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EFEITOS DO TREINAMENTO MUSCULAR VENTILATÓRIO SOBRE O ESTRESSE OXIDATIVO EM RATOS COM DIABETES MELLITUS TIPO 2

Santa Maria, RS
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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: Profª. Drª. Liliane de Freitas Bauermann

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Carlos Cassiano Figueiró da Silva

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Aprovado em 10 de Setembro de 2021:

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Santa Maria, RS
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RESUMO

EFEITOS DO TREINAMENTO MUSCULAR VENTILATÓRIO SOBRE O ESTRESSE OXIDATIVO EM RATOS COM DIABETES MELLITUS TIPO 2

AUTOR: Carlos Cassiano Figueiró da Silva
ORIENTADOR: Prof. Dr. Rodrigo Boemo Jaenisch
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O diabetes mellitus (DM) é uma doença crônica que está em ascensão em todo o mundo. É classificada em: diabetes mellitus tipo 1 (DM 1) e diabetes mellitus tipo 2 (DM 2). O DM 2 é a forma mais prevalente, e que resulta na inadequada secreção das células β pancreáticas e do aumento da resistência periférica à insulina, ocasionando hiperglicemia. A hiperglicemia corrobora para a formação excessiva de espécie reativas de oxigênio (EROs) e provoca o aumento estresse oxidativo (EO). O treinamento muscular ventilatório (TMV) é uma ferramenta não farmacológica, que determina benefícios em pacientes com DM, entretanto não são conhecidos os efeitos sobre o EO no modelo experimental. Assim, o objetivo do presente estudo foi avaliar os efeitos do TMV sobre o EO em ratos com DM 2. Foram utilizados ratos Wistar machos, alocados em 4 grupos: Grupo 1: animais sem DM sedentários (Sham-Sed); Grupo 2: animais sem DM treinados (Sham-TMV); Grupo 3 animais com DM sedentários (DM-Sed) e Grupo 4: animais com DM treinados (DM-TMV). O DM 2 foi induzido através de uma dieta de alta densidade energética e baixa dose de estreptozotocina (35 mg/kg), enquanto os animais dos grupos Sham receberam somente ração padrão comercial. O protocolo de TMV foi realizado durante 6 semanas, 5 dias/semana 30min/dia. Após 24 horas do último dia de intervenção os animais foram eutanasiados e amostras sanguíneas e de tecidos (coração, pulmões, rins, diafragma, gastrocnêmio e sóleo) foram coletados, pesados e armazenados para posterior análises. O protocolo de TMV reduziu os níveis de TBARS no plasma de ratos com DM 2. Em relação aos marcadores antioxidantes, quando comparamos os grupos Sham-Sed com DM-Sed, houve uma redução da MTT nos pulmões, de NPSH no coração, pulmões e diafragma, e da SOD no coração, rins, gastrocnêmio e plasma, caracterizando o modelo experimental de DM 2. Após o protocolo de TMV houve um aumento da atividade da SOD nos pulmões e rins quando comparamos os grupos DM-Sed com DM-TMV. Em conclusão, o protocolo de TMV reduziu a atividade oxidante no plasma, aumentou a antioxidante nos pulmões e rins.

Palavras chaves: Diabetes Mellitus, Exercício Respiratório, Dano Oxidativo, Antioxidantes.

ABSTRACT

EFFECTS OF VENTILATORY MUSCLE TRAINING ON OXIDATIVE STRESS IN RATS WITH MELLITUS DIABETES TYPE 2

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Diabetes mellitus (DM) is a chronic disease that is on the rise all over the world. It is classified as: type 1 diabetes mellitus (DM 1) and type 2 diabetes mellitus (DM 2). DM 2 is the most prevalent form, resulting in inadequate secretion of pancreatic β cells and increased peripheral insulin resistance, causing hyperglycemia. Hyperglycemia supports the excessive formation of reactive oxygen species (ROS) and causes increased oxidative stress (EO). Ventilatory muscle training (VMT) is a non-pharmacological tool that provides benefits in patients with DM, however the effects on OS and inflammation in the experimental model are not known. Thus, the aim of the present study was to evaluate the effects of VMT on EO in rats with DM 2. Male Wistar rats were used, divided into 4 groups: Group 1: sedentary animals without DM (Sham-Sed); Group 2: trained animals without DM (Sham-VMT); Group 3 sedentary DM animals (DM-Sed) and Group 4: trained DM animals (DM-VMT). DM was induced through a high energy density diet and a low dose of streptozotocin (35 mg/kg), while the animals in the Sham groups received only standard commercial chow. The VMT protocol was performed for 6 weeks, 5 days/week 30min/day. Twenty-four hours after the last day of intervention, the animals were euthanized and blood and tissue samples (heart, lungs, kidneys, diaphragm, gastrocnemius and soleus) were collected, weighed and stored for further analysis. The VMT protocol reduced plasma TBARS levels in DM 2 rats. Regarding antioxidant enzymes, when comparing the Sham-Sed and DM-Sed groups, there was a reduction in MTT in the lungs, NPSH in the heart, lungs and diaphragm, and SOD in the heart, kidneys, gastrocnemius and plasma, characterizing the model experimental DM 2. After the VMT protocol, there was an increase in SOD activity in the lungs and kidneys when comparing the DM-Sed with DM-VMT groups. In conclusion, the VMT protocol reduced oxidant activity in plasma, increased antioxidant activity in lungs and kidneys.

Keywords: Diabetes Mellitus, Breathing Exercises, Oxidative Stress, Antioxidants.

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LISTA DE ABREVIATURAS E SIGLAS

CAT	Catalase
CCS	Centro de Ciências da Saúde
CEUA	Comitê de Ética no uso de Animais
CHCM	Hemoglobina Corpuscular Média
COBEA	Colégio Brasileiro de Experimentação Animal
CONCEA	Conselho Nacional de Controle de Experimentação Animal
DCF-RS	Diclorofluoresceína
DCFH-RS	Diclorofluoresceína reduzida
DM	Diabetes Mellitus
DM 1	Diabetes Mellitus Tipo 1
DM 2	Diabetes Mellitus Tipo 2
DMSO	Sulfóxido de Dimetilo
DNA	Ácido Desoxirribonucléico
EDTA	Ácido Etilenodiamina Tetraacético
EO	Estresse Oxidativo
ER	Espécie Reativa
ERN	Espécie Reativa de Nitrogênio
ERO	Espécie Reativa de Oxigênio
GAP	Gabinete de Projetos
GPx	Glutationa Peroxidase
GSH	Glutationa Reduzida
H_2O_2	Peróxido de Hidrogênio
HOBr	Ácido Hipobromoso
HOCl	Ácido Hipocloroso
IC	Insuficiência Cardíaca
IL-6	Interleucina – 6
IL-b	Interleucina – b
LaFEx	Laboratório de Fisiologia Experimental
MDA	Malonaldeído
MTT	Metiltetrazólio
NaCl	Cloreto de Sódio
NO	Óxido Nítrico

NPSH	Níveis de SH não proteico
OH-	Radicais Hidroxila
O_2-	Ânion Superóxido
PPT	Proteína Plasmática
S1	Fração sobrenadante de baixa velocidade
SOD	Superóxido Dismutase
STZ	Estreptozotocina
TBARS	Substância reativa ao Ácido Tiobarbitúrico
TCA	Ácido Tricloroacético
TFK	Tampão K-Fosfato
TMV	Treinamento Muscular Ventilatório
TNFα	Fator de Necrose Tumoral α
UFSM	Universidade Federal de Santa Maria
VCM	Volume Corpuscular Médio

SUMÁRIO

1. INTRODUÇÃO

O diabetes mellitus (DM) é uma doença que está em ascensão no mundo todo. Em 2045, a estimativa de pessoas com DM é de 700 milhões, principalmente em países de baixa e média renda (INTERNATIONAL DIABETES FEDERATION, 2019; WORLD HEALTH ORGANIZATION, 2016). No ano de 2017, o Brasil ocupava a quinta posição entre os países com maior número de pessoas com DM. Projeções para o ano de 2045 demonstram que 20,3 milhões de brasileiros serão portadores dessa patologia (DIRETRIZES DA SOCEIDADE BRASILEIRA DE DIABETES, 2018).

O DM possui duas classificações principais: i) diabetes mellitus tipo 1 (DM 1), que tem como causa uma resposta autoimune, onde as células que produzem insulina são acometidas pelo sistema de defesa do organismo, o que resulta em pouca ou nenhuma produção de insulina; ii) diabetes mellitus tipo 2 (DM 2), que caracteriza-se pela resistência à insulina (INTERNATIONAL DIABETES FEDERATION, 2019).

O DM 2 é a forma mais prevalente, e acomete cerca de 90-95% dos casos. É resultante da secreção inadequada das células β pancreáticas e do aumento da resistência periférica à insulina (AMERICAN DIABETES ASSOCIATION, 2019; INTERNATIONAL DIABETES FEDERATION, 2017). A perda de sensibilidade à insulina pelos tecidos promove a falta de captação de glicose, ocasionando a hiperglicemia, que de forma crônica como ocorre no DM, favorece a formação de radicais livres, culminando em um dano oxidativo (BANDEIRA et al., 2013; MOHAMED et al., 2016).

Essas alterações podem danificar a integridade funcional e estrutural das células β pancreáticas, formando espécies reativas de oxigênio (EROs) juntamente com outros pró-inflamatórios. (AKASH et al., 2012; AKASH; REHMAN; CHEN, 2013). Quando há o desequilíbrio entre o sistema oxidante, predominantemente caracterizado pela produção de radicais livres, e o sistema antioxidante, denominamos de estresse oxidativo (EO). Visto que, quando produzido em excesso, os radicais livres ocasionam danos oxidativos, havendo a necessidade de o organismo desenvolver mecanismos de defesa, capaz de neutralizar as espécies reativas (ER) (REHMAN; AKASH, 2017).

Dessa forma, é importante que haja uma manutenção do equilíbrio entre os níveis de ER e a capacidade das enzimas antioxidantes, pois esse desequilíbrio pode ocasionar o desenvolvimento de várias patologias e doenças crônicas, dentre elas o DM (REHMAN;

AKASH, 2017). Diante disso, há inúmeras complicações associadas ao DM que podem ser causadas pela hiperglicemia e EO, como: disfunção endotelial, complicações cardiovasculares e hepáticas, retinopatia, neuropatia, nefropatia e alterações musculares (ASFANDIYAROVA et al., 2006; MAHBOOB; RAHMAN; GROVER, 2005; WERF et al., 2018).

