UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM REABILITAÇÃO FUNCIONAL

Larissa da Silva Tonetto

EFEITOS DO LASER DE BAIXA INTENSIDADE SOBRE O ESTRESSE OXIDATIVO EM RATOS COM DIABETES MELLITUS TIPO 2

Santa Maria, RS
2021

Larissa da Silva Tonetto

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

Orientador: Prof. Dr. Rodrigo Boemo Jaenisch
Coorientadora: Prof. Drª Liliane de Freitas Bauermann

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Tonetto, Larissa da Silva
EFEITOS DO LASER DE BAIXA INTENSIDADE SOBRE O ESTRESSE OXIDATIVO EM RATOS COM DIABETES MELLITUS TIPO 2 / Larissa da Silva Tonetto.- 2021.
78 p.; 30 cm

Orientador: Rodrigo Boemo Jaenisch
Coorientador: Liliane de Freitas Bauermann
Dissertação (mestrado) - Universidade Federal de Santa Maria, Centro de Ciências da Saúde, Programa de Pós Graduação em Reabilitação Funcional, RS, 2021

1. Laserterapia 2. Atividade Oxidativa 3. Atividade Antioxidante I. Jaenisch, Rodrigo Boemo II. Bauermann, Liliane de Freitas III. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

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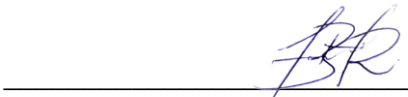
Aprovado em 8 de setembro de 2021



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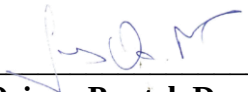

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Santa Maria, RS
2021

AGRADECIMENTOS

Gostaria de agradecer a todos que estiveram comigo durante esta jornada:

- A Deus, que me permitiu viver um sonho;
- Ao Programa de Pós-Graduação em Reabilitação Funcional e a Universidade Federal de Santa Maria que me permitiram ingressar e concluir o mestrado;
- Ao meu orientador Rodrigo Boemo Jaenisch e minha co-orientadora Liliane de Freitas Bauermann por acreditarem em mim e no meu trabalho;
- Aos meus amigos, Carlos Cassiano Figueiró da Silva e Nubia Gonzatti, pelo privilégio de poder contar com vocês em todas as horas;
- Aos laboratórios da professora Liliane de Freitas Bauermann (LAFEX) e dos professores Félix Alexandre Antunes Soares e Gustavo Orione Puntel (Laboratório de Neurotoxicidade e Neuroproteção Experimental), que nos ensinaram tanto durante o tempo em que estivemos ali;
- A todos os alunos que nos auxiliaram, mas, em especial, a Camille Gaube Guex e a Diane Hartmann, que nunca mediram esforços para que conseguíssemos concluir a pesquisa;
- A minha família e aos meus amigos que são a minha base. Em especial agradeço ao Mário Augusto Chagas Rother por todo o companheirismo e por toda a ajuda dada que me permitiu estudar e hoje concluir o sonho de ser mestre. Estendo este agradecimento a toda a sua família.
- A minha irmã Thaíse da Silva Tonetto e ao meu cunhado Renan Piveta que são exemplos de determinação, foco e garra, e que muito trabalharam e me ajudaram de todas as formas nos meus estudos. Eu amo vocês;
- Ao meu amor, Bruno Viegas Flôres, que me reencontrou nessa vida, que é quem me faz feliz todos os dias e que não poupa esforços para isso, que é com quem eu quero construir um lar e uma família, que é quem me inspira de tantas formas. Nosso amor é de outras vidas. Estendo aos meus sogros Paulo Roberto da Silva Flôres e Aurea Viegas Flôres, que me acolhem com tanto amor desde o primeiro dia em que estive com eles;
- A minha mãe, minha rainha, minha inspiração, eu te amo, obrigada por tanto, por renunciar a tantas coisas por mim, pela nossa relação de cumplicidade, por ser minha melhor amiga. Se eu for um pouquinho do que tu és como mãe, amiga, filha e irmã, eu já serei feliz. Eu te amo mais que tudo nesse mundo;
- Ao meu pai, meu anjo da guarda, que não está mais aqui nesse plano, mas que de onde estiver continua guiando meus passos e me abençoando. Pai, tu muito fizeste por mim e eu gostaria tanto que tu estivesse aqui para me ver mestre. Sinto tua falta todos os dias, meu amor por ti é para todo o sempre.

RESUMO

EFEITOS DO LASER DE BAIXA INTENSIDADE SOBRE O ESTRESSE OXIDATIVO EM RATOS COM DIABETES MELLITUS TIPO 2

AUTORA: Larissa da Silva Tonetto
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O DM tipo 2 é o mais prevalente, e caracteriza-se por defeitos na secreção e resistência periférica à insulina, o que determina o estresse oxidativo (EO). O laser de baixa intensidade (LBI) é uma ferramenta não farmacológica, e que vem sendo utilizada para o tratamento do DM, através da redução do EO e da e do aumento da atividade antioxidante. Assim, o objetivo do presente estudo foi avaliar os efeitos do LBI sobre o EO em órgãos, musculatura esquelética e soro de ratos com DM. Foram utilizados 31 ratos Wistar machos, divididos em dois grupos, com indução do DM 2 e sem indução do DM 2, considerando animais diabéticos com glicemia igual ou maior que 200 mg/dL, logo após os ratos foram alocados em 4 sub grupos: Grupo 1 - animais sem DM 2 SHAM (C-SHAM), Grupo 2 - animais com DM 2 SHAM (C-DM), Grupo 3- animais sem DM 2 com laser 21 J/cm^2 (L-SHAM), Grupo 4- animais com DM 2 com laser 21 J/cm^2 (L-DM). Após a indução ao DM 2 ou não, os animais receberam a LBI 5 dias/semana, durante 6 semanas. A dose foi irradiada em dois pontos no músculo gastrocnêmio direito. O laser utilizado foi de diodo de onda contínua tipo InGaAlP com potência de saída de 20 mW e comprimento de onda 660 nm. O tamanho do ponto foi de 0,035cm^2, dose de 21 J/ cm^2, tempo de 36 segundos em cada ponto e frequência contínua. Após 24 horas do último dia de intervenção, os animais foram anestesiados e eutanasiados. Coração, diafragma, fígado, gastrocnêmio direito, plasma, pulmões, rins e sóleo foram coletados, pesados e armazenados para posterior análise. O LBI reduziu os níveis plasmáticos de TBARS nos animais com DM 2. No coração, diafragma e gastrocnêmio, o LBI aumentou os níveis de NPSH no grupo DM 2. No coração, diafragma e plasma, o grupo L-DM aumentou a SOD quando comparado ao grupo C-DM. O grupo L-SHAM aumentou a SOD no coração, diafragma, gastrocnêmio e rins quando comparado ao grupo C-SHAM. Conclui- se que, o protocolo de LBI de 21 J/cm^2, com duração de 6 semanas, diminuiu a atividade oxidante e aumentou a antioxidante em ratos com DM 2.

Palavras-chave: Laserterapia. Atividade Oxidativa. Atividade Antioxidante.

ABSTRACT

EFFECTS OF LOW-LEVEL LASER THERAPY ON OXIDATIVE STRESS IN RATS WITH DIABETES MELLITUS TYPE 2

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Type 2 DM is the most prevalent and is characterized by defects in secretion and peripheral insulin resistance, which determine oxidative stress (OS). Low level laser therapy (LLLT) is a non-pharmacological tool that has been used for the treatment of DM, by reducing OS and inflammatory activity and increasing antioxidant activity. Thus, the aim of the present study was to evaluate the effects of LLLT on OS in organs, skeletal musculature and serum of rats with DM. Thirty-one male Wistar rats were used, divided into two groups, with DM 2 induction and without DM 2 induction, considering diabetic animals with blood glucose equal to or greater than 200 mg/dL, then the rats were allocated into 4 subgroups: Group 1 - animals without DM 2 SHAM (C-SHAM), Group 2 - animals with DM 2 SHAM (C-DM), Group 3- animals without DM 2 with 21 J/cm^2 laser (L-SHAM), Group 4- animals with DM 2 with 21 J/cm^2 laser (L-DM). After DM 2 induction or not, the animals received LLLT 5 days/week for 6 weeks. The dose was irradiated at two points in the right gastrocnemius muscle. The laser used was a continuous wave diode InGaAlP type with an output power of 20 mW and a wavelength of 660 nm. The stitch size was 0.035 cm^2, dose of 21 J/ cm^2, time of 36 seconds in each stitch and continuous frequency. Twenty-four hours after the last day of intervention, the animals were anesthetized and euthanized. Heart, diaphragm, liver, right gastrocnemius, plasma, lungs, kidneys and soleus were collected, weighed and stored for further analysis. LLLT reduced plasma levels of TBARS in animals with DM 2. In the heart, diaphragm and gastrocnemius, LLLT increased NPSH levels in the DM 2 group. In the heart, diaphragm and plasma, the L-DM group increased SOD when compared to the C-DM group. The L-SHAM group increased SOD in the heart, diaphragm, gastrocnemius and kidneys when compared to the C-SHAM group. It is concluded that the 21 J/cm^2 LLLT protocol, lasting 6 weeks, decreased the oxidant activity and increased the antioxidant and in rats with DM 2.

Keywords: Laser therapy. Oxidative Activity. Antioxidant activity.

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ATP	Adenosina Trifosfato
CAT	Catalase
CEUA	Comissão de Ética no Uso de Animais
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CONCEA	Conselho Nacional de Controle de Experimentação Animal
DM 1	Diabetes Mellitus tipo 1
DM 2	Diabetes Mellitus tipo 2
DM	Diabetes Mellitus
EDTA	Ácido Etilenodiamina Tetraacético
EO	Estresse Oxidativo
EREs	Espécies Reativas de Enxofre
ERNs	Espécies Reativas de Nitrogênio
EROs	Espécies Reativas de Oxigênio
FAPERGS	Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul
GAP	Gabinete de Apoio a Projetos
GPx	Glutationa Peroxidase
GRd	Glutationa Redutase
GSH	Glutationa Reduzida
H2O	Água
H2O2	Peróxido de Hidrogênio
I.P.	Intraperitoneal
LBI	Laser de Baixa Intensidade
LDL	Lipoproteínas de Baixa Densidade
MDA	Malonaldeído
NADPH	Fosfato de Dinucleótido de Nicotinamida Adenina
NO	Óxido Nítrico
NPSH	Níveis de Tiol Não Proteico
O2	Oxigênio
$O2^{\cdot}$	Superóxido
$OH^{\cdot}$	Hidroxila
PCR	Proteína C-Reativa
S1	Fração Sobrenadante de Baixa Velocidade
SDS	Dodecil Sulfato de Sódio
SOD	Superóxido Dismutase
STZ	Estreptozotocina
TBARS	Substâncias Reativas ao Ácido Tiobarbitúrico
TBA	Ácido Tiobarbitúrico
TCA	Ácido Tricloroacético
TFK	Tampão K-Fosfato
UFSM	Universidade Federal de Santa Maria

SUMÁRIO

1 INTRODUÇÃO

Frente ao avanço da urbanização, ao aumento do número de pessoas obesas e sedentárias, ao envelhecimento populacional e a maior sobrevida da população, o diabetes mellitus (DM) é considerado uma das principais doenças de evolução crônica. Esta doença apresenta altas taxas de prevalência e de mortalidade, o que a torna um importante problema de saúde pública (FLOR; CAMPOS, 2017; AMERICAN DIABETES ASSOCIATION, 2019).

