Control-HF ($P = 0.002$, effect size = 3.65, power analysis = 99%), NMES-HF ($P < 0.001$, effect size = 7.89, power analysis = 100%), PBMT-HF ($P < 0.001$, effect size = 4.10, power analysis = 99%), and NMES + PBMT-HF ($P < 0.001$, effect size = 3.07, power analysis = 99%) respectively. The infarct size did not influence ($p > 0.05$) any of the outcomes.

3.4 NMES and heart rate response

In the evaluation of the baroreflex by the logistic analysis of the sigmoidal baroreceptor curve, we verified that the animals of the NMES-HF group showed lower MAP_{50} than PBMT-HF group (90.8 ± 12.5 vs 104 ± 5.1, P = 0.075; effect size = 1.38; power analysis = 72%). Furthermore, the NMES-HF group showed lower HR range, and higher Maximum gain than other groups, but not significantly. The infarct size did not influence ($p > 0.05$) any of the outcomes. Data are shown in Table 5 and summarized in Figure 3. Blood pressure variability and heart rate during baroreflex sensitivity analysis are presented in Figure 4.

4. Discussion

The main novelties of this investigation are associated with the long-term changes in hemodynamic function that induces an autonomic imbalance and BRS adaptation to a new set point due to heart failure. Through the application of the different interventions in the peripheral muscles of rats with HF, we showed that NMES and PBMT induce: 1) a decrease in systolic blood pressure; 2) a reduction in mean arterial pressure; 3) and a reduction of the heart rate. Interestingly, although the animals submitted to the therapies have shown improvement in the parameters of autonomic control, by increasing the HF component and reducing the LF/HF ratio, suggesting greater participation of the parasympathetic nervous system, as well as improving baroreflex sensitivity, by the reduction of the HR range and MAP_{50}, and increased gain, these results were not significant. We believe in the hypothesis that, as it is a long-term protocol, there was an adaptation of peripheral receptors.

The model of rats with HF, induced by left descending coronary artery ligation surgery, presents as central characteristic hemodynamic dysfunction, which is directly related to the area of MI, especially when it is higher than 30% (Vítor Scotta Hentschke, Capalonga, Rossato, Perini, Alves, Quagliotto, et al., 2017; Jaenisch et al., 2011; Pfeffer et al., 1979). Previous studies have shown that rats submitted to MI induction surgery showed more significant impairment of cardiac function (lower ejection fraction and shortening fraction), increased chemoreflex activity, greater sympathetic activation, and reduced parasympathetic activity with a consequent reduction in HRV and increased LF/HF ratio, besides more significant impairment of baroreflex sensitivity, with areas of infarction of 38% of the LV (Del Rio, Marcus, & Schultz, 2013). In the present study, the NMES group had a larger MI area (36.2%) than the other experimental groups, associated with hemodynamic dysfunction. These changes are directly related to the surgical procedure itself, as they were all performed by the same researcher.

Muscle contraction generated by NMES improves the morphological and metabolic characteristics of the skeletal muscle, providing an increase in blood vessel density, GLUT-4 protein content, and cross-sectional muscle area of HF rats (de Leon et al., 2011).

Sympathetic nervous system hyperactivation is a strong predictor of morbidity and mortality in patients with HF. Changes in autonomic control are directly related to several cardiovascular reflexes and central adjustments that contribute to sympathetic hyperactivity in HF (Triposkiadis

et al., 2009). The attenuation of the baroreflex system is a determinant factor in the neural control of the cardiovascular system. It is used as a significant prognostic value and a predictor of sudden cardiac death in patients with HF (La Rovere, Bigger, Marcus, Mortara, & Schwartz, 1998; La Rovere, Pinna, & Raczak, 2008; Zhang & Anderson, 2014). As seen, rats of the NMES-HF and NMES + PBMT-HF groups increased the gain and reduced the HR range and MAP_{50} when compared to the other groups, but not significantly. These can be represented by a more significant variation of the heart rate to maintain blood pressure within normal parameters. A study from our laboratory has also found similar results; animals with HF undergoing the four-week NMES protocol had increased sensitivity of arterial baroreceptors, reduction in sympathetic modulation, increase in parasympathetic modulation, and a decrease in sympathovagal balance (Lazzarotto Rucatti et al., 2015). Another interesting study from our laboratory showed that an eight-week protocol of electroacupuncture (EA) was able to improve the baroreflex sensitivity of rats with HF; animals of the EA-HF group showed higher gain than the Sham group animals. However, it did not evaluate the sympathovagal balance, which could help to explain this result (Lima et al., 2015).

Importantly, the animals of the Control-HF group showed smaller MI areas than the NMES-HF group, which may influence the results. Smaller MI areas are related to lower structural cardiac impairment. Besides, BRS may lose function over time, being replaced by long-term changes that are triggered by other neuropeptides such as angiotensin II, vasopressin, and aldosterone (Gallinat, Busche, Raizada, & Sumners, 2000; Macova, Pavel, & Saavedra, 2009). It is well documented in the literature that the central overactivation of angiotensin AT1 receptors are responsible for baroreceptor desensitization, sympathetic hyperactivity, and decreased sympathetic inhibition after myocardial infarction in rats and rabbits (Szczepanska-Sadowska, Czarzasta, & Cudnoch-Jedrzejewska, 2018). Nevertheless, aldosterone has pro-fibrotic effects that are capable of reducing arterial compliance and compromising baroreflex activity, which is directly related to impaired autonomic control of cardiac and vascular functions that are generated by hypertension and heart failure (Struthers & MacDonald, 2004).

We are pioneers in the use of PBMT in rats with HF. To date, it has been possible to observe that PBMT improves the systemic and muscular inflammatory profile, increasing the levels of anti-inflammatory cytokines (Vítor S. Hentschke et al., 2013) and reducing oxidative stress when applied in low doses ($3J/cm^2$). Still, it can cause DNA damage in higher doses ($21J/cm^2$) (Biasibetti et al., 2014). Besides, PBMT improves functional capacity by increasing $\dot{V}O_2$basal, $\dot{V}O_2$max, $\dot{V}O_2$reserve, run distance, time to exhaustion, and maximum velocity reached in the cardiopulmonary exercise test (Capalonga et al., 2016; Vítor Scotta Hentschke, Capalonga, Rossato, Perini, Alves, Stefani, et al., 2017). It is also important to highlight that, so far, the main effects of PBMT (LEDT or LLLT) are related to the peripheral muscle tissue without significant influences at the central hemodynamic level, which was also confirmed in the present study.

Our findings confirm the effects of NMES, but it has not been possible to establish the impact of photobiomodulation therapy on the hemodynamic function of HF rats. However, it should be noted that NMES alone or combined with PBMT was not able to maintain its effects on baroreflex sensitivity and autonomic control for a longer-term. Thus, reinforcing information that these therapies, alone, do not provide the necessary benefits to improve hemodynamic function and autonomic control. At the same time, it has not yet been possible to describe the main mechanisms responsible for the improvement in hemodynamic function and arterial pressure, reinforcing the need for further studies to investigate these results.

CONCLUSIONS

In conclusion, the main finding of the present study was to show the effects of long-term NMES isolated and associated with PBMT in the rat model of heart failure. Here we showed that, despite the greater area of infarction, the animals of the NMES-HF group had improved their hemodynamic function and arterial pressure control, without influence on baroreflex sensibility and autonomic control. Besides that, the association with the PBMT did not bring additional effects on these variables.

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DISCLOSURES

The authors Cleber Ferraresi and Nivaldo Parizotto declare that they applied for a patent for the LEDT equipment used in the present study. There are no conflicts of interest to be declared by the other authors.

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Table 1. Photobiomodulation therapy (PBMTT) and Neuromuscular Electrical Stimulation (NMES) protocols

Protocols	
Photobiomodulation therapy (PBMT)	GaAlAs (850nm)
Frequency	Continuous output
Optical output	50 mW
Application area or spot size	0,2 cm^2
Power density	0,25 W/cm^2
Energy (per point)	3 J
Energy density (per point)	15 J/cm^2
Application time (per point)	60 s
Number of irradiation points per muscle group	2
Total energy delivered per muscle group	6 J
Application mode	LED device positioned at 90º angle in skin contact without moving and with light pressure
	Device parameters
NMES (FES VIF 995, Quark, Piracicaba, Brazil)	I = 70 mA; T = 40 µs - 250 µs; F = 4 Hz - 200 Hz; Alternating Current
Frequency (F)	30 Hz
Application time (t)	30 min
Duty cycle	8 s ON – 4 s OFF
Pulse duration (T)	250 µs
Waveform	asymmetric bipolar
Intensity (I)	Enough to generate visible muscle contraction

Table 2. Body, heart and ventricular weight ratio, tissue and infarct characteristics of Sham-operated rats and rats with Heart Failure

Measurement	Sham (n=6)	Control-HF (n=5)	NMES-HF (n=6)	PBMT-HF (n=6)	NMES + PBMT-HF (n=6)	*P* (GLM)
Initial Body Mass (g)	258 ± 18.2	248 ± 19.1	255.0 ± 25.9	232.0 ± 28	252 ± 9.67	0.27
Final Body Mass (g)	311 ± 7.97	306 ± 20.9	310 ± 36	291 ± 34	319 ± 11.5	0.427
Infarcted Area (%) §	---------	25.1 ± 4.55	36.2 ± 6.75 *	24.9 ± 2.59	27.1 ± 2.13	0.001
H/BM (mg/g)	3.45 ± 0.65	3.43 ± 0.62	3.86 ± 0.63	3.44 ± 0.36	3.76 ± 0.46	0.531
LV/BM (mg/g)	2.52 ± 0.36	2.55 ± 0.46	2.8 ± 0.35	2.62 ± 0.24	2.56 ± 0.16	0.606
RV/BM (mg/g)	0.94 ± 0.4	0.88 ± 0.21	1.06 ± 0.39	0.98 ± 0.16	1.2 ± 0.45	0.577
Pulmonary Congestion (%)	73.2 ± 1.6	75.6 ± 2.88	77.4 ± 1.62 †	75.6 ± 1.58	76.7 ± 1.35 †	0.005
Hepatic Congestion (%)	72.8 ± 1.79	72.3 ± 0.18	73.1 ± 0.9	72.1 ± 0.73	71.7 ± 1.18	0.26

Values are means ± SD. Control-HF = Heart Failure Control group; NMES - HF = Neuromuscular Electrical Stimulation Heart Failure group; PBMT-HF = Photobiomodulation Therapy Heart Failure group; NMES + PBMT-HF = Electrical Stimulation plus Photobiomodulation Therapy Heart Failure group. H/BM: heart-to-body mass ratio; LV/BM: left ventricular-to-body mass ratio; RV/BM: right ventricular-to-body mass ratio. GLM: General Liner Model followed by Bonferroni post hoc test. § The Sham group was not included in the analysis. * $P \leq 0.01$ compared to Control-HF, PBMT-HF, and NMES + PBMT-HF; † $P < 0.05$ compared to Sham;

Table 3. Power spectral analysis of cardiovascular parameters in awake rats

Measurement	Sham (n=6)	Control-HF (n=5)	NMES-HF (n=6)	PBMT-HF (n=6)	NMES + PBMT-HF (n=6)	*P* (GLM)
HRV, ms²	45.8 ± 14.5	44.6 ± 14.2	56.6 ± 18.7	33.5 ± 15.5	47.7 ± 39.9	0.557
$f_{LF,\ Hz}$	0.25 ± 0.05	0.22 ± 0.04	0.23 ± 0.05	0.23 ± 0.08	0.27 ± 0.12	0.87
LF, ms²	8.9 ± 1.85	10.3 ± 5.17	9.15 ± 4.8	7.22 ± 4.57	9.77 ± 9.91	0.923
Lfnu	0.42 ± 0.17	0.52 ± 0.16	0.32 ± 0.04	0.42 ± 0.15	0.4 ± 0.06	0.174
$f_{HF,\ Hz}$	1.5 ± 0.43	1.26± 0.25	1.53 ± 0.28	1.53 ± 0.75	1.35 ± 0.54	0.846
HF, ms²	16 ± 11.8	8.6 ± 2.16	20.9 ± 11.3	10.2 ± 6.04	18.1 ± 23.3	0.502
Hfnu	0.58 ± 0.17	0.48 ± 0.16	0.68 ± 0.04	0.58 ± 0.15	0.60 ± 0.06	0.174
LF/HF	1 ± 0.83	1.34 ± 0.71	0.48 ± 0.1	0.87 ± 0.4	0.77 ± 0.2	0.129
VLF, ms²	20.6 ± 5.03	25.4 ± 10.4	26.4 ± 7.23	15.8 ± 8.27	19.6 ± 7.71	0.161

Values are means ± SD. Control-HF = Heart Failure Control group; NMES-HF = Neuromuscular Electrical Stimulation Heart Failure group; LEDT - HF = Light Emitting Diode Therapy Heart Failure group; NMES + PBMT-HF = Neuromuscular Electrical Stimulation plus Photobiomodulation Therapy Heart Failure group. HRV: Heart Rate Variability; LF: Low Frequency Component; HF: High Frequency Component; LF/HF: Low and High Frequency Ratio; VLF: Very Low Frequency. GLM: General Liner Model followed by Bonferroni post hoc test.

Table 4. Hemodynamic variable

Measurement	Sham (n=6)	Control-HF (n=5)	NMES-HF (n=6)	PBMT-HF (n=6)	NMES + PBMT-HF (n=6)	*P* (GLM)
SAP (mmHg)	132 ± 7.97	120 ± 8.14	117 ± 5.31 †	123 ± 10.2	113 ± 5.39 *	0.003
DAP (mmHg)	90 ± 6.22	85.9 ± 5.79	85.9 ± 5.79	87.9 ± 5.83	81.7 ± 5.16	0.147
MAP (mmHg)	109 ± 6.54	101 ± 6.71	99.2 ± 6.07	104 ± 7.41	95.7 ± 5.37 *	0.025
HR (bpm)	458 ± 21.4	527 ± 52.1	446 ± 39.6 ¥	498 ± 38.2	444 ± 35.4 ¥	0.004
LVEDP (mmHg)	4.42 ± 1.07	19.8 ± 5.86 †	23.2 ± 3.19 †	22.7 ± 6.21 †	21 ± 7.55 †	<0.001
LVSP (mmHg)	120 ± 27.8	129 ± 28.2	115 ± 20.7	115 ± 22.2	105 ± 14.6	0.548
+dP/dtmax (mmHg/s)	6441 ± 1997	5403 ± 1339	4640 ± 582	4943 ± 1179	4357 ± 1172	0.089
-dP/dtmax (mmHg/s)	-4238 ± 1098	-3813 ± 1270	-2861 ± 283.8	-3208 ± 1162	-2803 ± 680.4	0.07

Values are means ± SD. Control-HF = Heart Failure Control group; NMES - HF = Neuromuscular Electrical Stimulation Heart Failure group; PBMT-HF = Photobiomodulation Therapy Heart Failure group; NMES + PBMT-HF = Neuromuscular Electrical Stimulation plus Photobiomodulation Therapy Heart Failure group. SAP: Systolic Arterial Pressure; DAP: Diastolic Arterial Pressure; MAP: Median Arterial Pressure; HR: Heart Rate; LVEDP: Left ventricular end-diastolic pressure; LVSP: Left ventricular systolic pressure; $+dP/dt_{max}$: Left ventricular maximum change in pressure over time; $-dP/dt_{max}$: Left ventricular minimum change in pressure over time. GLM: General Liner Model followed by Bonferroni post hoc test. † $P<0.01$ compared to Sham; * $P<0.05$ compared to Sham; ¥ $P<0.05$ compared to Control-HF; # $P<0.05$ compared to NMES + PBMT-HF.