Alguns estudos demonstraram que o EO tem uma forte ligação para a patogênese do DM, potencializando a geração de vários mediadores inflamatórios, além do aumento nos níveis de agentes oxidantes e redução da atividade das enzimas antioxidantes (BURGOS-MORÓN et al., 2019; GOBOZA et al., 2019; REHMAN; AKASH, 2016, 2017). A hiperglicemia é um dos fatores principais que corrobora para a formaçao excessiva de EROs, juntamente com a redução da defesa antioxidante provoca o aumento do EO no DM (BARSSOTTI et al., 2021). Estudos experimentais em modelo de DM já confirmaram o aumento da atividade oxidante decorrente do quadro hiperglicêmico (OGAR et al., 2019; SANKARANARAYANAN; KALAIVANI, 2020; SARANDOL et al., 2020), através dos níveis de substância reativa ao ácido tiobarbitúrico (TBARS) (SANKARANARAYANAN; KALAIVANI, 2020) e a redução da atividade das enzimas antioxidantes, como os níveis de SH não proteico (NPSH) e da superóxido dismutase (SOD) (DOS SANTOS et al., 2017; GOBOZA et al., 2019). Além de induzir processos crônicos de EO, o DM ocasiona inflamação vascular, aumentando a ativação de neutrófilos e a liberação de enzimas que elevam a atividade inflamatória (WEIHRAUCH et al., 2020).

O treinamento muscular ventilatório (TMV) é uma ferramenta não farmacológica que tem como objetivo aumentar a força e resistência dos músculos ventilatórios (LANGER et al., 2018, ARCHIZA et al., 2018 YEH 2019, AZAMBUJA 2020). Alguns estudos verificaram que, o TMV em indivíduos com DM 2 promoveu o aumento da pressão inspiratória máxima (CORREA et al., 2011; KAMINSKI et al., 2015, MOAWD 2020, ALBARRATI 2021), a redução dos níveis de glicose (CORRÊA et al., 2015; SCHEIN et al., 2020; SILVA et al., 2012), a melhora da resistência insulínica (SILVA et al., 2012), e consequentemente a melhora da capacidade funcional (MOAWD et al., 2020).

Estudos que utilizaram o TMV no modelo experimental animal encontraram resultados interessantes nos últimos anos. Em ratos saudáveis, pesquisadores verificaram o aumento da espessura e hipertrofia do músculo diafragma (BISSCHOP et al., 1997). O nosso grupo vem contribuindo com o conhecimento sobre o TMV no modelo experimental. Em ratos com insuficiência cardíaca (IC); verificamos que o protocolo de TMV, proporcionou melhora da função hemodinâmica, da sensibilidade barorreflexa e da mecânica respiratória (JAENISCH et

al., 2011), além de benefícios na variabilidade da frequência cardíaca (JAENISCH et al., 2017a). Quando analisamos os efeitos do TMV sobre o diafragma verificamos que, em ratos com IC, o TMV aumentou a enzima citrato sintase (JAENISCH et al., 2017b) e reduziu dano do DNA (JAENISCH et al., 2018). Recentemente publicamos um estudo que demonstrou que após o protocolo de TMV em ratos com DM, houve o aumento na atividade parassimpática, o que sugere benefícios frente o controle autônomo e função cardiovascular (TREVISAN et al., 2021).

Considerando que, o DM pode ocasionar complicações locais e/ou sistêmicas, por meio do aumento da atividade oxidante, redução da antioxidante, (ALHAIDER et al., 2011; GUEX et al., 2019), novas ferramentas terapêuticas não farmacológicas, como o TMV, no modelo experimental de DM, podem ser testadas frente a essas alterações.

Até o nosso conhecimento, há apenas um estudo que utilizou o TMV como forma de tratamento em ratos com DM. Diante disso, a hipótese do nosso estudo é a de que o TMV possa melhorar o EO em ratos com DM 2. Acreditamos que o presente estudo possa esclarecer e fortalecer os efeitos do TMV, além de elucidar os efeitos fisiopatológicos do DM 2 no modelo experimental.

Este projeto fez parte de um projeto guarda-chuva intitulado “Efeitos do treinamento muscular ventilatório e da laserterapia sobre o perfil inflamatório e o estresse oxidativo em ratos com diabetes mellitus tipo 2”, o qual foi registrado no Gabinete de Projetos (GAP) (ANEXO A) do Centro de Ciências da Saúde (CCS), aprovado pelo Comitê de Ética no Uso de Animais (CEUA) da UFSM (ANEXO B) sob o número 6622101118, autorizado pelo Departamento de Fisiologia e Farmacologia (APÊNDICE C) e do laboratório de Fisiologia Experimental (LaFEx) (APÊNDICE D). O pesquisador responsável teve o termo de compromisso (APÊNDICE A) e termo de responsabilidade (APÊNDICE B) assinados, seguindo as normas conforme diretrizes do Conselho Nacional de Controle de Experimentação Animal (CONCEA).

2. REVISÃO DE LITERATURA

2.1. DIABETES MELLITUS

O diabetes mellitus (DM) é uma doença que está em ascensão no mundo todo. Em 2045, a estimativa de pessoas com diabetes é de 700 milhões, principalmente em países de baixa e média renda (INTERNATIONAL DIABETES FEDERATION, 2019; WORLD HEALTH ORGANIZATION, 2016). O Brasil tem apresentado um aumento na incidência de DM e, nas últimas décadas, vem sendo considerado o país com o quarto maior número de indivíduos acometidos por essa patologia (DE ALMEIDA-PITITTO et al., 2015; DIRETRIZES DA SOCEIDADE BRASILEIRA DE DIABETES, 2018).

O DM é considerado uma doença metabólica crônica, e ocorre quando o pâncreas não produz insulina suficiente, ou essa é incapaz de exercer de forma adequada a sua função (INTERNATIONAL DIABETES FEDERATION, 2017, 2019). A perda de sensibilidade à insulina pelos tecidos promove a falta de captação de glicose, ocasionando a hiperglicemia, caracterizada por níveis elevados de glicose no sangue ocasionando um distúrbio no metabolismo dos carboidratos, lipídios e proteínas (INTERNATIONAL DIABETES FEDERATION, 2017, 2019). Além disso, a hiperglicemia crônica possui associação a longo prazo com disfunções, danos e falência de diferentes órgãos, como os olhos, nervos, rins e vasos sanguíneos (AMERICAN DIABETES ASSOCIATION, 2019).

O DM pode ser categorizado em duas formas principais: diabetes tipo 1 (DM 1) ou tipo 2 (DM 2) (AMERICAN DIABETES ASSOCIATION, 2019). O DM 1, que acomete de 5-10% dos casos, tem como causa a deficiência absoluta da secreção de insulina, há presença de autoanticorpos que destroem as células β pancreáticas, tornando o paciente insulinodependente. O DM 2, considerada a forma mais prevalente da doença, acometendo entre 90-95% dos casos, é resultante de um defeito progressivo da secreção de insulina e resistência à insulina, devido a um desgaste do pâncreas por um processo de hiperfuncionamento compensatório (AMERICAN DIABETES ASSOCIATION, 2019; INTERNATIONAL DIABETES FEDERATION, 2017).

Alguns estudos demonstraram que um dos principais fatores causais para o desenvolvimento de EO é a patogênese do DM 2, além da redução da secreção de insulina através das células β pancreáticas e o desenvolvimento da resistência insulínica (AKASH; REHMAN; CHEN, 2013; REHMAN; AKASH, 2017). Vários fatores estão envolvidos na

indução do EO, como: mediadores pró-inflamatórios, resistência à insulina, hiperglicemia e dislipidemia, inatividade física e tolerância à glicose reduzida. Esses fatores podem danificar a integridade funcional e estrutural das células β pancreáticas, além do mais, a formação de EROs juntamente com outros pró-inflamatórios são potencializadas pelo EO, podendo interromper o fluxo sanguíneo nas células β pancreáticas abolindo sua função (AKASH et al., 2012; AKASH; REHMAN; CHEN, 2013).

O EO é induzido nas ilhotas pancreáticas por diversos fatores, dentre eles, a hiperglicemia, que faz com que aumente o estresse celular (REHMAN; AKASH, 2017; TIEDGE et al., 1998). Segundo Grankvist et al., 1981 e Tiedge et al., 1998, nas células β pancreáticas os níveis de capacidade das enzimas antioxidantes são muito baixos quando comparadas a outros tecidos metabólicos, além de que, essas células são mais vulneráveis e suscetíveis à geração de EROs e EO (GRANKVIST; MARKLUND; TALJEDAL, 1981; TIEDGE et al., 1998). Alguns estudos experimentais, encontraram que a capacidade antioxidante total reduz significativamente em DM 2, indicando a diminuição dos níveis de componentes antioxidantes enzimáticos e não enzimáticos (DEMIRCAN et al., 2008; FORD et al., 2003), ao mesmo tem em que os níveis biomarcadores de EO são aumentados (DEMIRCAN et al., 2008).

Uma vez que o DM ocorre, há uma produção excessiva de radicais livres que é ocasionado por vários mecanismos, além da redução da atividade das enzimas antioxidantes. Diante disso, há inúmeras complicações associadas ao DM que podem ser causadas pela hiperglicemia e EO, como: disfunção endotelial, complicações cardiovasculares e hepáticas, retinopatia, neuropatia e nefropatia (ASFANDIYAROVA et al., 2006; MAHBOOB; RAHMAN; GROVER, 2005; WERF et al., 2018).

2.2. ESTRESSE OXIDATIVO

2.2.1. Espécies Reativas e Radicais Livres

O nosso organismo possui um sistema antioxidante complexo, que atua como mecanismo de defesa contra radicais livres, que constantemente são formados no metabolismo celular que em excesso ocasionam oxidação de moléculas biológicas (HALLIWELL, 2006). O

desequilíbrio de pró-oxidantes e antioxidantes que resultam em danos celulares é chamado de EO (HALLIWELL, 2006; HALLIWELL; WHITEMAN, 2004).

Os radicais livres podem ser produzidos de forma natural ou por alguma disfunção biológica sendo produto da oxidação, processo fundamental da vida aeróbica e do metabolismo celular. O que caracteriza um radical livre é um ou mais elétrons desemparelhados em sua órbita externa sendo moléculas altamente instáveis. Quando esses elétrons desemparelhados encontram-se centrados nos átomos de oxigênio ou nitrogênio, são denominadas espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN). As espécies reativas são moléculas que possuem uma alta reatividade e podem ser radicais livres ou compostos não radicalares (HALLIWELL, 2006).