Pacientes com DM apresentam duas formas clínicas: o DM tipo 1 ou o DM tipo 2. O DM tipo 2 é o mais prevalente, e caracteriza-se por defeitos na secreção e resistência periférica à insulina, o que determina o estresse oxidativo (EO) (FLOR; CAMPOS, 2017; AMERICAN DIABETES ASSOCIATION, 2019). Em estado hiperglicêmico, o organismo apresenta alterações em diferentes vias que levam ao EO e, consequentemente, a complicações do DM (DARYABOR et al., 2020).

O EO caracteriza-se pelo desequilíbrio entre radicais livres e defesas antioxidantes (ZHANG et al., 2020). Uma vez que, quando produzidos em excesso, determinam danos oxidativos, o que resulta no desenvolvimento de mecanismos de defesa durante os processos metabólicos (ZHANG et al., 2020). Esses mecanismos têm como objetivo restringir os níveis intracelulares de tais espécies reativas e comedir a ocorrência de danos decorrentes da produção excedente (ZHANG et al., 2020).

A cronicidade desse processo determina implicações sobre o processo etiológico de numerosas patologias crônicas, dentre elas o DM (ZHANG et al., 2020). O treinamento físico associado a modificações no estilo de vida reduz a progressão da resistência à insulina, tornando-se, assim, uma importante ferramenta não farmacológica para o tratamento do DM (SAMPATH KUMAR et al., 2018). O exercício aeróbio aumenta a sensibilidade à insulina, o que promove um maior efeito no controle glicêmico (AMERICAN DIABETES ASSOCIATION, 2019).

Outra ferramenta não farmacológica que vem sendo utilizada para o tratamento do DM é o laser de baixa intensidade (LBI). Este atua por meio de reações fotoquímicas em nível celular, através de um feixe de luz intenso (vermelho ou infravermelho), monocromático, colimado e de frequência pura, o que o torna útil na área biomédica (ABDEL-WAHHAB et al., 2018; SANTOS et al., 2018; MUSSTTAF; JENKINS; JHA, 2019).

Estudos evidenciaram que o LBI modifica o metabolismo celular e apresenta uma série de vantagens, tais como: alívio da dor, redução no tempo de cicatrização e reparo de lesões em pacientes que, por conta de alguma condição sistêmica, como no caso do DM, têm esse processo

prejudicado. Assim como, reduz danos e sequelas decorrentes do DM (ABDEL-WAHHAB et al., 2018; SANTOS et al., 2018).

Ahmed et al. (2018) avaliaram o efeito da quercetina associada ao LBI na cicatrização de feridas em ratos diabéticos por meio de reorganização estrutural e efeitos modulatórios sobre o EO. Os autores encontraram melhorias no estado glicêmico e no sistema de defesa antioxidante, bem como a reorganização estrutural (AHMED et al. 2018).

O estudo de Karkada et al. (2020) analisou o efeito do LBI sobre marcadores de EO na dinâmica de cicatrização de feridas neuropáticas diabéticas em ratos Wistar. Os resultados mostraram um efeito positivo do LBI sobre o estado do sistema de defesa antioxidante dos leucócitos sanguíneos em ratos com DM, um aumento na atividade da SOD e uma diminuição do TBARS (KARKADA et al., 2020).

No entanto, ainda existe uma escassez de estudos acerca dos efeitos do LBI sobre o EO. Desta maneira, espera-se contribuir para o tema, apontando o LBI como uma ferramenta não farmacológica para o tratamento dos distúrbios causados por essa patologia.

2 REVISÃO DE LITERATURA

2.1 DIABETES MELLITUS

O diabetes mellitus tipo 1 (DM 1) ocorre, principalmente, em crianças e adultos jovens. É causado por uma reação autoimune, com a destruição das células-beta no pâncreas e o aumento dos níveis de glicose sanguínea (EIZIRIK; PASQUALI; CNOP, 2020). A forma mais prevalente do DM é o diabetes mellitus tipo 2 (DM 2), que ocorre pela combinação do aumento da resistência periférica à insulina e a secreção inadequada de células-beta pancreáticas, levando a falência dessas células (EIZIRIK; PASQUALI; CNOP, 2020).

No mundo, atualmente, existem 463 milhões de adultos vivendo com DM. Estima-se que esse número chegue a 700 milhões em 2045 (IDF, 2019). O Brasil encontra-se na quinta posição entre os países com maior número de pessoas diabéticas, com aproximadamente 16 milhões de casos entre o público adulto (IDF, 2019).

A ausência, secreção deficiente ou resistência periférica de insulina desregulam o metabolismo de carboidratos, lipídeos e proteínas, e resultam em hiperglicemia e glicosúria (AMERICAN DIABETES ASSOCIATION, 2019). Esse desequilíbrio facilita a formação de placas e lesões ateroscleróticas, podendo gerar doenças cardiovasculares (DARYABOR et al., 2020). No fígado, ocorre um armazenamento de gordura nos hepatócitos, o que gera esteatose e, consequentemente, resistência à insulina (DARYABOR et al., 2020). O prejuízo na síntese de glicogênio nos músculos esqueléticos é um sinal prematuro do DM 2 (AMERICAN DIABETES ASSOCIATION, 2019).

O DM ocasiona diversas complicações graves em pacientes com esta patologia. O EO, através da produção exacerbada de espécies reativas de oxigênio (EROs), as quais tem a propriedade de destruir células e suas estruturas de membranas, é uma das principais causas da progressão do DM e das complicações associadas (KARMASH et al. 2020).

2.2 ESTRESSE OXIDATIVO

2.2.1 Espécies Reativas e Radicais Livres

As espécies reativas provenientes do oxigênio podem ser compostas tanto de átomos quanto de moléculas ou íons, possuindo alta reatividade em seu predomínio e constituindo três classes de compostos (DI MEO; VENDITTI, 2020). Sendo estas, espécies reativas de oxigênio

(EROs), reativas de enxofre (EREs) e reativas de nitrogênio (ERNs) (OTASEVIC et al., 2020). Essas, ainda, podem ser divididas entre radicais livres e compostos não radicalares (RODRIGUES et al., 2020).

Radicais livres são moléculas ou átomos com pelo menos um elétron desemparelhado de seus orbitais externos, que permitem a transferência de elétrons com moléculas vizinhas (DI MEO; VENDITTI, 2020). Esses, podem agir como aceptores ou doadores de elétrons modificando o ambiente molecular ao seu redor (DI MEO; VENDITTI, 2020). Em contrapartida, os compostos não radicalares, não dispõem de elétrons livres, fazendo com que em comparação aos radicais livres, sejam menos instáveis, ainda que também possam reagir com moléculas na sua redondeza (RODRIGUES et al., 2020).

2.2.2 Radicais Livres e Mecanismo de Geração de Radicais Livres

Radicais livres atuam como mediadores para a transferência de elétrons em várias reações bioquímicas e, quando em proporções adequadas, possibilitam a produção de adenosina trifosfato (ATP), por meio da cadeia transportadora de elétrons (YARIBEYGI; ATKIN; SAHEBKAR, 2019). Além disso, eles participam de mecanismos de defesa durante o processo de infecção, fazendo com que sua produção contínua seja um processo fisiológico que cumpre funções biológicas significativas (YARIBEYGI; ATKIN; SAHEBKAR, 2019). Quando produzidos em excesso podem gerar danos oxidativos, resultando no desenvolvimento de mecanismos de defesa antioxidante durante os processos metabólicos (YARIBEYGI; ATKIN; SAHEBKAR, 2019).

A geração de radicais livres ocorre nas membranas celulares, citoplasma e nas mitocôndrias, sendo a última a principal fonte geradora, em decorrência da cadeia transportadora de elétrons (YARIBEYGI; ATKIN; SAHEBKAR, 2019). Na parte final da cadeia transportadora de elétrons, a enzima catalisadora citocromo oxidase, oxida quatro moléculas de citocromo C retirando um elétron de cada uma delas (WATSON; MCSTAY, 2020). Esses elétrons se ligam ao oxigênio (O2) para formar água (H2O). Ou seja, o O2 sofre redução tetravalente, na qual ele aceita a ligação de quatro elétrons (WATSON; MCSTAY, 2020).

A função do citocromo C oxidase é controlar a geração de radicais livres, impedindo sua formação excessiva na mitocôndria (WATSON; MCSTAY, 2020). No entanto, uma pequena parcela do oxigênio metabolizado nas mitocôndrias é deslocada para outra via metabólica, e reduzido de modo univalente, dando origem aos radicais livres superóxido ($O2^{\cdot}$)

e hidroxila (OH˙) e ao composto não radicalar peróxido de hidrogênio (H2O2) (LIGUORI et al., 2018).

2.2.3 Sistema de Defesa Antioxidante e Balanço Redox

O sistema de defesa antioxidante pode ser dividido em enzimática e não-enzimática (LIGUORI et al., 2018). No sistema de defesa não-enzimática se destacam as vitaminas (vitamina C, o α-tocoferol e β-caroteno), minerais (zinco, cobre, selênio e magnésio) e compostos fenólicos, uma vez que esta é composta por antioxidantes de origem nutricional (LIGUORI et al., 2018). Carotenoides como licopeno, luteína e zeaxantina, também fazem parte desse sistema (LIGUORI et al., 2018).