Table 5. Effect of NMES and PBMT on baroreflex curve parameters in awake rats

Measurement	Sham (n=6)	Control-HF (n=5)	NMES-HF (n=6)	PBMT-HF (n=6)	NMES + PBMT-HF (n=6)	*P* (GLM)
Upper plateau (bpm)	495 ± 57.3	494 ± 26.7	451 ± 39.9	484.5 ± 15.3	438 ± 54.1	0.093
Lower plateau (bpm)	313 ± 31	320 ± 61.2	344 ± 56.4	320 ± 28.2	333 ± 28	0.725
HR range (bpm)	182 ± 80.2	175 ± 65.2	107± 46.9	164 ± 23.8	105 ± 48.2	0.055
MAP_{50} (mmHg)	103 ± 3.52	100 ± 6.72	90.8 ± 12.5	104 ± 5.1 *	95.6 ± 7.45	0.043
Maximum gain (beats·min^{-1}·mm Hg^{-1})	-9.07 ± 4.91	-7.69 ± 4.17	-12.5 ± 5.86	-6.8 ± 1.56	-9.83 ± 4.78	0.27

Values are means ± SD. Control-HF = Heart Failure Control group; NMES-HF = Neuromuscular Electrical Stimulation Heart Failure group; PBMT-HF = Photobiomodulation Therapy Heart Failure group; NMES + PBMT-HF = Neuromuscular Electrical Stimulation plus Photobiomodulation Therapy Heart Failure group. MAP_{50}, MAP that corresponds to the value found at half of the HR range evoked by the baroreflex response. GLM: General Liner Model followed by Bonferroni post hoc test. * Compared to NMES-HF, not significant difference; p = 0.075.

FIGURE LEGENDS

Figure 1. Flowchart of design

Figure 2. Experimental timeline of the study

Figure 3. Plots of logistic analysis of the sigmoidal baroreceptor curve, showing the mean values of the ratio between mean arterial pressure (MAP) and heart rate (HR) of rats Sham group; Control-HF = Heart Failure Control group; NMES-HF = Neuromuscular Electrical Stimulation Heart Failure group; PBMT-HF = Photobiomodulation Therapy Heart Failure group; NMES + PBMT-HF = Electrical Stimulation plus Photobiomodultaion Therapy Heart Failure group. In the upper right corner, they are showing the plot of baroreflex sensitivity between the same experimental groups. Data are presented as mean ± SD. GLM: General Liner Model followed by Bonferroni post hoc test.

Figure 4. Delta of blood pressure and heart rate during baroreflex sensitivity analysis of Sham group; Control-HF = Heart Failure Control group; NMES-HF = Neuromuscular Electrical Stimulation Heart Failure group; PBMT-HF = Photobiomodulation Therapy Heart Failure group; NMES + PBMT-HF = Electrical Stimulation plus Photobiomodulation Therapy Heart Failure group. Data are presented as mean ± SD.

Figure 1. Flowchart of design

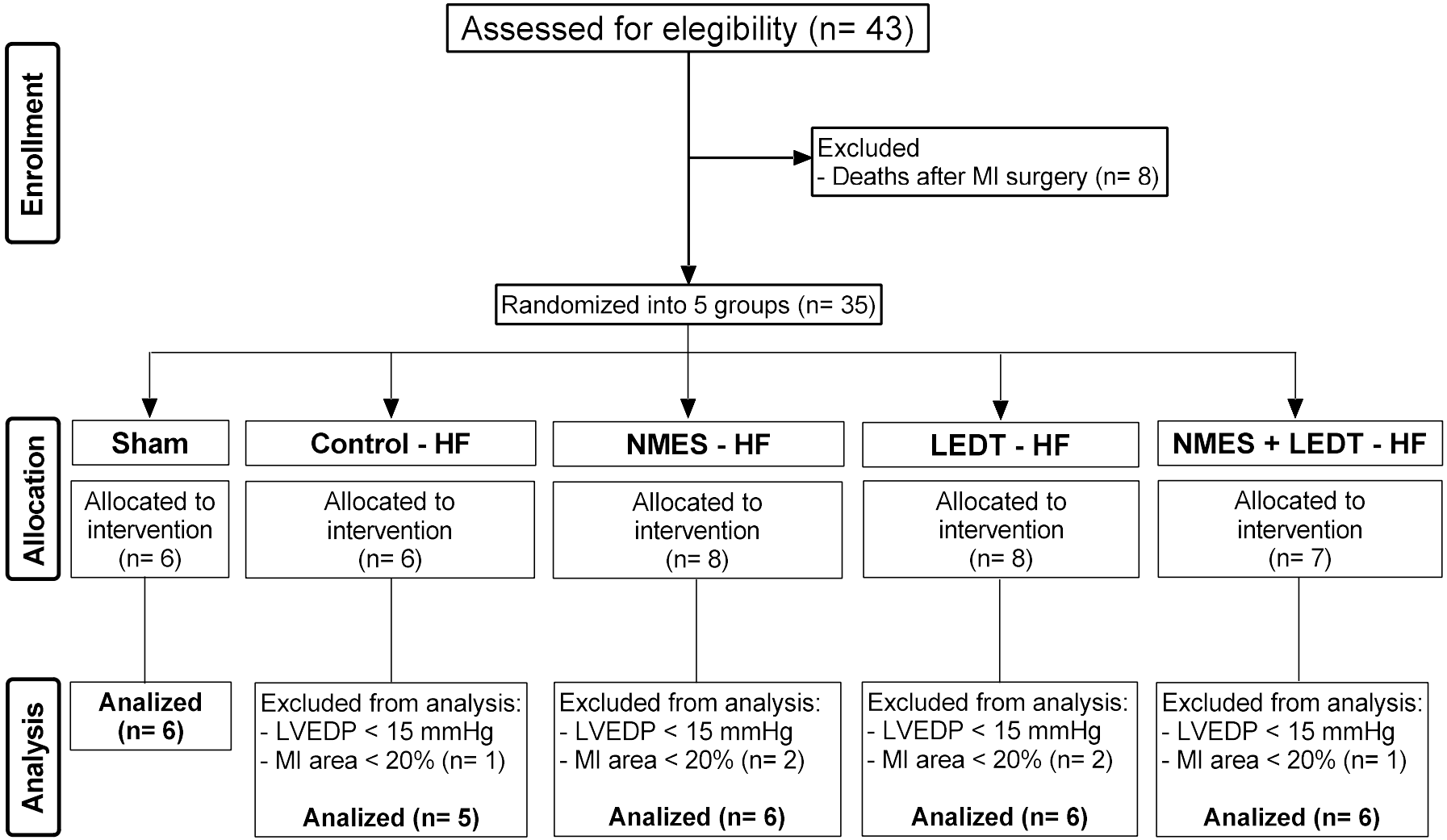


Figure 2. Experimental timeline of the study

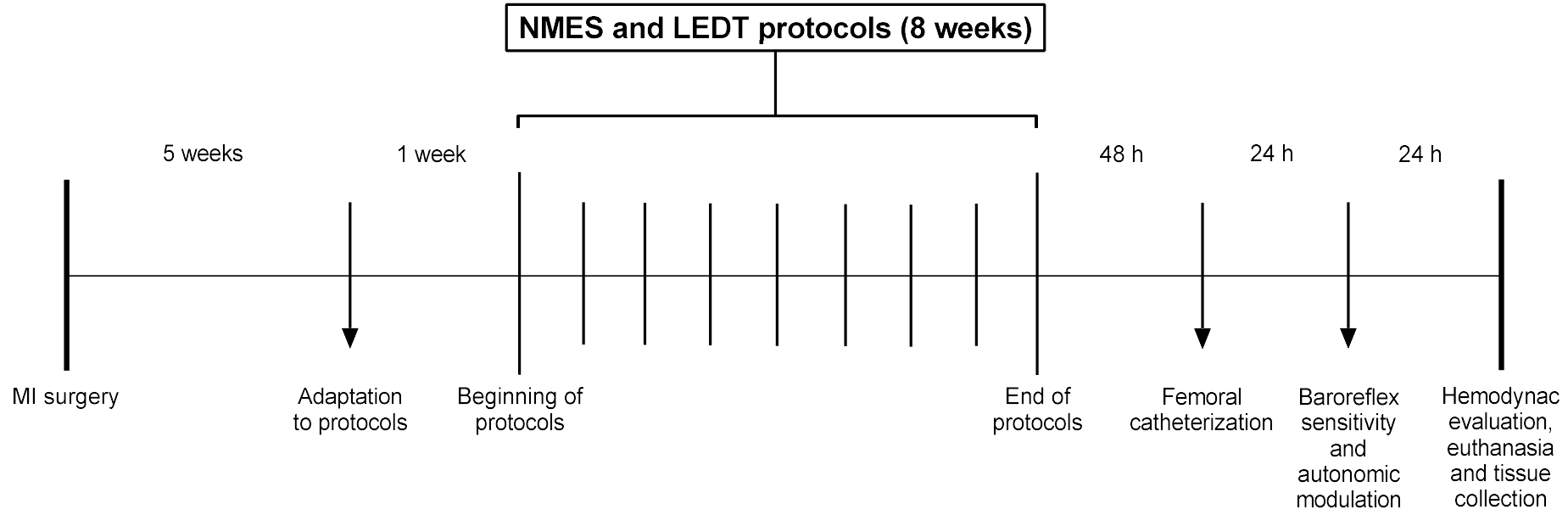


Figure 3. Baroreflex sensitivity

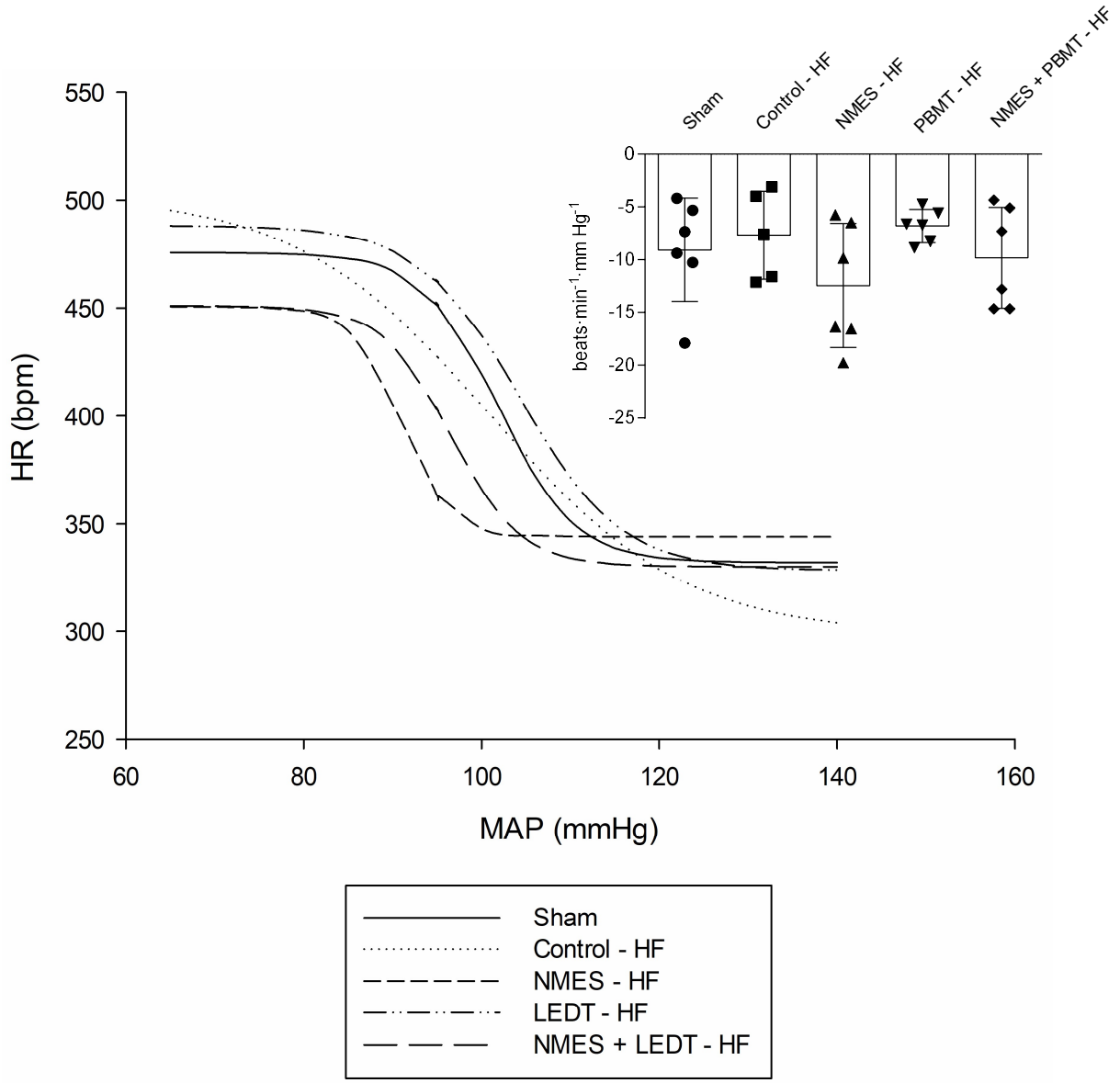
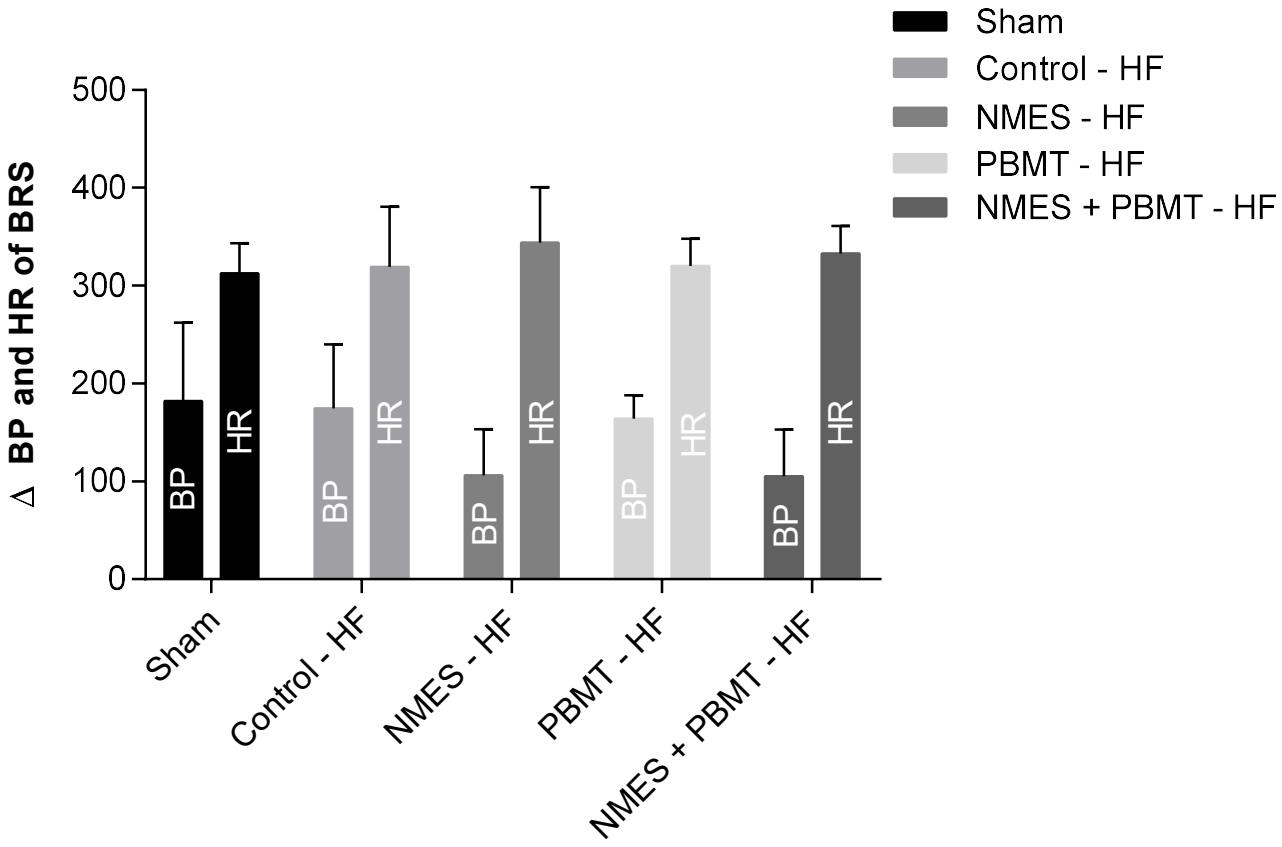