As EROs são um dos principais potenciadores do EO, já que sua formação é um subproduto inevitável do metabolismo (NOWOTNY et al., 2015), quando essas espécies não são removidas de maneira eficaz e segura, o EO pode ser prejudicial à saúde. Há diversas razões para a ocorrência do EO, uma delas é a exaustão dos sistemas antioxidantes, o que resulta na excessiva produção de EROs ou radicais livres, como: ânions superóxido (O_2-) e radicais hidroxila (OH-), além disso as EROs incluem compostos não radicalares, como: peróxido de hidrogênio (H_2O_2), ácido hipocloroso (HOCl) e ácido hipobromoso (HOBr) (PUPPEL; KAPUSTA; KUCZYŃSKA, 2015).

O desencadeamento de danos oxidativos a biomoléculas celulares pode acontecer sempre que a formação de espécies reativas, principalmente EROs, for superior a capacidade antioxidante celular de neutralizá-las, processo esse conhecido como EO. Inúmeras alterações celulares possuem associação com o EO, dentre elas: modificação nas funções enzimáticas, comprometimento na funcionalidade nuclear, alterações no funcionamento mitocondrial, além de participar ativamente no processo fisiopatológico de diversas doenças crônicas (BARBOSA et al., 2010; HALLIWELL; CROSS, 1994).

2.2.2. Sistemas de defesa antioxidantes

O dano celular induzido pelo EO, em condições fisiológicas normais é normalmente modulado através da capacidade antioxidante auto protetora da célula, chamado de sistema de defesa antioxidante, responsável por neutralizar as espécies reativas geradas em excesso que podem levar o organismo à situação de estresse. As duas classes principais desses mecanismos

antioxidantes celulares são: as defesas enzimáticas (catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx)) e as não – enzimáticas (glutationa reduzida (GSH) e as vitaminas A, C e E) (HALLIWELL; CROSS, 1994; REHMAN; AKASH, 2017).

É importante que haja uma manutenção do equilíbrio entre os níveis de espécies reativas (ER) e a capacidade das enzimas antioxidantes, para o funcionamento fisiológico normal de órgãos e sistemas. Diante disso qualquer desequilíbrio entre esses níveis pode ocasionar o desenvolvimento de várias patologias e doenças crônicas, dentre elas a DM 2 e suas complicações (REHMAN; AKASH, 2017).

2.2.3. Dano oxidativo

Como consequência da ação deletéria das ER sobre as biomoléculas, há a manifestação de danos oxidativos. Para que esse evento não ocorra, é necessário o equilíbrio entre a geração de ER e a capacidade de defesa do sistema antioxidante. Tanto a redução da capacidade de defesa do sistema antioxidante, quanto a produção excessiva de ER podem ocasionar o comprometimento biomolecular, mecanismo esse, conhecido como dano oxidativo (HALLIWELL, 2006). Os biomarcadores de dano oxidativo são classificados de acordo com a biomolécula sobre a qual as ER atuam: proteína, lipídios ou ácido desoxirribonucleico (DNA) (HALLIWELL; WHITEMAN, 2004), também podendo serem avaliados por métodos indiretos, baseados na capacidade antioxidante (HUANG; BOXIN; PRIOR, 2005).

2.3. TREINAMENTO MUSCULAR VENTILATÓRIO

O treinamento muscular ventilatório (TMV) é uma ferramenta não farmacológica que vem demonstrando diversos benefícios em diferentes populações. Os músculos ventilatórios, ao serem treinados, resultam em aumento da força e da resistência muscular ventilatória (LANGER et al., 2018; MCCONNELL, 2009), que determina uma redução da fadiga dos músculos respiratórios, a redução na sensação de dispneia (LAOUTARIS et al., 2008), aumento da eficiência ventilatória, da capacidade funcional (DALL'AGO et al., 2006) e, consequentemente a melhora da qualidade de vida (LIN et al., 2012; TURNER et al., 2012).

Além de promover benefícios em indivíduos saudáveis (ILLI et al., 2012) e em atletas de elite (JÚNIOR; GÓMEZ; NETO, 2016), o TMV proporciona efeitos benéficos em diferentes populações com diversas condições patológicas. Estudos verificaram bons resultados sobre o TMV em pacientes com doença pulmonar obstrutiva crônica (LANGER et al., 2018), no pré e pós operatório de cirurgia cardíaca (KODRIC et al., 2013; SAVCI et al., 2011), em pacientes com IC (SMART; GIALLAURIA; DIEBERG, 2013; WANG; YEH, 2019), com insuficiência renal (PELLIZZARO; THOMÉ; VERONESE, 2013) e em diabéticos (KAMINSKI et al., 2011; SILVA et al., 2012; ALBARRATI 2020).

Alguns pesquisadores verificaram os efeitos do TMV em indivíduos com DM sobre desfechos importantes. O TMV foi capaz de aumentar a força e a resistência muscular ventilatória (CORREA et al., 2011; KAMINSKI et al., 2015), reduzir os níveis de glicose (CORRÊA et al., 2015; SILVA et al., 2012; SCHEIN ET AL., 2020; PINTO ET AL., 2021, MOAWD 2020, ALBARRATI 2021), melhorar à resistência insulínica e os índices glicêmicos em idosos (SILVA et al., 2012), além de aumentar a capacidade funcional (MOAWD et al., 2020).

Com o intuito de analisar o efeito do TMV no modelo experimental, alguns autores desenvolveram estudos em ratos, tanto saudáveis quanto com doenças cardiometabólicas. Bisschop et al., (1997), realizaram um protocolo de TMV de 8 semanas em ratos saudáveis, e encontraram um aumento da espessura diafragmática e hipertrofia de fibras do tipo IIa e IIX/b. Jaenisch et al. (2011) encontraram, após um protocolo de TMV de 6 semanas em ratos com IC, a melhora da função hemodinâmica, da sensibilidade barorreceptora e da mecânica respiratória (JAENISCH et al., 2011), além do aumento da enzima citrato sintase (JAENISCH et al., 2017b) e redução ao dano do DNA no músculo diafragma, o que forneceu indiretamente a possível melhora do EO no modelo experimental (JAENISCH et al., 2018). Em um estudo que utilizou o TMV em ratos com DM induzido pela estreptozotocina, foi encontrado que essa ferramenta resultou na melhora da modulação parassimpática, o que contribui para o melhor entendimento dessa ferramenta no sistema nervoso autônomo (TREVISAN et al., 2021).

Até o momento, resultados interessantes foram obtidos utilizando o TMV em ratos com doenças cardiometabólicas. Entretanto, até o nosso conhecimento, nenhum estudo verificou o efeito do TMV sobre o estresse oxidativo e a atividade inflamatória em ratos com DM.

3. OBJETIVOS

3.1. OBJETIVO GERAL

Avaliar o efeito do TMV, por um período de 6 semanas, sobre o estresse oxidativo em ratos com DM 2.

3.2. OBJETIVOS ESPECÍFICOS

Analisar o impacto do TMV sobre o TBARS no plasma sanguíneo em ratos com DM 2.

Avaliar o impacto do TMV sobre a atividade oxidante por meio da MTT, NPSH e SOD no coração, pulmões, rins, diafragma, gastrocnêmio e plasma sanguíneo em ratos com DM 2.

4. ARTIGO

Effects of ventilatory muscle training on stress in rats with mellitus diabetes type 2

Ventilatory muscle training in rats with mellitus diabetes type 2

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Brief Summary

Our study demonstrated that, after induction of DM 2 in rats, there was an increase in oxidant activity, and a reduction in antioxidant activity. Furthermore, VMT in rats with DM 2 reduced plasma TBARS levels and increased SOD in the lungs and kidneys.

Abstract

Objective: To evaluate the effects of VMT on oxidative stress in rats with DM 2.

Method: Male Wistar rats were allocated into 4 groups: Group 1: animals without DM 2 sedentary (Sham-Sed); Group 2: animals without DM 2 trained (Sham-VMT); Group 3 sedentary animals with DM 2 (DM-Sed) e Group 4: animals with DM 2 trained (DM-VMT). The VMT protocol was performed for 6 weeks, 5 days/week 30min/day. After 24 hours of the last day of intervention, the animals were euthanized and blood and tissue samples (heart, lungs, kidneys, diaphragm, gastrocnemius and soleus) were collected, weighed and stored for further analysis.

Results: The VMT protocol reduced the levels of TBARS in the plasma of rats with DM 2. Regarding antioxidant enzymes, there was a reduction in NPSH levels of the DM group in the heart, lungs and diaphragm comparing the DM-Sed group with Sham-Sed. VMT increased SOD activity in the lungs and kidneys of rats with DM 2 when comparing the DM-Sed and DM-VMT groups.

Conclusion: The VMT protocol reduced plasma TBARS levels and increased SOD in the lungs and kidneys.

Keywords: Diabetes Mellitus, Breathing Exercise, Oxidative Damage.

Introduction

Diabetes mellitus (DM) is a disease that is on the rise worldwide. In 2045, the estimated number of people with DM is 700 million [1,2]. The loss of insulin sensitivity in the tissues promotes the lack of glucose uptake, causing the hyperglycemia that chronically occurs in DM. Favors the formation of free radicals, which culminates in oxidative damage [3,4].

These changes can damage the functional and structural integrity of pancreatic β cells, with consequent formation of reactive oxygen species (ROS) [5]. In addition, inflammatory activity is potentiated by the oxidative stress (EO) generated, which can cause damage to pancreatic β-cell blood flow, altering their function [5,6]. Therefore, there are numerous complications associated with DM that can be caused by hyperglycemia and EO, such as: endothelial dysfunction, cardiovascular and hepatic complications, retinopathy, neuropathy, nephropathy and muscle changes [7,9].

Some studies have shown that EO potentiates the increase in oxidant enzyme levels, reduces antioxidants, as well as exacerbates inflammatory mediators [10,12]. Experimental studies in the DM model confirmed the increase in oxidant activity resulting from the hyperglycemic condition [12,14], through levels of thiobarbituric acid reactive substance (TBARS) [13], and reduced activity of antioxidant enzymes, such as non-protein SH (NPSH) and superoxide dismutase (SOD) levels [11,15].

Ventilatory muscle training (VMT) is a non-pharmacological tool that aims to increase the strength and endurance of the ventilatory muscles. [16,17]. Some studies found that the VMT in individuals with DM 2 promoted an increase in maximal inspiratory pressure [18,19], the reduction of glucose levels [20,21], improving insulin resistance [22], in addition to improving autonomous control [19] and functional capacity [23].

Although our group has already demonstrated that VMT improves hemodynamic function, baroreflex sensitivity [24], in addition to heart rate variability [25] and reduced DNA [26] damage in rats with heart failure, only one study associated VMT in rats with DM [21]. We recently published a study that demonstrated that after the VMT protocol in rats with DM, there was an increase in parasympathetic activity, which suggests benefits in terms of autonomic control and cardiovascular function [21].

