UNIVERSIDADE NOVE DE JULHO

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOFOTÔNICA APLICADA ÀS CIÊNCIAS DA SAÚDE

MARCELO FRIGERO

EFEITOS DA LASERTERAPIA DE BAIXA INTENSIDADE SOBRE MARCADORES DE ESTRESSE OXIDATIVO DO MÚSCULO GASTROCNÊMIO EM RATOS DIABÉTICOS SUBMETIDOS A EXERCÍCIO DE ALTA INTENSIDADE.

SÃO PAULO, SP

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Orientador: Prof. Dr. Paulo de Tarso Camillo de Carvalho

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“ Nunca perca a fé na humanidade, pois ela é como um oceano. Só porque existem algumas gotas de água suja nele, não quer dizer que ele esteja sujo por completo. ” - Mahatma Gandhi

Resumo

Objetivo: O objetivo do presente estudo foi determinar se a terapia Laser de baixa intensidade (LBI), quando usado no músculo gastrocnêmio de ratos diabéticos, submetidos a exercícios de alta intensidade, até a fadiga, interfere com a expressão de marcadores de estresse oxidativo. **Materiais e Métodos**: Para realização do trabalho, a amostra foi composta por 24 ratos machos (*Norvergicos Albinus*), de linhagem Wistar. Os animais foram divididos aleatoriamente em três grupos designados da seguinte forma: Grupo Diabético Fadiga - Não recebeu irradiação para LBI e realizou o protocolo de fadiga; Grupo Diabético Fadiga Laser - recebeu irradiação com LBI e realizou o protocolo de fadiga e o Grupo Controle Não Diabético – não recebeu irradiação e realizou protocoloco de fadiga. A Diabetes foi induzida por estreptozotocina (STZ) (50 mg / kg, ip), e confirmado através da medida de glicose no sangue. Foi utilizado o Laser de 808 nm e energia de 4J, para os grupo indicado e os animais foram submetidos ao exercicio de corrida em esteira rolante com velocidade e inclinação gradual até a fadiga, observado através do VO_2máx e nível de lactato. Os ratos foram sacrificados, e o tecido muscular retirado para a análise dos marcadores de SOD; CAT; GPX e TBARS. **Resultados**: Os valores $VO_2^{0.75}$máx no Grupo Diabético Fadiga foram significativamente mais elevados do que aqueles no Grupo Controle Não Diabético ($p < 0,01$) e do Grupo Diabético Fadiga Laser ($p < 0,05$). Os resultados indicam que as atividades de CAT, SOD, e GPx foram maiores e estatisticamente significativas ($p < 0,05$) no do que as dos Grupo Diabético Fadiga. Resultados para o Grupo Diabético Fadiga Laser foram semelhantes ao Grupo Controle Não Diabético e estatisticamente ($p > 0,05$) significativas para as atividades de enzimas antioxidantes em comparação com o Grupo Diabético Fadiga. No Grupo Diabético Fadiga Laser poderia também minimizar a concentração de TBARS ($p > 0,05$). **Conclusão**: LBI pode reduzir o estresse oxidativo, bem como aumentar o $VO_2^{0.75}$máx. Além disso, nossos resultados indicam que a LBI pode ser uma alternativa para melhoria das condições musculares em relação a fadiga. Novos estudos clínicos devem ser realizados para verificar a eficácia da técnica.

Palavras Chave: Diabetes Mellitus, Laser Baixa Potência, Estresse Oxidativo

Abstract

Purpose: The aim of the present study was to determine whether low-level laser therapy (LLLT), when used in gastrocnemius muscle of diabetic rats subjected to high-intensity exercise to fatigue, Interferes with the expression of stress markers oxidative.

Materials and Methods: The total of 24 male Wistar rats (Norvergicos Albinos). Were used for this study: 24 diabetics rats. The animals were randomly divided into three groups designated as follows: Group Diabetic Fatigue - Did not receive irradiation to LLLT and performed the fatigue protocol; Group Diabetic Laser Fatigue - received irradiation to LLLT and performed the fatigue protocol and Group Non-Diabetic Control - Did not receive irradiation to LLLT and performed the fatigue protocol. Diabetes was induced in rats by streptozotocin (50 mg / kg, ip) was subsequently performed measure of blood lactate levels, LLLT laser was applied at 808 nm and 4 joules of energy to the Indicated groups and the animals were exercised groups submitted to treadmill running with speed and gradual slope until exhaustion noted by VO_2máx and lactate level. The rats were euthanized, and muscle tissue and presented the analysis of SOD markers; CAT; GPX and TBARS. **Results:** $VO_2^{0.75}$máx values in the Group Diabetic Fatigue were significantly higher from those in the control group ($p < 0.01$) and the Group Diabetic Laser Fatigue ($p < 0.05$). The results indicate that the activities of CAT, SOD, and GPx were higher and statistically significant ($p<0.05$) in the Group Diabetic Laser Fatigue than those in the diabetic groups. Outcomes for LBI group were similar than control group ($p>0.05$), statistically significant activities of antioxidant enzymes compared to the Group Diabetic Fatigue. The LBI group could also mitigate the concentration of TBARS ($p> 0.05$). **Conclusion:** LBI imay reduce oxidative stress, as well as increase $VO_2^{0.75}$máx. Furthermore, our outcomes indicate that LBI can be an alternative to maintain physical fitness in conditions where subjects aren't able to perform exercise. Which needs to be tested in further clinical studies.

Keywords: Diabetes mellitus, low level laser therapy, stress oxidative.

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LISTA DE ABREVIATURAS E SÍMBOLOS

ATP= Adenosina Trifosfato

AsGa= Arsenieto da Gálio

AsGaAl= Arsenieto de Gálio e Alumínio

CAT = Catalase

CK= Creatina Fosfato

cm/s= Centímetros por segundo

CO_2= Gás Carbônico

DM = Diabetes Mellitus

DM1=Diabetes Mellitus tipo 1

DM2=Diabetes Mellitus tipo 2

DNA= Ácido Desoxirribonucleico

ERO = Espécie Reativa de Oxigênio

GCND = Grupo Controle Não Diabético

GDF = Grupo Diabético Fadiga

GDFL= Grupo Diabético Fadiga Laser

GPX = Glutationa Peroxidase

J = Joules

J/cm² = Joules por centímetro quadrado

LASER = Amplificação de Luz por Emissão Estimulada de Radiação

LBI = Laserterapia de Baixa Intensidade

LDH = Lactato Desidrogenase

LLLT = Low-Level Laser Therapy

mM/l = Concentração Molar

mW= microwatts

nm= Nanometro

NO= Óxido Nítrico

OMS = Organização Mundial de Saúde

QR= Coeficiente Respiratório

RL = radicais livres

ROS= Reactive Species of Oxygen

rpm= Rotações por Minuto

SBD= Sociedade Brasileira de Diabetes

SOD = Superóxido Dismutase

STZ = Estreptozotocina

OMS= Organização Mundial de Saúde

$O2 \bullet^{-}$ = superóxido

TBARs = Ácido Tiobarbitúrico

TNF α = Fator de Necrose Tumoral alfa

VO_2máx= Consumo Máximo de Oxigênio

$VO_2^{0.75}$máx= Consumo Máximo de Oxigênio Alométrico

W/cm² = Watts por centímetro quadrado

% = Porcentagem

1. CONTEXTUALIZAÇÃO

1.1– Diabetes Mellitus (DM)

A Diabetes Mellitus, hoje, é um dos principais problemas de saúde pública em muitos países. Estima-se que cerca de 143 milhões de pessoas em todo o mundo sofram de diabetes e acredita-se que esse número possa dobrar até 2030, admitindo-se até mesmo uma pandemia diabética. O Brasil estaria entre os 10 países (32 países estudados) com maior número de pessoas com diabetes (King et al., 1998).

A diabetes se refere a um conjunto de diversas doenças, os tipos mais comuns de diabetes são: tipo 1, tipo 2 e diabetes gestacional. Todos os tipos têm sintomas semelhantes, pois todas as formas da doença se caracterizam por um aumento do açúcar, ou glicose, no sangue. Esse aumento ocorre devido a impossibilidade do organismo em retirar a glicose do sangue e direcioná-la para as células do corpo. Seu aparecimento está associado à diminuição ou alteração de um hormônio proteico (insulina) produzido pelo pâncreas, órgão responsável pela manutenção dos níveis normais de glicose no sangue (Bicudo, 1997).

Os sintomas que levam ao diagnóstico dessa doença são: fadiga, cansaço, sede e fome intensas, micção frequente e perda de peso, apesar da excessiva fome.

Geralmente, os jovens e as crianças acometidos pela doença são magros (Sociedade Brasileira de Diabetes - S.B.D, 1999). Esse estado de saúde caracterizado por sede excessiva e urina abundante que acometia algumas pessoas já era percebido pelos egípcios em 1500 a.C., que o denominaram ‘diabetes’, uma palavra latina que significa sifão, devido à urina abundante que parecia passar por um sifão.

Assim, no início da Era Cristã, quando a doença começou a ser estudada pelos gregos, já se utilizava a palavra diabetes.

A World Health Organization (WHO-1999) define DM como uma desordem metabólica de etiologia múltipla, caracterizada por uma hiperglicemia crônica que acarreta distúrbios no metabolismo de carboidratos, gorduras e proteínas resultantes de defeitos na secreção e/ou ação da insulina.

Mecanismos homeostáticos mantém os níveis de glicose sanguínea dentro de uma faixa estreita de 4,5 - 5,5 mM/l. Este controle é realizado por modulação hormonal, sendo, basicamente, dois os hormônios reguladores: glucagon que é o hormônio responsável pelo estímulo da gliconeogênese pelo fígado nos períodos de jejum e a insulina, o hormônio que estimula a captação e a utilização da glicose pelos músculos esqueléticos, músculo cardíaco e adipócitos, após a ingestão de alimentos ricos em carboidratos.

Esses dois hormônios são secretados e liberados pelo pâncreas: glucagon pelas células alfa das ilhotas de Langherans e a insulina pelas células β. Estados em que a homeostasia do metabolismo de carboidratos e lipídeos não é regulada de maneira apropriada pela insulina, resultam, primariamente, em um aumento dos níveis de glicose sanguínea em jejum e pós-prandial.

Se esse desequilíbrio homeostático não for restabelecido, mas sim continuar por um período de tempo longo, expondo o sistema endócrino a uma sobrecarga, ocorre uma exacerbação dos distúrbios metabólicos que resulta em hiperglicemia, a qual pode evoluir para a síndrome chamada Diabetes Mellitus (Tiwari e Rao, 2002).

Diabetes Mellitus é um distúrbio endócrino no qual o metabolismo da glicose está alterado devido a uma perda total de insulina após destruição das células β pancreáticas, responsáveis pela produção e liberação de insulina – (DM dependente de insulina ou tipo 1) ou devido a uma liberação inadequada de insulina pelas células β pancreáticas ou ainda uma insensibilidade à insulina pelos tecidos alvos (DM não dependente de insulina ou tipo 2).

A DM tipo 1, é uma desordem heterogênea e poligênica, representando 5 a 10% do total de casos. A DM tipo 2, é a mais comum, representa cerca de 90 a 95% dos casos e resulta de uma combinação de deficiência na secreção e/ou ação da insulina (Persaud et al, 1999).

Todas as formas de diabetes, inerentes ou adquiridas, são caracterizadas de acordo com Schmidt e Stern (2000), por: hiperglicemia; falta absoluta ou relativa de insulina (ou resistência insulina); desenvolvimento de patologias micro e macrovasculares específicas da diabetes (retinopatias, neuropatias, desordens neurológicas, amputação de membros inferiores).

A hiperglicemia prolongada na diabetes, causa uma variedade de mudanças patológicas em pequenos vasos, artérias e nervos periféricos. As células do endotélio vascular tornam-se alvos primários dos danos hiperglicêmicos por causa do fluxo contínuo de glicose através deles.

A hiperglicemia também leva a um aumento na produção de espécies reativas do oxigênio (EROs) dentro das células endoteliais da aorta. Além disso, a hiperglicemia induz a ativação de isoformas de PK-C, o aumento na formação de derivados glicosilados e estimula um aumento no fluxo de glicose na via das aldose-redutases, resultando em um acúmulo de sorbitol (Sakurai e Tsuchiya, 1988, Hunt et al, 1988).

Estes seriam os principais mecanismos metabólicos pelos quais a hiperglicemia causa a complicação da diabetes com destruição tecidual (Baynes, 1991, Schimidt e Stern, 2000).

O DM, tipo 1 (classificado anteriormente por - DM insulinodependente) desenvolve-se com maior frequência, entre crianças e adolescentes. É conhecido como insulinodependente, pois, devido à produção ineficiente de hormônio, torna-se necessária a injeção de insulina. Sem esta, pode ocorrer o fenômeno da cetoacidose, que causa um aumento de gordura no sangue e o consequente mau funcionamento dos rins.

Caso não seja tratada, a cetoacidose pode conduzir ao coma e, em questão de dias ou semanas, pode levar à morte (Bicudo, 1997; Sarafino, 1994; Thompson & Gustafson, 1996).

A DM insulinodependente, geralmente ocorre em crianças entre 5 a 6 anos e 11 a 13 anos (Thompson & Gustafson, 1996). Segundo estimativas, 2 a 3 % das crianças com DM Tipo 1, morrem nos primeiros 10 anos depois do diagnóstico, e 12 a 13% morrem 20 anos depois do diagnóstico, devido a

complicações como o excesso de episódios de hipo ou hiperglicemia (Johnson, 1995).

No Brasil, estima-se que cinco milhões de indivíduos sejam diabéticos, sendo que metade deles desconhece o diagnóstico, com uma incidência do Tipo 1, na infância e adolescência, na ordem de 1 ou 2 para cada 1000 jovens (Chipkevitch, 1994). É a quarta causa de morte no país, além de ser a segunda doença crônica mais comum na infância e adolescência (Chipkevitch,1994; S. B. D., 1999).

Isso mostra que, atualmente, o diabetes é um dos mais importantes problemas de saúde, em termos do número de pessoas afetadas, pela incapacitação produzida, mortalidade e custos do tratamento (Ministério da Saúde, Programa Harvard/Joslin/S.B.D., 1996; S.B.D., 1999).

1.2- Fadiga

A fadiga é um termo comumente usado em conversas diárias, com significados subjetivos, tão variados como as pessoas que o utilizam. Termos como fadiga, sonolência, cansaço, falta de energia e exaustão são usados alternadamente.

A fadiga muscular, pode ser definida como sendo a incapacidade do músculo para manter uma determinada potência (Edwards,1981), ou uma deficiência em sustentar um nível particular de desempenho durante um exercício físico (Davis,1997). Os mecanismos etiológicos responsáveis pela fadiga muscular têm recebido uma importante atenção dos fisiologistas e bioquímicos por mais de um século (Fitts,1994).

As principais dificuldades, ao investigar a fadiga, devem-se à natureza multifatorial e à complexidade dessa (Kirkendall, 2000; Mcardle,1994). A origem e extensão da fadiga muscular dependem da especificidade do exercício, tipo de fibra muscular e o nível de aptidão física individual (Fitts,1994).