Figure 4. Delta of blood pressure and heart rate during baroreflex sensitivity analysis



7 ARTIGO 2

Aerobic exercise and photobiomodulation therapy improve functional capacity of rats with type 2 diabetes and heart failure

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Atualmente estamos passando por uma enorme crise relacionada aos padrões alimentares, associados ao comportamento sedentário da população. Como consequência, há um crescimento exponencial no indice de doenças cardiometabólicas e cardiovasculares. A doença cardiovascular (DCV) é a principal causa de morte no mundo, podendo ser representada pela insuficiência cardíaca (IC) e o diabetes mellitus (DM) tipo 2. Quando associadas, geram comprometimento metabólico, cardiopulmonar, neuro-humoral e functional muito maior do que quando se apresentam separadamente. Estas disfunções são responsáveis por alterações tanto a nível sistêmico quanto muscular periférico, apresentando como desfecho final a redução na capacidade funcional dos indivíduos acometidos. O exercício aeróbio (EA) possui efeitos comprovados na melhora da capacidade funcional de pacientes com DM tipo 2 e IC bem controlados. No entanto, a terapia por fotobiomodulação (PBMT) surge como uma modalidade mais recente, que ainda necessita de maiores investigações para comprovar seus reais efeitos nessa população. Este artigo descreve os efeitos de um ptotocolo de oito semanas de EA e PBMT sobre a capacidade funcional de ratos com DM tipo 2 e IC.

Aerobic exercise and photobiomodulation therapy improve functional capacity of rats with type 2 diabetes and heart failure

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Abstract:

Purpose: To analyze the effects of aerobic exercise (AE) and photobiomodulation therapy (PBMT) alone or combined on functional capacity, hemodynamic function, and morphological characteristics of type 2 diabetes mellitus (DM-2) and heart failure (HF) rats. **Methods:** Male Wistar rats (~120g) were divided into three groups: rats with DM-2 and HF that did not received any intervention (DMHF-Control; n=4), rats with DM-2 and HF that received AE training (DMHF-AE; n=6), and rats with DM-2 and HF that received AE training and PBMT (DMHF-AE+PBMT; n=6). DM-2 was induced by a high-fat diet associated with streptozotocin (STZ) injection, and myocardial infarction (MI) surgery was used to induce HF. The animals were subjected to the AE and PBMT protocols (8 weeks). **Results:** Comparing group values only in the post-protocol period, both the AE and the AE+PBMT groups increased the time, distance, and maximal speed than the Control group ($p<0.001$) in the exercise test. From the delta (Δ) percentage evaluation, only the AE+PBMT group improved the time ($p=0.016$), distance ($p=0.019$), and maximal speed ($p=0.011$) compared to the Control group. AE group had a lower left ventricular posterior wall in diastole (LVPWd) than Control group ($p=0.02$). AE and PBMT were not able to change the morphological characteristics. AE and PBMT can control the mass body gain, but not influence the LEE index, and the glycemic control. **Conclusions:** An 8-week protocol of AE combined with PBMT was able to potentiate the effects of isolated AE on performance in the exercise test of rats with DM-2 and HF.

Keywords: Type 2 diabetes mellitus, Heart failure, Photobiomodulation, Myocardial infarction.

1. INTRODUCTION

Cardiovascular disease (CVD) remains the leading cause of death and disability among patients with diabetes mellitus (DM) [1]. DM exacerbates the mechanisms underlying atherosclerosis and heart failure (HF). Unfortunately, these mechanisms are not adequately modulated by therapeutic strategies that focus only on optimal glycemic control with drugs or approaches currently available [2]. In patients with DM, the prevalence of HF is higher than that in the general population, since a 1% increase in glycated hemoglobin is associated with an 8% increase in HF risk. Individuals with DM have a three- to sevenfold increased risk of developing CVD when compared to nondiabetic patients [3].

Animal models of HF and DM have been extensively used in the scientific literature to simulate the alterations caused by these pathologies in clinical settings. Currently, it is known that aerobic exercise (AE) can positively influence a wide range of cardiovascular and metabolic outcomes, including insulin sensitivity, lipid profile, vascular reactivity and cardiorespiratory fitness, primarily benefiting patients with DM-2 [4]. According to recent studies, patients with DM-2 who have suffered myocardial infarction (MI), or have undergone a coronary intervention, should be referred to a cardiac rehabilitation program, where physical exercise can be initiated under the supervision of a qualified professional [5].

Photobiomodulation therapy (PBMT) is a therapeutic modality widely used in clinical practice, with several pieces of evidence confirming its beneficial effects on the muscular performance of athletes and healthy people [6]. Recently, our research group has directed efforts to uncover the potential effects of photobiomodulation on improving functional capacity, inflammatory profile, and oxidative stress in rats with HF. So far, it has been shown that, in the short term, PBMT application can improve the inflammatory profile at both skeletal muscle and systemic levels [7], reducing oxidative stress and DNA damage when applied at low doses (3J/cm^2), but may cause DNA damage at higher doses (21J/cm^2) [8]. However, the long-term effects are related to the improvement of functional capacity, by increasing distance, time, and speed in the treadmill maximal effort test [9], as well as by increasing maximum oxygen uptake ($\dot{V}O_2$max), reserve of oxygen consumption ($\dot{V}O_2$reserve), and baseline oxygen uptake ($\dot{V}O_2$basal) when associated with resistance training [10]. The primary aim of this study was to analyze the effects of AE and PBMT on the functional capacity, hemodynamic and morphological characteristics of DM-2 rats with HF after MI surgery. The secondary aim was to verify whether the therapies were able to influence the glycemic and body mass control of these animals. The main hypothesis of this study was that the association of AE training with PBMT might potentiate the effects of functional capacity improvement.

2. MATERIAL AND METHODS

2.1 Animals, ethics and sample size calculation

The experiments were conducted in 54 male Wistar rats (±30 days of age and body mass ~70-150g) from the Animal Breeding Unit of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA). Three rats were housed per cage and received food and water ad libitum in an animal room under a 12-h light-dark cycle, at 22ºC. The study protocol followed the ethical rules established by the Guide for Care and Use of Experimental Animals published by the National Institutes of Health (NIH publication no 85-23, revised in 1996). The assembly and execution of the project followed the ARRIVE protocol. The

UFCSPA Ethics Committee on Animal Use approved all procedures (protocol 189/16). For the sample size calculation, we used as a primary outcome the maximal speed on the treadmill. To detect a minimum difference of 4 m/min [11], with two-tailed α=0.05, an effect size of 1.66, and a power of analysis of 95%, we estimated a total of 12 animals. The software used for the calculation was G*Power version 3.1.9.2 for Windows (University of Düsseldorf, Düsseldorf, Germany).

2.2 Experimental design

All animals were submitted to induction of DM-2 and MI with consequent HF. After 6 weeks of recovery, the animals were divided into three experimental groups: DM-2 with HF that did not receive intervention (DMHF-Control, n = 4), DM-2 with HF submitted to AE (DMHF-AE, n = 8), and DM-2 with HF subjected to AE associated with PBMT (DMHF-AE+PBMT, n = 8). Functional capacity was assessed before induction of MI, before and after interventions. Echocardiography was assessed before and after eight weeks of intervention, with the ejection fraction (EF) as the main variable. Glycemic tests were performed before MI, before and after interventions. The main glycemic control variables were fasting blood glucose levels and the result of the glucose tolerance test (GTT). To body mass control, the animals were weighed weekly. After the conclusion of the protocols, hemodynamic evaluation was performed, followed by euthanasia and tissue collection. From this, we noticed that four animals did not develop signs of HF, excluding them from the analyses. Thus, a total of 16 animals were distributed among the three experimental groups, totaling 70.3% of losses, as shown in Figure 1.

2.3 Induction of diabetes mellitus 2 (DM-2)

DM-2 was induced through a high-fat diet associated with an intraperitoneal injection of streptozotocin (STZ, Sigma, St. Louis, MO, 30 mg/kg). The high-fat diet was provided throughout the study and was composed of lard (37.4% w/w; total metabolizable energy 22.8 MJ/kg, with 58.3% of total fat, 24.5% of carbohydrate, and 17.2% of protein). To meet the standard (except for starch and lard), the quantities were adjusted to 13.7% albumin (Naturovos®, Rio Grande do Sul, Brazil), 7.4% amino acids (Aminomix®, São Paulo, Brazil), and 1.1% bone and oyster powder [12]. This diet was prepared weekly in the laboratory and stored in a refrigerator (4-8 °C) during the study period. Four weeks after the beginning of a high-fat diet, the animals were intraperitoneally injected with STZ. One week after STZ injection, the animals were fasted for 12 h for a blood glucose test, and blood was collected from the tail of the rats using test strips (Performa, Roche, USA). Animals that presented fasting blood glucose <7.8 mmol/L (<140.4 mg/dL) received another dose of STZ (30.0 mg/kg) in order to develop insulin resistance [13].

2.3.1 Glycemic control

Glycemic control was performed three times, that is, 5th week (baseline), 10th week, and 15th week from the beginning of the high-fat diet. As described in the previous item, for the glycemic tests, the animals fasted for 12 h, and from there, blood samples were collected from the tail tip of the rats using the test strips (Performa, Roche, USA).

2.3.2 LEE index and body mass control

Every 2 weeks, the LEE index was analyzed through the relationship between the nasal-anal length and body mass, as follows: $Body\ mass\ \frac{1}{3(g)} \div nasal - anal\ length\ (cm) \times 100$ [14]. The body mass was measured every week support weight control.

2.3.3 Glucose tolerance test (GTT)

The rats were fasted for 12 to 16 h to analyze fasting glycemia (FG). The success rate was calculated through the GTT, at the 5th and 13th week after STZ injection, that is, before and after 8 weeks of the protocol. For GTT, 40% glucose (2.0 g/kg body mass) was administered, intraperitoneally (IP). Blood samples were then taken from a needle prick at the tail tip of the rats at 0, 30, 60, 90, and 120 min. The glycemic response during the GTT was evaluated by calculating the area under the curve (AUC) [12].

2.4 Surgery to induce myocardial infarction (MI)

The procedures for acute myocardial infarction induction surgery were followed according to Katz et al. (2019) [15], already used in another study of our group [9].

2.5 Assessments

2.5.1 Evaluation of functional capacity

Functional capacity was assessed by the maximal speed, distance and time spent in an incremental exercise test, before-MI, before-protocol, and post-protocol, which followed a protocol previously used by our research group. More details can be found in previous studies [9, 16].

2.5.2 Echocardiography

All evaluations were performed by the same researcher and followed the recommendations of the American College of Echocardiography [19]. The following structural variables were measured: interventricular septum in diastole (IVSd, mm), interventricular septum in systole (IVSs, mm), left ventricular end-diastolic diameter (LVEdD, mm), left ventricular end-systolic diameter (LVESD, mm), left ventricular posterior wall in diastole (LVPWd, mm), and left ventricular posterior wall in systole (LVPWs, mm). From the primary variables, we obtained the secondary variables of LV systolic function: end-diastolic volume (LVEdV (ml) = $1.047(LVEdD)^3$ and end-systolic volume (LVEsV (ml) = $1.047(LVEsD)^3$) using a cubic or ellipsoid model; LV ejection fraction (EF (%) = [(LVEdV−LVEsV)−LVEdV] × 100), LV fractional shortening (FS (%) = [(LVEsD−LVEsD)/LVEdD] × 100), relative wall thickness (RWT = (IVSd + LVPWd)/LVEdD) and left ventricular end-diastolic diameter-to-body mass (LVEDD/body mass, mm/kg). We considered only animals with HF, characterized by an EF less than 50%. More details are in the supplementary material.