Já a defesa enzimática é composta por superóxido dismutase (SOD), catalase (CAT), fosfato de dinucleótido de nicotinamida adenina (NADPH), glutationa peroxidase (GPx), glutationa redutase (GRd) e por níveis de tiol não proteico (NPSH) (composto por 90 % de GSH –glutationa reduzida- intracelular e 10% de aminoácidos livres, como cisteína e metionina) (YANG; XIANGMING, 2017; SU et al., 2019). A defesa enzimática controla a formação de radicais livres e compostos não radicalares, envolvidos com as reações em cadeia que resultam na propagação e no desenvolvimento de danos oxidativos (SU et al., 2019).

O balanço redox celular é descrito pelo equilíbrio entre substâncias oxidantes e redutoras (LIU et al., 2018). Os principais oxidantes endógenos são: O2˙, OH˙ e H2O2. Em contrapartida, a mitocôndria possui um sistema antioxidante composto por SOD, CAT, NADPH, GPx, GRd e GSH além das vitaminas C e E (SU et al., 2019).

2.2.4 Estresse Oxidativo e Diabetes Mellitus

O organismo, em estado hiperglicêmico, visto em pacientes com DM, pode apresentar alterações em diferentes vias que levarão ao EO (LUC et al., 2019). Na via poliol (na qual há a conversão de glicose em sorbitol, que é um álcool originado do açúcar, o que leva ao acúmulo intracelular, com participação na etiologia da neuropatia diabética) ocorre o aumento da atividade da enzima NADPH oxidase e, consequentemente a redução da GSH, CAT e SOD. Outras duas vias são a auto oxidação da glicose e a glicação proteica. Na última, ocorre a ativação de macrófagos (IGHODARO, 2018).

Todas essas vias apontam para o EO que, consequentemente, determinam o aumento na relação de oxigênio e óxido nítrico (O2/NO), culminando na formação de peroxinitrito (RADI,

2018). Essa formação pode alterar a oxidação das lipoproteínas de baixa densidade (LDL), reduzir a vasodilatação por meio da diminuição do NO dependente, aumentar a atividade de coagulação, assim como a aderência de leucócitos e plaquetas e a permeabilidade endotelial, o que eleva os fatores trombóticos e ateroscleróticos (HUA; MALINSKI, 2019).

2.3 LASER DE BAIXA INTENSIDADE

A primeira vez que foi elucidado o princípio da emissão estimulada de fótons foi em 1917 por Albert Einsten, porém a aplicação prática foi realizada mais tarde por Townes et al. (1955), por meio da emissão estimulada de micro-ondas (SILVA NETO; JÚNIOR, 2017). Apenas em 1960, os aparelhos começaram a ser denominados de laser, após a primeira emissão estimulada de radiação de espectro visível ser obtida pelo físico Maiman (SILVA NETO; JÚNIOR, 2017). Na década de 70 o laser começou a ser utilizado em estudos experimentais após a comprovação de que a irradiação emitida pelo aparelho de baixa intensidade melhorava o processo de cicatrização em feridas (SILVA NETO; JÚNIOR, 2017).

O laser emite radiação que produz campos eletromagnéticos intensos, baseados no comprimento de onda e na frequência empregada, que transitam entre o infravermelho e o ultravioleta de acordo com amplitude ou intensidade, sendo aplicados de diferentes maneiras. Sendo classificados em alta e baixa intensidade (MUSSTTAF; JENKINS; JHA, 2019). O laser de alta intensidade produz efeito térmico, sendo utilizado para coagulação ou secção de tecidos (MUSSTTAF; JENKINS; JHA, 2019). O laser de baixa intensidade não produz efeito térmico, e é utilizado no reparo tecidual (MUSSTTAF; JENKINS; JHA, 2019).

Cada laser possui um comprimento de onda diferente, que possibilita uma interação distinta em cada tecido (MUSSTTAF; JENKINS; JHA, 2019). Os comprimentos de onda variam de 632,8 nm a 980 nm, sendo mais utilizados os de 630 a 700 nm (vermelho) e de 700 a 904 nm (infravermelho) (MUSSTTAF; JENKINS; JHA, 2019).

Após evidências cientificas serem constatadas sobre o efeito do laser como método terapêutico na década de 80, cientistas começaram a discutir os mecanismos de ação do laser em nível celular, assim como fisiológico (SILVA NETO; JÚNIOR, 2017). Estudos sobre o tratamento de queimaduras em ratos evidenciaram que, o comprimento de onda mais utilizado foi o de 660 nm, uma vez que os outros comprimentos de onda não apresentaram grandes penetrações teciduais (BRASSOLATTI et al., 2018).

No estudo de Chen et al. (2021) foi investigado o efeito do laser na homeostase de espécies reativas de oxigênio em células de fibroblastos de pele embrionária humana cultivadas

em altas concentrações de glicose. Os resultados demonstraram que, o alto nível de glicose destruiu as células induzindo alta concentração de EROs. Após a aplicação de laser, os autores verificaram um aumento na capacidade antioxidante celular, o que reduziu a concentração de EROs (CHEN et al., 2021). No estudo de Dos Santos et al. (2017), no qual ratos Wistar possuíam artrite reumatoide, o LBI demonstrou efeitos favoráveis na modulação do EO, com o aumento da atividade antioxidante avaliada pela SOD, CAT e GPx (DOS SANTOS et al., 2017).

Em ratos com DM, de Frigero et al. (2018) verificaram que, por meio de uma única aplicação de LBI (4 J/cm^2) e exercícios de alta intensidade, houve um aumento na atividade antioxidante, verificada pela SOD e GPx. Ainda, os autores observaram a redução da atividade oxidante, analisada pelos níveis das substâncias reativas ao ácido tiobarbitúrico (TBARS), com consequente redução do EO (FRIGERO et al. 2018).

O LBI é um tratamento não invasivo e não farmacológico, com eficácia comprovada em relação ao alívio de dores musculoesqueléticas e neuropáticas (M. A. et al., 2019). A luz emitida durante a laserterapia reage com o citocromo C oxidase aumentando a produção de ATP e reduzindo os níveis de EROs e a morte celular (M. A. et al., 2019).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar os efeitos do laser de baixa intensidade, aplicado no músculo gastrocnêmio, sobre o estresse oxidativo e a atividade antioxidante em um modelo experimental de DM 2, induzido por dieta e estreptozotocina.

3.2 OBJETIVOS ESPECÍFICOS

Verificar o impacto do LBI 21 J/cm^2 sobre a atividade oxidante através do TBARS no plasma sanguíneo em ratos com DM 2.

Analisar o impacto do LBI 21 J/cm^2 sobre a atividade antioxidante por meio do SOD e do NPSH em coração, diafragma, gastrocnêmio, rim e plasma sanguíneo em ratos com DM 2.

4 ARTICLE SUBMITTED TO LASERS IN MEDICAL SCIENCE JOURNAL

EFFECTS OF LOW-LEVEL LASER THERAPY ON OXIDATIVE STRESS IN RATS WITH TYPE 2 DIABETES MELLITUS

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Declarations

Funding: This study was funded in part by the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) and by the Foundation for Research Support of the State of Rio Grande do Sul (FAPERGS) - EDITAL FAPERGS/CAPES 05/2017 - Master's Degree.

Conflicts of interest: The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and materials: Not applicable

Code availability: Not applicable

Author contributions: Not applicable

Ethical approval: The approval was obtained from the CEUA of the UFSM of Santa Maria. The procedures used in this study are in accordance with the Animal Use Ethics Commission.

Consent to participate: Not applicable

Consent to publication: Not applicable

Compliance with ethical standards

Research involving human and/or animal participants: Study approved by the Ethics Committee in the Use of Animals (CEUA) of the Federal University of Santa Maria (UFSM) under number 6622101118.

EFFECTS OF LOW-LEVEL LASER THERAPY ON OXIDATIVE STRESS IN RATS WITH TYPE 2 DIABETES MELLITUS

Abstract

The present study aimed to evaluate LLLT effects on oxidative stress in type 2 DM. Thirty-one male Wistar rats were used and divided into 4 groups: Group 1 - C-SHAM, Group 2 - C-DM, Group 3 - L-SHAM, Group 4- L-DM. The protocol was performed 5days/week, for 6 weeks. The animals that received LLLT had one dose irradiated at two spots in the right gastrocnemius muscle for 36 seconds in each spot. Twenty-four hours after the last intervention day, the animals were euthanized. Heart, diaphragm, liver, right gastrocnemius, plasma, lungs, kidneys, and soleus were collected, weighed, and stored for further analysis. It is possible to observe the reduction in TBARS plasma levels in animals with DM. Concerning the heart, diaphragm, and gastrocnemius, Group L-DM had an increase in NPSH levels, after the protocol. In the heart, diaphragm, and plasma, Group L-DM had an increase in SOD, when compared to Group C-DM. There was a rise in SOD in Group L-SHAM regarding the heart, diaphragm, gastrocnemius, and kidneys, compared to Group C-SHAM. The 6 weeks-protocol with LLLT reduced oxidative stress in plasma levels and increased antioxidant activity in animals with DM.

Keywords: Laser therapy. Oxidative activity. Antioxidant activity.

Introduction

The worldwide prevalence of diabetes mellitus (DM) is approximately 463 million people, which may rise to 700 million in 2045, according to the International Diabetes Federation [1]. Such a scenario provokes an expansion in financial and social costs, not only for the patients but also for the health system [2]. Diabetes mellitus may present two clinical forms, namely DM type 1, or type 2.

Type 2 Diabetes Mellitus (DM 2) is the most prevalent form of the disease and may reach 91% of the cases in high-income countries [1]. This clinical form presents itself with an increase in peripheral resistance to insulin, the inadequate secretion of pancreatic beta-cells, and, consequentially, pancreatic beta-cell failure. This alters the molecular metabolism, causing hyperglycemia and glycosuria [2,3].

Chronic hyperglycemia and metabolic dysregulation, caused by DM, lead to chronic complications, such as oxidative stress (OS). It is characterized by an imbalance between the excess production of free radicals and the reduction of antioxidant defenses [4].

Free radicals are described as molecules of atoms that have at least one impaired electron in external orbitals, hence allowing electron transference among other molecules. They act in electron transference in numerous biochemical reactions and, when produced in adequate proportions, achieve significant biological functions. However, when excessively produced, free radicals may generate oxidative damages [5, 6].

The goal of antioxidant defense is the control of the formation of free radicals involved in the development of oxidative damages. It is mainly composed of superoxide dismutase (SOD), catalase (CAT), nicotinamide adenine dinucleotide phosphate (NADPH), glutathione peroxidase (GPx), glutathione reductase (GRd), and non-protein thiol levels (NPSH) [7].