Dentro da literatura científica, as definições de fadiga variam muito, e a definição torna-se mais difícil, porque há pouca diferenciação entre as causas,

indicadores e efeitos da mesma, porém é geralmente reconhecida como complexa e / ou multifacetada, englobando componentes fisiológicos, psicológicos e de eventos que envolvam situação de qualidade de vida e de trabalho (Bartley,1976; Winningham,1994)

Embora a fadiga seja considerada uma ocorrência normal na vida diária, é também um sintoma associado com uma variedade de condições fisiológicas e psicológicas. Fadiga normalmente ocorre após o esforço físico ou mental, sono inadequado, ou outros fenômenos temporários.

Às vezes chamada de aguda ou saudável, é geralmente temporária e aliviada com o repouso. Fadiga aguda serve como um mecanismo de proteção ao sinalizar a necessidade do corpo para o descanso.

Crônica ou patológica, no entanto, ocorre após pequeno esforço e continua apesar do descanço. Este tipo de fadiga é associada principalmente com doenças como o cancro, doença pulmonar obstrutiva crônica, artrite reumatóide, depressão, e fibromialgia, mas ele também pode desempenhar um papel significativo na diabetes (Rose,1998).

1.2.1 - Os efeitos da fadiga em pessoas com diabetes

Como em outras condições, a fadiga em pessoas com diabetes é provável que seja multidimensional, englobando fatores fisiológicos, psicológicos e de estilo de vida.

Pesquisa realizada em mulheres diabéticas, apontam que a fadiga estava presente entre os quatro primeiros sintomas encontrados que interferiam em sua qualidade de vida (Stover et al 2001). As pesquisas qualitativas tem sido a principal fonte de temas sobre os efeitos deletérios decorrentes da fadiga em adultos com diabetes (Hill-Briggs, 2003; Chasens, 2008). Por exemplo, pesquisa realizada com mulheres afro-americanos portadoras de diabetes, concluiu que a fadiga limita a capacidade das mesmas em sua atividades diarias (Hill-Briggs,2003). A fadiga estava presente também entre os preocupações predominantes nas mulheres diabetícas australianas,

que relataram que a mesma limitava suas atividades sociais à aquelas somente consideradas necessarias (Koch 1999).

De acordo com Fritschi (2010), em um grupo de discussão sobre as barreiras encontradas para a motivação do tratamento em pacientes com diabetes, através de um programa de exercicios, estaria a sonolência e o cansaço.

Em todos os estudos acima referenciados, os participantes notaram a sensação de que eles não eram mais capazes de participar das atividades e atribuiam essa deficuldade a fatiga (Hill-Briggs, 2003).

Esses achados sugerem que a fadiga tem grande importancia e consequências graves para os pacientes com diabetes, uma vez que demanda do paciente um desgaste físico e mental para realizar as sua atividades de vida diaria.

1.3 - Espécies Reativas de Oxigênio

O termo espécies reativas de oxigênio (EROs) inclui os átomos e moléculas que possuem alta radioatividade e efeitos oxidantes e regulatórios, portanto, contempla tanto as espécies químicas *radicais livres* (RL), que são os átomos ou moléculas que possuem um ou mais elétrons desemparelhados na camada de valência, quanto as *não radicais livres,* uma definição para os átomos e moléculas que geram alta radioatividade sem apresentarem o desemparelhamento de elétrons (Drödge, 2002; Powers e Jackson, 2008).

Os radicais livres primários mais relevantes na regulação biológica são o superóxido ($O2 \bullet^{-}$) e o óxido nítrico (NO). O ânion superóxido produzido pelas células, por enzimas e de forma não-enzimática, pode ser liberado no espaço extracelular por canais sensíveis à voltagem, mas muitos dos efeitos regulatórios ocorrem por ações de EROs, quimicamente derivadas do superóxido (Drödge, 2002; Jackson, 2008; Allen, Lamb e Esterlab, 2008).

A alta produção destas espécies reativas é responsável por várias ações deletérias, tais como aumento de peroxidação lipídica, aumento na oxidação de proteínas e danos ao DNA podendo resultar em morte celular.

Entre as diversas fontes de produção de EROs reconhecidas, podemos destacar: a cadeia de transporte de elétrons mitocondrial, músculo esquelético; e auto oxidação de catecolaminas (Javesghani *et al*., 2002; Zoppi *et al*., 2003; Urso e Clarkson, 2003; McAnulty *et al*., 2005).

Existem nos organismos vivos, substâncias que neutralizam as EROs – chamadas de antioxidantes - definidas como aquelas que são capazes de atrasar significativamente ou inibir a oxidação de um substrato. Essa definição inclui as substâncias presentes em baixas concentrações no organismo, mas com alta capacidade antioxidante como os antioxidantes enzimáticos e não enzimáticos. O grupo enzimático possui um número limitado de enzimas e são constituídas por Superóxido Dismutase (SOD), Catalase (CAT) e Glutationa Peroxidase (GPx). Estas enzimas constituem-se a defesa primária contra a geração de EROs durante o exercício e sua atividade é conhecida por aumentar em resposta ao exercício, tanto nos estudos em animais quanto em humanos (Powers, Ji e Leeuwenburgh*,* 1999; Drödge, 2002; Mentin *et al.,* 2003; Powers e Jackson, 2008).

As enzimas antioxidantes são importantes e desempenham papel complementar. De fato, as isoenzimas da SOD regulam a quantidade de superóxido presente nas células, mas promovem aumento na concentração intracelular de peróxido de hidrogênio. Em paralelo, as enzimas CAT e GPx degradam peróxido de hidrogênio e a glutationa peroxidase produzidos como subprodutos da ação lesiva de EROs sobre lipídios. Nos mamíferos, existem três isoenzimas da SOD, codificadas e reguladas de forma independente: a citosólica (Cu, Zn- SOD ou SOD1), a mitocondrial (Mn-SOD ou SOD2) e uma forma extracelular da Cu, Zn-SOD ou (SOD3), sendo que a CuZn-SOD-citosólica está presente em maior quantidade que a Mn-SOD- mitocondrial (Sankarapandi e Zweier, 1999).

O sistema antioxidante não enzimático inclui principalmente a glutationa reduzida (GSH), alfa-tocoferol (vitamina E), beta-caroteno (vitamina A), ascorbato (vitamina C), ubiquinona (Coenzima Q10) e cisteína); bem como contempla as substâncias que têm baixa atividade antioxidante, mas

encontram-se em altas concentrações no organismo, como os aminoácidos livres, os peptídeos e as proteínas (Silveira, 2004).

1.3.1- Estresse Oxidativo

O balanço entre as taxas de produção e de remoção das EROs determina sua concentração. Quando ambas as taxas estão equilibradas, as células e tecidos encontram-se em estado estável. Se esse estado é rompido pelo aumento da produção de EROs sem a concomitante compensação dos antioxidantes, ou pela redução apenas da concentração de antioxidantes, são gerados a sinalização redox e o estado de 'estresse oxidativo'.

O quadro de estresse oxidativo, definido como "um desequilíbrio entre oxidantes e antioxidantes, em favor dos oxidantes" resulta em peroxidação lipídica, um dano que pode alterar a permeabilidade da barreira celular e comprometer a sua integridade.

Para avaliação do estresse oxidativo são utilizados subprodutos provenientes da peroxidação lipídica. Um desses marcadores, comumente utilizado, é o malondialdeído (MDA), medido por sua reatividade ao ácido tiobarbitúrico (TBARS) (Drödge, 2002; Ramel, Wagner e Elmadfa, 2004; Lekhi Gupta e Singh, 2007; Powers e Jackson, 2008; Teixeira *et al*., 2009).

Uma situação que pode alterar o estado redox, por causar um aumento da produção de EROs, é o exercício físico; embora esse resultado não tenha sido encontrado em alguns estudos (Benoni *et al.,* 1995; Quadilátero *et al.,* 2010).

Durante o exercício físico, o aumento da produção de superóxido pode ser o resultado de sua maior geração pela fibra muscular – o que pode ocorrer em diversos locais, como na mitocôndria, no retículo sarcoplasmático, nos túbulos transversos, no sarcolema e nocitosol (Vassilakopoulos *et al.,* 2002; Chevion *et al*., 2003; Close *et al*., 2005; Lekhi, Gupta e Singh, 2007; Allen,

Lamb e Westerlab, 2008; Jackson, 2008; Jacobs, Donavan e Robinson, 2009; Powers *et al.,*2008).

Além da produção pela fibra muscular, é também considerada na literatura a geração de superóxido através da xantina oxidase, principalmente em situações em que há ocorrência de isquemia/reperfusão, pela ativação de leucócitos através das alterações hormonais, metabólicas e circulatórias promovidas pelo exercício e pelo aumento da concentração de lactato (Cooper *et al*., 2007; Silva, 2006; Allen, Lamb e Westerlab, 2008; Brooks, 2009).

1.4 - Diabetes Tipo I e Estresse Oxidativo

O papel do estresse oxidativo nas complicações diabéticas tem sido amplamente estudado, com estudos envolvendo bloqueio da via oxidante e terapias antioxidantes.

Reis e cols. (2006) relatam em seu estudo, que diferentes metodologias para quantificação do status oxidante e antioxidante vêm sendo utilizadas e aperfeiçoadas.

Dominguez e cols. (1998), avaliando 24 pacientes pré-púberes dentro de sete a dez dias após início clínico do diabetes, demonstraram concentrações elevadas de malonaldeído (MDA) plasmático, produto final da oxidação de ácidos graxos poli-insaturados, em relação ao grupo-controle ($p < 0,0001$), sugerindo que radicais livres de oxigênio possam exercer seus efeitos tóxicos em estágios precoces da doença, mantendo-se elevados no curso dela, quando comparados com um grupo de 30 pacientes portadores de DM1, que apresentavam a doença, com evolução variando de 2 a 22 anos, sem complicações.

Demonstraram ainda baixos níveis de glutationa peroxidase no grupo recém-diagnosticado em relação aos controles ($p < 0,0001$), com progressivo declínio no curso da doença.

Estes mesmos autores não encontraram correlação entre estes achados e os parâmetros de controle glicêmico e lipídico.

Vessby e cols. (2002) avaliando 38 pacientes DM1, com 16,1 ± 10,3 anos desde o diagnóstico, não demonstraram diferença significativa nas variáveis de peroxidação lipídica (8-iso-prostaglandina F2 e MDA) em relação ao grupo-controle. Neste grupo, a capacidade antioxidante total do plasma (quantificada por quimioluminescência), foi 16% menor no grupo de diabéticos ($p < 0,0005$), a despeito do simultâneo aumento nos níveis de tocoferol ($p < 0,05$), sem correlação com o controle glicêmico.

Hata e cols. (2006) demonstraram, em DM1 com 5,5 ± 4,4 anos de doença, correlação direta entre aumento de 8-OhdG (8-hidroxi-2-deoxiguanosina), outro marcador de estresse oxidativo, com o controle glicêmico e a presença de microalbuminúria, achados confirmados em outros estudos (Huvers,1999; Domingues,1998).

Gleisner e cols. (2006), avaliando um grupo de DM1 pré-púberes, com menos de cinco anos de diagnóstico, não observou diferença estatisticamente significante nos marcadores do estresse oxidativo em relação aos controles, com parâmetros bioquímicos similares entre os dois grupos.

Reis (2006), na avaliação do estresse oxidativo em um grupo de DM1 brasileiros, com 2,62 ± 2,24 anos de doença, sem comorbidades associadas e em tratamento intensivo com insulina, observou diferença estatisticamente significante na produção de EROs por granulócitos de DM1 em relação aos não-diabéticos ($p < 0,05$), quantificados por quimioluminescência dependente de luminol (o método se fundamenta na amplificação pelo luminol da luminescência natural emitida pelas espécies reativas de oxigênio).

O status antioxidante do plasma foi avaliado pela redução direta do MTT (sal de tetrazólio: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) pelo plasma, ensaio utilizado na medida de viabilidade e proliferação celular, que se fundamenta na redução do MTT por meio de redutases, que agem como doadora de elétrons na redução do MTT (Berridge,1996).

Esta redução, descrita inicialmente como fenômeno intracelular, envolve NADH2 e FADH2 e é primariamente uma medida da taxa de produção de

NADH na hiperglicemia. Não foi observada diferença significativa na capacidade de redução direta do MTT pelo plasma entre DM1 e não-diabéticos ($p > 0,05$), caracterizando manutenção do poder antioxidante do plasma neste grupo. Nenhuma correlação entre controle glicêmico e lipídico e os parâmetros avaliados foi encontrada.

1.5 - Laser de baixa intensidade (LBI)

O termo LASER significa Light Amplification by Stimulated Emission of Radiation (amplificação de luz por emissão estimulada de radiação). O Laser foi desenvolvido na década de 1960, e é uma luz com propriedades especiais, como monocromaticidade e baixa divergência. A laserterapia de baixa intensidade (LBI) é a aplicação de luz (geralmente um Laser de baixa potência na faixa de 1 mW – 500 mW) a uma patologia. Estudos recentes vêm demonstrando que a LBI promove a regeneração tecidual, modulação da inflamação e alívio da dor. A luz é tipicamente de largura espectral estreita, indo do espectro vermelho ao infravermelho próximo (600 nm – 1000 nm), com uma densidade de potência (irradiância) entre 1 mW a 5W/cm2 (Huang et al., 2009).

Para Pagès et al., (1999) e Tuby; Maltz; Oron, (2007), as energias depositadas pelo fóton nos tecidos biológicos podem provocar processos vibracionais, rotacionais e eletrônicos que prontamente se transforma em outro tipo de energia ou efeito biológico, o efeito bioelétrico impulsiona o aumento e a quantidade na produção de ATP produzida pela célula agindo diretamente na mobilidade iônica, dessa forma potencializa a bomba de sódio e potássio, mantendo com maior eficácia a diferença de potencial de ação intracelular e extracelular.

O Laser também pode exercer um efeito sobre os tecidos. A interação do Laser com os tecidos biológicos é dependente do comprimento de onda, da densidade de energia e da potência do Laser (Huang *et al*., 2009)

Os autores Albertini (2002) e Say, (2003) indicam que os componentes celulares podem absorver fótons fornecidos por meio da energia do Laser de

baixa intensidade (fotorreceptores) e ampliar a produção de ATP provendo energia para a célula, que por sua vez pode modular a resposta inflamatória.

Os elementos da cadeia respiratória são postulados como fotorreceptores primários, mormente o citocromo C oxidase. Quando as células são irradiadas com múltiplas faixas de luz, esta é absorvida pela cadeia respiratória. As primeiras ocorrências fotoquímicas e fotofísicas acontecem na mitocôndria de células eucariontes e na membrana citoplasmática (Karu, 1999).

A laserterapia pode provocar a fosforilação de proteínas quinases MAPK/ERK nas células, as quais estão associadas ao mecanismo de proliferação celular (Stein *et al*., 2005). Verifica-se um crescimento na produção de ATP e maior atividade da enzima fosfatase alcalina, o que incrementa a proliferação celular, e ainda maior expressão de citocinas como interleucina 6, quando a célula é submetida à irradiação Laser.