2.5.3 Hemodynamic evaluation

Briefly, the animals were anesthetized with xylazine (12 mg/kg, ip) and ketamine (90 mg/kg, ip), and then a polyethylene catheter (PE-50) was inserted into the right carotid artery. Thus, the catheter that was connected to a pressure gauge (Narco Biosystem Miniature Pulse Transducer RP-155, Houston, TX,

USA), coupled to a pressure amplifier (Stemtech), was positioned inside the left ventricle. Ventricular pressure pulse waves were recorded for 5 minutes. Records provide us the following variables: left ventricular systolic pressure (LVSP), left maximum ventricular change in pressure over time (dP/dt_{max}), left minimum ventricular change in pressure over time (dP/dt_{min}) and left ventricular end-diastolic pressure (LVEDP), according to Hentschke et al. [10].

2.5.4 Morphological analysis and tissue collection

Immediately after the hemodynamic evaluation, with the animals still anesthetized from previous analysis, euthanasia was performed by decapitation, and heart, lungs, and liver were collected, according to Hentschke et al. [16].

2.5.6 Heart hypertrophy, lung, and hepatic congestion

The heart, lungs, and liver were removed and weighed. The right ventricle (RV) and LV were dissected, separated, and weighed. The heart-to-body mass (H/BM), LV-to-body mass (LV/BM), and RV-to-body mass (RV/BM) were determined and used as an indication of heart hypertrophy. The lungs and liver of each animal were dehydrated (80°C) for 48 h and then reweighed to determine the water content. Lung and liver wet-to-dry weight ratios were used to determine the percentage of water in those tissues, as an indication of congestion, using the following formula, according to Alves et al. [20]:

$$weight\ wet - weight\ dry\ \div weight\ wet \times 100$$

2.5.7 Infarct size

MI scars and the total area of the LV were traced manually on the scanned images and measured automatically by a computer (Image Pro-Plus 6.1, Media Cybernetics, Silver Spring, USA), according to Alves et al. [20]. All procedures are resumed as in Figure 2, the study timeline.

2.6 Interventions

2.6.1 Aerobic exercise (AE) protocol

Six weeks after MI surgery, a group of trained animals underwent AE training sessions on a treadmill. A mean intensity ranging from 50 to 70% of the maximum speed reached in the maximal speed test was used. Initially, for the first 5 min at 50% (~10 m/min), after 5 min of heating, it increased to 60% (~15 m/min), and within 15 min the animals were running at 70% (~20 m/min). In the last 5 min the speed was reduced, which corresponded to the period of cooling. To avoid adaptation effects, the intensity was increased by 10% every 15 days [17, 18]. Animals in the DMHF-Control group were placed on the treadmill every day during the same protocol period, but the treadmill was turned off.

2.6.2 Photobiomodulation therapy (PBMT)

A GaALAs laser apparatus (ISO: 13485, model 2779, Chattanooga Group - Intelect® Mobile Laser, Austin, Texas, USA) was used for this study, according to Hentschke et al. [10]. Further information on the characteristics, parameters, and mode of application are showed in Table 1 and supplementary materials.

2.7 Statistical analysis

The Shapiro-Wilk test was used to check data distribution. The comparisons between baseline and post interventions (exercise time, distance, maximal speed) were analyzed with the generalized linear model, using the gamma model, and considering the groups (DMHF-Control, DMHF-AE, and DMHF-AE+PBMT) as a factor. The Bonferroni correction was used for all comparisons. Statistical difference between groups in the percentages of change (Δ%) of exercise time, distance, maximal speed was analyzed with the general linear model. The variables related to glycemic and body mass control (i.e. glycemic level, GTT, LEE index, and body mass) were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Hemodynamic and morphological variables (i.e., EF, LVEDP, infarct size, pulmonary and hepatic congestion) were analyzed only after the protocols using one-way ANOVA followed by Tukey's post hoc test. A $p < 0.05$ was considered statistically significant. To analyze the statistical difference obtained, we performed Cohen's d test to measure the effect size, followed by a power analysis of the comparison. We considered a reliable power analysis of at least 80.0%. All statistical analyses were performed using the Sigma plot software version 12.0 for Windows (San Jose, California, USA), GraphPad Prism software version 7.0 for Windows (San Diego, California, USA), and IBM SPSS Statistic Data Editor version 22. All images and graphs were created using GraphPad Prism software version 7.0 for Windows (San Diego, California, USA). Data are presented as mean ± standard deviation (SD).

3. RESULTS

3.1 Mortality, morphological characteristics, pulmonary and hepatic congestion

The mortality rate was 62.8%. Before starting the protocols, all animals had EF less than 50% and blood glucose above 140.4 mg/dL, proving the development of HF and DM-2. Table 2 summarizes the data on body mass, heart hypertrophy, infarct size, and pulmonary and hepatic congestion.

3.2 Metabolic variables

3.2.1 AE and PBMT can control the mass body gain, but not influence the LEE index.

Regarding the body mass gain curve, it was observed that all groups in the study had an ascending pattern up to the 10th week. DMHF-AE and DMHF-AE+PBMT groups maintained their body masses, whereas the DMHF-Control group showed a marked gain in body mass in the last 2 weeks. In the 20th week of follow-up, the DMHF-Control group had a higher body mass than that in the DMHF-AE group (p=0.024, effect size = 1.04, power analysis = 43%) (Figure 3A). Neither protocol was able to influence the LEE index, as shown in Figure 3B.

3.2.2 Glycemic control

Regarding the glycemic values, the DMHF-AE+PBMT group reached higher levels at the 11th week than at baseline (p=0.018, effect size = 1.22, power analysis = 63%). The DMHF-AE group reached higher levels at the 5th and 11th weeks when compared to baseline (p<0.001, effect size = 5.78, power analysis = 100% and p<0.001, effect size = 2.95, power analysis = 99%, respectively). However, the DMHF-Control

group reached higher levels only in the 5th week compared to baseline (p=0.016, effect size = 1.92, power analysis = 77%), as shown in Figure 4.

3.2.3 AE and PBMT do not influence GTT.

Data related to GTT of DM-2 with HF rats treated with AE or AE plus PBMT are shown in Figure 5. Regarding the GTT, there was no difference between the groups.

3.3 AE improves echocardiography parameters.

Table 3 shows the values of echocardiographic variables as well as hemodynamic evaluation. It was found that the DMHF-AE group had a lower LVPWd when compared to the DMHF-Control group at the end of the protocol, (p= 0.02, effect size = 2.53, power analysis = 97%). The values of echocardiographic variables collected before the protocols are available in the supplementary material.

3.4 AE and PBMT improve functional capacity

Functional capacity tests performed before the induction of MI showed that there was no difference between groups in this period. The DMHF-AE+PBMT group achieved a longer test time in the post-protocol than in the before-MI and before-protocol periods (p<0.001, effect size = 3.88, power analysis = 99% and p<0.001, effect size = 2.80, power analysis = 99%, respectively). The DMHF-AE group also reached a greater test time in the post-protocol than the before-MI and before-protocol periods (p=0.005, effect size = 1.08, power analysis = 54% and p=0.045, effect size = 2.40, power analysis = 98%, respectively). Also, DMHF-AE+PBMT group had a greater distance covered in the post-protocol period than in the before-MI and before-protocol periods (p<0.001, effect size = 2.04, power analysis = 94% and p<0.001, effect size = 2.45, power analysis = 78%, respectively). The DMHF-AE group also showed an increase in the distance covered in the post-protocol period (p=0.007, effect size = 2.45, power analysis = 99%). Maximal speed was higher in the DMHF-AE+PBMT group in the post-protocol period than that in the before-MI and before-protocol periods (p<0.001, effect size = 2.66, power analysis = 99% and p<0.001, effect size = 2.00, power analysis = 94%, respectively). However, the DMHF-AE group reached a higher maximal speed only in the post-protocol compared to the before-protocol period (p=0.014, effect size = 2.33, power analysis = 98%). In the before-MI period, the DMHF-AE group reached a higher maximal speed than the DMHF-AE+PBMT group (p=0.036, effect size = 1.29, power analysis = 67%). Comparing group values only in the post-protocol period, both the DMHF-AE and the DMHF-AE + PBMT groups increased the time, distance, and maximal speed in the exercise test, as shown in Figure 6 (A, B, and C). However, from the delta (Δ) percentage evaluation, only the DMHF-AE+PBMT group improved the time (p=0.016, effect size = 1.94, power analysis = 86%), distance (p=0.019, effect size = 1.96, power analysis = 86%), maximal speed (p=0.011, effect size = 2.23, power analysis = 93%) in the exercise test in relation to the DMHF-Control group. Further details are shown in Figure 6 D and Table 4 in the supplementary material.

4. DISCUSSION

This study revealed that the combination of therapies was not able to act on hemodynamic variables, since systolic and diastolic pressures were like those in the control group. The animals of the DMHF-AE group showed a reduction in left ventricular posterior wall thickness in the diastole (LVPWd). Our results showed that the interventions were not able to modify glycemic levels presented during GTT and in total values over time. To the best of our knowledge, through this study, it was possible to demonstrate that improvement in the functional capacity of groups that performed the training protocols, represented by a significant increase in time to exhaustion, greater run distance, and higher maximal speed reached during the exercise test. Interestingly, on delta percentual analysis, the group undergoing combination therapy showed a more considerable increase in test time, distance, and maximal speed during the exercise test compared with the DMHF-Control group, suggesting an additional benefit of PBMT versus only AE. Analyzing the figure 6D (delta Δ percentage), we noticed that the DMHF-AE+PBMT group was different from the DMHF-Control group, but it was not different from the DMHF-AE group. At the same time, the DMHF-AE group showed no difference when compared to the DMHF-AE+PBMT and DMHF-Control groups. Although the DMHF-AE group did not show any difference in relation to the other groups, we noticed that the isolated aerobic exercise brought some benefit related to the functional capacity of the DM-2 rats with HF, but with less magnitude than the DMHF-AE+PBMT group.

Previous studies, conducted by our research group, showed that PBMT, in a protocol similar to the present study, improved the $\dot{V}O_2$max and distance covered in healthy Wistar rats with a dose-dependent response [21], as well as to improved oxygen consumption ($\dot{V}O_2$basal, $\dot{V}O_2$max, and $\dot{V}O_2$reserve) and exercise tolerance (running distance, time to exhaustion and maximal velocity) of rats with HF when combined with resistance exercise [10]. To better understand how PBMT can influence functional capacity variables, we decided to enroll HF animals to an 8-week LEDT protocol. Thus, we were able to present exciting results, where LEDT improved functional capacity by increasing the distance covered, time, and speed of exercise, without influencing the $\dot{V}O_2$max [9].

A recent study showed that patients with DM-2 and HF suffer severe mitochondrial dysfunction, mainly related to the reduction in mitochondrial respiration, associated with the lower mitochondrial content as well as the lower absolute mitochondrial O_2 flow in complex I of the mitochondrial respiratory chain, characterizing an intrinsic dysfunction. In addition, mitochondrial complex I dysfunction is associated with a greater amount of mitochondrial reactive oxygen species (ROS) and less gene expression of the antioxidant enzyme superoxide dismutase 2 (SOD2). The higher mitochondrial production of ROS can trigger muscle atrophy, especially in type II fibers. Altogether, the changes responsible for mitochondrial muscle impairment are directly related to a greater limitation to exercise [22]. Although the study has shown metabolic changes in humans, it should be noted that these changes are also found in animals.

Several mechanisms may explain the effects of PBMT on exercise performance. Among them, there is an increase in the activity of cytochrome c oxidase, a terminal enzyme of the mitochondrial respiratory chain, which mediates the transfer of electrons from cytochrome c to molecular oxygen. It is also considered a photoreceptor able to modify cellular metabolism, increasing the concentration of adenosine triphosphate (ATP) and Ca^{2+} as well as the synthesis of DNA and RNA [23]. According to Santos et al. (2014) [24], PBMT can increase mitochondrial cytochrome c oxidase activity in the intact skeletal muscle, directly contributing to the improvement in muscle performance, as well as acting as a protective

factor against the development of fatigue and tissue damage. Other possible mechanisms are the improvement in the inflammatory profile and oxidative stress. Currently, both DM-2 and HF are known to trigger a systemic inflammatory cascade associated with increased oxidative stress [25, 26]. Previous studies conducted in our laboratory have shown that PBMT improves the inflammatory profile by reducing tumor necrosis factor alpha (TNF-α) and interleukine-6 (IL-6), increasing interleukine-10 (IL-10) at the muscle level [7], and reducing the oxidative stress in rats with HF [8]. Also, the results of a recent study showed that PBMT was able to maintain oxidative activity similar in type 1 diabetic rats compared to that of the control group, indicating an increase in antioxidant activity [27]. Clinically, we believe that PBMT can be another adjunctive therapeutic modality in the rehabilitation of patients affected by chronic diseases, through its anti-inflammatory effects and in the reduction of oxidative stress. Curiously, from the 16th week, animals in the DMHF-Control group showed a marked gain in body mass, suggesting that protocols were effective in controlling animals' body mass. It should be noted that, between the 12th and 20th weeks, the animals were in the training period. Similar to these reports, a study with type 1 diabetic rats subjected to 6 weeks of AE (high and low intensity) and resistance exercise revealed that the gain of body mass in the control group was higher from the beginning of the study, regardless of the exercise modality [28].

The present study has some limitations that reinforce the need for discussion, but do not invalidate the results. The absence of two other experimental groups, the sham group, which could provide further information on the efficacy of the DM-2 induction method, and a DM-2 group submitted only to PBMT, which would reinforce the effects of this therapy specifically, aside from the lack of biochemical analyses that could help in the understanding of physiological mechanisms (e.g., inflammatory profile, oxidative stress, and enzymatic activity) responsible for the results.

5. CONCLUSIONS

In conclusion, this study is the first to report on the effects of combined AE and PBMT on the functional capacity of DM-2 rats induced by a high-fat diet and HF. Here, we showed that the two therapies provide an improvement in functional capacity, but the addition of PBMT to AE was able to potentiate the effects of improved performance on the exercise test compared to that in the isolated AE.

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FIGURE LEGEND

Figure 1. Flowchart of the study.

Figure 2. Experimental timeline.

Figure 3. Metabolic variables. Values are means ± SD. DMHF-Control = Type 2 Diabetes Mellitus and Heart Failure Control group; DMHF-AE = Type 2 Diabetes Mellitus and Heart Failure and Aerobic Exercise; DMHF-AE+PBMT = Type 2 Diabetes Mellitus and Heart Failure with Aerobic Exercise and Photobiomodulation Therapy. A. Body mass control (g); B. LEE index. * $p<0.001$ compared to Baseline, 2nd, 4th, 6th and 8th week; $^{\#}$ $p<0.05$ compared to DMHF-AE at 20th week; ‡ $p<0.05$ compared to DMHF-AE at 2nd week (two-way repeated measure ANOVA followed by Tukey post hoc test).