Therefore, the present study aimed to evaluate whether the VMT protocol can improve OS in rats with DM 2, in addition, we believe that it is able to clarify and strengthen the effects

of VMT, in addition to elucidating the pathophysiological effects of DM 2 in the model experimental.

Materials and Methods

Use of experimental animals

The research was carried out at the Experimental Physiology Laboratory of the Federal University of Santa Maria (LaFEx, UFSM) – RS. For this study, 32 male Wistar rats, 7 weeks old, weighing approximately 200 - 250g were used. The animals came from the Central Animal Facility of UFSM, isolated in polypropylene boxes (41 x 33 x 16 cm) in a group of 3 animals per box, with a floor covered with shavings. The animals were kept in the rat vivarium of building 21 of the UFSM in a room with controlled temperature and humidity (22 ± 2ºC and 50 to 60%), with air exhaustion and a 12-hour "light-dark" cycle. The animals were acclimatized for 14 days prior to the beginning of the experiment and they received water and food ad libitum. All animals were maintained and handled in accordance with the ethical principles in animal experimentation developed by the Brazilian College of Animal Experimentation (COBEA), and following principles determined in law nº 11,794, of October 08, 2008.

DM 2 induction

After the acclimatization period, the animals had their basal blood glucose checked with a manual glucometer (G TECH FREE Lite, Infopia Co., Ltd., South Korea), through a small puncture in the caudal vein, being collected 0.1 – 0.5 ml of blood [27]. For DM 2 induction, the animals were fed a high-energy-density diet (70% standard commercial chow, 15% sucrose, 10% lard and 5% yolk powder) [28] for an initial period of four weeks.

The animals in the control group received standard commercial chow and all animals received water ad libitum. The animals were submitted to a 12-hour fast and on the 29th day the blood glucose was checked again, on the 30th day the animals of the DM 2 group were administered a single dose of streptozotocin (STZ) - 35 mg/kg - intraperitoneally (ip) , dissolved in vehicle (0.01 M sodium citrate solution, pH = 4.5) with a volume of 1 ml/kg [29,30]. The animals in the Sham group that consumed a standard diet received only the vehicle intraperitoneally. One week after the administration of STZ or vehicle, the fasting blood glucose was checked again and the animals that presented blood glucose greater than or equal to 200 mg/dL were considered diabetic. The groups received their respective diets for another four weeks and then

the VMT protocol was started. The diets were followed as described above until the end of the experiment [31] and blood glucose was checked again one day before euthanasia.

Animals that did not become diabetic after drug infusion and a hypercaloric diet received humane euthanasia with anesthetic overload according to the regulations of the National Council for the Control of Animal Experiments (CONCEA).

Experimental draw

The animals were divided into 4 experimental groups:

Group 1: Sedentary Sham rats (Sham-Sed; n=8).

Group 2: Trained Sham rats (Sham-VMT; n=8).

Group 3: DM 2 Sedentary rats (DM-Sed; n=8).

Group 4: Rats with DM 2 trained (DM-VMT; n=8).

Ventilatory Muscle Training Protocol

To perform the VMT, the animals were conditioned to breathe through a one-way valve connected to a mask. Conditioning was performed so that the position of the animal's head is adequate (at the anterior end of the equipment where resistance is carried out during breathing, which makes the training effective) [24].

The VMT protocol was performed for 30min/day, 5 days/week, for 6 weeks [21,24]. The load offered, for the training of the ventilatory muscles, was generated by means of an alinear resistor, coupled to an acrylic cylinder, with an initial hole diameter of 0.8 mm. At the end of the first week, the diameter was reduced to 0.3 mm, imposing maximum resistance to the training [21,32].

Euthanasia

At the end of the 6-week protocol, 24 hours after the last day of intervention, the animals were euthanized under deep anesthesia with isoflurane (4% - until the animal showed no reflex) [33]. To complete the euthanasia of the animals, blood was collected by cardiac puncture (1 to 2mL). To complete the euthanasia of the animals, blood was collected by cardiac puncture (1 to 2mL). Confirmation of the death of the animals was made by observing the respiratory arrest for a time greater than 180 seconds. In addition, the absence of respiratory movement (apnea);

absence of heartbeat (asystole); absence of pulse, pale mucous membranes, and loss of corneal reflex [34]. The same protocol was used in case of humanitarian euthanasia.

The organs heart, lungs, liver and kidneys, in addition to the diaphragm, gastrocnemius and soleus muscles, were collected and weighed. The samples were homogenized and stored in a freezer at -80ºC and the blood was centrifuged and plasma extracted for further biochemical analysis.

Tissue preparation

Heart, lung, liver and kidney organs were removed, weighed and homogenized (UltraTurrax, Staufen, Germany) in phosphate buffer (heart 1:4, lung 1:5, liver 1:10 and kidney 1:10 – g/mL). The diaphragm, gastrocnemius and soleus muscles were removed, weighed and homogenized (UltraTurrax, Staufen, Germany) in sodium chloride (NaCl) (0.9%) (diaphragm 1:10 and gastrocnemius 1:10 – g/mL). After homogenization, the organ and muscle samples were centrifuged at 3000 rpm for 10 minutes (SPIN MAX 80-2B, Didactic SP, SP), in order to obtain a low-speed supernatant fraction (S1), which was used for different assays. biochemicals [35].

Protein quantification

Protein content was measured according to the method described by Lowry et al., using bovine serum albumin as a standard. Aliquots of 20 μL of the supernatant fraction from the organs and muscles and 180 μL of comassie were pipetted into a cell culture plate – 90 wells and measured at 595 nm (SpectraMax® i3x Multi-Mode Microplate Reader) [36].

Determination of TBARS levels

TBARS levels were determined according to the method described by Ohkawa et al. Plasma homogenate (40 μL of sample) was placed in an eppendorf and 20 μL of distilled water, 100 μL of acetic acid, 100 μL of thiobarbituric acid (TBA) and 40 μL of sodium dodecyl sulfate (SDS) were added. Then, they were incubated for 120 minutes at 100°C. 500 μL aliquots of the S1 plasma supernatant fraction were added to the color reaction. TBARS levels were measured at 532 nm (SpectraMax® i3x Multi-Mode Microplate Reader) using malondialdehyde (MDA) standard curve and corrected for protein content [37].

Determination of mitochondrial dehydrogenase activity (MTT reduction assay)

The homogenates of organs (heart and lungs), muscles (diaphragm and gastrocnemius) and plasma (27 μL of sample) are incubated with 2.7 μL of MTT in eppendorf at 34ºC for 60min. After this period, 270 μL of dimethyl sulfoxide (DMSO) are added to extract colored components, transferred to a cell culture plate – 90 wells and measured at 570 nm (SpectraMax® i3x Multi-Mode Microplate Reader). Results are expressed as a percentage of control values [38].

Determination of non-protein SH (NPSH) levels

NPSH levels were determined in organ homogenates (heart and lungs), muscle (diaphragm and gastrocnemius) and plasma through 134 μL of sample precipitated with 67 μL of TCA (5%) and subsequently centrifuged in eppendorf at 2000 rpm for 10 minutes in a microtube centrifuge (Hitachi Medical Systems - CF15RX II). 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 a cell culture plate. – 90 wells. Measurements at 412 nm (SpectraMax® i3x Multi-Mode Microplate Reader). The results were calculated in relation to a standard curve constructed with GSH (reduced glutathione) at known concentrations (98, 58, 48, 38, 28 μL of distilled water, respectively; 100 μL of TFK; 0, 40, 50, 60 μL of GSH, respectively and 2 μL of DTNB) and corrected for the protein content [39].

Superoxide dismutase (SOD)

In the SOD analysis, the homogenates of the samples were added to a medium containing ethylenediamine tetraacetic acid (2mM EDTA) and bicarbonate buffer (50mM NaHCO3/Na2CO3, pH 10.3). Epinephrine (4mM) was added at the time of plate reading to initiate SOD kinetic activity for 5 minutes. The colored product of epinephrine degradation (which was inhibited by SOD cellular activity) was verified spectrophotometrically at 480 nm. SOD enzyme activity was expressed in units of enzyme activity per milligram of protein [40].

Statistical analysis

The program used was GraphPad Prism (GraphPad Software, CA, USA). Data distribution was evaluated using the Kolmogorov-Smirnov normality test. Variables with more than two measurements were compared by two-way ANOVA for repeated measurements followed by post hoc Bonferroni. The significance level of 5% was considered significant ($p<0.05$).

Results

The experiment started with 32 animals. During the DM 2 and VMT induction protocol, there were two losses, 1 from the DM-Sed Group and 1 from the DM-VMT Group. 30 animals completed the experiment. Body weight and blood glucose are shown in Table 1. In relation to body weight, in all groups there was an increase ($p<0.05$) at the end of the experiment, compared to the initial weight. In addition, the groups with DM, at the end of the experiment, showed an increase ($p<0.05$) in blood glucose (Table 1).

Determination of TBARS levels

Regarding plasma TBARS levels, DM groups showed higher levels ($p<0.001$) when compared to Sham groups. When comparing DM-Sed versus DM-VMT diabetic groups, the trained group had lower ($p<0.001$) levels of TBARS, indicating that VMT was able to reduce this marker in the plasma of rats with DM (Figure 2).

Determination of mitochondrial dehydrogenase activity (MTT reduction assay)

The DM-Sed group showed a lower MTT activity in the lungs when compared to the Sham-Sed group ($p<0.01$), characterizing the experimental model of DM. In the gastrocnemius muscle, when we compared the Sham-VMT group with the DM-VMT, the DM group showed higher ($p<0.05$) MTT activity. No differences were observed in heart, diaphragm and plasma (Table 2).

Determination of non-protein SH (NPSH) levels

In the heart, when we compared Sham-Sed with DM-Sed, the DM group showed lower levels ($p<0.001$). Comparing the DM-VMT group to the Sham-VMT group, the DM group had lower levels ($p<0.05$) of this marker. The DM groups had lower levels in relation to NPSH. The

Sham-VMT group had lower levels ($p<0.001$) compared to the Sham-Sed group, showing that the VMT group reduced NPSH levels when compared to the untrained group.

In the lungs, the DM-Sed group presented lower ($p<0.05$) levels of NPSH when compared to the Sham-Sed group, characterizing the experimental model of DM. When comparing the Sham-VMT group with the Sham-Sed group, the trained group showed lower levels ($p<0.01$).

Regarding the NPSH levels in the diaphragm muscle, the DM-Sed and DM-VMT groups, when compared to the Sham-Sed and Sham-VMT groups, respectively, the DM groups showed lower levels ($p<0.001$), confirming the reduction of antioxidant markers in an experimental model of DM.