Dessa maneira, foi sugerido que a irradiação Laser tem um efeito terapêutico a mais por fomentar a produção citocinas, causando a comunicação intercelular, migração e proliferação, para auxiliar no processo de cicatrização tecidual (Hawking-Evans; Abrahanse, 2006).

1.5.1 - Laser de baixa intensidade e Estresse Oxidativo

Conforme estudos realizados por Lubart et al., (2005); Fillipini et al., (2005); Huang et al., (2009); Liu et al., (2009) existem evidências na literatura revelando que a LBI aumenta a atividade da enzima SOD e estimula a sua síntese pela célula, o que poderia explicar a diminuição dos danos oxidativos a lipídios e proteínas constatados depois da aplicação de LBI.

Basso *et al*., (2012) descrevem que o LBI reduz a produção de espécies reativas de oxigênio (EROs) que acontece durante a fase aguda de lesão epitelial e muscular. Isso acontece, mormente por acelerar o processo inflamatório e diminuir a exposição celular a EROs.

A avaliação dos efeitos da aplicação de LBI em lesões induzidas, em ratos, por 3 horas de isquemia seguida de reperfusão foi realizada com a

aplicação de LBI (AsGa, 810nm) imediatamente e após 1 hora de oclusão do suprimento sanguíneo.

Identificou-se que a aplicação de LBI protegeu o músculo contra os efeitos deletérios causados pela lesão isquêmica, proporcionando um aumento na atividade da creatina fosfoquinase na capacidade antioxidante total sérica e em proteínas de choque térmico.

Os autores descreveram aumento na capacidade antioxidante total sérica quando a LBI foi aplicada em ratos sem a indução de lesão por isquemia/reperfusão (Avni *et al.* 2005).

Segundo Fillipin *et al.* 2005 e Rizzi *et al.* 2006, que pesquisaram os efeitos da LBI (AsGa 904nm, contínuo, 45 mW e 5 J/cm^2, 35 segundos de aplicação) sobre o estresse oxidativo em modelo experimental de trauma no tendão de *Aquiles* e músculos de ratos, a LBI reduziu a perda da arquitetura normal (histologia) e a resposta inflamatória, assim como os níveis de TBARS, quando comparados ao grupo que não recebeu Laser.

Observaram ainda um aumento na atividade enzimática da SOD, indicando que um dos mecanismos de redução da resposta inflamatória pela LBI possa estar relacionado à modulação da atividade da SOD.

Estudo realizado por Liu et al. (2009), com indução da lesão muscular em ratos por meio de exercícios em esteira, foi aplicado LBI em 3 momentos: imediatamente após, 18 horas após e 42 horas após o protocolo de exercícios. Identificou-se que os grupos que receberam a aplicação de LBI exibiram uma melhora significativa na avaliação histológica (menor quantidade de infiltrados inflamatórios), diminuição nas concentrações de CK e da peroxidação lipídica e aumento na atividade de SOD.

Segundo Servetto et al., (2010), em uma pesquisa que tinha como foco investigar o efeito fotobiomodulador da LBI aplicada por dois Lasers distintos (He-Ne, 632,8nm, contínuo, 5mW, 1min de aplicação/sessão e AsGa (904nm), pulsado, 12mW, 47 segundos de aplicação/sessão) em um modelo experimental de miopatia, que foi induzido por meio da infiltração de adrenalina (0,05 mg/rato/dia) no músculo durante 5 dias; no grupo, em que a lesão foi desenvolvida, observou-se aumento significativo de L-citrulina e SOD

e uma diminuição de óxido nítrico (NO). A aplicação de LBI diminuiu significativamente a L-citrulina e a atividade da SOD e aumentou a concentração de NO.

Foram observados os efeitos da LBI em lesões de pele induzidas experimentalmente; a LBI foi aplicada sete vezes (2, 12, 24, 48, 72, 96 e 120 horas) após a formação da lesão, empregando equipamentos e doses distintas (AsGa, 904nm, pulsado, 70 mW, 60 segundos de aplicação/sessão e He-Ne, 660nm, contínuo, 30 mW, 60 segundos de aplicação/sessão). Os resultados apontaram que a aplicação da LBI foi capaz de diminuir a peroxidação lipídica, os danos às proteínas e a atividade de SOD e CAT, potencializando a cicatrização da ferida, mormente nas doses de 1 e 3 J/cm² do Laser de He-Ne e na dose de 3 J/cm² do Laser de AsGa (Silveira *et al.,* 2013).

Estudo realizado em indivíduos não treinados e irradiados com LBI por 5 minutos, antes do exercício na esteira avaliou a fadiga e o estresse oxidativo, indicando que o uso do Laser antes da execução do exercício, pode estar relacionado com o atraso na fadiga muscular, aumentando o desempenho e o consumo máximo de oxigênio absoluto relativo, diminuindo a indução do estresse oxidativo e lesões musculares. As atividades da LDH, lesão muscular (CK) e danos lipídios (substâncias reativas ao TBARS) foram reduzidas (De Marchi *et al.,* 2012).

1.6 - Determinação da fadiga através da capacidade funcional

A capacidade funcional e/ou o desempenho físico durante o exercício de um indivíduo pode ser avaliada através de um procedimento não invasivo, que concilia a análise de gases expirados, variáveis respiratórias e oximetria, chamado de teste de ergoespirometria (Serra, 1997).

A ergoespirometria caracteriza-se como um exercício de intensidade progressiva, tendo em vista que a velocidade de corrida é aumentada gradativamente durante a sua realização. O indivíduo que está executando a

atividade é instruído a realizá-la até a fadiga, a fim de obter-se uma avaliação mais fidedigna da sua capacidade cardiopulmonar (Bearden & Moffatt, 2000).

Esse método tem sido útil na determinação de fatores ligados a indicadores de performance, identificação de intolerância ao exercício, determinantes de transição metabólica, avaliação clínica e terapêutica de diversas patologias, prescrição de intensidade do exercício, índices de eficiência respiratória e cardiovascular e custo energético (Silva et al., 1998, Denis et al., 1984).

Diferentes parâmetros podem ser avaliados em um teste de ergoespirometria, dentre eles, o tempo total de execução do exercício, o consumo máximo de oxigênio (VO_2máx), os limiares aeróbios e anaeróbios, tempo de teste em que o indivíduo atinge estes limiares, entre outros (Baroni & Leal Junior, 2010).

A diminuição do desempenho pode estar intimamente ligada à instalação do processo de fadiga muscular (Terrados & Fernandez, 1997), sendo assim, a mensuração do tempo total de execução do exercício durante o teste de ergoespirometria, torna-se um ótimo parâmetro para avaliação da fadiga, pois a execução do mesmo se dá até a exaustão (Serra, 1997).

Apesar dos resultados positivos obtidos em estudos recentes, diversos fatores ainda permanecem desconhecidos, tais como: doses e parâmetros ideais de aplicação, mecanismos de ação e os efeitos em relação a fadiga muscular. Acreditamos que a LBI aplicada com o objetivo de atenuar a fadiga apresente o mesmo padrão bifásico de dose-resposta já observado em outras desordens musculoesqueléticas, e que a potência e o tempo de irradiação também influenciem na eficácia da terapia, conforme também observado na terapêutica destas desordens.

2. HIPÓTESE DE PESQUISA

Com base em estudos anteriores, apresentamos a hipótese de que a utilização da LBI é capaz de retardar o processo fisiológico de fadiga muscular em ratos diabéticos. Além disso, acreditamos que, diferentemente da terapia farmacológica, a terapia Laser apresenta um padrão bifásico ou ondulatório de curva dose-resposta.

3 - OBJETIVO

3.1 - Objetivo Principal

O presente projeto teve por objetivo avaliar os aspectos bioquímicos e biomoleculares da ação do LBI (λ 808nm), em ratos diabéticos submetidos ao exercício de alta intensidade.

3.2 - Objetivos Específicos

Avaliar se a LBI é capaz de alterar os seguintes aspectos:

I- Avaliar a ação do LBI na expressão proteica: SOD (superóxido dismutase), GPx (Glutathione Peroxidase), TBARS (Ácido Tiobarbitúrico) e CAT (Catalase)

II - Avaliar a ação LBI nos níveis lactato e no consumo de oxigênio (VO_2-máx) durante o processo de fadiga.

4 – MATERIAL E MÉTODOS

4.1 - Modelo Animal

A amostra foi composta por 24 ratos (*Norvergicos Albinus*), de linhagem Wistar, machos, com peso corporal médio de ±180 g provenientes do Biotério da Universidade Nove de Julho (Uninove), mantidos em condições controladas de luminosidade e temperatura, com água e alimentação *ad libitum*. Todos os procedimentos experimentais foram realizados em conformidade com as normas do Colégio Brasileiro de Experimentação Animal (COBEA), e do comitê de ética em pesquisa da Universidade Nove de Julho – UNINOVE. Este estudo foi aprovado no comitê de ética em pesquisa em animais da Universidade Nove de Julho sob o número de parecer AN0035/2014.

4.2 - Grupos Experimentais

Os animais foram randomizados e distribuídos em 3 grupos, de oito (8) animais:

1 - Grupo controle não diabético – Não recebeu irradiação à LBI e realizou protocolo de fadiga.

2 - Grupo diabético fadiga – Não recebeu irradiação à LBI e realizou protocolo de fadiga

3 - Grupo diabético fadiga Laser – Recebeu irradiação à LBI e realizou protocolo de fadiga.

4.3 - Calculo da Amostra

O processamento do cálculo amostra foi realizado por meio do software Ene®, versão 3.0 (Barcelona, Espanha). O tamanho da amostra foi calculado com base no ensaio experimental conduzido por Rodrigues et al (2014). Assim, elegeu-se como variável desfecho o blood glucose nos grupos controle e

diabetes submetidos a treinamento físico. O cálculo foi baseado na detecção de diferenças de 223,60 mg/dL entre os grupos, assumindo um desvio-padrão de 6,80 mg/dL. Assim, considerando-se um poder estatístico de 80% e alfa de 0,05, foi estimado um número de 8 animais por grupo.

4.4 - Indução da diabetes

Os animais foram anestesiados com uma mistura de Ketamina+Xilasina (87 e 13 mg/kg respectivamente), por via intraperitoneal, e o diabetes foi induzido por uma única injeção também intraperitoneal de estreptozotocina (STZ, 50mg/kg, Sigma S/A). A estreptozotocina foi dissolvida em tampão citrato (0,01M, pH 4,5) e injetada cerca de cinco minutos após a diluição. Os animais foram mantidos em jejum de 12 horas antes da indução. No grupo controle (8 animais), foi injetado intraperitoneal somente tampão citrato. Foi realizada a indução inicialmente em 26 animais, mas só foram incluídos nos grupos diabéticos, animais que apresentarem glicemia de jejum ≥ 250 mg/dl (Carvalho *et al*,2010), mensurada através do equipamento AccuCheck, Advantage-Roche.

A diabetes foi induzida em um numero maior de ratos, em relação aos grupos, pois de acordo com Deeds et al (2011), devemos levar em consideração a perda de animais devido à toxicidade da STZ, que segundo seus relatos gira em torno de 30%.

4.5 - Desenho Experimental

Foram organizados três grupos: um grupo controle de ratos não diabético fadigado, um grupo de ratos diabéticos que não foi irradiado o LBI, mas realizou exercício até atingir a fadiga e um terceiro grupo que recebeu

irradiação e realizou exercício até atingir a fadiga. A figura 1, representa o tempo experimental dos grupos.

O termo *1° dia* refere-se ao primeiro dia de treinamento dos animais na esteira para adaptação. Foram realizados três dias de adaptação, no quarto dia foi realizada a terapia Laser em três pontos da pata traseira dos animais, bilateralmente, sendo que o Grupo Diabético Fadiga Laser recebeu o tratamento com o LBI e fez o exercício físico até atingir a fadiga e o Grupo Diabético Fadiga fez somente exercícios até atingir a fadiga e o Grupo Controle Não Diabético realizou o protocolo de fadiga, sem estimulação do LBI.

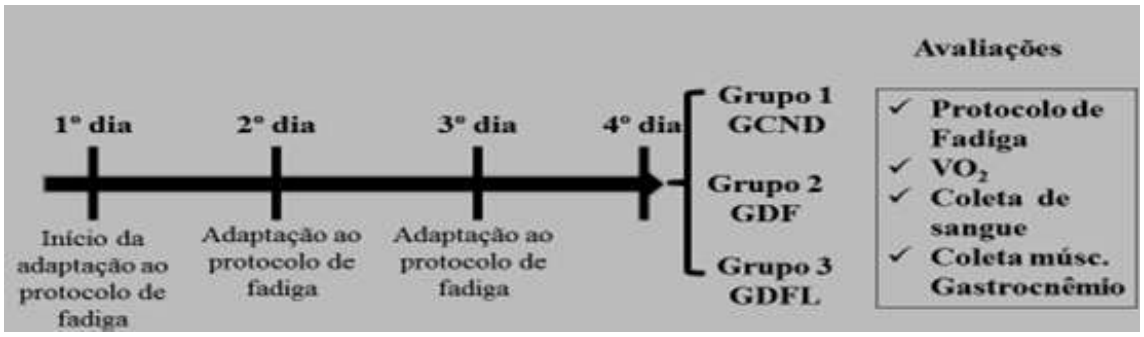

Figura 1. Representação esquemática do tempo experimental.

No quarto dia, antes do procedimento dos exercícios de alta intensidade, foi coletado sangue da cauda do animal de todos os grupos e ainda houve a irradiação do Laser no Grupo Diabético Fadiga Laser. Após o procedimento na esteira, foi realizada coleta de sangue imediatamente após o exercício, e uma outra amostra foi coletada após um minuto da primeira. Os animais foram identificados, pesados e posteriormente eutanasiados administrando tiopental (THIOPENTAX - Cristália) dose de 100mg/kg (DL) por via intraperitoneal associado à lidocaína 10mg/ml (Xylestesin - Cristália). A seguir foram retirados os músculos Gastrocnêmios bilateralmente.

4.6 - Aplicação do Laser e Parâmetros utilizados

O Laser utilizado foi o Arsenieto de Gálio de Alumínio (AsGaAl) da marca DMC® (São Carlos, Brasil), modelo Photon Laser III, com potência de 30mW, área do feixe de 0,028cm², e comprimento de onda de λ 808nm.

Dados do Equipamento	Parâmetros
Laser	AsGaAl 808nm
Fabricante / tipo	DMC® Photon Laser III
Frequência	Modo contínuo
Potência de saída	30mW
Densidade de potência	1,071 W/cm²
Diâmetro do Diodo	0,028cm²
Densidade de energia	142,4 J/cm²
Energia dose	4J
Tempo de tratamento	133 segundos
Número de pontos	3 pontos Bilaterais
Método	Transcutâneo
Energia (dose) Total	24J

Figura 02. Parâmetros do Laser.

A aplicação foi realizada em três pontos, bilateralmente, pelo método transcutâneo na pata traseira na região posterior sobre o músculo gastrocnêmio, com densidade de energia de 107J/cm^2 com duração de 1minuto e 40 segundos, esta dose foi escolhida por apresentar redução do CK e melhora da resistência à fadiga (Sussai et al. 2006).