Figure 4. Glycemic curve. Values are means ± SD. DMHF-Control = Type 2 Diabetes Mellitus and Heart Failure Control group; DMHF-AE = Type 2 Diabetes Mellitus and Heart Failure and Aerobic Exercise; DMHF-AE+PBMT = Type 2 Diabetes Mellitus and Heart Failure with Aerobic Exercise and Photobiomodulation Therapy. * $p<0.01$ compared to Baseline; $^{\#}$ $p<0.05$ compared to Baseline; ‡ $p<0.05$ compared to DMHF-Control at week 11 (two-way repeated measure ANOVA followed by Tukey post hoc test).

Figure 5. Glucose tolerance test (GTT) of type 2 DM with HF rats. Values are means ± SD. DMHF-Control (n=4) = Type 2 Diabetes Mellitus and Heart Failure Control group; DMHF-AE (n=6) = Type 2 Diabetes Mellitus and Heart Failure and Aerobic Exercise; DMHF-AE+PBMT (n=6) = Type 2 Diabetes Mellitus and Heart Failure with Aerobic Exercise and Photobiomodulation Therapy. Graphs A and B show variations in blood glucose and insulin levels in the before-protocol period. Graphs C and D show the same variations in the post-protocol period. Graphs B and D show data related to the area under the curve (AUC).

Figure 6. Effects of AE and PBMT on functional capacity. Values are means ± SE. DMHF-Control = Type 2 Diabetes Mellitus and Heart Failure Control group; DMHF-AE = Type 2 Diabetes Mellitus and Heart Failure and Aerobic Exercise; DMHF-AE+PBMT = Type 2 Diabetes Mellitus and Heart Failure with Aerobic Exercise and Photobiomodulation Therapy. Graphics A, B and C, effect of the interventions on exercise time, distance and maximal speed analyzed with the generalized linear model (GZLM). Graphs D, delta Δ percentage of change of exercise time, distance, and speed after intervention with general linear model (GLM), with Bonferroni correction.

Table 2. Body, heart and ventricular mass ratio, tissue and infarct characteristics of Type 2 Diabetic and Heart Failure rats.

Measurement	DMHF-Control (n=4)	DMHF-AE (n=6)	DMHF-AE+PBMT (n=6)	*p* (ANOVA)
Initial Body Mass (g)	73.7 ± 12.5	76.6 ± 6.0	117.5 ± 3.0 *‡	0.004
Final Body Mass (g)	441.5 ± 8.1	379.2 ± 2.8	395.7 ± 4.9	0.22
Infarcted Area (%)	22.7 ± 11.9	33.7 ± 8.6	25.3 ± 14.5	0.42
H/BM (mg/g)	2.62 ± 0.3	2.91 ± 0.3	2.91 ± 0.4	0.38
LV/BM (mg/g)	2.05 ± 0.2	2.34 ± 0.3	2.24 ± 0.4	0.38
RV/BM (mg/g)	0.58 ± 0.1	0.57 ± 0.1	0.67 ± 0.09	0.19
Pulmonary Congestion (%)	82.4 ± 1.9	79.8 ± 3.02	68.4 ± 14.9	0.06
Hepatic Congestion (%)	69.5 ± 0.7	70.6 ± 0.7	68.3 ± 2.61	0.11

Values are means ± SD. DMHF-Control = Type 2 Diabetes Mellitus and Heart Failure Control group; DMHF-AE = Type 2 Diabetes Mellitus and Heart Failure and Aerobic Exercise; DMHF-AE+PBMT = Type 2 Diabetes Mellitus and Heart Failure with Aerobic Exercise and Photobiomodulation Therapy. H/BM: heart-to-body mass ratio; LV/BM: left ventricular-to-body mass ratio; RV/BM: right ventricular-to-body mass ratio. * $p<0.01$ compared to DMHF-Control; ‡ $p<0.05$ compared to DMHF-AE (one-way ANOVA followed by Tukey post hoc test).

Table 3. Echocardiographic and hemodynamic variables after experimental protocols

Measurement	DMHF-Control (n=4)	DMHF-AE (n=6)	DMHF-AE+PBMT (n=6)	*p* (ANOVA)
Echocardiography				
HR (beats min^{-1})	368.3 ± 25.3	346.8 ± 44.5	360.8 ± 19.8	0.58
IVSd (mm)	1.7 ± 0.26	1.5 ± 0.15	1.6 ± 0.29	0.46
IVSs (mm)	2.5 ± 0.39	2.2 ± 0.37	2.0 ± 0.47	0.31
LVEdD (mm)	9.3 ± 1.47	9.2 ± 0.40	9.3 ± 0.8	0.97
LVEsD (mm)	7.7 ± 1.65	7.7 ± 0.6	8.0 ± 0.98	0.85
LVPWd (mm)	1.5 ± 0.19	1.2 ± 0.12*	1.3 ± 0.13	0.03
LVPWs (mm)	1.5 ± 0.25	1.3 ± 0.09	1.4 ± 0.28	0.29
LVEdD/BW	20.8 ± 2.43	23.5 ± 2.55	22.8 ± 3.37	0.28
FS (%)	17.5 ± 4.84	16.9 ± 3.02	14.5 ± 3.84	0.41
EF (%)	43.5 ± 10.0	42.5 ± 6.4	37.2 ± 8.2	0.40
RWT	0.35 ± 0.07	0.29 ± 0.02	0.31 ± 0.06	0.26
E' (cm s^{-1})	0.41 ± 0.03	0.51 ± 0.08	0.41 ± 0.08	0.05
A' (cm s^{-1})	0.26 ± 0.04	0.26 ± 0.02	0.22 ± 0.06	0.31
E/A	1.56 ± 0.27	1.98 ± 0.41	1.95 ± 0.53	0.24
Hemodynamic evaluation				
LVEDP (mmHg)	13.7 ± 4.0	14.1 ± 1.7	10.1 ± 4.2	0.13
LVSP (mmHg)	94.9 ± 11.6	99.7 ± 11.1	99.5 ± 21.16	0.87
$+dP/dt_{max}$ (mmHg s^{-1})	3159 ± 296.9	3568 ± 403.8	2891 ± 632.0	0.08
$-dP/dt_{min}$ (mmHg s^{-1})	-2179 ± 284.5	-2732 ± 309.2	-2355 ± 937.9	0.37

Values are means ± SD. DMHF-Control = Type 2 Diabetes Mellitus and Heart Failure Control group; DMHF-AE = Type 2 Diabetes Mellitus and Heart Failure and Aerobic Exercise; DMHF-AE+PBMT = Type 2 Diabetes Mellitus and Heart Failure with Aerobic Exercise and Photobiomodulation Therapy. HR: heart rate; IVSd: interventricular septum in diastole; IVSs: interventricular septum in systole; LVEdD: left ventricular end-diastolic diameter; LVEsD: left ventricular end-systolic diameter; LVPWd: left ventricular posterior wall in diastole; LVPWs: left ventricular posterior wall in systole; LVEdD/BW: left ventricular end-diastolic diameter-to-body weight ratio; FS: left ventricular fractional shortening; EF: left ventricular ejection fraction; RWT: relative wall-thickness; A: late peak velocity; E: maximal early diastolic peak velocity; LVEDP:

Left ventricular end-diastolic pressure; LVSP: Left ventricular systolic pressure; $+dP/dt_{max}$: Left ventricular maximum change in pressure over time; $-dP/dt_{min}$: Left ventricular minimum change in pressure over time. * $p<0.05$ compared to DM+HF-Control (one-way ANOVA followed by Tukey post hoc test).

Figure 1. Flowchart of the study.

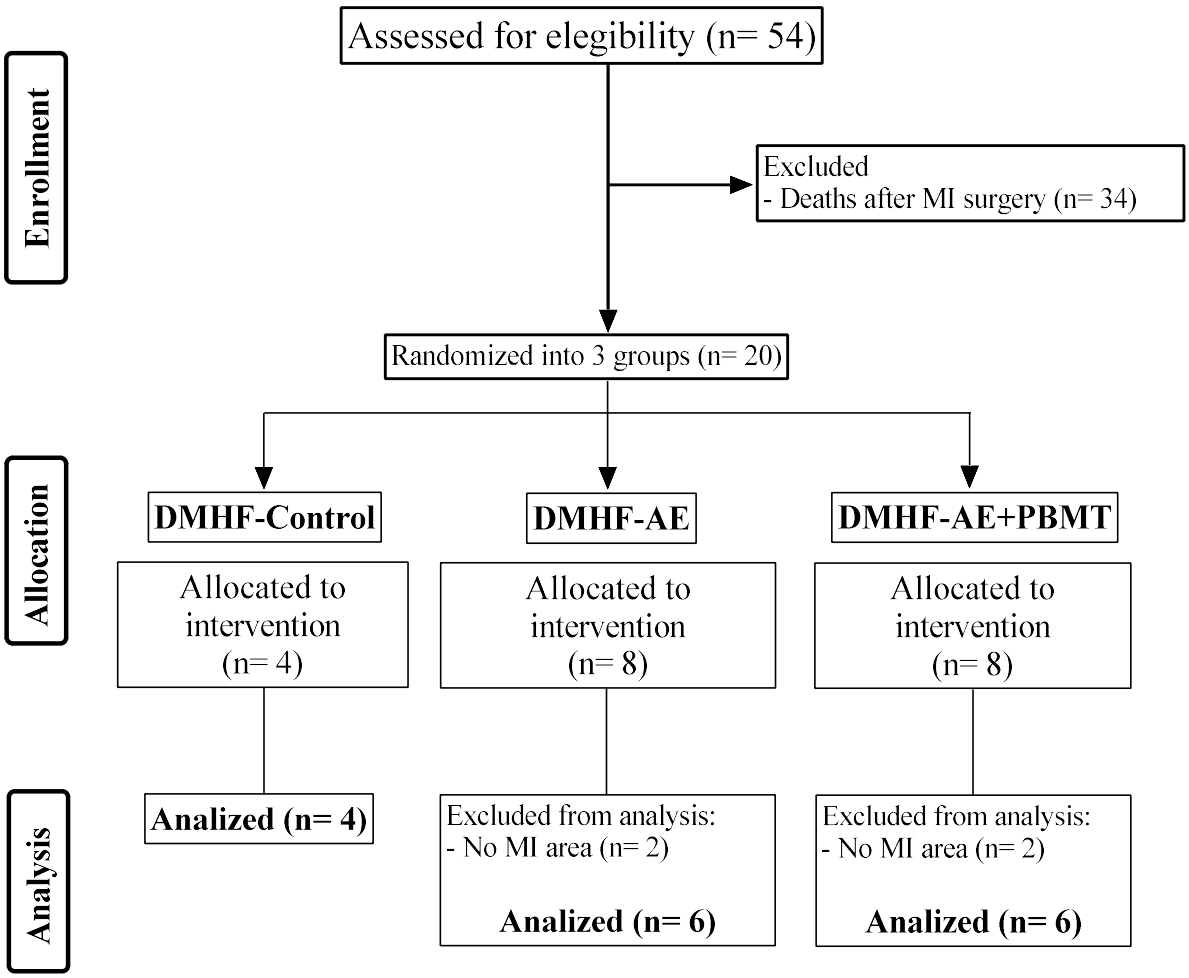


Figure 2. Experimental timeline.

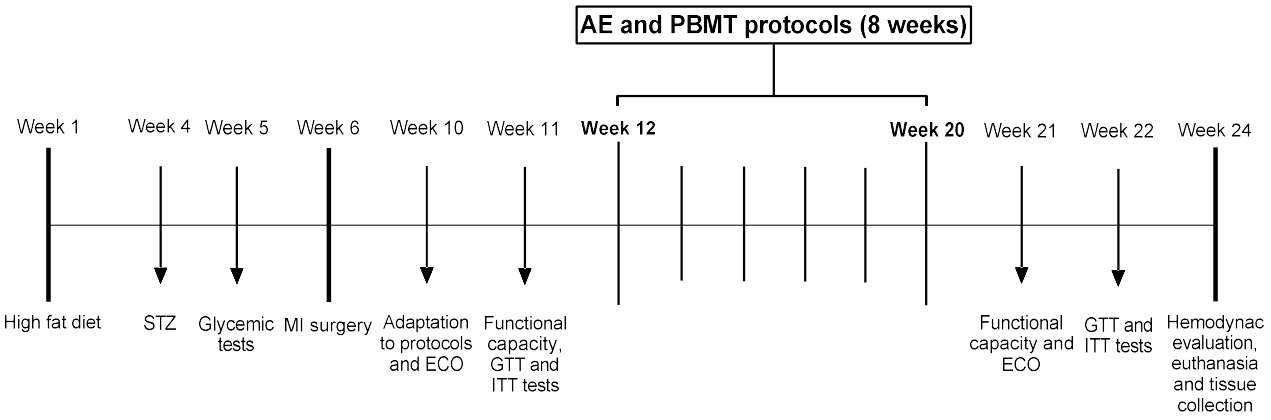


Figure 3. Metabolic variables.

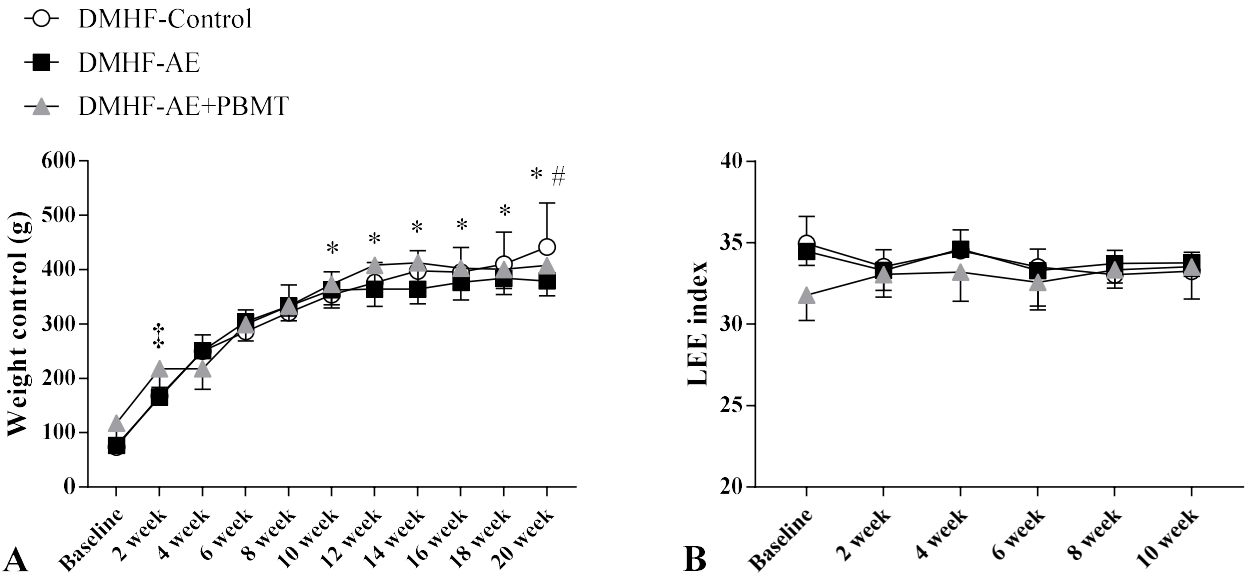


Figure 4. Glycemic curve.