In a hyperglycemic state, the organism may present alterations in different metabolic pathways. The main one is the polyol pathway, where there is an increase in the activity of the NADPH oxidase enzyme and, consequently, a reduction in GSH, CAT, and SOD. Two other pathways are glucose auto-oxidation and protein glycation. In the latter, there is the activation of macrophages [8, 9].

Low-intensity laser therapy (LLLT) is a non-invasive therapeutic intervention that amplifies light by stimulating the emission of radiation. This resource may stimulate enzymatic activity by reducing stress. The emitted light during laser therapy reacts with cytochrome C oxidase, increasing ATP production and reducing ROS levels and cellular death [10].

Low-level laser therapy modifies cellular metabolism and has advantages, such as pain relief, healing time reduction, and repair of injuries in patients who, as in the case of DM, due to some systematic condition, have this process impaired. It also reduces damage and sequelae resulting from DM [11]

Considering that DM may lead to local or systemic complications, such as the increase of OS markers and the decrease of antioxidant defenses in rats with DM [12, 13], new therapeutic tools as LLLT can be tested, in the experimental model of LLLT. Therefore, the present study verified LLLT effects on oxidative stress in rats with DM 2.

Materials and methods

Animals

Thirty-two male Wistar rats were used (with a sample loss of 1 on them, which did not respond to DM 2 induction and underwent humane euthanasia). They were 7 weeks old and weighed 200 to 250g. The animals were placed in boxes (coated with shavings that were daily changed) in groups of three rats per box, under controlled temperature (21ºC) and humidity (50 to 60%) with air exhaust and a 12h- "light-dark" cycle. The animals were acclimated for 14 days before the beginning of the experiment and all of them were kept and handled under the ethical principles in animal experimentation by the National Council for the Control of Animal Experimentation (CONCEA).

After the experiment was carried out, the materials that were not used, just as the carcasses, were properly discarded. The activities involving the animals in this research began after the approval of the project by the Project Support Office (GAP), under the register 050439 (ANNEX A), and by the Ethics Committee on the Use of Animals (CEUA), under number 6622101118 (ANNEX B). As well as the work only began after signing the Term of Responsibility (APPENDIX A), the Consent Form of the Department of Physiology and Pharmacology (APPENDIX B), and the Consent Form of the Laboratory of Experimental Physiology (APPENDIX C) from the Federal University of Santa Maria (UFSM).

Experimental groups

The animals were divided into two groups, animals with and without DM 2 induction, considering diabetic animals with blood glucose equal to or greater than 200 mg/dL. Afterward, the rats were allocated into 4 subgroups, which are described below, based on the study by Frigero et al. [13].

Grup 1 - 8 animals without DM 2 without laser 21 J/cm^2 (C-SHAM)

Grup 2 - 7 animals with DM 2 without laser 21 J/cm^2 (C-DM)

Grup 3 - 8 animals without DM 2 with laser 21 J/cm^2 (L-SHAM)

Grup 4 - 8 animals with DM 2 with laser 21 J/cm^2 (L-DM)

Induction of DM 2

After acclimatization, the basal blood glucose of the animals was assessed by means of a small puncture in the tail vein with subsequent collection of a "drop" of blood (0.1 to 0.5 ml) [14] inserted into a manual glucometer (G Tech Free Lite, Infopia Co., LTDA, South Korea). For DM 2 induction, the animals were fed a high energy density diet, composed of 70% conventional animal feed, 15% sucrose, 10% fat, and 5% powdered egg yolk for the initial period of four weeks. The animals in the control group received standard commercial animal feed [15] and the diets were maintained throughout the experiment. All animals received water ad libitum.

On the 29th day, the animals underwent a 12h fast and their blood glucose was once again measured. On the 30th day, a single dose of intraperitoneal (i.p.) streptozotocin (STZ) – 35 mg/kg– was administered, dissolved in vehicle (0,01 M sodium citrate solution, pH - 4.5) with a volume of 1mL/kg. Only the vehicle was administered via i.p to the animals in the control group [16, 17].

One week after STZ or vehicle administration, the fasting blood glucose of the animals was measured again to confirm DM 2 induction. The animals that presented blood glucose equal to or greater than 200 mg/dL were considered diabetic. The groups received their respective diets for another four weeks and then, after 10 weeks, the LLLT protocol was started. Animals that did not become diabetic after drug infusion and a hypercaloric diet, or that presented any pathology during the protocol, received humane euthanasia with anesthetic overload, following CONCEA regulations.

Low-level laser therapy protocol

After DM 2 confirmation, the LLLT or placebo protocol was initiated on the rats. The used laser consisted of a diode continuous-wave type InGaAlP (model Endophoton-llt-0107; KLD Biossistemas equipamentos eletrônicos LTDA., São Paulo, Brazil) with an output power of 20 mW and a wavelength of 660 nm (visible red). The size of the spot was 0.035cm², 21 J/ cm² dose, for 36 seconds in each spot, with a continuous frequency. Before starting the experiments, the laser equipment was calibrated using an energy meter (Optical multimeter ILX Lightwave omn-6810b; ILX Lightwave Lasers MED SCI Corporation, Bozeman, MT, USA).

In LLLT groups, one single dose was irradiated in each animal, in two spots in the gastrocnemius muscle (medially and laterally; approximately 3cm from the beginning of the paw), on 5 days/week, for 6 weeks [18]. For this purpose, the skin was shaved and cleaned every day before application. The laser was irradiated with the probe held in contact with the skin at a 90º angle, maintaining a slight pressure [19]. The animals from the placebo groups (with and without DM 2) underwent the same handling procedures, asepsis and trichotomy, although without laser treatment. Besides, they were used as the control group (Fig. 1).

Euthanasia

Twenty-four hours after the last intervention day, the animals received deep anesthesia with isoflurane (4%) [20]. Also, blood was collected by cardiac puncture (10mL), which concluded their euthanasia. After this process, the heart, diaphragm, liver, right gastrocnemius, plasma, lungs, kidneys, and right soleus were collected and weighed.

Tissue preparation

Sections of these organs were homogenized so that analyzes about the OS could be developed. Muscle tissue samples were collected and stored for further homogenization and analysis. All samples were stored in an ultra freezer at -80ºC. The collected blood was centrifuged and the plasma extracted for analysis.

The extracted organs were homogenized in phosphate buffer (Ultraturrax, Staufen, Germany), while the muscles were homogenized in sodium chloride (0.9%) [21]. After homogenization, the organ samples were centrifuged (Clinical centrifuge - Spin Max 80-2b, didática SP, SP), so that a low-speed supernatant fraction (S1), which was used for different analyses [21], could be obtained.

Protein quantification

Protein content was measured according to the method described by Lowry et al. [22], by the use of bovine serum albumin as a standard. In a microplate, 20 µL of sample and 180 µL of coomassie were pipetted, then measurements were taken at 595 nm in a microplate reader (SpectraMax® i3x Multi-Mode Microplate Reader) [22].

Thiobarbituric acid reactive substance levels determination

The thiobarbituric acid reactive substance (TBARS) levels were determined following the method described by Ohkawa et al. [23]. Organ, muscle and serum homogenates (40 µL of the sample) were placed in Eppendorf with 20 µL of distilled water, 100 µL of acetic acid, 100 µL of thiobarbituric acid (TBA) and 40 µL of sodium dodecyl sulfate (SDS), and right after incubated for 120 minutes at 100°C. Aliquots of 200 µL of organs and muscles and 500 µL of serum were transferred to the microplates, where measurements were taken at 532 nm in a microplate reader (SpectraMax® i3x Multi-Mode Microplate Reader). TBARS levels were measured using standard malonaldehyde curve (MDA) and corrected by protein content [23].

Measurement of non-protein thiol levels

Non-protein thiol (NPSH) levels were determined by 134 µL of precipitated samples of previously homogenized organs and muscles, with 67 µL of trichloroacetic acid (TCA) (5%), and subsequently centrifuged in Eppendorf at 2000 rpm for 10 minutes in a microtube centrifuge (Hitachi Medical Systems - CF15RX II) [24]. The supernatant fraction (60 µL) was added to a reaction medium containing K-phosphate buffer (TFK) (100 µL, pH 7.4), distilled water (38 µL), and DTNB (2 µL) in microplates. Spectrophotometric measurements were taken at 412 nm in a microplate reader (SpectraMax® i3x Multi-Mode Microplate Reader). The results were calculated regarding a standard curve constructed with GRd at known concentrations (98, 58, 48, 38, 28 µL os distilled water, respectively; 100 µL of TFK; 0, 40, 50, 60 µL of GSH, respectively, and 2 µL of DTNB) and corrected by protein content [24].

Measurement of superoxide dismutase activity

In SOD analysis, the homogenate of the organ, muscle, and serum samples was added to 2mM EDTA (ethylenediaminetetraacetic acid) and bicarbonate buffer (NaHCO 3 / Na 2 CO 3 50 mM, pH 10.3) [26]. At the time of the reading, epinephrine (4mM) was added to the plate to initiate SOD for 5 minutes. The product color from adrenaline degradation (which was inhibited by SOD cellular activity) was verified spectrophotometrically (SpectraMax® i3x Multi-Mode Microplate Reader) at 480 nm. Superoxide dismutase enzyme was expressed in units of enzymatic activity per milligram of protein [25].

Statistical analysis

In order to verify the normality of the data, the Kolmogorov-Smirnov test was used. Variables from more than two measurements were compared by two-way analysis of variance for repeated measures, followed by Bonferroni's Post hoc. A significance level of 5% was considered for all tests. The Graphpad Prism 5 program (Graphpad Software, CA, USA) was used for data analysis.

Results

Bodyweight and blood glucose of the groups

In table 1, it is possible to observe that, after the protocol period (6 weeks), the final weight of the animals in the 4 groups had increased when compared to the initial weight ($p < 0,05$). The diabetic animals (C-DM and L-DM), at the end of the protocol, had high blood glucose levels when compared to their initial values ($p<0,05$), characterizing the hyperglycemia. There was no statistical difference when comparing Group C-DM to Group L-DM (Table 1).

Oxidant Activity Marker

Determination of the TBARS levels

It was possible to observe an increase in the TBARS plasmatic levels in Group C-DM, when compared to Group C-SHAM ($p<0,001$). There was a reduction in TBARS plasmatic levels when analyzing the laser effect on the animals with DM ($p<0,001$) (Fig. 2).