4.7 - Protocolo de Exercício forçado. Capacidade aeróbia em esteira - consumo máximo de oxigênio (VO_2máx.) e fadiga

O consumo de oxigênio (VO_2) e a produção dióxido de carbono (VCO_2) foram avaliados em esteira rolante sem inclinação (Panlab/Harvard-Apparatus, Harvard Bioscience Company, Massachusetts, USA), com os animais permanecendo dentro de uma caixa conectada ao analisador de gases (Panlab/Harvard-Apparatus Oxylet System, Harvard Bioscience Company, Massachusetts, USA), equipado com sensor de O_2 diodo LBI (resolução: 0,01%) e sensor de CO_2 infravermelho (resolução: 0,01%). Antes e após todos os testes, o sistema foi calibrado com concentrações conhecidas de O_2, CO_2 e N_2, como recomendado pelo fabricante, de acordo com o protocolo estabelecido anteriormente (Amadio *et al,* 2015).

O fluxo de ar na caixa sobre a esteira foi mantido entre 1,2 l/min e 2,0 l/min, conforme o peso do animal. A fração de oxigênio no ar efluente foi registrada a cada segundo. Todos os animais foram submetidos a três dias de familiarização ao exercício em esteira antes da determinação do VO_2máx. Em cada dia de adaptação, os animais realizaram o exercício por 15 minutos da seguinte forma: 1º dia: 25 cm/s (5 minutos), 35 cm/s (5 minutos) e 45 cm/s (5 minutos); 2º dia: 25 cm/s (5 minutos), 45 cm/s (5 minutos) e 55 cm/s (5 minutos); 3º dia: 25 cm/s (5 minutos), 55 cm/s (5 minutos) e 65 cm/s (5 minutos). Para determinação do VO_2máx, os animais realizaram aquecimento por 2 minutos com velocidade da esteira fixada a 25 cm/s.

Por seguinte, a velocidade de corrida foi aumentada em 9 cm/s a cada 2 minutos até inabilidade do animal em manter o exercício. Os seguintes critérios foram adotados para caracterizar o VO_2máx (Wisloff et al., 2001): (1) estabilidade do VO_2 na vigência de aumento da intensidade do exercício; (2) razão de troca respiratória acima de 1,05. O VO_2máx será expresso em ml.kg< >-1. min-1 e, segundo a escala alométrica, em ml/kg^{0,75}/min-1, que representa o VO_2max por unidade de massa magra do animal.

4.8 - Coleta do Sangue e Eutanásia

Imediatamente após a atividade física e a fadiga provocada nos animais, os mesmos foram submetidos à coleta de sangue, que foi realizada retirando uma amostra da cauda do animal.

Após a retirada do sangue os animais foram eutanasiados, administrando tiopental (THIOPENTAX - Cristália) dose de 100mg/kg (DL) por via intraperitoneal associado à lidocaína 10mg/ml (Xylestesin - Cristália). Após a eutanásia, os músculos Gastrocnêmios foram cirurgicamente removidos. As amostras resultantes foram congeladas em nitrogênio líquido e armazenadas a -80 ° C e distribuídas, sendo uma para a realização dos procedimentos análise da expressão proteica Western blotting (SOD, GPX), e analise bioquímica (TBARS, CAT).

4.9 - Biomarcadores de Peroxidação de Lipídios: Concentração TBARS

Para a determinação das concentrações de TBARS, as amostras de tecido muscular foram colocadas em Eppendorf® gelados contendo 1,5 ml de tampão fosfato 0,05 N (composição, em g/L: KH_2PO_4, 1,34 e $NaHPO_4$ $2H_2O$, 7,1), homogeneizadas em Polytron® e centrifugadas durante cinco minutos a 10.000 rpm. Feito isso, o sobrenadante foi separado e armazenado a -20°C para análise posterior por meio de *kits* comerciais (Cayman Chemical®, Michigan, EUA).

4.10 - Concentração de lactato sanguíneo

O nivel de lactato no sangue foi determinado através de um analisador portátil de lactato (modelo de medidor de lactato Accutrend® Roche Diagnostics, Mannheim, Alemanha) (Baldari,2009). A análise da atividade basal (em repouso) foi realizada antes da aplicação do LBI. Imediatamente apòs o

animal atingir a fadiga, que foi associada a verificação do VO_2máx, uma nova medição foi realizada. Decorrido um minuto dessa medição, mais uma amostra de sangue foi coletada. O grupo controle não realizou o protocolo de exercicios forçados, mas foram submetidos ao mesmo processo de avaliação do lactato sanguíneo (Rocha et al,2013).

4.11 - Preparação do tecido

Os músculos foram homogeneizados (Homogeneizador Omni International HEC) em tampão de lise RIPA (25 mM Tris-HCl pH 7,6, 150 mM de cloreto de sódio, 1% de NP-40, 1% desoxicolato de sódio, 0,1% de SDS) suplementado com cocktail inibidor de protease (1 mM AEBSF, aprotinina 800 μ M, 40 μ M bestatina, 14 μ M E-64, 20 μ M de leupeptina, 15 μ M de pepstatina A; Sigma Aldrich, Irlanda) e inibidores de fosfatase de fluoreto de sódio 10 mM, 1 mM de ortovanadato de sódio, utilizando um 10% w rácio / v.

As amostras foram homogeneizadas com recurso a 8 × 10 s rajadas e deixadas em gelo durante 20 min. Os homogenatos foram então centrifugados durante 15 min a 11.000 g e os sobrenadantes foram armazenados a -80 ° C.

A concentração de proteína foi determinada utilizando um ensaio bicinconínico (BCA) (Thermo Scientific, Irlanda), conforme descrito no protocolo do fabricante com absorvências medidas a 562 nm utilizando um espectrofotómetro-M3 SpectraMax (Molecular Devices, EUA) (Williams et al, 2015).

4.12 - Western Blotting

O teor de proteína da GPx e SOD foram medidas nas fracções musculares. Trinta microgramas de proteína foram carregadas em cada poço de gel de poliacrilamida com de gradiente 4% -12% (Novex, Invitrogen) e separados como de rotina, por eletroforese em gel de sulfato de sódio poliacrilamida dodecil (SDS-PAGE) durante 1,5 horas a 20°C, seguido por transferência para uma membrana de nitrocelulose. Todas as membranas

foram bloqueadas em 5% de proteína do leite desnatado (NFM) durante 1 hora à temperatura ambiente.

Em geral, as membranas foram incubadas em diluições apropriadas de anticorpos primários (diluídos em 1% em solução salina NFM tamponada com Tris a 0,05% de Tween-20 [TBST] durante a noite num quarto frio a 4° C). As membranas foram lavadas em TBS-T, seguido por incubação em diluições apropriadas de anticorpos secundários (diluídos em 5% NFM em TBS-T) conjugado com peroxidase de rábano.

Os sinais foram desenvolvidos utilizando um substrato chemilumines-cent (Lumigen TMA-6; Lumigen, Southfield, MI) e visualizados por exposição das membranas. Os registros digitais foram capturadas por uma câmera Kodak 290, e bandas de proteínas foram quantificadas utilizando o software de análise 1D (Eastman Kodak). As bandas foram quantificadas como densidade óptica × área banda e expressa em unidades arbitrárias.

4.13 - Análise Estatística

Os dados obtidos foram tabulados em Software Microsoft Excel 2007 e inicialmente foi utilizado o teste de Shapiro-Wilk para verificar a normalidade. Os biomarcadores de estresse oxidativo foram comparados usando análise de uma via de variância (ANOVA) com o teste de Tukey post-hoc *e teste t student para* a análise do lactato. Para tanto foi utilizado Software Graph-pad 4.0 (Software Inc., San Diego, CA, EUA). Os dados foram expressos em média ± desvio padrão, e as diferenças com valores de $p<0,05$ foram considerados significativos.

5 – RESULTADOS

Artigo submetido a revista Plos One

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Effects of Low-Level Laser on Oxidative Stress Markers of the Gastrocnemius Muscle in Diabetic Rats Subjected to High-Intensity Exercise

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Full Title:	Effects of Low-Level Laser on Oxidative Stress Markers of the Gastrocnemius Muscle in Diabetic Rats Subjected to High-Intensity Exercise
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Corresponding Author:	Paulo de Tarso Camillo de Carvalho, Ph.D Univesidade Nove de Julho São Paulo, São Paulo BRAZIL
Keywords:	Oxidative Stress; Low Level Laser; Diabetic Rats; Exercise
Abstract:	Purpose: The aim of the present study was to determine whether low-level laser therapy (LLLT) in diabetic rats subjected to high-intensity exercise, interferes with the expression of oxidative stress markers in the gastrocnemius muscle. Materials and Methods: A total of 32 male Wistar rats (norvergicos albinus) were used for this study: 24 diabetic rats and 8 control rats. The animals were randomly allocated into four groups designated as follows: diabetic control group (GCD)-did not receive irradiation LLLT and was not subjected to the fatigue protocol; diabetic fatigue group (GDF)-did not receive irradiation LLLT but performed the fatigue protocol; diabetic laser fatigue group (GDFL)-received irradiation LLLT and performed the fatigue protocol, and; non-diabetic control group (GCND)-did not receive irradiation LLLT but performed the fatigue protocol. Diabetes was induced via intraperitoneal administration of streptozotocin (50 mg/kg). We subsequently assessed blood lactate levels, and LLLT was applied at 808 nm and 4 joules of energy in the indicated groups. The animals in the appropriate exercise groups were submitted to treadmill running with speed and gradual slope until exhaustion as noted by VO2max and lactate level. The animals were euthanized, and the muscle tissue was excised for the analysis of SOD markers: CAT, GPX, and TBARS. Results: VO20.75max values in the Diabetic group were significantly higher from those in the control group ($p < 0.01$) and the Diabetic LLLT group ($p < 0.05$). The results indicate that the activities of CAT, SOD, and GPx were higher and statistically significant ($p < 0.05$) in the Diabetic LLLT group than those in the diabetic groups. Outcomes for LLLT group were similar than control group ($p > 0.05$), statistically significant activities of antioxidant enzymes compared to the diabetic group. The LLLT group could also mitigate the concentration of TBARS ($p > 0.05$). Conclusion: LLLT imay reduce oxidative stress, as well as increase VO2 0.75max, . Furthermore, our outcomes indicate that LLLT can be an alternative to maintain physical fitness in conditions where subjects aren't able to perform exercise. Which needs to be tested in further clinical studies.
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animal sacrifice is part of the study, please include briefly in your statement which substances and/or methods were applied. Please enter the name of your Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board, and indicate whether they approved this research or granted a formal waiver of ethical approval. Also include an approval number if one was obtained. **Field Permit** Please indicate the name of the institution or the relevant body that granted permission.	
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Programa de Pós-Graduação em **Ciências da Reabilitação**
Stricto Sensu
Avaliação e Intervenção Terapêutica

Subject: **Submission of a manuscript for evaluation**

Title: **Effects of Low-Level Laser on Oxidative Stress Markers of the Gastrocnemius Muscle in Diabetic Rats Subjected to High-Intensity Exercise**

Authors: Marcelo Frigero, Andrey Jorge Serra, Ernesto Cesar Leal-Junior, Cid André Fidelis de Paula Gomes, Evela Aparecida Pereira da Silva, Anna Cristina de Farias Marques, Leslie Andrews Portes, Flavio Silva, Paulo José Ferreira Tucci, Paulo de Tarso Camillo de Carvalho

Dear editor in chief,

I, Paulo de Tarso Camillo de Carvalho, corresponding author of the manuscript, am enclosing herewith a manuscript entitled: **Effects of Low-Level Laser on Oxidative Stress Markers of the Gastrocnemius Muscle in Diabetic Rats Subjected to High-Intensity Exercise ,** for possible evaluation.

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2. All authors of this paper have read and approved the final version submitted;

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Best regards.

Paulo de Tarso Camillo de Carvalho, PhD
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Effects of Low-Level Laser on Oxidative Stress Markers of the Gastrocnemius Muscle in Diabetic Rats Subjected to High-Intensity Exercise

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Abstract

Purpose: The aim of the present study was to determine whether low-level laser therapy (LLLT) in diabetic rats subjected to high-intensity exercise, interferes with the expression of oxidative stress markers in the gastrocnemius muscle.

Materials and Methods: A total of 24 male Wistar rats (*norvergicos albinus)* were used for this study: 16 diabetic rats and 8 control rats. The animals were randomly allocated into three groups designated as follows: control group (GCD)-did not receive irradiation LLLT and was not subjected to the fatigue protocol; diabetic fatigue group (GDF)-did not receive irradiation LLLT but performed the fatigue protocol; diabetic laser fatigue group (GDFL)-received irradiation LLLT and performed the fatigue protocol, and; non-diabetic control group (GCND)-did not receive irradiation LLLT but performed the fatigue protocol. Diabetes was induced via intraperitoneal administration of streptozotocin (50 mg/kg). We subsequently assessed blood lactate levels, and LLLT was applied at 808 nm and 4 joules of energy in the indicated groups. The animals in the appropriate exercise groups were submitted to treadmill running with speed and gradual slope until exhaustion as noted by VO_2max and lactate level. The animals were euthanized, and the muscle tissue was excised for the analysis of SOD markers: CAT, GPX, and TBARS. **Results:** VO20.75max values in the Diabetic group were significantly higher from those in the control group ($p < 0.01$) and the Diabetic LLLT group ($p < 0.05$). The results indicate that the activities of CAT, SOD, and GPx were higher and statistically significant ($p < 0.05$) in the Diabetic LLLT group than those in the diabetic groups. Outcomes for LLLT group were similar than control group ($p > 0.05$), statistically significant activities of antioxidant enzymes compared to the diabetic group. The LLLT group could also mitigate the concentration of TBARS (p>

0.05). **Conclusion:** LLLT may reduce oxidative stress, as well as increase VO2 0.75max,. Furthermore, our outcomes indicate that LLLT can be an alternative to maintain physical fitness in conditions where subjects aren't able to perform exercise. Which needs to be tested in further clinical studies.

Results: significant differences in statistically.

Conclusion: These results suggest laser therapy

Introduction

Type 1 diabetes accounts for only about 5–10% of all cases of diabetes; however, as its incidence continues to increase worldwide there are serious short-term and long-term implications. The disorder has a strong genetic component, inherited mainly through the human leukocyte antigen (HLA) complex; however, factors that can trigger the onset of clinical disease remain largely unknown. The management of type 1 diabetes is best undertaken in the context of a multidisciplinary approach, by a team of health specialists, and requires continual attention to many aspects [1]. Currently, regular exercise, is considered one of the three main approaches to the treatment of type 1 diabetes alongside insulin therapy and dietary regimens [2]. Exercise should theoretically be an attractive option for people who prefer not to use medication, or wish to obtain additional blood glucose control benefits [3]. However, strenuous exercise results in muscle fatigue and frequently causes post-exercise muscle damage that does not induce metabolic improvement, but rather impairs it. Growing evidence shows that muscle damage suppresses insulin sensitivity in comparison to the resting state (non-exercise condition), and this is caused by oxidative stress and inflammatory factors generated by the damaged muscle [4]. Appropriate exercise promotes the generation of physiological reactive oxygen species (ROS), enhances antioxidative potential, promotes exercise performance, improves metabolism, as well as, retards aging and related diseases. On the other hand, an exercise overload can cause excess generation of ROS, resulting in exercise-induced fatigue or even exercise-induced injury. Mitochondria are the main cellular sites for ROS production and act as the key organelles that modulate intracellular redox homeostasis [5].