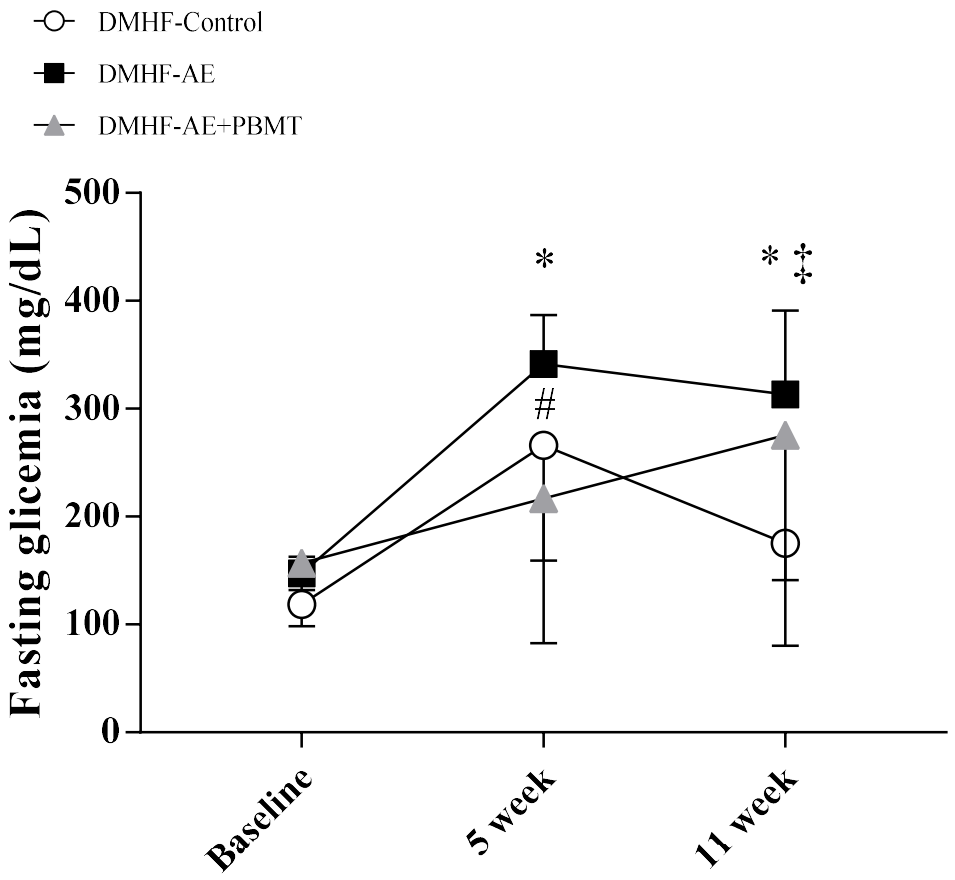


Figure 5. Glucose tolerance test (GTT) of type 2 DM with HF rats.

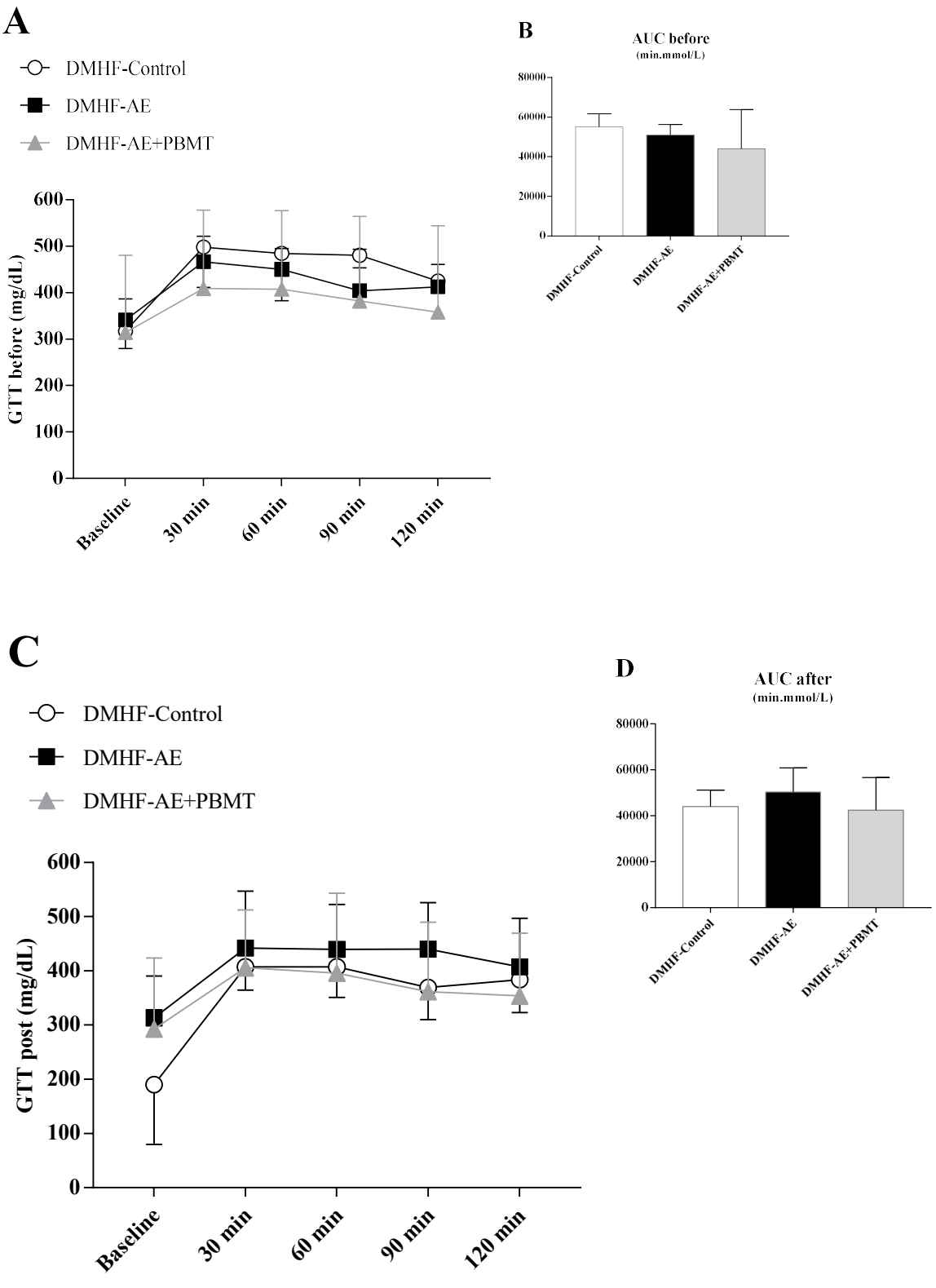
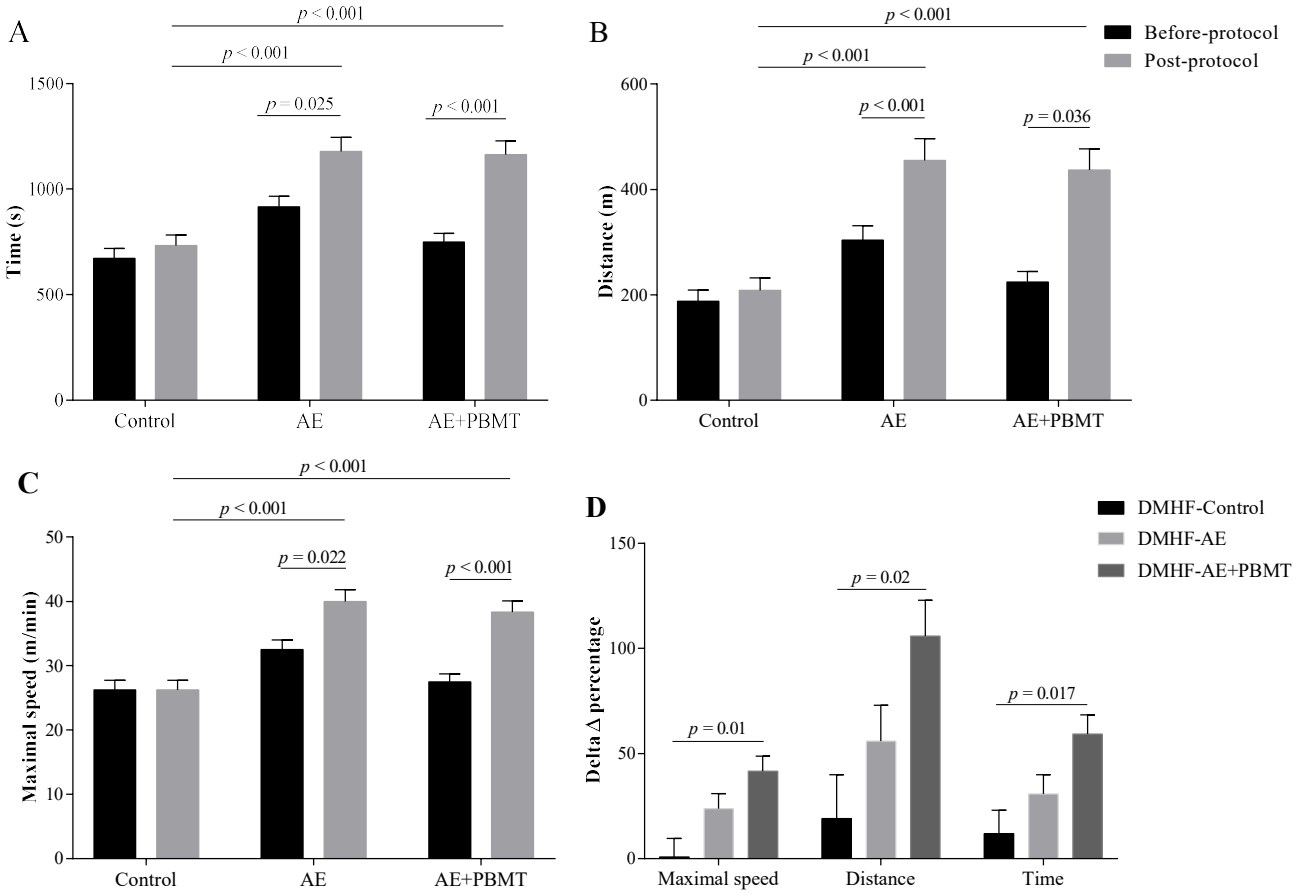


Figure 6. Effects of AE and PBMT on functional capacity



Lasers in Medical Science – supplementary material

Aerobic exercise and photobiomodulation therapy improve functional capacity of rats with type 2 diabetes and heart failure

Abstract word count: 250

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Tables: 4

Figures: 6

Supplementary files: 1

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2. MATERIAL AND METHODS

2.5.2 Echocardiography

Twenty-four hours after the functional capacity test, the animals were subjected to noninvasive echocardiography evaluation equipped with an 8–13-MHz electronic transducer. The animals were anesthetized with isoflurane (2%) throughout the procedure, the same protocol used for the MI surgery. The chest was previously shaved, and an ultrasound-transmitted gel was applied. M-mode tracings were derived from a 2D mode obtained from parasternal short-axis views of the left ventricle (LV) at three levels: basal (at the tip of the mitral valve leaflets), middle (at the papillary muscle level), and apical (distal to the papillary muscle but before the final curve cavity). The following structural variables were measured: interventricular septum in diastole (IVSd, mm), interventricular septum in systole (IVSs, mm), left ventricular end-diastolic diameter (LVEdD, mm), left ventricular end-systolic diameter (LVESD, mm), left ventricular posterior wall in diastole (LVPWd, mm), and left ventricular posterior wall in systole (LVPWs, mm). These measures were obtained from the means of at least three cardiac cycles at each of the three levels, and the final value of each rat was the mean of all three described planes. From the primary variables, we obtained the secondary variables of LV systolic function: end-diastolic volume (LVEdV (ml) = $1.047(\text{LVEdD})^3$ and end-systolic volume (LVEsV (ml) = $1.047(\text{LVEsD})^3$) using a cubic or ellipsoid model; LV ejection fraction (EF (%) = [(LVEdV−LVEsV)−LVEdV] × 100), LV fractional shortening (FS (%) = [(LVEsD−LVEsD)/LVEdD] × 100), relative wall thickness (RWT = (IVSd + LVPWd)/LVEdD) and left ventricular end-diastolic diameter-to-body mass (LVEDD/body mass, mm/kg). LV diastolic function was obtained through the measurement of mitral diastolic inflow by pulsed Doppler, obtained from the four-chamber view, and the sample volume was positioned at the tip of the mitral valve to obtain the mitral diastolic flow velocity, which was used to measure the peak E and A wave velocities (cm/s) and the ratio between them (E/A ratio). The heart rate was calculated using the average of three consecutive cycle intervals [1].

2.6.2 Photobiomodulation therapy (PBMT)

A GaALAs laser apparatus was used for this study with: wavelength 850 nm, continuous mode, cluster with three diodes, power of output 300 mW (100/diode), spot size 0.012 cm^2, and power density 8.33 W/cm^2. There were three simultaneous irradiation points, applied bilaterally, with three medial and three lateral points, totaling twelve application points in each animal [2]. Further information on the characteristics, parameters, and mode of application are summarized in Table 1. The protocol was applied for 8 weeks, five times a week, in which PBMT was applied immediately after the AE protocol. The animals of the DMHF-control group, which will be controlled for both PBMT and AE, followed the same protocol already described, with 29.2 s of application in each animal, but with the device turned off.

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2. Hentschke VS, Capalonga L, Rossato DD, et al (2017) Maximal oxygen uptake and exercise tolerance are improved in rats with heart failure subjected to low-level laser therapy associated with resistance training. Lasers Med Sci 32:73–85. https://doi.org/10.1007/s10103-016-2088-3

Table 1. Photobiomodulation therapy (PBMT).