In the gastrocnemius muscle, the DM-VMT group compared to the Sham-VMT group showed lower levels ($p<0.001$), evidencing that VMT in healthy rats increases NPSH levels. When comparing two healthy groups, the Sham-VMT group compared to the Sham-Sed group showed higher levels ($p<0.01$) of NPSH. The NPSH results are shown in Table 2.

Superoxide dismutase (SOD)

When comparing the Sham-Sed and Sham-VMT groups with the DM-Sed and DM-VMT groups, respectively, the DM groups showed lower levels ($p<0.01$) in SOD activity in the heart.

Regarding SOD activity in the lungs, the DM-Sed group, when compared to the Sham-Sed group, showed higher levels ($p<0.001$), we also found higher levels ($p<0.001$) in the DM-VMT group compared to the Sham-VMT group . When comparing the two diabetic groups, the DM-VMT group had a higher ($p<0.001$) SOD activity.

In the kidneys, when comparing DM-Sed with Sham-Sed and DM-VMT with Sham-VMT, the DM groups showed lower levels ($p<0.05$) in SOD activity. When we compared the Sham-Sed group with Sham-VMT and DM-Sed with DM-VMT, the groups that received VMT showed higher levels ($p<0.001$) in SOD activity, showing benefits of VMT.

In the diaphragm muscle, the DM-VMT group compared to the Sham-VMT group showed lower levels ($p<0.001$) in SOD activity, while when we compared the two Sham groups, the VMT group showed higher levels ($p<0.01$).

Regarding SOD activity in the gastrocnemius muscle, when we compared DM-Sed and DM-VMT with Sham-Sed and Sham-VMT, respectively, lower levels ($p<0.05$) were found in

the DM groups. On the other hand, when comparing Sham-Sed with Sham-VMT, the sedentary group presented higher levels ($p<0.01$).

In plasma comparing the DM-Sed groups with Sham-Sed and DM-VMT with Sham-VMT, the DM groups showed lower levels ($p<0.001$). When comparing the two Sham groups, the VMT group showed higher levels of this marker ($p<0.05$). The results of SOD activity are shown in Table 2.

Discussion

Some studies have already evaluated the effect of this VMT protocol in an experimental model in healthy rats and, in recent years, our group has found interesting results from the use of this tool in rats with heart failure [32] and, in recent years, our group has found interesting results from the use of this tool in rats with heart failure [24,26,41]. We recently published a study that verified the effects of VMT on hemodynamic function, heart rate variability and muscle morphology in rats with DM [21]. We believe that the present study is the first to investigate the effects of VMT on oxidative stress in rats with DM 2. Therefore, we demonstrated the effectiveness of the DM 2 induction protocol, since the final glycemia of the DM-Sed and DM-VMT groups increased at the end of the experiment compared to the Sham groups. The induction of DM 2 was through feeding with a high energy density diet, associated with a low dose of STZ, other studies confirm that this combination induces DM in rats, increasing glycemic indices and other markers [21,29,30,42].

The beneficial effects of VMT in different populations and pathologies are already well established in the literature [48,51]. Regarding the use of this tool in patients with DM 2, some studies show that VMT caused an increase in maximal inspiratory pressure, a reduction in glucose levels, an improvement in insulin resistance, in addition to an improvement in autonomous control and functional capacity [18,19], a reduction in glucose levels [20,22], an improvement in insulin resistance [22], in addition to an improvement in autonomous control [19] and functional capacity [23].

Based on the determination of TBARS levels, in our study we found an increase in plasma in the DM groups. Hyperglycemia increases the formation of ROS which, in combination with reduced antioxidant defense, leads to increased oxidative stress in diabetics [46]. In addition, experimental studies in a DM model found an increase in oxidant activity as a consequence of the hyperglycemic state that induces lipid peroxidation [13,14,47]. These findings

are in agreement with our study, which showed an increase in oxidant markers in STZ-induced DM rats [13,48,49], as Sankaranarayanan et al. found an increase in plasma TBARS levels [13].

Our results showed that after 6 weeks of VMT, it was possible to reduce plasma TBARS levels in the DM-VMT group compared to the DM-Sed group, demonstrating that VMT possibly causes systemic benefits. It is believed that this finding may be due to the attenuation of the metaboreflex, but we did not evaluate the redirection of blood flow in our study. In patients with DM 2, there is a reduction in the bioavailability and activity of nitric oxide, which causes micro and macrovascular changes [50]. Regarding muscle changes, there is an impairment of skeletal muscles, as a result of endothelial wall injuries and a reduction in the capillary density of this muscle group, causing a reduction in mass, peripheral muscle strength and respiratory dysfunctions [51], causing a reduction in mass, peripheral muscle strength and respiratory dysfunctions [52]. We recently published a study that showed that in rats with DM there was a reduction in the cross-sectional area in the diaphragm, tibial anterior and soleus muscles [21], confirming that DM reduces muscle mass.

Faced with these muscle changes, fatigue of the diaphragm muscle can occur, causing an accumulation of metabolites, which in turn causes the metaboreceptors to accumulate by-products of metabolism. From this, information is sent to the central nervous system causing sympathetic hyperexcitation. As a result, there is an increase in sympathetic activity that establishes peripheral vasoconstriction, redirecting blood to the diaphragmatic musculature, which results in reduced peripheral blood flow [17,53,54]. We know that the VMT increases diaphragmatic muscle strength [16], so the fatigue perception threshold also increases, through the activation of the metaboreflex, which causes a reduction in the perception of effort, preserving peripheral blood flow [53,54]. The increase in blood flow provides the release of nitric oxide, which, in turn, exerts the function of vasodilation and smooth muscle relaxation, in addition to inducing the response of antioxidants that can inhibit or balance the action of oxidant enzymes [55]. Thus, in the present study, the reduction in plasma TBARS levels in the DM-VMT group may be related to the mechanisms mentioned above.

Another interesting finding in our study is that DM is associated with a decrease in antioxidant markers, consistent with previous findings [46,56,57]. Comparing the Sham-Sed and DM-Sed groups, the DM group showed a reduction in antioxidant activity through the levels of MTT in the lungs, NPSH in the heart, lungs and diaphragm, and SOD in the heart, kidneys, gastrocnemius and plasma, going to the meeting of other studies that confirmed that the

experimental model of DM 2 reduces the activity of antioxidant enzymes [11,25]. When we compared the two DM groups, VMT increased SOD levels in the lungs and kidneys. Previous studies have shown that prolonged and/or high-intensity exercise promotes oxidative stress and skeletal muscle is the main source of ROS production. However, when this production occurs at moderate levels during exercise, there is a positive physiological adaptation in skeletal muscles through mitochondrial biogenesis and synthesis of antioxidant enzymes, whereas the production of ROS at high levels can result in damage to macromolecular structures [58,59].

Regarding the antioxidant markers in the skeletal muscles, especially in the diaphragm and gastrocnemius, when we compared the Sham-Sed with Sham-VMT groups, we found an increase in the trained group. However, we were unable to confirm whether this tool is capable of increasing antioxidant activity in general. Recent studies published by our group confirm that VMT effects on diaphragmatic musculature in rats with heart failure increased the citrate synthase enzyme [41] and reduced DNA damage [26], suggesting a reduction in oxidative activity and a possible increase in antioxidant. In addition, the VMT protocol in DM rats was able to potentiate parasympathetic modulation [21], although the impact of VMT on the activity of oxidant and antioxidant markers has not been evaluated.

There are some limitations in relation to the present study that may help to better understand the effects of VMT. First, we did not analyze other parameters of oxidative stress, which would allow a better understanding of the effect of VMT in this model. Second, we did not perform histological analysis, which could have confirmed some findings on the effect of VMT on the muscles analyzed in our study.

Conclusion

We concluded that VMT reduced the oxidant activity, through the levels of TBARS in the plasma of rats with DM 2. In addition, VMT was able to increase the activity of SOD in the lungs and kidneys of rats with DM 2, which infers in benefit of antioxidant activity.

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Interest conflicts:

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

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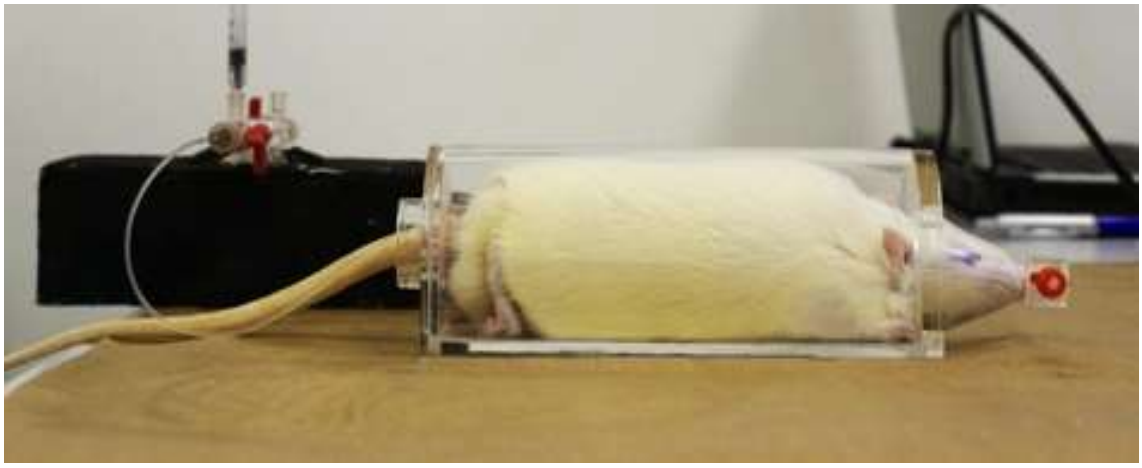

Figure 1. Instrument developed by Jaenisch et al. 2011, for ventilatory muscle training in rats.

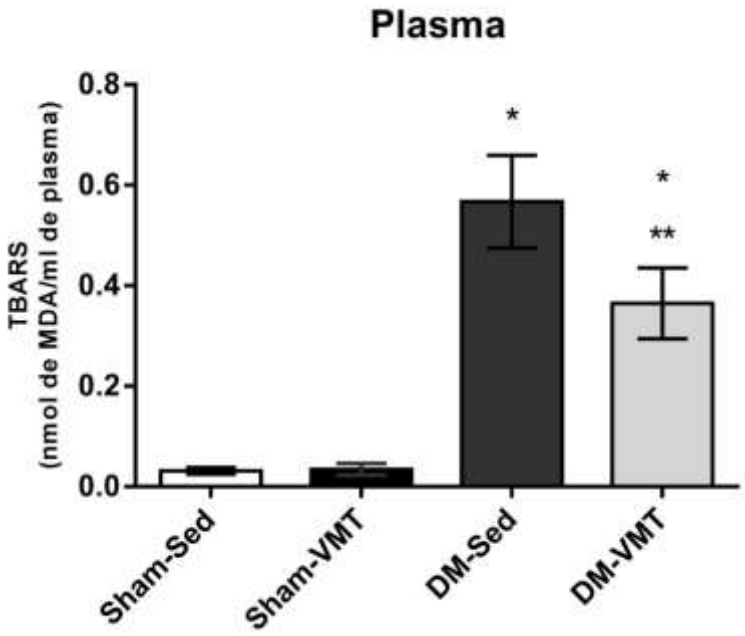

Figure 2. Plasma TBARS levels.