Oxidative stress, referring to the prevalence of oxidant factors over antioxidant mechanisms, plays a central role in the pathogenesis and progression of diabetes and its complications [6]. Diabetes is usually accompanied by an increase in the production of free radicals or impairment in antioxidant defenses. Mechanisms by which increased oxidative stress is involved in diabetic complications are partly known, including the activation of transcription factors, advanced glycated end products (AGEs), and protein kinase C [7].

The levels of ROS are increased by hyperglycemia, which occurs in both, type 1 and type 2 diabetes and, to a lesser extent, during insulin resistance, resulting in oxidative stress. Free fatty acids, which may be elevated during inadequate glycemic control, may also be contributory factors [8].

Acute exercise has the capacity to increase the generation of ROS and to induce muscle damage, particularly during single bouts of exhaustive exercise [9, 10]. These results could be more pronounced in a diabetic population where a sedentary life style and diabetes-related dysfunction could impair antioxidant defenses and increase susceptibility to oxidative stress and muscle damage.

Since the mid-1960s, the use of light energy as a therapy for inflammatory processes and cell trophism has opened up a new field of research with regards to understanding the interaction between electromagnetic energy and biological tissue [11]. More recently, low-level laser therapy (LLLT) and light-emitting diode therapy (LEDT) have been used to mitigate and delay muscle fatigue [12] in clinical [13, 14] and experimental [15, 16] studies, which demonstrate the ability of laser therapy to improve mitochondrial function and combat ROS and reactive nitrogen species (RNS) generated during physical exercise [17].

We, therefore, hypothesized that irradiation with LLLT could prevent increased oxidative stress and have a protective effect on the muscle by augmenting cellular antioxidant mechanisms. In the current study, we examined the relationship between LLLT, muscle fatigue, and oxidative stress, independent of changes in blood glucose levels.

Materials and methods

Animal Model

The study sample consisted of 24 male Wistar rats (*Norvegicus albinus*) with a mean body weight of 180± 40g. The animals were acquired from the animal facility of the University Nove de Julho (UNINOVE), where they were housed and kept under controlled lighting and temperature conditions, and had *ad libitum* access to water and food. All experimental procedures were carried out in accordance with the standards established by the Brazilian College for Animal Experimentation (COBEA). The animals were handled in compliance with the national guidelines for the humane treatment of laboratory animals, and the UNINOVE Research Ethics Committee approved all experimental procedures.

Sample Calculation

The sample calculation was carried out using the Ene® software, version 3.0 (Barcelona, Spain). Sample size was determined based on a previous experimental study by Berkowitz et al. [18]. The blood glucose concentration in the control and diabetic animals undergoing physical training was elected as the outcome variable. The calculation was based on the detection of differences in glucose concentrations of 223.60 mg/dL between groups, assuming a standard deviation of 6.80 mg/dL. Thus,

considering a statistical power of 80% and alpha level of 0.05, the number of animals required was determined as 8 per group.

Induction of Diabetes

The animals were maintained on a 12-hour fasting cycle and subsequently anaesthetized with a mixture of ketamine + xylazine (87 and 13 mg/kg, respectively) administered intraperitoneally. Diabetes was also subsequently induced via a single intraperitoneal injection of streptozotocin (STZ, 50 mg/kg, Sigma S/A). Prior to the injection, STZ was dissolved in citrate buffer (0.01M, pH 4.5) and administered approximately 5 minutes after dilution [19].

Experimental Groups

Two weeks after the STZ injection, the animals were considered to have type 1 diabetes if the plasma glucose level was > 250 mg/dL and diabetic features, such as polyuria, polydipsia, and hyperphagia, were observed [20]. Four weeks after the STZ injection, animals were randomly allocated to two groups with 8 animals each: (i) control group with 8 normal rats injected intravenously with only citrate buffer was also included in the study (ii) diabetic fatigue group did not receive irradiation LLLT but performed the fatigue protocol; (iii) diabetic laser fatigue group received irradiation LLLT and performed the fatigue protocol.

Blood lactate concentration

Blood lactate concentrations were determined using a portable lactate analyzer (model Accutrend® lactate meter Roche Diagnostics, Mannheim, Germany) using a drop of blood taken from the animal's tail [21]. A baseline measurement (at rest) was performed prior to application of the LLLT, and a repeat measurement was performed

immediately after fatigue had been reached, as determined by analysis of the maximum volume of oxygen (VO_2max) level. The control group underwent the same blood lactate evaluation process, but was not subjected to exercise on the treadmill [22].

Low-level laser application

The DMC Photon Laser III® aluminum gallium arsenide diode laser (AlGaAs) (DMC, Sao Carlos, SP, Brazil) system was used for irradiation under the parameters listed in Table 1. The laser was applied transcutaneously, and irradiation was performed at three anatomical locations for the duration of 44 s/location in the central, proximal, and distal areas of the gastrocnemius muscle [23]. The laser therapy was applied prior to each training session for both groups (those that underwent exercise training and the group that received LLLT only).

Table 1. Summary of the laser parametersP.D., power density; **E.D.,** energy density

High-intensity exercise protocol and determination of VO_2max

High-intensity exercise was assessed using a motorized treadmill coupled with a gas analyzer (Panlab; Harvard Bioscience Company, MA, USA), which continuously recorded the volume of oxygen (VO_2) and carbon dioxide (VCO_2). Three days prior to testing, the rats were introduced to the treadmill for 15 min in three 5-min stages as follows: Day 1 - 25 cm/s, 35 min/s, and 35 cm/s; Day 2 - 25 cm/s, 45 min/s, and 55 cm/s; and Day 3 25 cm/s, 55 min/s, and 65 cm/s. To evaluate VO_2max, each rat

performed a 2-min warm-up at 25 cm/s, with the treadmill speed increased by 9 cm/s every 2 min until physical exhaustion occurred. The VO_2max was performed before and after exercise training and was calculated as an allometric score (mL/kg 0.75/min), which is the VO_2max/lean body mass ratio [23].

Following the procedure on the treadmill and the blood sampling for lactate analysis, the animals were identified, weighed, and euthanized via intraperitoneal thiopental administration (THIOPENTAX - Cristália 100 mg/kg) (DL) with lidocaine 10 mg/ml (Xylestesin - Cristália). Tissue samples were then taken from the gastrocnemius muscles.

Tissue preparation

Muscles were homogenized (GLH Homogeniser Omni International) in RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM sodium chloride, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (1 mM AEBSF, 800 μM aprotinin, 40 μM bestatin, 14 μM E-64, 20 μM leupeptin, 15 μM pepstatin A; Sigma Aldrich, Ireland) and phosphatase inhibitors 10 mM sodium fluoride, 1 mM sodium orthovanadate, using a 10% w/v ratio. Samples were homogenized using 8 × 10 s bursts and left on ice for 20 min. Homogenates were then centrifuged for 15 min at 11,000 g and the supernatants were stored at −80°C. Protein concentration was determined using a bicinchoninic (BCA) assay (Thermo Scientific, Ireland) as described in the manufacturer's protocol, and absorbance was measured at 562 nm using a SpectraMax-M3 spectrophotometer (Molecular Devices, USA)

Activity of the enzyme Catalase (CAT)

Catalase activity was measured as described by Weydert and Cullen [24]. To determine the level of CAT activity, tissue samples were placed on ice in Eppendorf tubes containing 1 mL of 0.05 N phosphate, sonicated and centrifuged at 10,000 rpm for 5 minutes. The supernatant was separated and stored at -20°C for subsequent analysis using commercial kits (Cayman Chemical®, Michigan, USA). Which is based on the reaction of ammonium molybdate with H_2O_2 to form a light yellow complex compound. Adduct was measured spectrophotometrically at 405 nm with a plate reader (Cary 300 Bio, UV–vis spectrophotometer, Varian Instruments, CA, USA).

Concentrations of thiobarbituric acid

A modified version of the 2-thiobarbituric acid-reactive substances (TBARS) procedure by Draper and Hadley [25] . For the determination of TBARS concentrations, tissue samples were placed on ice in an Eppendorf container containing 1.5 mL of 0.05N phosphate buffer , homogenized and centrifuged for 5 minutes at 10,000 rpm. The supernatant was then separated and stored at -20 ° C for subsequent analysis using commercial kits (Cayman Chemical®, Michigan, USA). Absorbance was measured at 532 nm against the blank using a spectrophotometer (Cary 300 Bio, UV–vis spectrophotometer, Varian Instruments, CA, USA)

Western blotting

The protein content of glutathione peroxidase (GPx) and superoxide dismutase (SOD) was measured in the muscle fractions. Thirty microgram protein was loaded into each well of a 4%–12% gradient polyacrylamide gel (Novex; Invitrogen) and separated

by routine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for 1.5 hours at 20°C followed by transfer to a nitrocellulose membrane. All membranes were blocked in 5% non-fat milk protein (NFM) for 1 hour at room temperature. In general, membranes were incubated in appropriate dilutions of primary antibodies (diluted in 1% NFM in Tris-buffered saline with 0.05% Tween-20 [TBST] overnight in a 4°C cold room). Membranes were washed in TBS-T followed by incubation in appropriate dilutions of secondary antibodies (diluted in 5% NFM in TBS-T) conjugated to horseradish peroxidase. Signals were developed using a chemiluminescent substrate (Lumigen TMA-6; Lumigen, Southfield, MI) and visualized by exposing the membranes. Digital records were captured with a Kodak 290 camera, and protein bands were quantified using 1-dimensional (1D) analysis software (Eastman Kodak). Bands were quantified as optical density × band area and expressed in arbitrary units [26].

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). Biomarkers of oxidative stress were compared using a one-way analysis of variance (ANOVA) with Tukey's *post-hoc* testing. Data were expressed as mean ± standard deviation, and differences with p-values < 0.05 were considered significant.

Results

Functional fitness assessment (maximal oxygen uptake, $VO_2max^{0.75}$ Allometric)

The evaluation of cardiopulmonary function was performed using a standard approach for the $VO_2max^{0.75}$ measurement (maximum VO_2 corrected for body mass of the animals), during the muscle fatigue protocol. The objective of this measure was to ensure that animals submitted to the protocol had declining functional fitness characterizing the effectiveness of the protocol. No statistically significant difference in VO_2max values was observed when comparing the control group (non-diabetic) 54.1 ± 3.4 ml/ kg/min and the group of diabetic animals subjected to fatigue treated with LLLT 53.1 ± 5.4 ml/kg/min ($p > 0.05$). However, comparisons between the control group and the group of diabetic animals subjected to fatigue 40.10 ± 2.7 ml/kg/min showed a statistically significant difference ($p < 0.05$). When comparing the fatigued groups with and without LLLT, or the diabetic group versus the diabetic fatigue group treated with LLLT, no statistically significant differences were observed ($p > 0.05$, Fig 1).

The respiratory quotient ($RQ = VCO_2/VO_2$) was also used as an indicator that animals subjected to the treadmill protocol had reached the maximum effort. These results are depicted in Fig 2B and show a statistical difference when comparing the fatigued groups with and without intervention by the LLLT ($p < 0.05$).

Fig 1. Comparison of the functional fitness assessment (maximal oxygen uptake, allometric VO_2 $max^{0.75}$)

Data are expressed as mean ± standard deviation. Panel A: * $p < 0.05$ & ** $p < 0.01$ using Tukey's test for comparisons with the control group; # $p < 0.05$ and ## $p < 0.01$ using Tukey's test for comparisons with the diabetic fatigue group. Panel B: Respiratory Quotient ($RQ = VCO_2{}^{0.75}/VO_2{}^{0.75}$); * $p < 0.05$ and ** $p < 0.01$ using Tukey's

test for comparisons with the control group; # $p < 0.05$ and ## $p < 0.01$ using Tukey's test for comparisons with the diabetic fatigue group.

Maximum speed treadmill running

With regards to the maximum speed achieved during the fatigue protocol the results were as follows: control group 55.20 ± 3.3 m/me; LLLT group 49.20 ± 8.8; and diabetic fatigue group 42.16 ± 9.0. There was a statistical difference between the control group and the diabetic fatigue group ($p < 0.05$) (Fig 2).

Fig 2. Comparative analysis of the effect of treadmill running between the control group, diabetic fatigue group, and diabetic fatigue group with low-level laser therapy (LLLT) * $p < 0.05$ and ** $p < 0.01$ using Tukey's test for comparisons with the control group; # $p < 0.05$ and ## $p < 0.01$ using Tukey's test for comparisons with the diabetic fatigue group.

Determination of Blood lactate

Blood lactate levels were determined at rest (baseline) and immediately after the treadmill exercise. These measures were used as the scope parameter and maximum effort was determined as the level that surpassed twice that of baseline. In intra-group statistical analysis (pre- and post-exercise) blood lactate levels showed the following results. There were statistically significant differences between baseline (2.80 ± 0.4) and

post-exercise (10:58 ± 4.10) levels in the control group ($p < 0.05$). Similarly, there were significant differences in baseline and post-exercise levels in the diabetic fatigue group (4.6 ± 0.4 vs.15.4 ± 16.4, $p < 0.05$), as well as, the LLLT group (3:02 ± 0:29 vs. 12.86 ± 2:37, $p < 0.05$) (Fig 3).

Fig 3. Comparison of blood lactate levels between groups (baseline and post-exercise) Data are expressed as mean ± standard deviation. Control group, diabetic fatigue group, diabetic fatigue with low-level laser therapy (LLLT) group. * $p < 0.05$, t-test compared with the control group basal level; # $p < 0.05$, t-test compared with diabetic fatigue group basal level; Φ $p < 0.05$, t-test compared with the diabetic fatigue with low-level laser therapy (LLLT) group.

Western blot: Superoxide dismutase (SOD) and Glutathione peroxidase (GPx)

The levels of SOD and GPx in the gastrocnemius muscle were determined with Western blotting and the results were as follows: the control group (3.40 ± 0.4); diabetic fatigue group (1.70 ± 0.3); and LLLT (3.10 ± 0.27). The SOD protein expression in the gastrocnemius muscle of the control rats was significantly different from the diabetic fatigue group ($p < 0.01$), before exercise. Comparisons between the diabetic fatigue group before exercise and the intervention group (LLLT) showed significantly higher values ($p < 0.01$).We also observed a significant increase in the protein expression of SOD in the LLLT group in comparison to diabetic fatigue group however, there was no significant difference between the LLLT group and the control group ($p > 0.05$) (Fig 4).

The same pattern of protein expression as that of SOD was observed for GPx in the control group (6.60 ± 0.6), diabetic fatigue group (10.4 ± 0.7), and LLLT (30.6 ± 0.7). We noted a statistically significant difference between the control group and diabetic fatigue group ($p < 0.01$), and between the diabetic fatigue group and LLLT group ($p < 0.01$), which demonstrates the antioxidant potential of LLLT. This observation can be seen when comparing the control group and LLLT, which showed no statistical difference ($p > 0.05$, Fig 4).