Low-level laser therapy (LLLT)	GaAlAs (850nm)
Number of diodes	3
Pulse frequency	Continuous output
Output power (mW)	300 (100/diode)
Spot size (cm^2)	0,012
Power density (W/cm^2)	8.33
Protocol regime	Immediately after aerobic exercise protocol; bilaterally in hind limbs.
Irradiation sites per application	3 (simultaneously)
Irradiation regions per hind limb	2 (one application medially and other laterally)
Irradiation sites per hind limb	6 (3 sites medially and 3 sites laterally)
Irradiation sites per animal	12
Dose per treatment (J/cm^2)	61.25
Total energy per point (J)	0,735
Total energy per region (J)	2.205
Total energy per hind limb (J)	4.410
Total energy per animal (J)	8.820
Time per point (s)	7.3
Time per region (s)	7.3
Time per hind limb (s)	14.6
Time per animal (s)	29.2
Frequency treatment	1x / day
Number of treatments per week	5
Protocol duration (weeks)	8
Total number of treatments	40
Application mode	Spot held stationary in skin contact at 90º angle with slight pressure

Table 5. Echocardiographic and hemodynamic variables before experimental protocols

Measurement	DMHF-Control (n=4)	DMHF-AE (n=6)	DMHF-AE+PBMT (n=6)
Echocardiography			
HR (beats min^{-1})	372.0 ± 24.3	352.0 ± 36.1	402.2 ± 26.1
IVSd (mm)	1.5 ± 0.20	2.4 ± 0.40	1.7 ± 0.20
IVSs (mm)	2.4 ± 0.50	3.4 ± 0.30	2.3 ± 0.47
LVEdD (mm)	9.4 ± 0.60	9.1 ± 0.30	9.9 ± 0.16
LVEsD (mm)	7.7 ± 0.90	7.3 ± 0.60	8.5 ± 0.54
LVPWd (mm)	1.4 ± 0.10	1.3 ± 0.10	1.4 ± 0.18
LVPWs (mm)	1.4 ± 0.10	1.3 ± 0.10	1.5 ± 0.21
LVEdD/BW	23.8 ± 1.50	25.1 ± 1.60	24.4 ± 3.10
FS (%)	18.0 ± 4.23	20.3 ± 4.40	14.2 ± 4.76
EF (%)	44.6 ± 8.55	49.0 ± 8.3	36.4 ± 10.4
RWT	0.31 ± 0.03	0.41 ± 0.03	0.32 ± 0.03
E' (cm s^{-1})	0.49 ± 0.11	0.49 ± 0.05	0.48 ± 0.13
A' (cm s^{-1})	0.22 ± 0.02	0.30 ± 0.08	0.25 ± 0.09
E/A	2.21 ± 0.62	1.70 ± 0.37	2.26 ± 0.71

Values are means ± SD. DMHF-Control = Type 2 Diabetes Mellitus and Heart Failure Control group; DMHF-AE = Type 2 Diabetes Mellitus and Heart Failure and Aerobic Exercise; DMHF-AE+PBMT = Type 2 Diabetes Mellitus and Heart Failure with Aerobic Exercise and Photobiomodulation Therapy. HR: heart rate; IVSd: interventricular septum in diastole; IVSs: interventricular septum in systole; LVEdD: left ventricular end-diastolic diameter; LVEsD: left ventricular end-systolic diameter; LVPWd: left ventricular posterior wall in diastole; LVPWs: left ventricular posterior wall in systole; LVEdD/BW: left ventricular end-diastolic diameter-to-body weight ratio; FS: left ventricular fractional shortening; EF: left ventricular ejection fraction; RWT: relative wall-thickness; A: late peak velocity; E: maximal early diastolic peak velocity.

Table 4. Functional capacity

Measurement	DMHF-Control (n=4)			DMHF-AE (n=6)			DMHF-AE + PBMT (n=6)			*P* (GLZM)
	Before-protocol	Post-protocol	Δ (%)	Before-protocol	Post-protocol	Δ (%)	Before-protocol	Post-protocol	Δ (%)	
Time (s)	672.75 ± 46.2	733 ± 50.34	12.03 ± 11.09	749.16 ± 42.01	1163.83 ± 65.27 *‡	30.88 ± 9.06	915.66 ± 51.35	1179 ± 66.12 *#	59.35 ± 9.06 †	0.016
Distance (m)	188.25 ± 20.99	208.87 ± 23.29	19.07 ± 20.85	224.23 ± 20.42	437.06 ± 39.8 *#	55.94 ± 17.02	303.9 ± 27.67	454.95 ± 41.43 *‡	105.91 ± 17.02 †	0.021
Maximal speed (m/min)	26.25 ± 1.47	26.25 ± 1.47	0.83 ± 8.77	27.5 ± 1.25	38.33 ± 1.75 *‡	23.81 ± 7.16	32.5 ± 1.48	40 ± 1.82 *#	41.70 ± 7.16 †	0.005

Values in estimates ± SE. DMHF-Control = Type 2 Diabetes Mellitus and Heart Failure Control group; DMHF-AE = Type 2 Diabetes Mellitus and Heart Failure and Aerobic Exercise; DMHF-AE+PBMT = Type 2 Diabetes Mellitus and Heart Failure with Aerobic Exercise and Photobiomodulation Therapy. * $p<0.001$ compared to DMHF-Control; † $p<0.05$ compared to DMHF-Control; # $p<0.001$ compared to before-protocol; ‡ $p<0.05$ compared to before-protocol (generalized linear model – GZLM, with Bonferroni correction).

8 DISCUSSÃO GERAL

As diversas formas de exercício aeróbio contínuo ou intervalado de baixa, média e moderada intensidade, assim como o exercício restistido, fornecem grandes benefícios à população que sofre com algum tipo de doença crônia, bem como às pessoas saudáveis como modalidade preventiva. A eletroestimulação neuromuscular há algum tempo vem sendo utilizada como alternativa coadjunvante no tratamento de pacientes acometidos por Insuficiência Cardíaca e Doença Pulmonar Obstrutiva Crônic (DPOC) em níveis mais avançados, algumas vezes associada ao exercício e à terapia farmacológica otimizada. Com relação à terapia por fotobiomodulação, embora tenhamos encontrado resultados instigantes relacionados à melhora da capacidade funcional, perfil inflamatório e estresse oxidativo tanto de pessoas saudáveis quanto de pacientes crônicos, ainda não temos nenhum estudo com potencial para afirmar e confirmar seus benefícios.

O nosso grupo de pesquisas vem trabalhando há pelo menos 10 anos com modalidades terapêuticas não farmacológicas tanto em nível experimental como clínico, relacionadas à recuperação das variáveis inerentes à IC. Portanto, a proposta desta tese foi ampliar alguns conceitos, incluindo o diabetes mellitus (DM) tipo 2.

O desafio foi enorme, pois o modelo animal de DM tipo 2 exige um controle rigoroso de muitas variáveis. Além disso, esse modelo ainda não tinha um protocolo estabelecido em nosso laboratório. Estudamos e analisamos algumas possibilidades e definimos aquela que mais se assemelha à população em geral, definido através de dieta hiperlipídica, associado à injeção intraperitoneal de estreptozotocina.

Ao iniciarmos o trabalho, nos deparamos com alguns problemas, tais como, a falta de animais no biotério da instituição e falta de alguns equipamentos necessários. Isso acabou atrasando o andamento das pesquisas e, consequentemente, não conseguimos atingir alguns objetivos propostos no projeto.

Durante as coletas, a partir de estudos prévisos, sabíamos que a mortalidade dos animais diabéticos submetidos à cirurgia de infarto era maior do que de animais saudáveis. Porém, percebemos uma característica que nos deixou intrigado: os animais passavam e suportavam bem à cirurgia, mas dentro das primeiras 4 a 6 horas do período de recuperação, mesmo já se movimentando e se alimentando em suas caixas, acabavam sofrendo parada cardiorrespiratória e indo à óbito. Acreditamos que este foi o principal problema encontrado, pois reduziu muito a nossa amostra, impossibilitando um maior

número de grupos experimentais, que poderíam ter auxiliado no melhor entendimento dos resultados.

Finalizadas as coletas e analisando os resultados, foi possível reforçar a importância da eletroestimulação na melhora dos parâmetros cardiovasculares de animais com IC. Infelizmente não encontramos efeitos da terapia por fotobiomodulação sobre o controle autonômico central, o que nos leva a pensar na hipótese que, pelo fato da fotobiomodulação atuar diretamente nos tecidos periféricos, sem possibilitar qualquer estímulo mecânico, não seja capaz de influenciar no controle central. Porém, como existem muitas variáveis que ainda não foram totalmente elucidadas em relação à terapia por fotobiomodulação, as pesquisas devem continuar sendo realizadas.

Seguindo essa linha, decidimos associar o exercício aeróbio com a terapia por fotobiomodulação em animais diabéticos tipo 2 e com IC. Através desse estudo foi possível verificar melhora na capacidade funcional dos animais. Embora os grupos submetidos às intervenções apresentaram melhora na capacidade funcional, percebemos que a associação da PBMT foi capaz de potencializar os efeitos do exercício aeróbio, demonstrado pelo maior delta de tempo do teste de exaustão quando comparado ao grupo controle no final do protocolo e pelo aumento da velocidade do teste no período pós-protocolo em relação aos dois tempos anteriores (pré-infarto e pré-protocolo). Isso nos mostra que a fotobiomodulação pode influenciar positivamente na capacidade funcional.

Outro importante resultado do nosso trabalho foi perceber que, tanto o exercício aeróbio quanto à terapia por fotobiomodulação não foram capazes de influenciar no controle glicêmico dos animais. Atualmente, a grande maioria dos estudos mostra que o exercício físico é um importante aliado no controle e na reabilitação do DM tipo 2 e da IC. No entanto, acreditamos que o nosso modelo tenha desenvolvido um comprometimento metabólico muito grande, ao ponto de nem mesmo o exercício aeróbio ser capaz de melhorar esse aspecto. Talvez aí esteja a grande questão levantada pelo nosso trabalho, o que nos instiga a seguir estudando, a fim de desvendar os reais mecanismos envolvidos no exercício aeróbio, na eletroestimulação e na terapia por fotobiomodulação, capazes de influenciar sobre capacidade funcional dos animais diabéticos e com IC.

9 CONCLUSÃO GERAL

Com todos os estudos clínicios e experimentais disponíveis na literatura, acreditamos que está na hora de ampliarmos nossos horizontes em busca da confirmação ou não desses efeitos na população, em especial, a terapia por fotobiomodulação. Assim, poderemos ampliar o leque de possibilidade e recursos terapêuticos capazes de beneficiar os pacientes que, por vezes, se encontram em situações de extrema incapacidade física.

9.1 CONCLUSÕES ESPECÍFICAS

Através dos resultados do primeiro estudo, demonstramos que a NMES tem efeitos benéficos sobre a melhora do balanço autonômico e da atividade barorreflexa arterial de ratos com IC e que a PBMT não foi capaz de influenciar sobre essas variáveis. O segundo estudo possibilitou verificar que o exercício aeróbio de moderada intensidade e a PBMT são capazes de melhorar a capacidade funcional de ratos com DM tipo 2 e IC, porém não foram capazes de atuar sobre o controle glicêmico desses animais.

10 PERSPECTIVAS

Seguiremos as pesquisas com esse modelo de DM tipo 2 e IC, pois ainda temos muitas questões a serem respondidas. O desafio agora é analisar os efeitos do exercício aeróbio e da terapia por fotobiomodulação sobre o controle autonômico, através da análise barorreflexa, e tentar entender melhor os mecanismos de ação dessas terapias sobre a musculatura esquelética, através das análises bioquímicas, no que se refere ao perfil inflamatório, o estresse oxidativo e o dano ao DNA, bem como através das análises histológicas.

ANEXOS

ANEXO A – Parecer de aprovação do Comitê de Ética da UFCSPA

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO

UFCSPA
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 189/16

2) DATA DO PARECER: 09/11/2016

3) TÍTULO DO PROJETO:

Exercício aeróbico associado a fototerapia: efeitos sobre a capacidade funcional, a função hemodinâmica e o perfil inflamatório de ratos diabéticos com insuficiência cardíaca após infarto do miocárdio.

4) PESQUISADOR RESPONSÁVEL:

Pedro Dal Lago

5) RESUMO DO PROJETO:

O projeto propõem-se a analisar os efeitos de um protocolo de exercício físico aeróbico associado à fototerapia sobre a capacidade funcional, a função hemodinâmica, os biomarcadores inflamatórios, o estresse oxidativo, o dano ao DNA bem como sobre as alterações morfológicas nos músculos cardíaco e esquelético de ratos diabéticos e com insuficiência cardíaca. Serão utilizados 51 ratos Wistar machos submetidos à dieta hiperlipídica e injeção de estreptozotocina (30mg/Kg) em dose única para promover o diabetes tipo II. A indução do infarto será através da ligadura da coronária descendente esquerda e a potência aeróbica será avaliada por um teste de esforço em esteira ergométrica para ratos. A fototeraspia será aplicada no músculo gastrocnêmio das duas patas. O treinamento aeróbico em esteira e a fototerapia serão realizados durante oito semanas. Serão realizadas avaliações ecocardiográficas e hemodinâmicas com posterior retirada dos músculos cardíaco e gastrocnêmio para análises bioquímicas e morfológicas.

6) OBJETIVOS DO PROJETO:

- Analisar os efeitos do exercício físico aeróbico e da fototerapia sobre os níveis de creatina quinase no miocárdio, no plasma e no músculo esquelético de ratos diabéticos em com insuficiência cardíaca.

- Analisar os efeitos do exercício físico aeróbico e da fototerapia sobre os níveis de citocromo c oxidase no músculo esquelético de ratos diabéticos e com insuficiência cardíaca.
- Analisar os efeitos do exercício físico aeróbico e da fototerapia sobre os níveis de TNFa, IL-1b, IL-6 e IL-10 no miocárdio, no plasma e no músculo esquelético de ratos diabéticos e com insuficiência cardíaca.
- Analisar se o exercício físico aeróbico e a fototerapia
- Analisar se o exercício físico aeróbico e a fototerapia modificam os biomarcadores de estresse oxidativo (TBARS e atividade da SOD e CAT) no miocárdio, no plasma e no músculo esquelético de ratos diabéticos e com insuficiência cardíaca.
- Analisar se o exercício físico aeróbico e a fototerapia causam dano ao DNA das células do miocárdio e do músculo esquelético de ratos diabéticos e com insuficiência cardíaca.
- Analisar se o exercício físico aeróbico e a fototerapia modificam a composição do colágeno das células do miocárdio e do músculo esquelético de ratos diabéticos e com insuficiência cardíaca.
- Analisar se o exercício físico aeróbico e a fototerapia modificam as variáveis morfológicas (densidade e número total de fibras musculares, densidade e número total de vasos sanguíneos, área total do músculo e hipertrofia) do músculo esquelético de ratos diabéticos e com insuficiência cardíaca.