Values expressed as mean ± SD. The groups were compared by two-way ANOVA with Post Hoc Bonferroni. Sedentary control group (Sham-Sed, n=8); Control group ventilatory muscle training (Sham-VMT, n=8); Sedentary diabetic group (DM-Sed, n=7); Diabetic group ventilatory muscle training (DM-VMT, n=7).
* $p<0.001$ compared to Sham-Sed and Sham-VMT.
** $p<0.05$ compared to DM-Sed.

Table 1 - Weight and blood glucose of animals

Weight and Blood Glucose

Groups	Starting Weight (g)	Last Weight (g)	Basal Blood Glucose (mg/dL)	Post STZ blood glucose and vehicle (mg/dL)	Last Blood Glucose (mg/dL)
Sham-Sed	266±18,29	486±23,93*	125±7,85	113±12,35	124±21,56
Sham-VMT	267±18,22	411±56,94*	130±14,06	127±33,66	127±31,11
DM Sed	248±17,23	362±45,57*	139±16,91	407±70,33*	428±44,28*
DM VMT	251±26,73	381±57,80*	130±10,9	403±63,70*	434±67,99*

Values expressed as mean ± SD. Sedentary control group (Sham-Sed, n=8); Control group ventilatory muscle training (Sham-VMT, n=8); Sedentary diabetic group (DM-Sed, n=7); Diabetic group ventilatory muscle training (DM-VMT, n=7).
* $p<0.05$ comparing initial and final values.

Table 2 - Antioxidant Activity Markers in the heart, lungs, kidneys, diaphragm, gastrocnemius and plasma.

MTT 570 nm					
Groups	Heart	Lungs	Diaphragm	Gastrocnemius	Plasma
Sham-Sed	90,68±14,51	155,54±27,11	103,08±11,92	98,48±7,53	113,32±20,98
Sham-VMT	67,54±2,24	119,22±24,43	102,41±9,16	92,97±10,96	119,37±14,42
DM-Sed	96,34±23,79	93,92±3,05 [a]	111,52±8,43	115,52±11,30	108,62±6,45
DM-VMT	85,25±0,57	104,67±26,27	104,29±12,88	111,16±15,91 [b]	103,74±5,93
NPSH (nmol SH/mg of protein)					
Groups	Heart	Lungs	Diaphragm	Gastrocnemius	Plasma
Sham-Sed	136,40±1,35	74,62±9,36	48,50±1,31	42,50±14,04	13,10±2,96
Sham-VMT	13,04±2,43 [c]	36,88±10,69 [c]	46,60±12,56	62,50±14,53 [c]	13,00±2,19
DM-Sed	15,83±7,18 [a]	44,50±17,32 [a]	19,00±4,80 [a]	35,70±1,76	13,20±4,52
DM-VMT	29,33±15,62 [b]	47,37±13,96	17,10±5,15 [b]	24,80±1,22[b]	18,20±1,45 [b]
SOD (U/mg of protein)					
Groups	Heart	Lungs	Kidneys	Diaphragm	Gastrocnemius	Plasma
Sham-Sed	0,0017±0,00	0,0006±0,00	0,0014±0,00	0,0007±0,00	0,03±0,01	0,03±0,00
Sham-VMT	0,0020±0,00	0,0007±0,00	0,0021±0,00 [c]	0,0013±0,00 [c]	0,01±0,00 [c]	0,04±0,00 [c]
DM-Sed	0,0007±0,00 [a]	0,0016±0,00 [a]	0,0005±0,00 [a]	0,0007±0,00	0,00±0,00 [a]	0,00±0,00 [a]
DM-VMT	0,0010±0,00 [b]	0,0027±0,00 [b, d]	0,0015±0,00 [b, d]	0,0006±0,00 [b]	0,00±0,00 [b]	0,00±0,00 [b]

Values expressed as mean ± SD. Sedentary control group (Sham-Sed, n=8); Control group ventilatory muscle training (Sham-VMT, n=8); Sedentary diabetic group (DM-Sed, n=7); Diabetic group ventilatory muscle training (DM-VMT, n=7); Determination of Mitochondrial Dehydrogenase (MTT) activity; Determination of non-protein SH (NPSH) levels; Superoxide dismutase (SOD). [a] P<0.05 comparing Sham-Sed; [b] P<0.05 comparing Sham-VMT; [c] P<0.05 comparing Sham-Sed; [d] P<0.05 comparing DM-Sed; (Two-way ANOVA with Bonferroni post hoc).

5. CONCLUSÃO

O presente estudo teve como objetivo avaliar os efeitos do treinamento muscular ventilatório sobre o estresse oxidativo em ratos com DM 2. Confirmamos que, o protocolo de indução do DM 2 foi eficaz, comprovado pelo aumento dos índices glicêmicos, parâmetros de atividade oxidativa e antioxidante nos animais com DM 2.

Em conclusão, o protocolo de TMV, aplicado durante 6 semanas, por 5 dias consecutivos, foi capaz de reduzir a atividade oxidante no plasma e aumentar a antioxidante nos pulmões e rins de ratos com DM 2.

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LISTA DE APÊNDICES

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

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.

LISTA DE ANEXOS

ANEXO A – REGISTRO GABINETE DE PROJETOS (GAP)

UNIVERSIDADE FEDERAL DE SANTA MARIA - UFSM

PROJETO NA ÍNTEGRA

Data/Hora: 21/08/2019 09:51
Autenticação: D391.285D.2A5F.C4A0.03E3.7714.1EC6.2D73
Consulte em http://www.ufsm.br/autenticacao

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: 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	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
2016520019	VANESSA ORTIZ DE ANDRADE	Aluno de Graduação	Participante	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

CANADIAN JOURNAL OF CARDIOLOGY

Journal of the Canadian Cardiovascular Society

AUTHOR INFORMATION PACK

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ISSN: 0828-282X

DESCRIPTION

The *Canadian Journal of Cardiology (CJC)* is the official journal of the Canadian Cardiovascular Society (CCS). The *CJC* is a vehicle for the international dissemination of new knowledge in **cardiology** and **cardiovascular science**, particularly serving as the major venue for **Canadian cardiovascular medicine**.

The *CJC* publishes original reports of clinical and basic research relevant to cardiovascular medicine, as well as editorials, review articles, and case reports. Papers on health outcomes, policy research, ethics, medical history, and political issues affecting practice, as well as letters to the editor, are welcomed. The *CJC* accepts and publishes articles in the English language only. Manuscripts are received with the understanding that they are submitted solely to the *Canadian Journal of Cardiology* and that none of the material contained in the manuscript has been published previously or is under consideration for publication elsewhere, with the exception of abstracts. Redundant or duplicate publications will not be considered. All statements and opinions are the responsibility of the authors. The CCS reserves copyright on all published material, and reproduction of the material, even by the authors, requires written permission. With submission of a manuscript, a letter of transmittal must indicate that all authors have participated in the research and that they have reviewed and agree with the content of the article. You are also welcome to submit to the *CJC*?s open access companion title, *CJC Open*.

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Micha Maeder, St Gallen, Switzerland
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Alexis Matteau, Montreal, Quebec, Canada
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Luc L. Mertens, Toronto, Ontario, Canada
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Arnold Ng, Brisbane, Australia
Jens Cosedis Nielsen, Aarhus, Denmark
Stéphane Noble, Geneva, Switzerland
Manoj Obeyesekere, Richmond, Australia
Erwin Oechslin, Toronto, Ontario, Canada
Morton Olesen, Copenhagen, Denmark

GUIDE FOR AUTHORS

INTRODUCTION

CANADIAN JOURNAL OF CARDIOLOGY INSTRUCTIONS FOR AUTHORS

The *Canadian Journal of Cardiology (CJC)* is the official journal of the Canadian Cardiovascular Society (CCS). The *CJC* is a vehicle for the international dissemination of new knowledge in cardiology and cardiovascular science, particularly serving as the major venue for Canadian cardiovascular medicine. The *CJC* publishes original reports of clinical and basic research relevant to cardiovascular medicine, as well as editorials, review articles, and case reports. Papers on health outcomes, policy research, ethics, medical history, and political issues affecting practice, as well as letters to the editor, are welcomed. The *CJC* accepts and publishes articles in the English language only. Manuscripts are received with the understanding that they are submitted solely to the *CJC* and that none of the material contained in the manuscript has been published previously or is under consideration for publication elsewhere, with the exception of abstracts. Redundant or duplicate publications will not be considered. Duplicate submission is a significant breach of scientific ethical principles and may result in sanctions. All statements and opinions are the responsibility of the authors. The CCS reserves copyright on all published material, and reproduction of the material, even by the authors, requires written permission. With submission of a manuscript, a letter of transmittal must include the following 4 statements:

1. All authors have participated in the work and have reviewed and agree with the content of the article.

2. None of the article contents are under consideration for publication in any other journal or have been published in any journal.

3. No portion of the text has been copied from other material in the literature (unless in quotation marks, with citation).

4. I am aware that it is the author's responsibility to obtain permission for any figures or tables reproduced from any prior publications, and to cover fully any costs involved. Such permission must be obtained prior to final acceptance.

Editorial Policy

Each issue of the *CJC* carries the following statement, to which the authors agree when they submit a manuscript for consideration:

Statements and opinions expressed in the articles and communications herein are those of the author(s) and not necessarily those of the Editor(s), Society, or publisher, and the Editor(s), Society, and publisher disclaim any responsibility or liability for such material.

Article Classifications

At the discretion of the Editor-in-Chief, submissions may be accepted for either print or online publication. Case Reports and Images in Cardiology papers are generally published online only. Word-count limits (see below) generally refer to all elements of the article, including the abstract, acknowledgements, references, tables, and figure legends.

Original Papers are generally limited to 5,000 words, including all elements (title page, abstract, text, references, tables, and figure legends) in the principal Microsoft Word file, except for brief summary, word count, and short title. Rare exceptions to the word length limit may be granted by the Editor-in-Chief for specific reasons.