Fig 4. Comparison of Superoxide Dismutase (SOD) activity in the gastrocnemius muscle of diabetic and non-diabetic animals. Data are expressed as mean ± standard deviation. Panel A represents the activity of the SOD after the fatigue protocol, whereby * denotes $p < 0.05$ using Tukey's test for comparisons with the control group; # denotes $p < 0.05$ and ## denotes $p < 0.001$ using Tukey's test for comparisons with the diabetic fatigue group. Panel B represents comparisons of the activity of glutathione peroxidase (GPx) in the gastrocnemius muscle of diabetic and non-diabetic animals and after the fatigue protocol, whereby * denotes $p < 0.05$ using Tukey's test for comparisons with the control group; # denotes $p < 0.05$ and ## denotes $p < 0.001$ using Tukey's test for comparisons with the diabetic fatigue group.

Activity of the enzyme Catalase (CAT)

The mean values of the enzyme catalase (CAT) activity (μmol/min/mg protein) were 6.10 ± 1.1 (control group), 8.40 ± 1.5 (diabetic fatigue group) and 6.30 ± 1.4 (LLLT group)The enzyme activity of CAT in the skeletal muscle of the control rats

was significantly different than that of the diabetic fatigue group rats ($p < 0.01$), before exercise. The results also showed an increase in the activity of CAT in the LLLT group in comparison to the diabetic fatigue group ($p < 0.01$, Fig 5).

Concentration of Thiobarbituric Acid (TBARS)

The mean concentrations of thiobarbituric acid (TBARS) (nmolMDA/mg protein) were as follows: control group (10.0 ±1.1); diabetic fatigue group (18.2 ± 2.4); and LLLT (7.3 ± 1.8). The activities of the oxidant TBARS in the skeletal muscle of the control rats was significantly different than that in the diabetic fatigue group rats ($p < 0.01$), before exercise. There was also a significant difference between the control group and the LLLT group ($p > 0.05$). Comparisons of the mean concentration in the diabetic fatigue group (before exercise) with the LLLT group showed a statistical difference ($p < 0.01$). We also observed that the LLLT had an influence on oxidant activity when compared with the diabetic fatigue group ($p < 0.01$, Fig 6).

Fig 5. Comparison of enzyme Catalase (CAT) activity and concentrations of Thiobarbituric Acid (TBARS) in the gastrocnemius muscle of the diabetic and non-diabetic animals Data are expressed as mean ± standard deviation. Panel A represents the activity of the enzyme CAT after the fatigue protocol where * denotes $p < 0.05$ and ** denotes $p < 0.001$ using Tukey's test for comparisons with the control group; # denotes $p < 0.05$ and ## denotes $p < 0.001$ using Tukey's test for comparisons with the diabetic fatigue group. Panel B shows the concentrations of Thiobarbituric Acid (TBARS) in the gastrocnemius muscle of the diabetic and non-diabetic animals and after the fatigue protocol where * denotes $p < 0.05$ and ** denotes $p < 0.001$ using Tukey's test for comparisons with the control group; # denote $p < 0.05$ and ##

denotes $p < 0.001$ using Tukey's test for comparisons with the diabetic fatigue group.

Discussion

The main axis of the present study revolves around current evidence that implicates oxidative stress as a participant in the development and progression of diabetes and its complications, and; recent studies demonstrating the potential antioxidant capacity of diverse types of LLLT in situations where increases in oxidative stress levels may occur due to muscle fatigue and high-intensity exercise.

Diabetes is usually accompanied by an increase in the production of free radicals or impaired antioxidant defenses. STZ-induced diabetes in rats represents a well-established animal model of type 1 insulin-dependent diabetes mellitus. Increased production of high levels of oxygen free radicals has been linked to glucose oxidation and non-enzymatic glycation of proteins, which contribute to the development of diabetic complications [25]. The protective antioxidant effects of LLLT have not been studied in many animal models, however, existing studies that have verified these effects show that LLLT can act as an effective anti-oxidant therapy. In the present study, we set out to evaluate the effect of LLLT in an animal model of type 1 diabetes, induced by STZ, whereby diabetic animals were subjected to muscular fatigue through high-intensity exercise on a treadmill electric and the exercise intensity was adjusted based on VO_2max.

In our study, VO_2max levels were monitored continually during the treadmill exercise and we found consumption to be lower in the diabetic group than in the other groups. Although VO_2 decreased in these animals, a lower value for RQ ($VCO_2^{0.75}/VO_2^{0.75}$), a marker of substrate utilization, indicates higher lipid oxidation than carbohydrate oxidation [26]. A similar finding was observed in the study by Wohl et al. [27] that showed a reduction in RQ in type 1 diabetes. Our findings also indicate that the diabetic group treated with LLLT and subjected to fatigue had $RQ^{0.75}$ values closer to $VO_2max^{0.75}$ while the control group showed higher $RQ^{0.75}$ values. Although these findings are important, the $VO_2max^{0.75}$ in this study was used to confirm the intensity of the fatigue test.

In other clinical studies evaluating the effect of LLLT on exercise performance (VO_2max, time to exhaustion, aerobic threshold, and anaerobic threshold), oxidative stress, the levels of antioxidant enzymes (SOD and CAT), and muscle status in humans (creatine kinase and lactate dehydrogenase), the results show increases in VO_2max in the groups treated with LLLT [28].

In the present study, we also observed that the group of irradiated diabetic animals subjected to LLLT showed superior performance when compared to the group of diabetic animals with fatigue levels closer to the non-diabetic control group. However, these findings contradict those of previous works depicting LLLT as modulators of performance agents in normal individuals and athletes [29, 30, 31].

According Ferraresi [32], some studies have demonstrated the use of LLLT to accelerate metabolic and structural changes in the muscle with the goal of preventing or reducing muscle fatigue. Studies on this issue have used experimental models to identify possible effects of LLLT on muscle tissue subjected to mechanical and metabolic stress from exercise. In particular, resistance to fatigue and improved muscle

energy metabolism has been the focus of these pioneering studies. In our study, this capability is established when associating the results of previous studies with the observed ability of LLLT to reduce lactate levels. These results further corroborate the findings Assis et al. Leal-Junior et al. [31, 32].

To verify the effects of LLLT on the oxidant activity and anti-oxidative potential, we evaluated the protein expression of SOD and GPx, and CAT activity following completion of the high-intensity exercise protocol. As shown in our results, the effects of the LLLT in the diabetic group were comparable to oxidant activity in the control group, which indicates a reduction in lipid peroxidation in the muscle fiber membrane of the intervention group. Our findings also point to an increase in anti-oxidant activity in these animals after treatment with LLLT. These results corroborate those of De Marchi et al. [28] as the authors previously concluded that the use of LLLT before a progressive-intensity running exercise increased exercise performance and decreased exercise-induced oxidative stress and muscle damage. This suggests that the modulation of the redox system by LLLT could be related to the delay in skeletal muscle fatigue observed following LLLT.

ROS, such as superoxide anions ($\bullet O_2^-$) and hydroxyl radicals ($\bullet OH$), cause the oxidation of membrane phospholipids, proteins, and DNA, and such modifications have been implicated in a variety of pathological conditions. However, when there is an excess production of free radicals, oxidative stress has deleterious effects on the structural and functional integrity of cells and tissues [33]. CAT, found in peroxisomes, plays a specific role in metabolizing hydrogen peroxide, a highly toxic substance to the cell. SOD has three isoforms in the human body: SOD1 in the cytoplasm, SOD2 in the mitochondria, and SOD3 in the extracellular fluid. This enzyme catalyzes the dismutation (oxidation and reduction redox reaction) of the superoxide in oxygen and

hydrogen peroxide. GPx, found in the mitochondria, reduces hydro-peroxides to alcohols and hydrogen peroxide to water [34].

Recently, it has been demonstrated that phototherapy with differing wavelengths, doses, and time windows can improve cytochrome C oxidase activity in intact skeletal muscles [35], which has a direct influence on ROS and RNS generated during physical exercise. Therefore, phototherapy improves mitochondrial function, which contributes to the reduction of muscle fatigue and subsequently enhances muscle performance. Biasibetti et al. [33] conducted a study with the aim of evaluating the influence of LLLT on parameters of oxidative stress and DNA damage in skeletal muscle and plasma of rats with heart failure (HF). They concluded that laser therapy appears to reduce SOD activity and DCFH oxidation levels, changing the oxidative balance in the skeletal muscle of HF rats, whereas high doses of LLLT seem to increase DNA damage.

da Fonseca et al. [36] reported that lipid peroxidation can be assessed by TBARS, a biochemical index of oxidative damage by free radicals. The authors conducted a study to evaluate the effects of the low-intensity infrared laser on plasma protein content and oxidative stress in blood, and concluded that LLLT increases plasma protein content and causes oxidative stress, lipid peroxidation, as well as, increases myeloperoxidase activity, which could depend on the fluency and frequency in blood samples.

Although the effects of LLLT on ROS production are still controversial, it has been suggested that LLLT could enhance ROS production in human neutrophils by activating the superoxide converting system. LLLT irradiation enables a more rapid activation of this system; however, laser irradiation has been demonstrated to reduce oxidative stress in different situations, such as isolated human neutrophils during

abdominal surgery and *in vitro* in liposome membranes [37]. Finally, since outcomes in the group undergoing LLLT were similar to the control group, and both were better than the control group and diabetic fatigue group, we believe LLLT could be a suitable alternative to maintain antioxidant activity in conditions where subjects are unable to maintain this activity, such as in type 1 diabetes. Nevertheless, our findings remain to be evaluated in further clinical studies.

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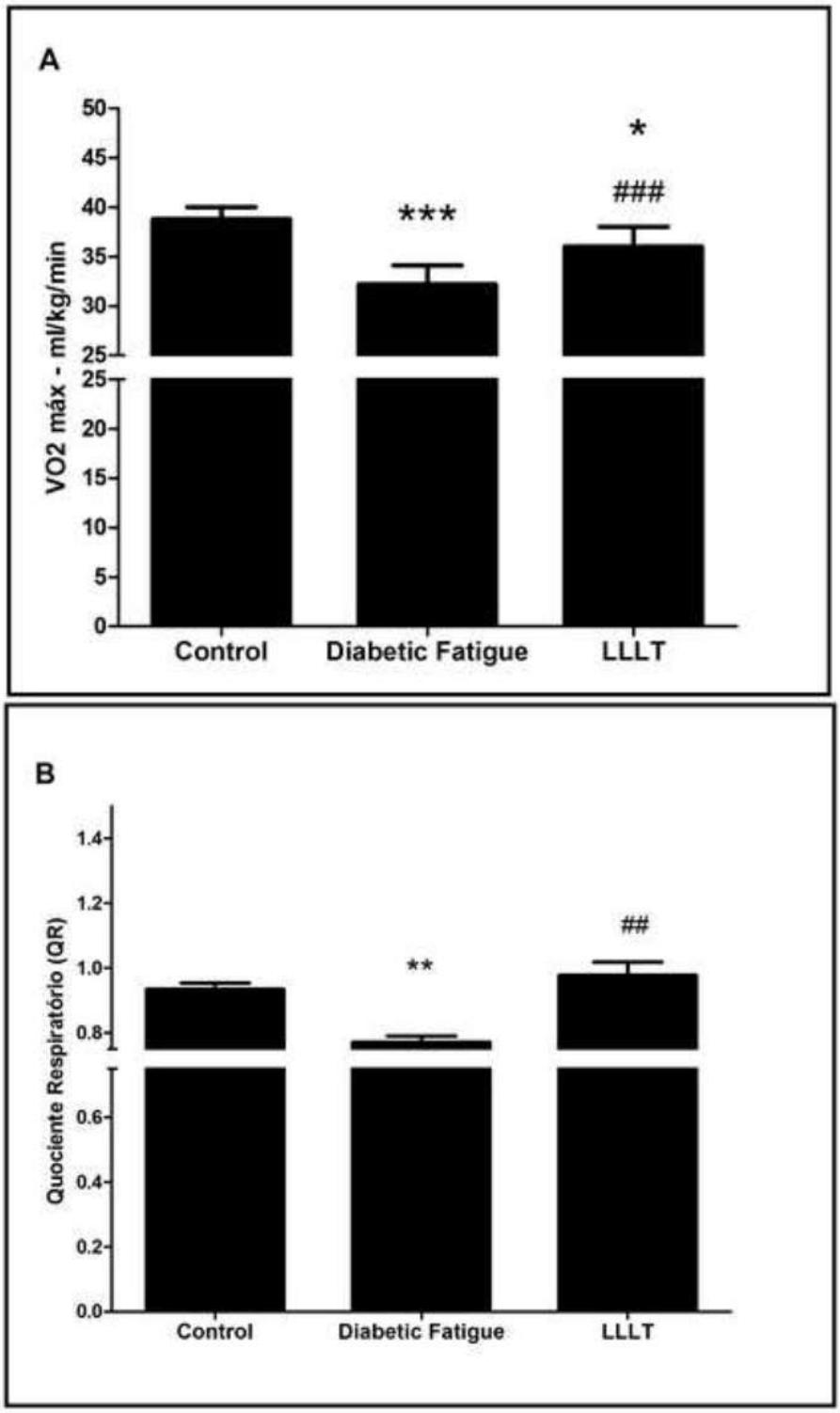
Table 1. Summary of the laser parameters.