7) FINALIDADE DO PROJETO: [ ] Ensino [X] Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título	[X] Adequado	[ ] Comentários
Introdução	[X] Adequada	[ ] Comentários
Objetivos	[X] Adequados	[ ] Comentários
Relevância e Justificativa	[X] Adequados	[ ] Comentários
Materiais e Métodos	[X] Adequados	[ ] Comentários
Cronograma para execução da pesquisa	[X] Adequado	[ ] Comentários
Orçamento e fonte financiadora	[X] Adequados	[ ] Comentários
Referências Bibliográficas	[X] Adequadas	[ ] Comentários

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE:
[X] Sim [ ] Não

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

Grau de dor/estresse: **B** | **C** ☐ **D** ☐ **E** ☐

Justifique:

GI 3 – Não foi informada a classificação no protocolo.

Espécie: Ratos Wistar **Número Amostral:** 51

Redução Amostral: ☐ Sim ☒ Não

Justifique:

Substituição de Metodologia: ☐ Sim ☒ Não

Se achar necessário, justifique e sugira uma nova metodologia:

Aprimoramento da Metodologia: ☐ Sim ☒ Não

Se achar necessário, justifique e sugira aprimoramentos da metodologia:

Acomodação e manutenção dos animais: ☒ Adequada ☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Especificar quando 2 ou 4 por caixa

Manipulação dos animais: ☒ Adequada ☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Analgesia dos animais (se aplicável): ☒ Adequada ☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com analgésico substituto:

Indicar a origem do medicamento

Anestesia dos animais (se aplicável): ☒ Adequada ☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com anestésico substituto:

Eutanásia dos animais (se aplicável): [x] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Local de Realização (Biotério/Labotarório):
Biotério
Outra instituição. Qual?
UFCSPA

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade

12) RECOMENDAÇÃO:

[X] Aprovado

[ ] Com Pendência

[ ] Não aprovado

Data de início 03/10/2016 Data de Término 31/12/2019

Comentários gerais sobre o projeto:

Todos os comentários foram adequadamente respondidos.

ANEXO B – Normas *Canadian Journal of Physiology and Pharmacology*

Prepare your manuscript

Format and style

Manuscript text must:

- be in English or French
- be double-spaced
- be single-column
- include page numbers
- include continuous line numbers (before acceptance only)
- be 8.5 x 11 inches in page size (or ISO A4)
- follow this order: title page, abstract, keywords, body text (Introduction, Materials and methods, Results, Discussion), acknowledgements, references, tables, figure captions, figures, appendices

Abbreviations and acronyms

Define abbreviations and acronyms when they are first mentioned in the text.

Footnotes

In body text, try to avoid footnotes. If unavoidable, cite footnotes using superscript Arabic numbers (^{1,2,3}), in order of appearance (starting with the title page), and include the footnote at the bottom of the page on which it is cited. Do not include footnotes in the reference list.

In tables, cite footnotes using symbols (in the order *, †, ‡, §, ||, ¶, #) or superscript lowercase italic letters (*a,b,c*).

Mathematical expressions

- Identify equations by calling out with numbers in parentheses placed flush with the left margin (for the *Canadian Journal of Physics*, place on the right).
- A letter or symbol should represent only one entity and be used consistently throughout the paper.
- Each variable (including those representing vectors, matrices, and tensors) must be clearly identified and defined in the text.
- Supply complex equations in an editable format by using LaTeX or a math editor (MathType).
- Supply simple, inline equations in Word, without using MathType. Insert symbols from Word's "Symbol" palette, using "normal text" or "Symbol" fonts only. Insert symbols using MathType ONLY if they cannot be found in the "Symbol" palette under one of those two fonts.

Reporting guidelines

Study reporting guidelines can help authors report their work transparently and accurately. We encourage their use. Up-to-date guidelines can be found at the EQUATOR Network, where authors can consult the flow chart or wizard to identify which guideline(s) to use. A completed copy of the guideline checklist may be submitted with the manuscript as a Supplementary file.

Spelling

Spelling should follow that of *Webster's Third New International Dictionary* or the *Oxford English Dictionary*. Authors are responsible for consistency in spelling.

Statistical analyses

The assumptions and (or) the model underlying any statistical analysis should be clearly stated. Do not use symbols such as * and ** to denote levels of significance unless accompanied by actual *p* values.

Units of measure

Use SI units of measure (Système international d'unités). If non-SI units are used, at first mention, supply the equivalent in SI units in parentheses.

Parts of the manuscript

Title page

Title

Should be accurate, informative, and brief. Include keywords in the title to optimize search engine discovery.

Author list

List all author names on the title page: check author order, spelling, capitalization, initials, and hyphens.

- Format names as: first name (or initial) middle name (or initial) last name (surname/family name).
- List affiliation(s) for each author that include: institution (department and university or organization), city, state or province, country. An author's affiliations should reflect where the research was conducted. If an author changes institution, the new affiliation can be listed in a footnote.
- Do not include academic degrees and professional titles.

Authorship criteria: We subscribe to the ICMJE definition of authorship. Any person listed as an author must meet each of these authorship criteria, and anyone who meets these authorship criteria must be listed as an author. Contributors who do not meet authorship criteria should be listed in the Acknowledgements section. See our **Publishing Policy** for details.

Author group names: If a group name appears on a paper in place of an author:

- if there is space, all group members will be included individually as authors, or
- if there are too many authors to fit on the first page of a paper, an Editor will decide who is included (e.g., only the Chair of a committee); or that
- the group name and the full list of names in the group will appear in the Acknowledgements section.

Corresponding author

Clearly identify the corresponding author and their email address on the title page. Note that the corresponding author must supply their ORCID iD during submission; however, it is not required on the title page.

Abstract

Provide the abstract in the manuscript text file on page 2, after the title page. Abstracts will be translated by the journal translator.

The abstract should be a single paragraph that summarizes the article. It helps readers decide whether to keep reading. Briefly describe the study rationale, objectives, methods, findings, and impact. Use keywords (and their plain-language synonyms) in the abstract to optimize search engine discovery. Do not include headings, reference citations, tables, figures, or acronyms.

Keywords

List keywords after the abstract. Good keywords are common to your field and accurately describe your topic. Use keywords in the title, abstract, and manuscript text to optimize search engine discovery. (Consider what words you would enter in a search box to find your work online.)

Graphical abstract

(Optional) Authors are encouraged to submit an illustration, diagram, equation, or other informative visual that explains the central message of the article and entices readers. The maximum allowable size is 40 mm (150 pixels) high by 85 mm (320 pixels) wide. Graphical abstracts appear online only.

Main body of the article

Introduction

In 1–3 paragraphs, explain the study rationale and objective(s).

- What is the problem, and why is it important?
- What is known on the topic? Establish context: provide background, briefly review the key literature, and mention existing gaps or controversies in the field.
- What is the overall aim of the study? State your research question, hypotheses, and predictions.

Materials and methods

Describe what you did (and how you did it) clearly and comprehensively enough for the study to be replicated.

- Include as appropriate the study design; primary and secondary outcome measures; computational, experimental, and statistical methods; materials; locations.
- Avoid long descriptions of known procedures; provide relevant references instead.
- Specify materials used (e.g., laboratory or field equipment, chemicals, biologic materials) and their sources (provide company name, city, and country in parentheses).

Ethics approval

For studies involving human participants:

- name the institutional ethics review committee that approved the study; and
- confirm that participants gave informed consent before participating in the study.

For studies involving animals:

- name the animal care review committee that approved the study;
- name the animal care guidelines that were followed (must be in line with the Canadian Council on Animal Care guidelines or the Guide for the Care and Use of Laboratory Animals);
- confirm that research involving endangered species was conducted in accordance with all applicable laws; and
- if permits were required for fieldwork, supply the research permit and (or) license numbers.

For studies involving collection of specimens:

- confirm that research involving collection of specimens was conducted in accordance with all applicable laws, guidelines, regulations; and
- if permits were required for fieldwork, supply the research permit and (or) license numbers.

Results

Report results that are directly relevant to your research question. Raw data and other observations may be submitted with the manuscript as Supplementary files or uploaded to a recognized data repository.

- Use subheadings, tables, and figures to organize and communicate your findings.
- Begin sections and (or) sentences with high-level observations, followed by statistical data.
- State the statistical tests used (if applicable), and when reporting numbers:

- define the values provided (e.g., mean and standard deviation or standard error, median, and interquartile range), and
- include the absolute value of *N* when describing frequencies (i.e., percentages, proportions, ratios).

Discussion or Conclusion

Compare your findings with previously published work; include points of agreement and difference. Describe the limitations and main contributions of your work. Propose avenues of future study. Speculation should be clearly identified and based on observations related to the manuscript.

Author statements

Acknowledgements

The names and contributions of institutions and people who contributed to the work but do not meet authorship criteria may be listed in the Acknowledgements section. This section should be written in the third person. Authors are responsible for ensuring that people named in the Acknowledgements agree to be named.

Competing interests statement

Authors are responsible for disclosing all financial and non-financial relationships that might bias or be seen to bias their work. Supply a statement of competing interests during submission and on the article title page. Authors who are unsure what to list may wish to consult the ICMJE form for disclosure of competing interests.

- If there is nothing to declare, the statement should read: “Competing interests: The authors declare there are no competing interests.”
- If there are competing interests to declare, specify authors by full name. Statements should take the form of “Competing interests: AUTHOR is an employee and shareholder of COMPANY. AUTHOR has received speaker fees and travel honoraria from COMPANY.”

Contributors’ statement

Supply an author contribution statement, identifying authors by their initials, and specifying contributions according to the CRediT roles selected for each author in the peer-review system during submission.

Funding statement

Supply a funding statement that lists what support the authors received to carry out the research.

- If the study was unfunded, state “Funding: The authors declare no specific funding for this work.”
- If the study received funding, include each funding agency name written out in full, followed by its grant or award number in parentheses. E.g., “Funding: This research was supported by FUNDING AGENCY NAME (grant No. ###).”

Data availability statement

Supply a data availability statement that says whether the data underpinning the work are available. If yes, specify how data can be accessed and under what conditions data can be reused. Supply repository name, persistent unique identifier (PID: DOI/compact identifier/accession number), and hyperlink.

References

See journal-specific guidelines for information on preferred citation style. Note the following:

- Unpublished reports, private communications, and submitted manuscripts do not belong in the reference list. Include them as footnotes or parenthetically in the body text, e.g., (J. Jones, personal communication, 2010).
- For books and articles that are in press (accepted but not yet published), include them in the reference list.

Tables and Figures

In general:

- Cite each figure and table, and name them in order of first appearance in the text (Figure 1, Figure 2, Table 1, Figure 3, Table 2, etc.)
- Figures and tables should add information to the article, not duplicate results that are (or could be) explained briefly in the text.
- Figures and tables should be understandable without having to read the article. Any abbreviations or symbols used should be defined in the caption (figures) or in the Note (tables).

Table specifications:

- Supply tables in an editable format, i.e., Word (table function in DOC or DOCX) or Excel (spreadsheet in XLS or XLSX). Each data point should be in a separate cell.
- Provide a table caption (e.g., Table 1. Xxx).
- Footnotes should be designated by symbols (order: *, †, ‡, §, ||, ¶, #) or superscript lowercase italic letters (*a,b,c*).
- Use a Note under the table for additional information.

Figure specifications:

See How to prepare figures for figure specifications and guidance.

A caption explains how to read a figure, independent of the article text. Follow the guidelines below:

- Terms, abbreviations, and symbols used must be defined and correspond with those in the text.
- List tools and settings used for image capture.

- Maps: captions must attribute the source (e.g., Google Map attribution guidelines) or, if original, list the software used to create the map.
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Appendices

An appendix should be able to stand alone, as a separate, self-contained document. Figures, equations, and tables used in an appendix should be numbered sequentially but separately from those used in the main body of the paper, e.g., Fig. A1, eq. (A1), Table A1.

Supplementary files

Supplementary files consist of extra tables, figures (maps), detailed calculations, and data sets produced by the authors as part of their research, but not essential for understanding or evaluating the manuscript.

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Authors may incorporate audio and video clips into their paper; these are published online, adding a dimension to the paper that cannot be achieved in print. For submission guidelines and accepted formats, see technical specifications under How to prepare a video abstract.

Data availability

We encourage authors to make their primary research data publicly available for all articles reporting original research.

- See journal-specific instructions for recommended repositories by discipline. You may also identify an appropriate repository with org or re3data.org, or use a generalist repository like Dryad or figshare.
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- Cite the data in your manuscript, e.g.:
    - In-text citation: (data are available in the Dryad Digital Repository, DOI: ###)

- Reference list entry: Authors. Year. Title [dataset]. Data repository. Version. Persistent unique identifier.

ANEXO C – Normas *Laser in Medical Science*

Instructions for Authors

Types of papers

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

Manuscript Submission

Manuscript Submission

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

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

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

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

Title page

Title Page

Please use this **template title page** for providing the following information.

The title page should include:

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- The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country
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For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

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

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

For life science journals only (when applicable)

Trial registration number and date of registration

Trial registration number, date of registration followed by “retrospectively registered”

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

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All manuscripts must contain the following sections under the heading 'Declarations'.

If any of the sections are not relevant to your manuscript, please include the heading and write 'Not applicable' for that section.

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Funding (information that explains whether and by whom the research was supported)

Conflicts of interest/Competing interests (include appropriate disclosures)

Availability of data and material (data transparency)

Code availability (software application or custom code)

Authors' contributions (optional: please review the submission guidelines from the journal whether statements are mandatory)

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Ethics approval (include appropriate approvals or waivers)

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Consent for publication (include appropriate statements)

Availability of data and material (data transparency)

Code availability (software application or custom code)

Authors' contributions (optional: please review the submission guidelines from the journal whether statements are mandatory)

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

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Text Formatting

Manuscripts should be submitted in Word.

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

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Please use no more than three levels of displayed headings.

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

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

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Always use footnotes instead of endnotes.

Acknowledgments

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

Scientific style

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

Units and abbreviations

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

Drugs

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

References

Citation

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

1. Negotiation research spans many disciplines [3].

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

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

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

The entries in the list should be numbered consecutively.

- Journal article

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

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

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086

- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

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

- Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

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

ISSN.org LTWA

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For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

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

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- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

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- Avoid effects such as shading, outline letters, etc.
- Do not include titles or captions within your illustrations.

Figure Numbering

- All figures are to be numbered using Arabic numerals.
- Figures should always be cited in text in consecutive numerical order.
- Figure parts should be denoted by lowercase letters (a, b, c, etc.).
- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures,"A1, A2, A3, etc." Figures in online appendices [Supplementary Information (SI)] should, however, be numbered separately.

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- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
- Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

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