Antioxidant Activity Makers

Measurement of NPSH levels

The results of the antioxidant markers are described in Table 2. In the heart, there was a decrease in NPSH levels in Group C-DM, when compared to Group C-SHAM ($p<0,05$). The animals with DM had increased NPSH levels after the LLLT protocol ($p<0,05$).

In the diaphragm muscle, NPSH levels decreased in Group C-DM, when compared to Group C-SHAM. The laser produced an increase in the levels of this marker when comparing Group L-DM to Group C-DM ($p<0,05$).

In the gastrocnemius muscle, Group L-SHAM presented a reduction in NPSH levels after treatment ($p<0,05$). In contrast, the diabetic group irradiated with LLLT of 21 J/cm2 showed an increase in the levels of this marker ($p<0,05$). In the kidney, Group L-DM showed a reduction in NPSH levels when compared to Group L-SHAM ($p<0,05$) (Table 2).

Measurement of SOD antioxidant enzyme activity

In table 2, it is possible to observe that, in the heart, there was a reduction in the SOD levels in the C-DM group when compared with the C-SHAM ($p<0,05$). After the LLLT protocol, the laser groups presented an increase in SOD levels when compared to the control groups, both in the SHAM and diabetic rats groups ($p<0,05$).

In the diaphragm, the L-SHAM and L-DM groups showed an increase in SOD levels when compared to the animals in the control groups ($p<0,05$). In the gastrocnemius muscle, the DM groups (C-DM and L-DM) presented a reduction in SOD levels when compared to the SHAM groups (C-SHAM and L-SHAM) ($p<0,05$). There was an increase in the marker level in the laser group when comparing the C-SHAM to the L-SHAM ($p<0,05$).

In the kidney, Group L-SHAM showed an increase in SOD levels when compared to Group C-SHAM. In the diabetic group treated with LLLT, the laser induces the reduction of SOD levels compared to Group L-SHAM ($p<0,05$). Regarding blood plasma, Group C-DM had a reduction in SOD levels when compared to Group C-SHAM, whilst Group L-DM presented an increase when compared to Group C-DM ($p<0,05$).

Discussion

In the present study, an increase in blood glucose in diabetic groups was verified (C-DM and L-DM) after DM induction. When analyzing the effects of the laser on the rats with DM on TBARS, it was possible to observe a reduction in plasma levels of the animals with DM. In the heart, diaphragm, and gastrocnemius, the laser group presented, after the treatment, an increase in NPSH levels when compared to its control group. In the heart, diaphragm, and plasma, Group L-DM had an increase in SOD levels when compared to Group C-DM. Group L-SHAM showed an increase in SOD levels in the heart, diaphragm, gastrocnemius, and kidney when compared to Group C-SHAM.

Hyperglycemia contributes to the increase of the reactive oxygen species (ROS) [26]. Furthermore, OS can lead to insulin resistance, with consequent compromise in different systems. The therapeutic tools must act on the DM 2, so that the hyperglycemic and insulin resistance can be improved [27].

Bahwal et al. [28] verified that, after the treatment with LLLT 5 J/cm2 on the experimental DM model, there was a reduction in the blood glucose level. In a similar study, Gong et al. [27] investigated the effect of LLLT 8 J/cm2 in the improvement of glucose metabolism in skeletal muscle in type 2 DM models. The authors identified a reduction in blood glucose and insulin resistance, with reversal of metabolic abnormalities in skeletal muscle,

once the it is responsible for glucose metabolism. Besides, the laser acted on its metabolic improvement through glucose uptake and accelerated glycogen synthesis [27, 28].

Li et al. [29] demonstrated that the compound laser acupuncture-moxibustion had positive effects on the regulation of hyperglycemia, fasting insulin, and blood lipids levels in male Wistar rats with type 2 diabetes mellitus. In our studies, we did not verify the glucose level reduction in the animals with DM after the LLLT 21 J/cm2 protocol.

The combination of diabetes and hyperglycemia affects the redox balance, which increases the oxidant and reduces the antioxidants substances [30]. Hyperglycemia stimulates ROS production, which in turn can determine the OS. LLLT is believed to act on OS through the absorption of red and infrared light by cytochrome C oxidase, stimulating its performance. In our study, hyperglycemia has increased in C-DM and L-DM groups when comparing the initial and final values, confirming the effectiveness of DM 2 induction through the STZ. At the same time, the LLLT did not induce the reduction of hyperglycemic levels.

Regarding oxidative activity, analyzing the plasma TBARS levels in our study, it is verified a reduction in the diabetic group after the LLLT, demonstrating the treatment efficiency. In the study of Tatmatsu-Rocha et al [3] with twenty DM mouses, the animals received the LLLT treatment over 5 days, and it was verified a reduction in the TBARS levels in the irradiated diabetic group when compared with the non-irradiated diabetic group. This is due to the fact that the membrane is constantly subjected to lipid lipoperoxidation, once in OS, and LLLT acts through the activation of the antioxidant defense mechanism, protecting the lipid membranes against oxidative damages [31].

Frigero et al. [13] verified that the laser therapy in diabetic rats submitted to high-level intensity exercises determined, in the gastrocnemius muscle, a reduction on the TBARS levels on the treated DM group. Although the studies do not evaluate the TBARS in the plasma specifically, they corroborate the findings of the present study. In our study, this marker showed an increase in diabetic individuals when comparing the C-SHAM and C-DM groups and, later, a reduction after the treatment with LLLT application when comparing C-DM to L-DM groups.

The antioxidants defenses, whether enzymatic or non-enzymatic, are produced under normal physiological conditions by the body, as well as the free radicals. However, the imbalance between the production of free radicals and the production of antioxidants results in the toxic effects of the former and in oxidative stress, consequently [32, 33].

In our study, a reduction in NPSH levels was found in the heart, diaphragm, and gastrocnemius when comparing C-SHAM to C-DM groups. After the LLLT protocol with 21 J/cm², an increase in Group L-DM was verified when compared to the control group with diabetics animals. Similarly, analyzing SOD after the laser, the heart and diaphragm levels increased in both L-SHAM and L-DM groups when compared to their respective control groups. After the LLLT application, in Group L-SHAM, SOD presented an increase both in the gastrocnemius muscle and kidneys. In Group L-DM, plasma showed an increase in oxidant activity through SOD.

The studies by Asghari et al. [34], designed to explore the possible effect of LLLT on kidney damage in twenty diabetic rats, and Frigero et al. [13], quote above, corroborate the results found in this study in the two antioxidant analyses. Although the first study only evaluated the renal system and the second study the gastrocnemius muscle, both verify a reduction in SOD and NPSH in the diabetic groups and an increase in these markers in groups submitted to the LLLT.

Oliveira et al. [35], in a study with 48 healthy female rats that aimed to determine whether or not the OS markers were influenced by LLLT in rats that underwent a high intensity resisted exercises session, found a reduction in the oxidative stress markers, as well as an increase in the antioxidant capacity. Sunemi et al. [36] also evaluated the effect of LLLT, applied to the gastrocnemius muscle, in healthy female Wistar rats, before and after a high-intensity resistance exercise session, on oxidative stress and corroborated with the findings in the study carried out by Oliveira et al. [35].

Low-level laser therapy attenuated oxidative stress by increasing antioxidant activity, enhancing mitochondrial function, oxygen dismutation, and, consequently, reducing peroxynitrite formation [37]. After LLLT performance, there was a metabolic increase, as well as in SOD and NPSH synthesis, which aims to inhibit ROS production and, consequently, generate cellular protection [37]. LLLT acts through cytochrome C oxidase, increasing ATP production and decreasing ROS levels, besides cellular death [10].

The present study has some limitations. It was not possible to perform the analysis of other oxidant and antioxidant activity parameters, which would provide greater knowledge on laser effects on OS.

Conclusion

In the present study, it was possible to conclude that the 21 J/cm² LLLT reduced oxidative activity in rats with DM 2, verified by TBARS, and increased the antioxidant activity, which was analyzed by NPSH and SOD indicators.

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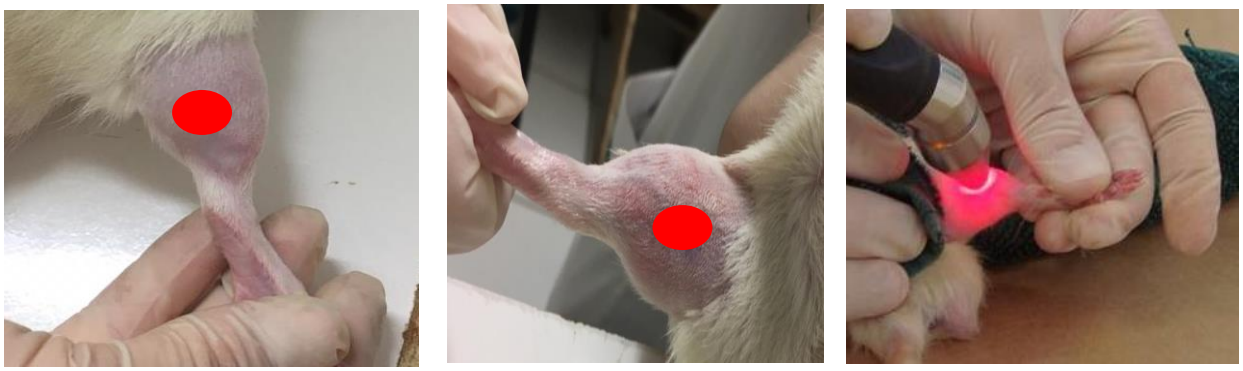
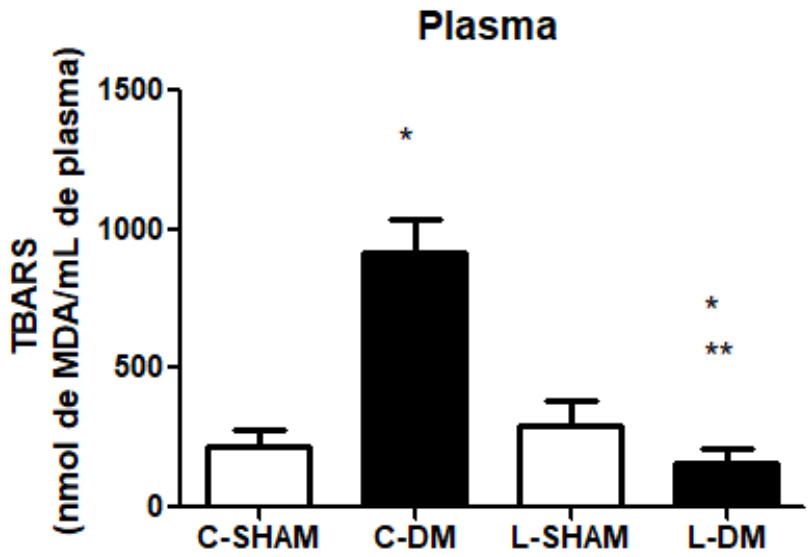