Laser λ = nm	Power (mW)	Spot size (cm^2)	P.D. (mW/cm^2)	Energy (Joule)	E.D. (J/cm^2)	Time (s)	Energy total (Joule)
808	30	0.028	1.071	4	142.4	133	24

P.D., power density; **E.D.,** energy density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###
Control
Diabetic Fatigue
LLLT
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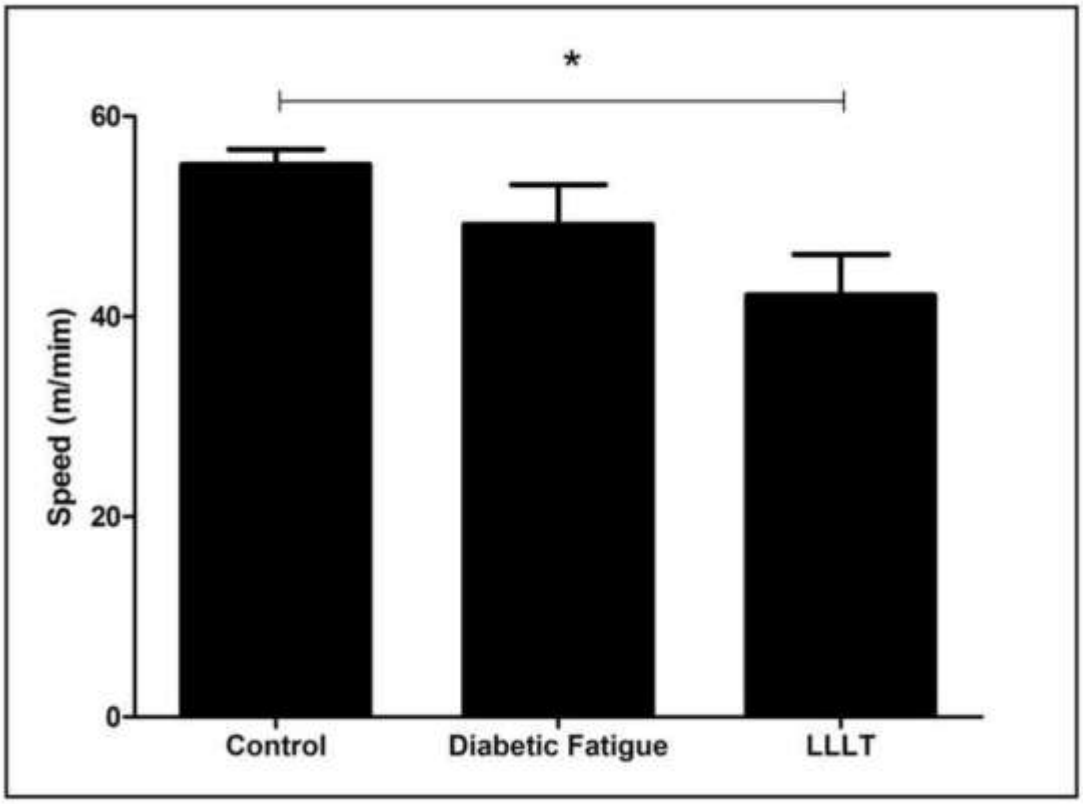
Control
Diabetic Fatigue
LLLT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60
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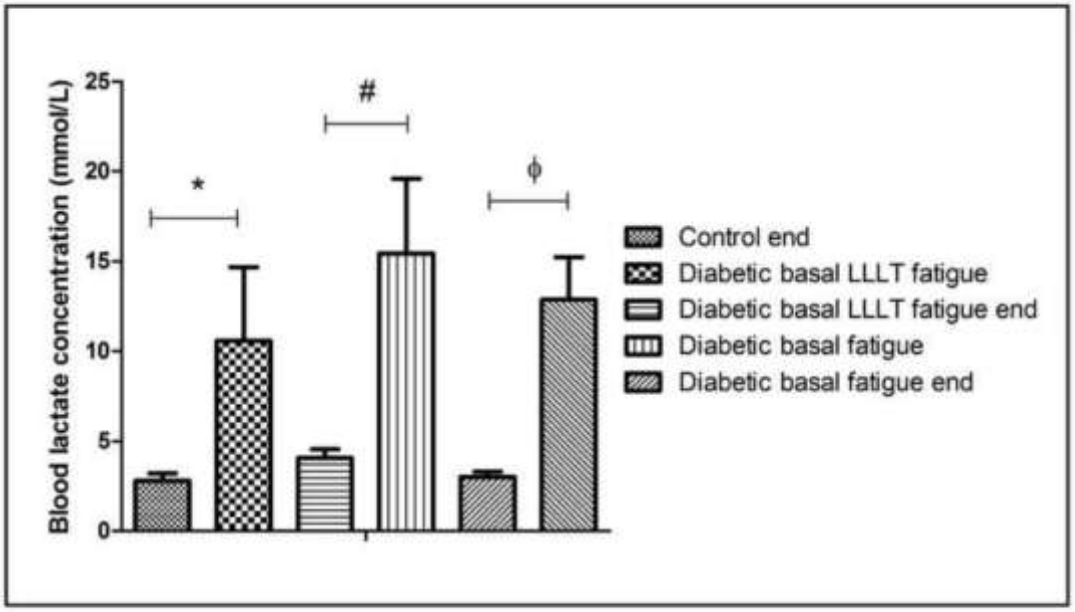
Control
Diabetic Fatigue
LLLT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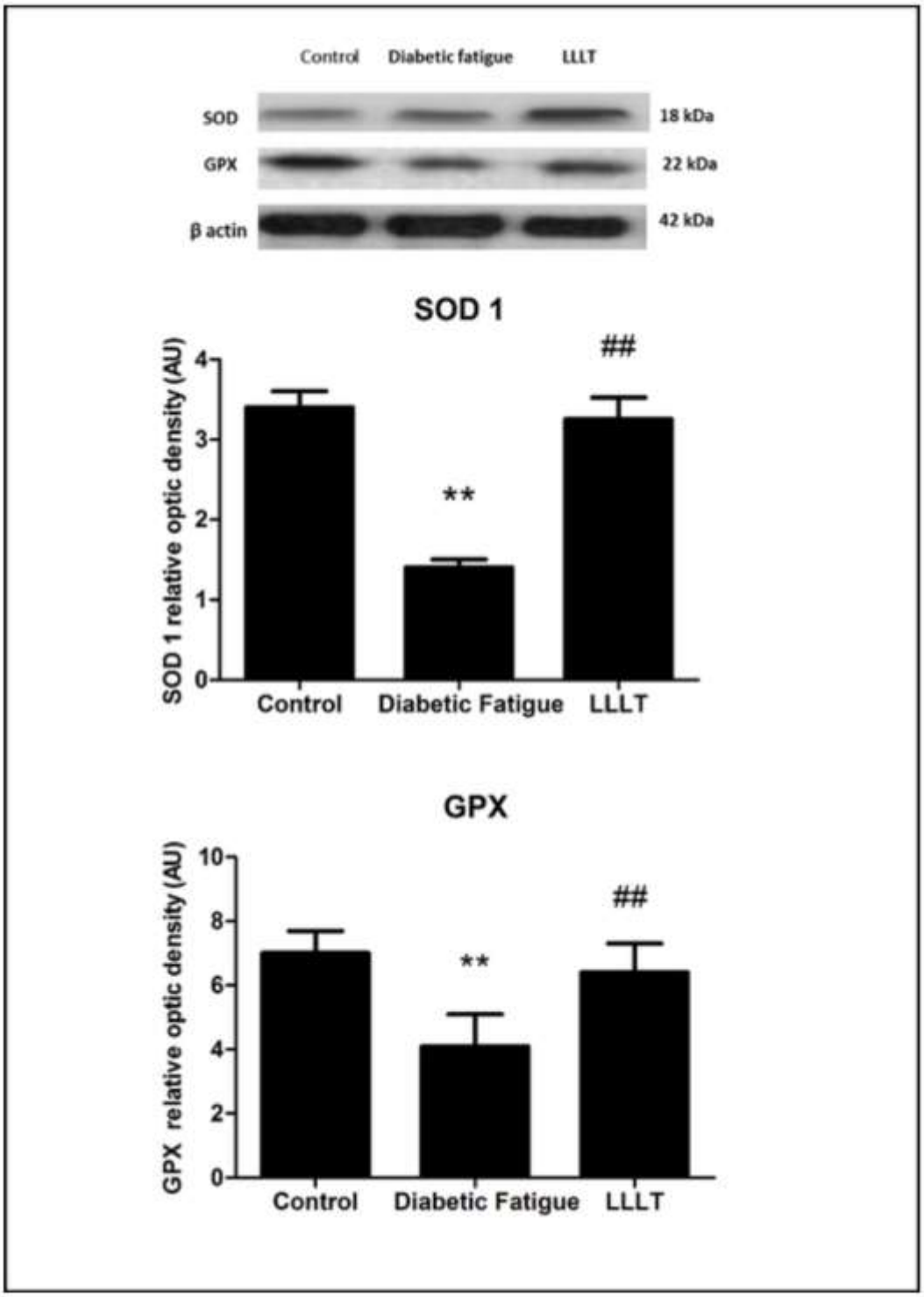
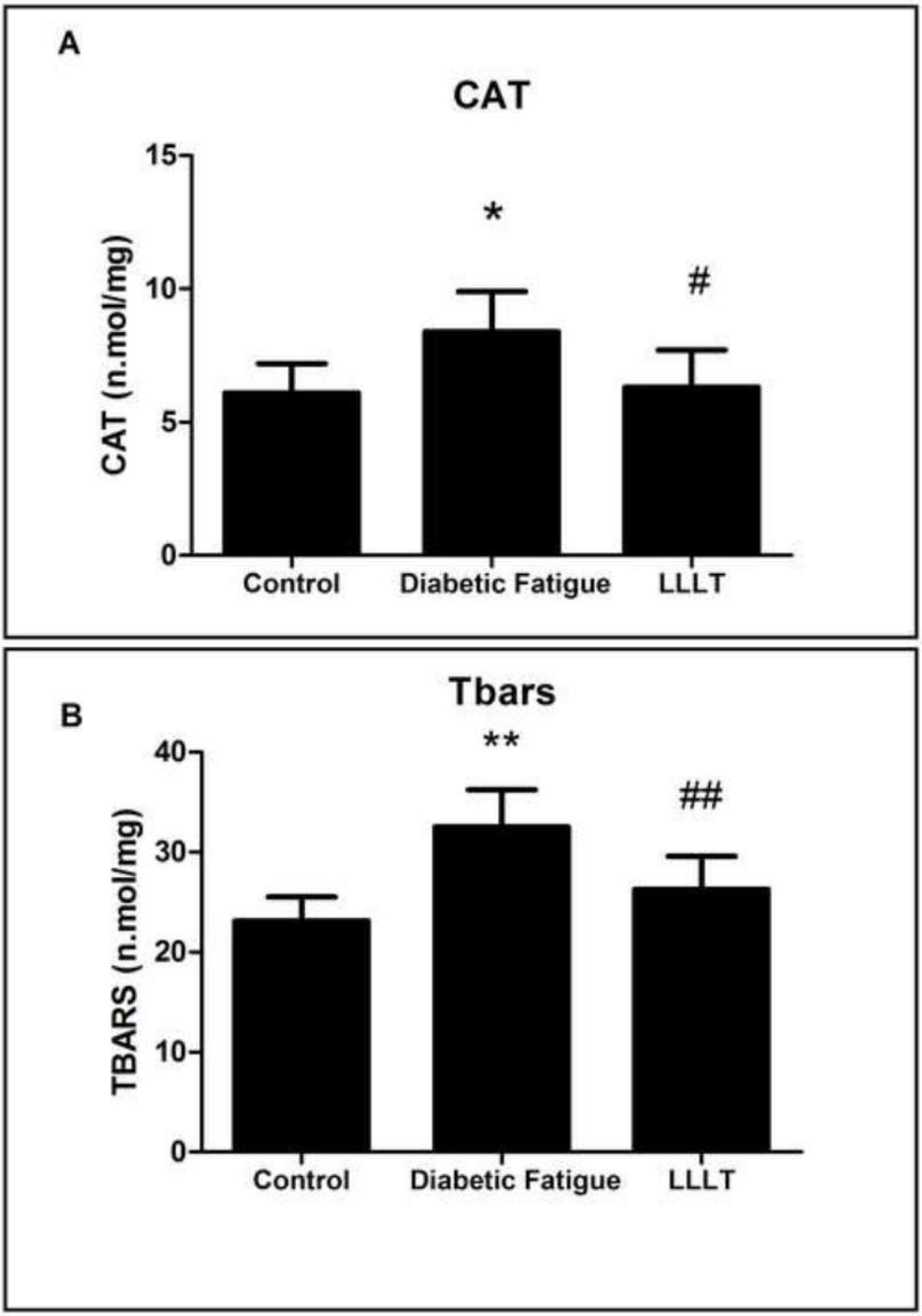

Control
Diabetic fatigue
LLLT
SOD
GPX
β actin
18 kDa
22 kDa
42 kDa
SOD 1
SOD 1 relative optic density (AU)
0
1
2
3
4
**
##
Control
Diabetic Fatigue
LLLT
GPX
GPX relative optic density (AU)
0
2
4
6
8
10
**
##
Control
Diabetic Fatigue
LLLT


A
CAT
CAT (n.mol/mg)
15
10
5
0
*
#
Control
Diabetic Fatigue
LLLT
B
Tbars
TBARS (n.mol/mg)
40
30
20
10
0
**
##
Control
Diabetic Fatigue
LLLT

6 - DISCUSSÃO

O presente estudo se baseia nas evidências de que as doenças crônicas, como a diabetes, são causadoras de danos teciduais e estresse oxidativo, e em estudos recentes que demostraram a capacidade antioxidante de diversos tipos de LBI, em situações em que houve um aumento do estresse oxidativo devido a fadiga muscular ou a realização de exercícios de alta intensidade.

A resistência à insulina ou a falta absoluta da mesma, resulta no comprometimento do controle metabólico da glicemia, culminando com um quadro de hiperglicemia que é considerado o fator de risco para o desenvolvimento das chamadas complicações da diabetes (Tiwari, 2002).

A diabetes é normalmente acompanhada por um aumento na produção de radicais livres ou as defesas antioxidantes são prejudicadas pela doença. O aumento na produção ou níveis elevados de radicais livres de oxigênio, tem sido associada a oxidação de glicose e glicação não enzimática de proteínas, os quais contribuem para o desenvolvimento de complicações diabéticas (Draper,1990).

Os efeitos protetores antioxidantes do LBI não tem sido alvo de estudos em modelos animais, entretanto os estudos realizados apresentam estes efeitos protetores e apontam que a LBI pode atuar como uma terapia antioxidante eficaz.

No presente estudo, optamos por avaliar o efeito do LBI em um modelo animal de DM1, induzida por STZ. Os animais diabéticos, foram submetidos a fadiga muscular através de exercícios de alta intensidade em uma esteira elétrica, com a intensidade do exercício sendo ajustada baseando-se no VO_2máx.

Em nosso estudo, os níveis de VO_2máx foram monitorados continuamente durante o exercício na esteira e encontramos que o consumo tende a ser menor no Grupo Diabético Fadiga do que nos outros grupos.

Embora o VO_2 tenha diminuído nestes animais, um valor menor para QR ($VCO_2^{0,75}$ / $VO_2^{0,75}$), indica maior oxidação de lipídeos do que de oxidação de carboidratos (Maritim,2003).

Uma descoberta semelhante, foi observada no estudo de Wohl et al. (2004) que demonstraram uma redução na QR na DM1. Nossos resultados também indicam que o grupo de animais diabéticos tratados com LBI e submetido a fadiga, teve um $QR^{0.75}$ com valores mais próximos de $VO_2máx^{0.75}$, enquanto o Grupo Controle apresentou valores mais elevados $QR^{0.75}$.

Estes resultados são importantes pois o $VO_2máx^{0,75}$, em nosso estudo, foi utilizado inicialmente para confirmar se o protocolo de exercícios de alta intensidade levou os animais ao estágio de fadiga.

No entanto, outros estudos clínicos que avaliaram os efeitos do LBI no desempenho do exercício (VO_2máx, tempo de exaustão, de limiar aeróbio, e limiar anaeróbio), o efeito sobre estresse oxidativo (analisando os níveis de enzimas antioxidantes SOD e CAT) e o estado muscular (creatina quinase e lactato desidrogenase) em seres humanos, encontraram resultados que mostram um aumento no VO_2máx nos grupos tratados com LBI (Wohl et al. (2004).

No presente estudo, também observamos que, o grupo de animais diabéticos irradiados com LBI demonstrou um desempenho superior quando comparado com o grupo de animais diabéticos não irradiados e com o grupo controle não diabético, em relação ao nível de fadiga. No entanto, estes resultados contradizem os achados de trabalhos anteriores, que descrevem o LBI como agente modulador de desempenho em indivíduos normais e atletas (De Marchi et al, 2012; Patrocinio T et al, 2013; Assis L, et al 2015).