Editorials and Viewpoint Papers. Editorials are normally invited. However, unsolicited Editorials and Viewpoint articles are welcomed and will be submitted for peer review. The distinction between Editorials and Viewpoints is that an Editorial will generally present comments on an article (usually accompanying it in the same issue of the Journal), whereas Viewpoints will present comments on a topical and/or controversial issue in clinical or basic cardiovascular medicine. Editorials should cite the paper commented on as one of the references in the paper. Length for both Editorials and Viewpoint papers should be no more than 2,000 words including all elements (title page, text, references, tables

and figure legends). No abstract or brief summary should be provided for Editorials. Viewpoint articles should include a 250-word unstructured abstract as well as a 60-word summary for online listing. Conflict of interest guidelines apply.

Cardiovascular Controversies - Point/Counterpoint. These are short articles presenting opposite positions of an area of controversy in cardiovascular medicine. They are usually invited, with 2 articles (1 for each side of the argument) invited at the same time, to be published together in the same issue of the journal. Length should be no more than 3,000 words including all elements (title page, abstract, text, references, tables, and figure legends). The abstract should be under 100 words and unstructured. A brief summary (< 60 words) for electronic TOCs should be provided, but is not included in word count. Conflict of interest guidelines apply.

Review Articles may be invited but unsolicited articles are also welcome. Reviews should not exceed 7,000 words including all elements (title page, abstract, text, references, tables, and figure legends). They should include a 250-word unstructured abstract as well as a 75-word summary should be provided for online listing.

Systematic Review/Meta-analysis papers follow the same length and structure guidelines as Review articles, except their abstract should be structured (Background, Methods, Results, Conclusions), and they are executed according to standards for the appropriate article type.

New Methods in Cardiovascular Research and Practice. This category will include reviews of important current methods as well as newly developed techniques and approaches. The focus will be mostly on new and evolving methods in clinical research and/or practice (e.g. new forms of trial design, biostatistical approaches, etc) but may also include fundamental work.

The guidelines will follow those for original articles if the manuscript describes the development of a specific new technique or method (see Original Articles in Article Classifications section). If the article is a review of a method(s) used, it will follow guidelines for review articles (see Review Articles in Article Classifications section). These articles are generally invited, but the editors will also consider author-initiated submissions.

CCS Guidelines and Position Statements are definitive positions taken by CCS-mandated committees on areas of clinical importance for which there is a need of guidance on diagnostic and therapeutic management. The word limit is generally 10,000 words for CCS Guidelines and 6,000 words for Position Statements, including all elements (title page, abstract, text, references, tables, and figure legends). Additional materials can be included as Online Supplementary Materials (see below). Additional options for publication of more extensive documents that must be approved prior to submission are: 1) publication of the Executive Summary in the print journal with the full document available as an externally funded journal supplement, which will generally be industry-sponsored (see guidelines for *CJC* supplements at www.onlinecjc.ca); 2) exceptionally, a series of papers in a theme issue of the Journal. If funds available are sufficient for typesetting but not printing, the full document can be published online. In some instances for which the size and focus of a series of guidelines papers can be accommodated in a specific appropriate theme issue of the *CJC*, option 2) may apply. In case of doubt, the authors should consult directly with the Editor-in-Chief. All CCS Guidelines and Position Statements published in *CJC* should have an unstructured 250-word abstract. Because of the extensive review that CCS Guidelines and Position Statements undergo at the level of the Secondary Review Panel and the CCS Guidelines Committee, these papers will generally be reviewed by the Editor-in-Chief and his/her designate rather than being sent to external peer-reviewers.

Guidelines and Position Statements from other societies and groups. These must deal with an issue of interest in cardiovascular medicine and can be considered for publication in *CJC* based on scientific merit and pertinence to the mission of *CJC*. The word limit is 6,000 words including all elements (title page, abstract, text, references, tables, and figure legends). Additional materials can be included as Online Supplementary Materials (see below). Additional options for publication of more extensive documents that must be approved prior to submission are: 1) publication of the Executive Summary in the print journal with the full document available as an externally funded journal supplement, which will generally be industry-sponsored (see guidelines for *CJC* supplements at www.onlinecjc.ca); 2) publication of the full article in print with printing costs (established by the *CJC* publisher Elsevier in consultation with CCS) defrayed by the submitting society or body. In case of doubt, the authors should consult directly with the Editor-in-Chief. All Guidelines and

Position Statements published in *CJC* should have an unstructured 250-word abstract. Depending on the internal review process that these Guidelines and Position Statements undergo (e.g., Secondary Review Panel, etc), these papers may be reviewed by the Editor-in-Chief and his/her designate rather than being sent to external peer-reviewers. The final decision on review process will be made by the Editor-in-Chief, based on information provided at submission.

Co-publication with other journals of Guidelines and Position Statements. In general, *CJC* does not favor co-publication. In instances in which another society or organization is involved intimately and officially with CCS in elaboration of the Guidelines or Position Statements, co-publication will be considered. In such instances, agreements regarding co-publication should be made by the parties concerned (CCS, *CJC*, and other participating societies/journals) at the onset of Guidelines/Position Statement committee deliberation.

CCS Clinical Practice Updates (CPUs) are the results of reflections undertaken by writing groups mandated by the Canadian Cardiovascular Society in areas of clinical importance for which there is a need for guidance on diagnostic and/or therapeutic management, but for which the evidence base is less developed and/or is insufficient for a formal Guidelines reflection. In many instances, these will be rapidly emerging areas for which clinical guidance is needed but for which extensive randomized trials are lacking. CPUs are evidence-based but are more narrowly focused and more concise than guidelines, do not have formally-voted recommendations and may have time sensitive elements that warrant "fast track" translation into clinical practice. In some cases, they may be documents presenting an approach to a particular clinical problem, based on the expert opinion of the writing group, in situations where either the evidence base is extensive and well-known but summary clinical guidance would be useful, or where there is no clear evidence (generally for very new approaches and/or techniques) and expert opinion is needed.

All CPUs must be approved and guided by the CCS Guidelines committee, but CPUs do not include formally voted guideline recommendations or the use of the GRADE system. CPUs should be carefully evidence based, and the evidence base should be clear in the published document in CJC. Rather than having formal recommendations, CPUs will have expert advisory suggestions for clinical practice.

Depending on the nature of the material covered, CPUs will be published in one of 3 general formats: 1) General CPUs, covering a specific area, will have a Word Count limit of 8,000 words, including all elements (title page, abstract, text, references, tables, and figure legends); 2) Focused CPUs, dealing with more focused/better circumscribed areas, will have a Word Count limit of 6,000 words, including all elements (title page, abstract, text, references, tables, and figure legends); 3) Practical CPUs, with an overall maximum of 4,000 words , including all elements (title page, abstract, text, references, tables, and figure legends; strict maximum of 5 references and 2 display items), providing practical instruction in well recognized and documented areas for which expert guidance would be helpful, are submitted as Contemporary Issues in Cardiology Practice or Health Promotion and Policy papers. The latter are described in detail elsewhere in the Instructions for Authors, but in brief have a maximum of 1500 words of text in the main section, 5 references and 2 display items. The specific format appropriate for a given CPU will be defined early in the process for each CPU, based on discussions between the chair(s) of the specific CPU committee and the chair of the CCS Guidelines Committee.

All CPUs should have an unstructured 250-word abstract, as well as a 75-word Brief Summary (which is not counted towards the total word count). These papers will generally be sent for evaluation by external peer-reviewers, like virtually all other CJC papers.

Case Reports must be informative to those in clinical practice. Case Reports should address uncommon presentations and/or treatments of common conditions, provide new insights into pathogenesis, or represent a newly recognized condition. The author(s) should provide sufficient literature review to place the report into context. No more than 5 references and 2 figures will be accepted, and the length should not exceed 1,000 words including all elements (title page, abstract, text, references, tables, and figure legends). An abstract of no more than 100 words should accompany the article and a 60-word summary should be provided for online listing. **For all Case Reports, conclude the Abstract and Discussion with a concise statement of the Novel Teaching Point(s) emerging from the case.**

Images in Cardiology papers demonstrate particularly insightful images used in the detection of cardiovascular disease. The imaging modality may be old or new. The text of submissions for this section should be limited to that necessary to describe the context and importance of the image(s) and should not exceed 500 words including all elements (title page, text, references, and figure legends). No more than 5 references and 2 figures will be accepted. No abstract should be included, but a 60-word summary (not included in word-count limit) should be provided for online listing. **For all Images in Cardiology manuscript, conclude the text with a concise statement of the novel element(s) or teaching point(s) that the image report adds to the literature.**

In general, both Case Reports and Images in Cardiology are published online only. If the authors cannot include all materials they would like to make available within the word count/figure limits, additional figures, tables, text, etc. can be provided in a Supplementary Material section (see below).

Journal News and Commentary papers are short non-scholarly papers that comment on the state of the journal or an issue. For example, this would include brief Forewords to supplement issues or comments by the editor about progress of the journal, new features being planned, changes to policies, etc. Such papers are limited to a maximum of 1,200 words and 5 references. They do not normally have display items, but a maximum of 1 figure or table can be included in exceptional cases to make specific points in a clearer fashion. No abstract or summary are to be included.

Training/Practice papers present information of interest to practitioners, such as practical technical and patient management instruction or matters relating to health policy and promotion, as well as guidelines for Canadian cardiovascular training programs. These papers are primarily intended for guidance in practice, health promotion and/or training and are not detailed scholarly items--scholarly analyses should be submitted in the appropriate category (Clinical Research, Systematic Review/Meta-analysis, Review papers, Translational Medicine, or Viewpoint). The text of submissions for this section should be no more than 1,500 words. No more than 5 references and 2 display items (figures and/or tables) will be accepted. An unstructured abstract of no more than 250 words should accompany the article, and a 60-word summary should be provided for online listing. Submissions are divided into 3 subsections: 1) Contemporary Issues in Cardiology Practice, which will highlight issues of relevance to clinical practice in the face of rapidly-advancing technologies and new medical knowledge, 2) Training in Cardiovascular Medicine and Research, which deal aspects relevant to cardiovascular clinical and research training programs, and 3) Health Policy and Promotion, which deal with matters relating to health policy and promotion.

Translational Medicine articles are generally invited, but unsolicited articles are also welcome. This section is intended to present reviews or meta-analyses dealing with novel scientific findings or concepts with important clinical relevance or application. Areas of potential application include (but are not limited to) physiology, pharmacology, molecular biology, genetics, genomics, pharmacogenomics, population science, etc. Word length and other guidelines are the same as for Review articles.