Fig. 1 – Application of LLLT in the gastrocnemius muscle



Values in mean ± SD. The groups were compared by two-way ANOVA with Post Hoc Bonferroni. TBARS: levels of thiobarbituric acid reactive substance. Control-Sham (C-SHAM, n=8); Laser-SHAM (L-SHAM, n=8), Control-Diabetes (C-DM, n=7) and Laser-Diabetes (L-DM, n=8).
* compared to C-SHAM and L-SHAM ($p<0.001$)
** compared to C-DM ($p<0.001$)

Fig. 2 – Plasma oxidizing activity marker

Table 1 - Body weight and blood glucose of the groups

Body weight and blood glucose of the groups					
Groups	Initial weight (g)	Final weight (g)	Initial blood glucose (mg/dL)	Post STZ and vehicle blood glucose (mg/dL)	Final blood glucose (mg/dL)
C-SHAM	266±18	486±23*	125±7	113±12	124±21
L-SHAM	283±29	467±32*	141±23	115±11	134±23
C-DM	248±17	362±45*	139±16	407±70*	428±44*
L-DM	242±19	382±55*	137±21	416±94*	412±53*

Values in mean ± SD. The groups were compared by two-way ANOVA with Post Hoc Bonferroni. STZ: streptozotocin. Control-Sham (C-SHAM, n=8); Laser-SHAM (L-SHAM, n=8), Control-Diabetes (C-DM, n=7) and Laser-Diabetes (L-DM, n=8).

* $p<0.05$ comparing initial and final values

Table 2 - Markers of antioxidant activity in heart, diaphragm, gastrocnemius, kidney and plasma

NPSH				
Groups	Heart (nmol SH/mg de prot.)	Diaphragm (nmol SH/mg de prot.)	Gastrocnemius (nmol SH/mg de prot.)	Kidney (nmol SH/mg de prot.)
C-SHAM	136±6	48±1	42±14	25±9
L-SHAM	87±10[c]	40±12	28±3[c]	30±10
C-DM	16±6[a]	20±5[a]	36±2	13±2
L-DM	50±6[bd]	76±15[bd]	62±7[bd]	18±2[b]

SOD					
Groups	Heart (U/mg)	Diaphragm (U/mg)	Gastrocnemius (U/mg)	Kidney (U/mg)	Plasma (U/mg)
C-SHAM	0,002±0,000	0,001±0,000	0,031±0,010	0,001±0,000	0,035±0,006
L-SHAM	0,004±0,000[c]	0,006±0,001[c]	0,048±0,011[c]	0,009±0,002[c]	0,034±0,011
C-DM	0,001±0,000[a]	0,001±0,000	0,002±0,000[a]	0,000±0,000	0,001±0,001[a]
L-DM	0,004±0,001[d]	0,003±0,001[bd]	0,003±0,001[b]	0,001±0,000[b]	0,036±0,011[d]

Values in mean ± SD. The groups were compared by two-way ANOVA with Post Hoc Bonferroni. Prot.: protein. NPSH: non-protein thiol levels. SOD: superoxide dismutase activity. Control-SHAM (C-SHAM, n=8); Laser-SHAM (L-SHAM, n=8), Control-Diabetes (C-DM, n=7) and Laser-Diabetes (L-DM, n=8).

a $p<0.05$ comparing C-SHAM and C-DM;

b $p<0.05$ comparing L-SHAM and L-DM;

c $p<0.05$ comparing C-SHAM and L-SHAM;

d $p<0.05$ comparing C-DM and L-DM.

5 CONCLUSÃO

O presente estudo teve como objetivo avaliar os efeitos do laser de baixa intensidade, sobre o estresse oxidativo em ratos com DM 2. Verificou-se que o protocolo com laser de baixa intensidade, aplicado durante 6 semanas por 5 dias consecutivos, na musculatura do gastrocnêmio, com intensidade de 21 J/cm^2, em ratos com DM 2, induzidos por STZ, foi eficaz na redução do estresse oxidativo. Da mesma forma, no aumento da atividade antioxidante em órgãos, músculos e plasma.

Demonstrando, desta maneira, que o LBI é uma importante ferramenta não farmacológica que pode ser utilizada para o tratamento de indivíduos com DM 2. Este estudo apresentou como limitações a escassez de publicações que corroborassem de alguma forma com os achados desta pesquisa. Assim como, outras análises acerca do estresse oxidativo, outros grupos com diferentes intensidades de LBI e um maior número de animais.

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APÊNDICES

APÊNDICE A - TERMO DE RESPONSABILIDADE

Universidade Federal de Santa Maria
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

TERMO DE RESPONSABILIDADE

Mediante este termo eu, Rodrigo Boemo Jaenisch, pesquisador da UFSM, e coordenador do projeto submetido à CEUA, comprometo-me em providenciar as autorizações necessárias ao desenvolvimento do projeto, tais como IBAMA, ICMBio, CTNBio CNPq, CGEN, FUNAI e Polícia Federal, quando for o caso, bem como verificar as condições de biossegurança necessárias.

Santa Maria, 10 de novembro de 2018.

APÊNDICE B - TERMO DE CONSENTIMENTO DO DEPARTAMENTO DE FISIOLOGIA E FARMACOLOGIA

TERMO DE CONSENTIMENTO DO DEPARTAMENTO DE FISIOLOGIA E FARMACOLOGIA

Eu, William Schoenau, abaixo assinado, responsável pelo Departamento de Fisiologia e Farmacologia da Universidade Federal de Santa Maria (UFSM), autorizo a realização do estudo intitulado "EFEITOS DO TREINAMENTO MUSCULAR VENTILATÓRIO E DA LASERTERAPIA SOBRE O PERFIL INFLAMATÓRIO E O ESTRESSE OXIDATIVO EM RATOS COM DIABETES MELLITUS TIPO II " a ser conduzido pelos pesquisadores Camille Gaube Guex, Carlos Cassiano Figueiró da Silva, Jhulie Anne Pinheiro Kemerich, Larissa da Silva Tonetto, Maria Elaine Trevisan, Nandiny Paula Cavalli, Nubia Gonzatti e Vanessa Ortiz de Andrade, orientado pelo professor Rodrigo Boemo Jaenisch e contando com colaboração da professora Liliane de Freitas Bauermann.

Fui informado, pelo responsável do estudo sobre as características e objetivos da pesquisa bem como das atividades que serão realizadas na instituição a qual represento.

Esta instituição está ciente de suas responsabilidades como instituição coparticipante do presente projeto de pesquisa e seu compromisso no resguardo da segurança e bem-estar dos sujeitos nela recrutados, dispondo de infraestrutura necessária para garantia de tal bem-estar.

Assinatura e carimbo

Prof. WILLIAM SCHOENAU
Chefe do Departamento de Fisiologia
e Farmacologia
CCS/UFSM

Santa Maria, 30 de ABRIL de 2019

APÊNDICE C - TERMO DE CONSENTIMENTO DO LABORATÓRIO DE FISIOLOGIA EXPERIMENTAL

APÊNDICE B - TERMO DE CONSENTIMENTO DO LABORATÓRIO DE FISIOLOGIA EXPERIMENTAL

Eu, Liliane de Freitas Bauermann, abaixo assinado, responsável pelo Laboratório de Fisiologia Experimental da Universidade Federal de Santa Maria (UFSM), autorizo a realização do estudo intitulado "EFEITOS DO TREINAMENTO MUSCULAR VENTILATÓRIO E DA LASERTERAPIA SOBRE O PERFIL INFLAMATÓRIO E O ESTRESSE OXIDATIVO EM RATOS COM DIABETES MELLITUS TIPO II " a ser conduzido pelos pesquisadores Camille Gaube Guex, Carlos Cassiano Figueiró da Silva, Jhulie Anne Pinheiro Kemerich, Larissa da Silva Tonetto, Maria Elaine Trevisan, Nandiny Paula Cavalli, Nubia Gonzatti e Vanessa Ortiz de Andrade, orientado pelo professor Rodrigo Boemo Jaenisch.

Fui informado, pelo responsável do estudo sobre as caracteristicas e objetivos da pesquisa bem como das atividades que serão realizadas na instituição a qual represento.

Esta instituição está ciente de suas responsabilidades como instituição coparticipante do presente projeto de pesquisa e seu compromisso no resguardo da segurança e bem-estar dos sujeitos nela recrutados, dispondo de infraestrutura necessária para garantia de tal bem-estar.

Liliane de F. Bauermann

Assinatura e carimbo

Liliane de F. Bauermann
CRB: 17045 - 03D
MEC: LP 02218/89
UFSM - MAT 2227178

Santa Maria, 30 de Abril de 2019.