Brief Rapid Reports are brief papers reporting the results of clinical or basic research that is limited in scope but time-sensitive and of unusual interest. Articles for this section will receive rapid editorial attention, with a decision generally provided within 2 weeks of submission, rapid (within 6 weeks of acceptance) online publication, and print publication in the next available issue. Papers submitted for this section will be accepted with at most minor revision. If major revision is needed, the paper will subsequently fall into the Original Papers category. The submission cover letter should explain why the article is considered appropriate for this category. Maximum length is 3,000 words (including title page, abstract, text, references, tables, and figure legends; but excluding Brief summary), with a 100-word abstract and a maximum of 3 illustration items (figures plus tables). A 60-word Brief Summary should be provided for online listing.

Letters to the Editor may deal with any subject of current interest to cardiovascular medicine. If the subject concerns a recent publication in *CJC*, the letter will normally be forwarded to the authors for comment. Both the letter and the response may be edited for clarity or brevity. Letters should not exceed 400 words, with no more than 4 references and 1 figure or table. Conflict of interest guidelines apply. Only one institutional affiliation should be listed on Letters to the Editor or none at all.

Contact details for submission

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BEFORE YOU BEGIN

Ethics in publishing

Please see our information on Ethics in publishing.

Declaration of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Authors must disclose any interests in two places: 1. A summary declaration of interest statement in the title page file (if double anonymized) or the manuscript file (if single anonymized). If there are no interests to declare then please state this: 'Declarations of interest: none'. 2. Detailed disclosures as part of a separate Declaration of Interest form, which forms part of the journal's official records. It is important for potential interests to be declared in both places and that the information matches. More information.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic thesis, see 'Multiple, redundant or concurrent publication' for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service Crossref Similarity Check.

Use of inclusive language

Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Content should make no assumptions about the beliefs or commitments of any reader; contain nothing which might imply that one individual is superior to another on the grounds of age, gender, race, ethnicity, culture, sexual orientation, disability or health condition; and use inclusive language throughout. Authors should ensure that writing is free from bias, stereotypes, slang, reference to dominant culture and/or cultural assumptions. We advise to seek gender neutrality by using plural nouns ("clinicians, patients/clients") as default/wherever possible to avoid using "he, she," or "he/she." We recommend avoiding the use of descriptors that refer to personal attributes such as age, gender, race, ethnicity, culture, sexual orientation, disability or health condition unless they are relevant and valid. These guidelines are meant as a point of reference to help identify appropriate language but are by no means exhaustive or definitive.

Authorship

All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Copyright

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For subscription articles

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You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

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Language (usage and editing services)
Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's Author Services.

Studies on patients or volunteers require ethics committee approval and informed consent, which should be documented in the paper. Appropriate consents, permissions and releases must be obtained where an author wishes to include case details or other personal information or images of patients and any other individuals in an Elsevier publication. Written consents must be retained by the author and copies of the consents or evidence that such consents have been obtained must be provided to Elsevier on request. For more information, please review the *Elsevier Policy on the Use of Images or Personal Information of Patients or other Individuals*, https://www.elsevier.com/patient-consent-policy. Unless you have written permission from the patient (or, where applicable, the next of kin), the personal details of any patient included in any part of the article and in any supplementary materials (including all illustrations and videos) must be removed before submission.

If experimental animals are used, provide a statement in the text to indicate that all procedures followed were approved by an institutional animal research ethical review board. (This is a requirement for such studies to be published in the CJC). If human subjects are involved, the text must indicate that all gave informed consent and that the protocol was approved by the institutional human research ethics review committee.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Submit your article

Please submit your article via https://www.editorialmanager.com/CJC/.

PREPARATION

General Guidelines

The manuscript should conform to the guidelines in "Uniform Requirements for Manuscripts Submitted to Biomedical Journals," 5th edition, prepared by the International Committee of Medical Journal Editors and published in N Engl J Med 1997;336:309-15 and Can Med Assoc J 1997;156:270-7, available online at http://www.cma.ca/mwc/uniform.htm.

Manuscript Preparation

The *CJC* will accept online submissions of original manuscripts through the Editorial Manager at https://www.editorialmanager.com/CJC/. Manuscripts submitted through this online system can easily be tracked by the authors, editors, and reviewers through final disposition. The corresponding author of the manuscript will receive automatic email notifications as the manuscript proceeds through the system. To begin using this system, go to https://www.editorialmanager.com/CJC/. Click the "Register" link on the toolbar at the top left to input author demographics and set up an account. After registration is complete, a notice will be sent via email indicating a user ID and password. Use this information to log in as an author by choosing the "Login" link on the toolbar, and select "Submit A Manuscript." Follow the prompts to complete the submission according to the specifications below. Be aware that the manuscript (with abstract included within), each table, and each figure need to be prepared as separate files following the guidelines listed below. Contact the editorial office if you have any problems or questions. Your user ID and password can be changed at any time by logging into https://www.editorialmanager.com/CJC/ with your user ID and password and then clicking the "Update My Information" link at the top of the page.

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Cover Page

With submission of a manuscript, a letter of transmittal must be provided, including the following 4 statements: 1. All authors have participated in the work and have reviewed and agree with the content of the article. 2. None of the article contents are under consideration for publication in any other journal or have been published in any journal. 3. No portion of the text has been copied from other material in the literature (unless in quotation marks, with citation). 4. I am aware that it is the author's responsibility to obtain permission for any figures or tables reproduced from any prior publications, and to cover fully any costs involved. Such permission must be obtained prior to final acceptance.

Title Page

Include the title (up to 150 characters in length, including spaces), authors' names (including full first name, middle initial, and family name), academic degrees and academic affiliations, and a short title of up to 45 characters (including spaces). Be sure that authors' names are listed as they should

appear in MEDLINE. Provide the word count on a separate line. Provide the full name, exact mailing address with postal code, telephone and fax numbers, and email address of the corresponding author to whom communication, proofs, and requests for reprints should be sent.

Brief Summary

For Original Papers, CCS Guidelines or Position Statements, Clinical Practice Updates, Translational Medicine articles, and Review articles, on a separate page, provide a brief summary of no more than 75 words, which will appear in the online contents listing (and in the print issue for the e-only articles section, if applicable) to inform readers of the principal contents of each paper. For Case Reports, Images in Medicine, Training/Practice papers, and Brief Rapid Reports, a 60-word summary should be provided.

Abstract

On a separate page provide an abstract of no more than 250 words (for Original Papers, CCS Guidelines or Position Statements, Clinical Practice Updates, Translational Medicine, and Review articles) or 100 words (for Case Reports and Brief Rapid Reports) that summarizes the study and conclusions, with clinical implications indicated as appropriate. Original Papers should have a structured abstract, with the following sections: Background, Methods, Results, Conclusions. Abstracts for CCS Guidelines or Position Statements, Clinical Practice Updates, Translational Medicine, Training/Practice papers, and Review articles, while the same length (250 word maximum) as those for Original Papers, should be unstructured (no Background, Methods, Results, Conclusions headings). **In all cases, conclude the abstract with a succinct sentence that summarizes the most novel finding(s) and/or contributions of the paper and its (their) relevance.**

Manuscript Text

Text files must be saved as Microsoft Word files. To ensure that the final, published version matches the electronic file, use one of the following fonts: Arial, Courier, or Times. The use of other fonts may result in missing symbols. The font size should be 12 points. Abbreviations must be defined at first mention in the text and should follow the form recommended in "Uniform Requirements for Manuscripts Submitted to Biomedical Journals." Appropriate headings and subheadings should be provided in the Methods, Results, and Discussion sections. References, tables, and figures should be numbered in the order of mention in the text. **Authors should conclude the Discussion with a brief Conclusions paragraph summarizing the most important novel elements of the study and their relevance to cardiovascular medicine.**

Article Structure

Subdivision

Divide your article into clearly defined sections. Each subsection is given a brief heading. Each heading should appear on its own separate line. Subsections should be used as much as possible when cross-referencing text: refer to the subsection by heading as opposed to simply 'the text'.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results

Results should be clear and concise.

Western blots

For all papers given the opportunity to resubmit their work to the journal, authors need to provide full-length, unmanipulated images of all Western blots including molecular weight markers used in all figures. These should be incorporated within the Online Supplement and any bands shown in the paper should be clearly identified on the original images in the supplement, along with the corresponding Figure number for reference.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Funding Sources

Any and all relevant funding sources for the study should be listed, including funding from industry and peer-review funding (with grant numbers if available). Funding Sources should be indicated in a separate paragraph after the Acknowledgements section.

Disclosures

All potential conflicts of interest must be identified in this section. Potential conflicts of interest that should be disclosed include: relationships with pharmaceutical and biomedical device companies or other corporations whose products or services are related to the subject matter of the article, from which any of the authors may obtain potential financial benefits. Such relationships include, but are not limited to, employment by an industrial concern, equity or stock ownership by authors or family members, membership on a standing advisory council or committee, being on the board of directors or publicly associated with the company or its products, where the concern produces products whose value or perception could be influenced by the content of the article. Other areas of real or perceived conflict of interest could include receipt of honoraria or consulting fees or receiving grants or funds from such corporations or individuals representing such corporations. Intellectual property rights held by the authors for inventions relevant to the subject of the article should also be declared. Funding by peer-review grant agencies does not generally constitute a conflict of interest, unless the funding is for intellectual property development related to the material covered in the paper from which the authors stand to obtain potential financial gain. Peer-review funding relevant to the material covered in the paper that does not constitute a conflict of interest should be indicated in the Funding Sources section that precedes the Disclosures section. If there are no potential conflicts of interest, this should be designated by indicating "none" in the Disclosures section. Disclosures should be indicated in a separate paragraph after the Funding Sources section.

Math formulae
Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Artwork

Figure artwork must be submitted in electronic format.

Western blots
For all papers given the opportunity to resubmit their work to the journal, authors need to provide full-length, unmanipulated images of all Western blots including molecular weight markers used in all figures. These should be incorporated within the Online Supplement and any bands shown in the paper should be clearly identified on the original images in the supplement, along with the corresponding Figure number for reference.

Electronic Artwork
General points
- Make sure you use uniform lettering and sizing of your original artwork.
- Embed the used fonts if the application provides that option.
- Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Provide captions to illustrations separately.
- Size the illustrations close to the desired dimensions of the printed version.
- Submit each illustration as a separate file.

A detailed guide on electronic artwork is available on our website:

https://www.elsevier.com/artworkinstructions

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

If your electronic artwork is created in a Microsoft Office application (e.g., Word, PowerPoint) then please supply 'as is' in the native document format.

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Book
Svensson LG, Crawford ES. Cardiovascular and Vascular Disease of the Aorta. Toronto: WB Saunders Company, 1997:184-5.

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Trehan S, Anderson JL. Thrombolytic therapy. In: Yusuf S, Cairns JA, Camm AJ, Fallen EL, Gersh BJ, eds. Evidence Based Cardiology. London: BMJ Books, 1998:419-44.

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