ANEXOS

ANEXO A – REGISTRO NO GABINETE DE APOIO A PROJETOS DO CENTRO DE CIÊNCIAS DA SAÚDE

UNIVERSIDADE FEDERAL DE SANTA MARIA - UFSM

Data/Hora: 22/12/2019 22:11
Autenticação: 4E9E.3151.3D37.8DB3.D2C4.0C31.C8BD.38A2
Consulte em http://www.ufsm.br/autenticacao

PROJETO NA ÍNTEGRA

Título: EFEITOS DO TREINAMENTO MUSCULAR VENTILATÓRIO E DA LASERTERAPIA SOBRE O PERFIL INFLAMATÓRIO E O ESTRESSE OXIDATIVO EM RATOS COM DIABETES MELLITUS TIPO II

Número: 050439 **Classificação**: Pesquisa **Registrado em**: 10/11/2018

Situação: Em andamento **Início**: 10/11/2018 **Término**: 01/12/2020

Avaliação: Não avaliado **Última avaliação**:

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

Supervisor financeiro: *Não se aplica*

Proteção do conhecimento: Projeto não gera conhecimento passível de proteção

Tipo de evento: Não se aplica **Carga Horária**: *Não se aplica*

Alunos matriculados: *Não se aplica*
Alunos concluintes: *Não se aplica*

Palavras-chave: treinamento ventilatório, laserterapia, exercício

Resumo: O Diabetes Mellitus do tipo II (DM II) ocorre pela combinação do aumento da resistência periférica à insulina e a secreção inadequada de células-beta pancreáticas. A exposição de altas concentrações de glicose determina o estresse oxidativo, o aumento de citocinas pró-inflamatórias e a diminuição de citocinas anti-inflamatórias, o que contribui para complicações locais ou sistêmicas. O treinamento muscular ventilatório (TMV) e a laserterapia (LT) de baixa intensidade são ferramentas não farmacológicas que promovem benefícios em pacientes com DM II, entretanto nenhum estudo foi desenvolvido em ratos com DM II, com a finalidade de esclarecer os mecanismos fisiológicos. Assim, o presente projeto utilizará ratos Wistar machos, alocados para um dos grupos experimentais descritos abaixo, perfazendo um n=8 animais por grupo: Grupo 1 - animais sem DM II sedentários, Grupo 2 - animais sem DM II com TMV, Grupo 3 - animais com DM II sedentários, Grupo 4 - animais com DM II e com TMV, Grupo 5- animais sem DM II com laser 3J, Grupo 6- animais com DM II com laser 3J, Grupo 7- animais sem DM II com laser 21J, Grupo 8- animais com DM II com laser 21J. O protocolo de TMV será realizado pelo período de 30min/dia, 5 dias/semana, durante 6 semanas. Duas doses diferentes serão irradiadas, em dois pontos no músculo gastrocnêmio direito, por 10 dias consecutivos, em cada animal dos quatro grupos que receberão a laserterapia. A hipótese inicial é que o TMV e a laserterapia de baixa intensidade possa diminuir o estresse oxidativo e melhorar o perfil inflamatório em ratos com DM II.

Objetivos: Avaliar os efeitos do treinamento muscular ventilatório e da laserterapia de baixa intensidade, sobre o perfil inflamatório e o estresse oxidativo em ratos com diabetes mellitus tipo II.

Justificativa: Considerando que o DM II pode conduzir a complicações locais ou sistêmicas, como o aumento da atividade pró-inflamatória, a diminuição da anti-inflamatória, o aumento de marcadores de estresse oxidativo e a redução das defesas antioxidantes de ratos com DM II, novas ferramentas terapêuticas como o TMV e a laserterapia de baixa intensidade, no modelo experimental de DM II, podem ser testadas frente essas alterações.

Resultados esperados: Esperamos que o treinamento muscular ventilatório (TMV) e a laserterapia de baixa intensidade possam melhorar o perfil inflamatório, por meio da diminuição de citocinas pró-inflamatória e o aumento de citocinas anti-inflamatórias. Ainda, espera-se reduzir o estresse oxidativo pela diminuição de enzimas oxidativas e aumento de anti-oxidativas em ratos com DM II. Além disso, esse estudo pode fortalecer e esclarecer os efeitos do TMV e da laserterapia de baixa intensidade sobre os aspectos fisiopatológicos da DM II no modelo experimental.

PARTICIPANTES

MATRÍCULA	NOME	VÍNCULO	FUNÇÃO	C.H.*	INÍCIO	TÉRMINO
201660457	CAMILLE GAUBE GUEX	Aluno de Pós-graduação	Participante	8	10/11/2018	19/11/2019
201660457	CAMILLE GAUBE GUEX	Aluno de Pós-graduação	Participante	2	20/11/2019	01/12/2020
201870544	CARLOS CASSIANO FIGUEIRÓ DA SILVA	Aluno de Pós-graduação	Participante	8	10/11/2018	01/12/2020
201510855	JHULIE ANNE PINHEIRO KEMERICH	Aluno de Graduação	Participante	8	10/11/2018	01/12/2020
201870534	LARISSA DA SILVA TONETTO	Aluno de Pós-graduação	Participante	8	10/11/2018	01/12/2020
2227178	LILIANE DE FREITAS BAUERMANN	Docente	Colaborador	8	27/03/2019	01/12/2020
378922	MARIA ELAINE TREVISAN	Docente	Colaborador	8	10/11/2018	01/12/2020
201511092	NANDINY PAULA CAVALLI	Aluno de Graduação	Participante	8	10/11/2018	01/12/2020
201870535	NUBIA GONZATTI	Aluno de Pós-graduação	Participante	8	10/11/2018	01/12/2020
2395822	RODRIGO BOEMO JAENISCH	Docente	Orientador	8	10/11/2018	01/12/2020

* carga horária semanal

UNIDADES VINCULADAS

UNIDADE	FUNÇÃO	VALOR	INÍCIO	TÉRMINO
04.37.00.00.0.0 - DEPARTAMENTO DE FISIOTERAPIA E REABILITAÇÃO	Responsável		10/11/2018	01/12/2020

CLASSIFICAÇÕES

TIPO DE CLASSIFICAÇÃO	CLASSIFICAÇÃO
Classificação CNPq	4.08.00.00-8 - FISIOTERAPIA E TERAPIA OCUPACIONAL
Linha de pesquisa	02.06.00 - FISIOTERAPIA
Quanto ao tipo de projeto de pesquisa	2.05 - Projeto de Pesquisa e Ensino

ANEXO B – COMPROVANTE DE APROVAÇÃO NA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Comissão de Ética no Uso de Animais da *Universidade Federal de Santa Maria*

CERTIFICADO

Certificamos que a proposta intitulada "EFEITOS DO TREINAMENTO MUSCULAR VENTILATÓRIO E DA LASERTERAPIA SOBRE O PERFIL INFLAMATÓRIO E O ESTRESSE OXIDATIVO EM RATOS COM DIABETES MELLITUS TIPO II", protocolada sob o CEUA nº 6622101118, sob a responsabilidade de **Rodrigo Boemo Jaenisch** *e equipe; Jhulie Anne Pinheiro Kemerich; Camille Gaube Guex; Carlos Cassiano Figueiró da Silva; Larissa da Silva Tonetto ; Liliane de Freitas Bauermann ; Maria Elaine Trevisan; Nandiny Paula Cavalli; Nubia Gonzatti; Vanessa Ortiz de Andrade* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 26/03/2019.

We certify that the proposal "EFFECTS OF VENTILATORY MUSCLE TRAINING AND LASER THERAPY ON INFLAMMATORY PROFILE AND OXIDATIVE STRESS IN RATS WITH DIABETES MELLITUS TYPE II", utilizing 64 Heterogenics rats (64 males), protocol number CEUA 6622101118, under the responsibility of **Rodrigo Boemo Jaenisch** *and team; Jhulie Anne Pinheiro Kemerich; Camille Gaube Guex; Carlos Cassiano Figueiró da Silva; Larissa da Silva Tonetto ; Liliane de Freitas Bauermann ; Maria Elaine Trevisan; Nandiny Paula Cavalli; Nubia Gonzatti; Vanessa Ortiz de Andrade* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 03/26/2019.

Finalidade da Proposta: 2

Vigência da Proposta: de 04/2019 a 12/2020 Área: Fisiologia

Origem:	Biotério Central UFSM						
Espécie:	Ratos heterogênicos	sexo:	Machos	idade:	80 a 100 dias	N:	64
Linhagem:	Wistar			Peso:	400 a 450 g		

Resumo: O Diabetes Mellitus do tipo II (DM II) ocorre pela combinação do aumento da resistência periférica à insulina e a secreção inadequada de células-beta pancreáticas. A exposição de altas concentrações de glicose determina o estresse oxidativo, o aumento de citocinas pró-inflamatórias e a diminuição de citocinas anti-inflamatórias, o que contribui para complicações locais ou sistêmicas. O treinamento muscular ventilatório (TMV) e a laserterapia (LT) de baixa intensidade são ferramentas não farmacológicas que promovem benefícios em pacientes com DM II, entretanto nenhum estudo foi desenvolvido em ratos com DM II, com a finalidade de esclarecer os mecanismos fisiológicos. Assim, o presente projeto utilizará ratos Wistar machos, alocados para um dos grupos experimentais descritos abaixo, perfazendo um n=8 animais por grupo: Grupo 1 - animais sem DM II sedentários, Grupo 2 - animais sem DM II com TMV, Grupo 3 - animais com DM II sedentários, Grupo 4 - animais com DM II e com TMV, Grupo 5- animais sem DM II com laser 3J, Grupo 6- animais com DM II com laser 3J, Grupo 7- animais sem DM II com laser 21J, Grupo 8- animais com DM II com laser 21J. O protocolo de TMV será realizado pelo período de 30min/dia, 5 dias/semana, durante 6 semanas. Duas doses diferentes serão irradiadas, em dois pontos no músculo gastrocnêmio direito, por 10 dias consecutivos, em cada animal dos quatro grupos que receberão a laserterapia. A hipótese inicial é que o TMV e a laserterapia de baixa intensidade possa diminuir o estresse oxidativo e melhorar o perfil inflamatório em ratos com DM II.

Local do experimento: Laboratório de Fisiologia Experimental (LAFEX), prédio 21, UFSM

Santa Maria, 07 de novembro de 2019

Avenida Roraima, 1000, Reitoria, 2º andar - CEP 97105-900 Santa Maria, RS - tel: 55 (55) 3220-9362 / fax:
Horário de atendimento: das 8:30 às 12h e 14h às 17hs : e-mail: ceua.ufsm@gmail.com
CEUA N 6622101118

Comissão de Ética no Uso de Animais da *Universidade Federal de Santa Maria*

Profa. Dra. Patrícia Severo do Nascimento
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Saulo Tadeu Lemos Pinto Filho
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

ANEXO C – NORMAS DA REVISTA

Lasers 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

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Manuscript Submission

Manuscript Submission

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

Permissions

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

Online Submission

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

Please ensure you provide all relevant editable source files. Failing to submit these source files might cause unnecessary delays in the review and production process.

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Editorial Procedure

Double-blind peer review

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

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

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

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Title page

Title Page

Please make sure your title page contains the following information.

Title

The title should be concise and informative.

Author information

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

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

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

Abstract

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

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

For life science journals only (when applicable)

Trial registration number and date of registration

Trial registration number, date of registration followed by "retrospectively registered"

Keywords

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

Declarations

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.

To be used for all articles, including articles with biological applications

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)

Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals

Ethics approval (include appropriate approvals or waivers)

Consent to participate (include appropriate statements)

Consent for publication (include appropriate statements)

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

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

LaTeX macro package (Download zip, 190 kB)

Headings

Please use no more than three levels of displayed headings.

Abbreviations

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

Footnotes

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

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

Always use footnotes instead of endnotes.

Acknowledgments

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

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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.

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References

Citation

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

1. Negotiation research spans many disciplines [3].

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

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

Reference list

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

The entries in the list should be numbered consecutively.

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

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

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

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

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

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

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

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

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

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

ISSN.org LTWA

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

Authors preparing their manuscript in LaTeX can use the bibtex file spbasic.bst which is included in Springer's LaTeX macro package.

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Tables

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

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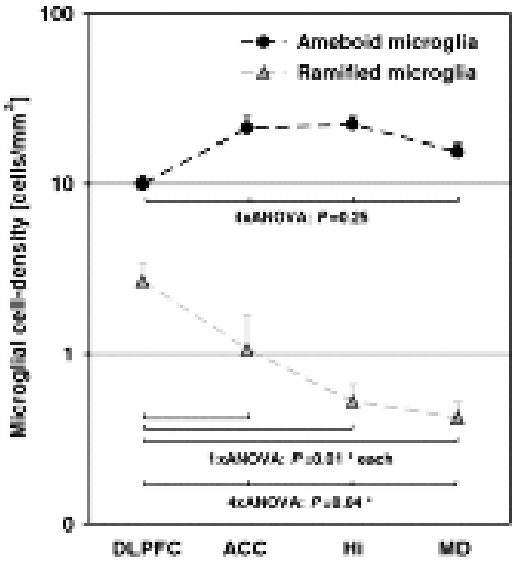
Artwork and Illustrations Guidelines

Electronic Figure Submission

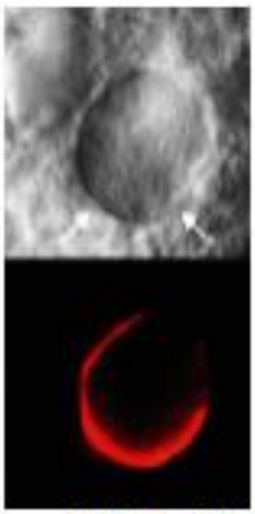
- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.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

Line Art



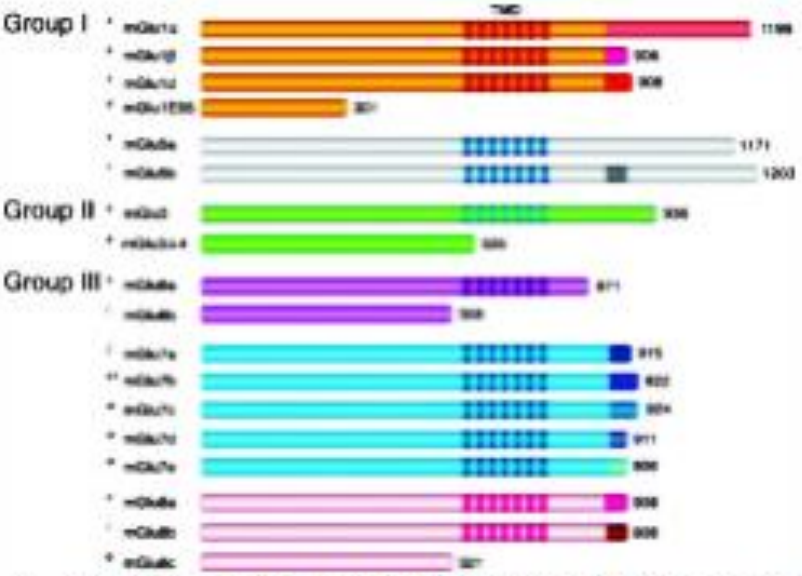
- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should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

Halftone Art



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

Combination Art



- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

Color Art

- Color art is free of charge for online publication.
- If black and white will be shown in the print version, make sure that the main information will still be visible. Many colors are not distinguishable from one another when converted to black and white. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent.
- If the figures will be printed in black and white, do not refer to color in the captions.
- Color illustrations should be submitted as RGB (8 bits per channel).

Figure Lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).
- Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).
- Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.
- 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.

Figure Captions

- 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.

Figure Placement and Size

- Figures should be submitted separately from the text, if possible.
- When preparing your figures, size figures to fit in the column width.
- For large-sized journals the figures should be 84 mm (for double-column text areas), or 174 mm (for single-column text areas) wide and not higher than 234 mm.
- For small-sized journals, the figures should be 119 mm wide and not higher than 195 mm.

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If you include figures that have already been published elsewhere, you must obtain permission from the copyright owner(s) for both the print and online format. Please be aware that some publishers do not grant electronic rights for free and that Springer will not be able to refund any costs that may have occurred to receive these permissions. In such cases, material from other sources should be used.

Accessibility

In order to give people of all abilities and disabilities access to the content of your figures, please make sure that

- All figures have descriptive captions (blind users could then use a text-to-speech software or a text-to-Braille hardware)
- Patterns are used instead of or in addition to colors for conveying information (colorblind users would then be able to distinguish the visual elements)
- Any figure lettering has a contrast ratio of at least 4.5:1

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Supplementary Information (SI)

Springer accepts electronic multimedia files (animations, movies, audio, etc.) and other supplementary files to be published online along with an article or a book chapter. This feature can add dimension to the author's article, as certain information cannot be printed or is more convenient in electronic form.

Before submitting research datasets as Supplementary Information, authors should read the journal's Research data policy. We encourage research data to be archived in data repositories wherever possible.

Submission

- Supply all supplementary material in standard file formats.
- Please include in each file the following information: article title, journal name, author names; affiliation and e-mail address of the corresponding author.
- To accommodate user downloads, please keep in mind that larger-sized files may require very long download times and that some users may experience other problems during downloading.
- High resolution (streamable quality) videos can be submitted up to a maximum of 25GB; low resolution videos should not be larger than 5GB.

Audio, Video, and Animations

- Aspect ratio: 16:9 or 4:3
- Maximum file size: 25 GB for high resolution files; 5 GB for low resolution files
- Minimum video duration: 1 sec
- Supported file formats: avi, wmv, mp4, mov, m2p, mp2, mpg, mpeg, flv, mxf, mts, m4v, 3gp

Text and Presentations

- Submit your material in PDF format: .doc or .ppt files are not suitable for long-term viability.
- A collection of figures may also be combined in a PDF file.

Spreadsheets

- Spreadsheets should be submitted as .csv or .xlsx files (MS Excel).

Specialized Formats

- Specialized format such as .pdb (chemical), .wrl (VRML), .nb (Mathematica notebook), and .tex can also be supplied.

Collecting Multiple Files

- It is possible to collect multiple files in a .zip or .gz file.

Numbering

- If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables.
- Refer to the supplementary files as "Online Resource", e.g., "... as shown in the animation (Online Resource 3)", "... additional data are given in Online Resource 4".
- Name the files consecutively, e.g. "ESM_3.mpg", "ESM_4.pdf".

Captions

- For each supplementary material, please supply a concise caption describing the content of the file.

Processing of supplementary files

- Supplementary Information (SI) will be published as received from the author without any conversion, editing, or reformatting.

Accessibility

In order to give people of all abilities and disabilities access to the content of your supplementary files, please make sure that

- The manuscript contains a descriptive caption for each supplementary material
- Video files do not contain anything that flashes more than three times per second (so that users prone to seizures caused by such effects are not put at risk)

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Ethical Responsibilities of Authors

This journal is committed to upholding the integrity of the scientific record. As a member of the Committee on Publication Ethics (COPE) the journal will follow the COPE guidelines on how to deal with potential acts of misconduct.

Authors should refrain from misrepresenting research results which could damage the trust in the journal, the professionalism of scientific authorship, and ultimately the entire scientific endeavour. Maintaining integrity of the research and its presentation is helped by following the rules of good scientific practice, which include*:

- The manuscript should not be submitted to more than one journal for simultaneous consideration.
- The submitted work should be original and should not have been published elsewhere in any form or language (partially or in full), unless the new work concerns an expansion of previous work. (Please provide transparency on the re-use of material to avoid the concerns about text-recycling ('self-plagiarism').
- A single study should not be split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time (i.e. 'salami-slicing/publishing').
- Concurrent or secondary publication is sometimes justifiable, provided certain conditions are met. Examples include: translations or a manuscript that is intended for a different group of readers.
- Results should be presented clearly, honestly, and without fabrication, falsification or inappropriate data manipulation (including image based manipulation). Authors should adhere to discipline-specific rules for acquiring, selecting and processing data.
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* Based on/adapted from:

ICMJE, Defining the Role of Authors and Contributors,

Transparency in authors' contributions and responsibilities to promote integrity in scientific publication, McNutt at all, PNAS February 27, 2018

Disclosures and declarations

All authors are requested to include information regarding sources of funding, financial or non-financial interests, study-specific approval by the appropriate ethics committee for research involving humans and/or animals, informed consent if the research involved human participants, and a statement on welfare of animals if the research involved animals (as appropriate).

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A Graduate Student's Guide to Determining Authorship Credit and Authorship Order, APA Science Student Council 2006

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Research involving human participants, their data or biological material

Ethics approval

When reporting a study that involved human participants, their data or biological material, authors should include a statement that confirms that the study was approved (or granted exemption) by the appropriate institutional and/or national research ethics committee (including the name of the ethics committee) and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. If doubt exists whether the research was conducted in accordance with the 1964 Helsinki Declaration or comparable standards, the authors must explain the reasons for their approach, and demonstrate that an independent ethics committee or institutional review board explicitly approved the doubtful aspects of the study. If a study was granted exemption from requiring ethics approval, this should also be detailed in the manuscript (including the reasons for the exemption).

Retrospective ethics approval

If a study has not been granted ethics committee approval prior to commencing, retrospective ethics approval usually cannot be obtained and it may not be possible to consider the manuscript for peer review. The decision on whether to proceed to peer review in such cases is at the Editor's discretion.

Ethics approval for retrospective studies

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If human cells are used, authors must declare in the manuscript: what cell lines were used by describing the source of the cell line, including when and from where it was obtained, whether the cell line has recently been authenticated and by what method. If cells were bought from a life science company the following need to be given in the manuscript: name of company (that provided the cells), cell type, number of cell line, and batch of cells.

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Further information is available from the International Cell Line Authentication Committee (ICLAC).

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The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Ethics approval'.

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• This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No. ...).

• Approval was obtained from the ethics committee of University C. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

• The questionnaire and methodology for this study was approved by the Human Research Ethics committee of the University of D (Ethics approval number: ...).

Examples of statements to be used for a retrospective study:

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

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

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Informed consent

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Summary of requirements

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The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1a, 1b and 1c.

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