Segundo Ferraresi (2012), alguns estudos têm demonstrado o uso do LBI para acelerar alterações metabólicas e estruturais no músculo, com o objetivo de prevenir ou reduzir a fadiga muscular. Estudos neste sentido têm usado modelos experimentais para identificar possíveis efeitos da LBI no tecido muscular submetido ao estresse metabólico do exercício, em particular, a resistência a fadiga muscular e a melhora do metabolismo energético são as variáveis avaliadas nestes estudos.

Para verificar os efeitos do LBI sobre o potencial de atividade oxidante e antioxidante, avaliamos a expressão da proteína SOD e GPx e a atividade CAT, após a conclusão do protocolo de exercícios de alta intensidade. Como mostrado nos nossos resultados, o efeito da LBI na atividade oxidante no Grupo Diabético Fadiga Laser pode ser comparável ao Grupo Controle Não Diabético, o que indica uma redução na peroxidação lipídica na membrana da fibra muscular do grupo de intervenção. Nossos resultados também apontam para um aumento na atividade antioxidante nestes animais após o tratamento com LBI.

Estes resultados corroboram com De Marchi et al. (2012), em que os autores concluíram que a utilização de LBI antes de um exercício progressivo de alta intensidade, pode levar a uma melhora do desempenho no exercício, diminuição do estresse oxidativo induzido pelo exercício e a uma menor possibilidade de lesão muscular. Sugerindo que a modulação do sistema redox por LBI pode estar relacionada com o atraso na fadiga muscular esquelética observada após a laserterapia.

As EROs, tais como superóxido (O2 •-) e radicais hidroxila (• OH), causam a oxidação de fosfolipídios na membrana, proteínas e DNA, e essas modificações podem implicar numa variedade de condições patológicas. No entanto, quando existe o excesso de produção de radicais livres, o estresse oxidativo tem efeitos deletérios sobre o a integridade estrutural e funcional de células e tecidos (Leal-Junior et al, 2013). A CAT, quando encontrado em peroxissomos, desempenha um papel específico no metabolismo de peróxido de hidrogénio, uma substância altamente tóxica para a célula.

Já o SOD tem três isoformas no corpo humano: SOD1 no citoplasma, SOD2 mitocôndrias e SOD3 no fluido extracelular. Esta enzima catalisa a dismutação (oxidação e redução redox reação) do superóxido em oxigênio e peróxido de hidrogênio. O GPx, encontrado nas mitocôndrias, reduz hidro peróxidos para álcoois e peróxido de hidrogênio a água (Biasibetti et al. 2014).

Recentemente Vieira et al., (2013), demostraram que a fototerapia com diferentes comprimentos de onda, doses e janelas de tempo pôde melhorar a atividade do citocromo C oxidase em músculos esqueléticos intactos, que tem

uma influência direta sobre as ERO e RNS, que é gerado durante o exercício físico. Portanto, a fototerapia melhora a função mitocondrial, o que contribui para a redução da fadiga muscular e subsequentemente aumenta o desempenho músculo.

Biasibetti et al., (2014) realizaram um estudo com o objetivo de avaliar a influência do LBI sobre parâmetros de estresse oxidativo e danos no DNA em músculo esquelético e no plasma de ratos com insuficiência cardíaca. Os autores concluíram que a terapia a Laser parece reduzir a atividade da SOD e níveis de oxidação DCFH, mudando o equilíbrio oxidativo no músculo esquelético de ratos, enquanto que doses elevadas da LBI parecem aumentar os danos no DNA.

Fonseca et al., (2012) relataram que a peroxidação lipídica pode ser avaliada pela TBARS, um índice bioquímico de danos oxidativos por radicais livres.

Embora os efeitos da LBI sobre a produção de EROs ainda são controversos, foi sugerido que a laserterapia pode aumentar a produção de EROs em neutrófilos humanos ativando o sistema de conversão de superóxido. A irradiação com LBI, permite uma ativação mais rápida deste sistema. No entanto, a irradiação com Laser foi demonstrada para reduzir estresse oxidativo em diferentes situações, tais como neutrófilos humanos isolados durante cirurgia abdominal e in vitro em membranas de lipossomas.

Finalmente, uma vez que os resultados do Grupo Diabético Fadiga Laser foram semelhantes aos do Grupo Controle Não Diabético, e ambos foram melhores do que o Grupo Diabético Fadiga, acreditamos que a laserterapia pode ser uma alternativa adequada para manter a atividade antioxidante em condições as quais os indivíduos são incapazes de manter, tal como na DM1. No entanto, os nossos resultados devem continuar a serem avaliados em estudos clínicos.

7 – CONCLUSÃO

A aplicação prévia de LBI ao exercício de alta intensidade, preveniu os danos oxidativos a lipídios (TBARS) e evitou a diminuição da atividade de proteína SOD e GPx e a atividade CAT, induzida pelo exercício físico.

A aplicação prévia de LBI ao exercício retardou a fadiga muscular e aumentou a performance dos voluntários (aumento no tempo total de execução do exercício e valores mais altos de VO_2máx.

Entendemos que para a utilização e consolidação da LBI em relação a fadiga e danos musculares mais estudos devam ser realizados, pois temos muitas variáveis ainda para considerar na aplicação da LBI.

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Photobiostimulation in the modulation of cytokines IL-6, IL-8 (cytokine-inducedneutrophiçç chemoattractant; CINC-1) ande the production of type I and III collagen in skin wounds in aged rats.

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Abstract:

Previous studies have discussed an inverse correlation between age and wound healing, because it relates to the association of aging with a gradual decrease in healing capacity. Treatment with low level laser therapy (LLLT) improve wound healing by inducing increases in mitotic activity, numbers of fibroblasts, collagen synthesis, and neovascularization. Therefore, this study aimed to evaluate the effects of LLLT in cutaneous wound healing in aged rats. A punch biopsy of 8 mm in diameter was performed to produce a skin wound. The study included 45 male rats, of which 15 were young (30 days) and 30 were elderly (500 days). The 45 animals were distributed into 3 experimental groups, which were subjected to skin wounds and 1aged group received LLLT, with a 30 mW laser beam (power density of 1.07 W/cm2), beam area of 0.028 cm2, and λ660nm produced through active phosphide Gallium-Aluminum-Indio (InGaAlP). The LLLT application took the form of a single-point transcutaneous method, with a total energy of 2 joules per wound site, energy density of 72 J/cm2, and time of 1 minute and 7 seconds. Analysis was performed to verify the effect of LLLT on the quantity of collagen I and III,at the wound site by immunohistochemistry, cytokine-induced neutrophil chemoattractant (CINC)-1, by enzyme-linked immunosorbent assay (ELISA) and interleukin (IL)-6 real time-polymerase chain reaction (RT-PCR). That we conclude LLLT is effective in the modulation of inflammatory mediators IL-6, CINC-1, as well as increased collagen production in aged animals during different phases of the tissue regeneration process. However, the effects of LLLT obtained in the aged animals (aged LLLT group) suggest that new dosimetries should be tested to achieve

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Abstract

Previous studies have discussed an inverse correlation between age and wound healing, because it relates to the association of aging with a gradual decrease in healing capacity. Treatment with low level laser therapy (LLLT) improve wound healing by inducing increases in mitotic activity, numbers of fibroblasts, collagen synthesis, and neovascularization. Therefore, this study aimed to evaluate the effects of LLLT in cutaneous wound healing in aged rats. A punch biopsy of 8 mm in diameter was performed to produce a skin wound. The study included 45 male rats, of which 15 were young (30 days) and 30 were elderly (500 days). The 45 animals were distributed into 3 experimental groups, which were subjected to skin wounds and 1aged group received LLLT, with a 30 mW laser beam (power density of 1.07 W/cm^2), beam area of 0.028 cm^2, and λ660nm produced through active phosphide Gallium-Aluminum-Indio (InGaAIP). The LLLT application took the form of a single-point transcutaneous method, with a total energy of 2 joules per wound site, energy density of 72 J/cm^2, and time of 1 minute and 7 seconds. Analysis was performed to verify the effect of LLLT on the quantity of collagen I and III by immunohistochemistry, cytokine-induced neutrophil chemoattractant (CINC)-1, by enzyme-linked immunosorbent assay (ELISA) and interleukin (IL)-6 real time-polymerase chain reaction (RT-PCR). That we conclude LLLT is effective in the modulation of inflammatory mediators IL-6, CINC-1, as well as increased collagen production in aged animals during different phases of the tissue regeneration process. However, the effects of LLLT obtained in the aged animals (aged LLLT group) suggest that new dosimetries should be tested to achieve better results.

1. Introduction

Successful wound healing requires function of many cellular and molecular events that are coordinated to result in tissue repair or regeneration. Disturbances in any of these functions result in impaired wound healing. Wound repair can be thought of as the culmination of 3 major overlapping phases: inflammation, proliferation, and remodeling [1, 2].

Delayed wound healing in the aged is associated with impaired inflammatory responses with alterations in chemokine production, reduced macrophage phagocytic capacity, enhanced proteolysis and degradation of matrix constituents due to excessive leukocytes and inflammation, delayed re-epithelialization and neovascularization and impaired fibroblast migration are other characteristics of wound healing in elderly subjects [3, 4].

Overall, there are global differences in wound healing between young and aged individuals [5]. It has long been thought that pro-inflammatory cytokines, including interleukins (IL) 1α and 1β, IL-6, and tumor necrosis factor (TNF)-α, play an important role in wound repair. They likely influence various processes at the wound site, including stimulation of keratinocyte and fibroblast proliferation, synthesis and breakdown of extracellular matrix proteins, fibroblast chemotaxis, and regulation of the immune response [6].

Chemokines, among which IL-8 are active participants in the healing process because it stimulates the migration of various cell types into the wound site; particularly inflammatory cells. IL-8 expression is increased in acute wounds and play a role in re-epithelialization by the increase in migration and proliferation of keratinocytes. It also induces the expression of matrix metalloproteinases (MMPs) by leukocytes, and it is a strong chemoattractant for neutrophils, participating thus the inflammatory response.

However, IL-8 at high levels decreases the proliferation of keratinocytes and the contraction of the collagen by fibroblasts structure [7].

MMPs play an important role in all stages of wound healing by degrading all components of the extracellular matrix (ECM) and have the capacity to synthesize collagen type III and IV (MMP-3), collagen type IV, V, VII, X, denatured collagen (MMP-9) and other MEC members who contribute to the remodeling of the wound [8]. MMPs have its release controlled by TIMPs. [9]. Many MMPs can also regulate the activity of chemokines and are also involved in neovascularization process due to interaction with vascular endothelial growth factor (VEGF) in the formation of new blood vessels [10].

Angiogenesis is another complex process that could play a role in wound healing. The aging-induced decrease in tissue perfusion and impairment of angiogenesis are known to affect wound healing [4].

Various treatments to reduce the delay of repair and problems in healing that occur with age have been studied both under normal conditions and under conditions involving altered wound healing [7,11]. LLLT has been shown to be able to change the delay time and normalize wound healing parameters, mainly involving the modulation of inflammatory cytokines [12,13] and enhanced production of collagens type I and III [14, 15].

We propose, therefore, that LLLT can positively influence the healing process of cutaneous wounds during aging and can also positively modulate mediators of this process, such as vascular endothelial growth factor (VEGF), MMPs, TIMP, collagen, and the pro-inflammatory cytokines IL-6 and CINC-1 (a homolog of human IL-8).

2. Materials and methods

2.1. Animals

The study animals consisted of 45 male Wistar rats (*Rattus norvegicus albinos*), of which 15 were young (30 days) with body weights ranging from 130–150 g and 30 were aged (500 days) with body weights ranging from 400–450 g. The animals were obtained from the animal housing facility of the Universidade Nove de Julho (Brazil) and kept under controlled conditions of light and temperature, with free access to water and chow. All experimental procedures were approved by the Institutional Research Ethics Committee (AN 0028/2014), and they were conducted according to the guidelines of the Brazilian College for Animal Experimentation as well as the standards of the International Council for Laboratory Animal Science.

2.2. Experimental groups

Animals (15 young and 30 elderly) were randomly divided into a total of 3 groups each of 15 animals, which were further separated to 3 subgroups each containing 5 animals, according to the experimental time points of 3, 7, and 14 days. The groups were split as follows: "control aged", elderly animals that only underwent skin wounding; "LLLT aged", elderly animals that underwent skin wounding and LLLT; and "control young", young animals that only underwent skin wounding.

The animals were anesthetized by an intramuscular injection of a 7% ketamine solution (Cetamin, Syntec, Cotia, SP, Brazil) and a 0.3% xylene solution (Xilazin, Syntec, Cotia, SP, Brazil) at a proportion of 2:1 and a total injection volume of 0.2 mL per 100 g body weight. All possible care was taken to avoid any discomfort to the animals. Once anesthetized, the animals were placed in the prone position; the site was sterilized with alcohol-iodine, and the dorsum of the animal was shaved. Skin wounding was performed using an 8 mm diameter 'punch' with a circular blade, allowing the

removal of a circular area of skin. Four wounds were inflicted on each animal, with the site of wounding located in the middle portion of the median sagittal plane. After wounding, the injured animals were placed in clean cages, 5 animals in each cage, and freely provided with water and chow. The analgesic dipyrone was administered for 2 days after wounding at a dose of 0.1 mL/animal, 4 times daily, with a minimum of 4 hours between doses.

2.3. Laser application

We used the Photon Laser III® (DMC Equipment's LTDA, SP, Brazil) for LLLT, with a λ660nm laser beam produced through active phosphide Indio-Gallium-Aluminum (InGaAIP) according to the following parameters: frequency continuous, power density 1.07W/cm^2, power output 30 mW, spot size 0.028 cm^2, energy density 72 J/cm^2,total energy delivered 2 Joules, irradiation time per treatment 67 seconds. The application of LLLT was initiated immediately after skin wounding, and then on alternate days until the day of euthanasia for each group. The control group underwent the same experimental procedures, but did not receive LLLT.

2.4. Euthanasia

At the end of each period of 3, 7, and 14 days, the animals were weighed and then euthanized via intracardiac injection with thiopental sodium (Cristália Lab, SP, Brazil) at the dose of 0.05 mL per 100 g body weight. After euthanasia, following the indicated times, the wounded areas were surgically removed with a 1 cm margin of skin around the lesion depth to the fascia. The resulting samples, which were taken from 2 animals per time point, were frozen in liquid nitrogen and stored at -80 °C. Of the 2 samples per time point, 1 sample was allocated for imunohistochemical procedures and

the other sample was allocated for the analysis of protein expression by enzyme-linked immunosorbent assay (ELISA) and gene expression by RT-PCR.

2.5. Histological procedures and quantification of collagen

Samples frozen in liquid nitrogen were placed in OCT™ (Sakura, Finetechnical Co. Ltd., Japan) and 4 mm sections were prepared using a cryostat (CM 1850, Leica, Germany) and histological sections stained not willing to slides silanized for immunohistochemistry.

2.6. Immunohistochemistry

The frozen sections were washed in phosphate-buffered saline (PBS) and endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min. The sections were washed in PBS (6 × 5 min washes) and mounted with 1% normal goat serum in PBS for 30 min. Subsequently, the slides were incubated in the presence of primary antibody applied overnight at 4°C. Primary antibodies used were: Collagen I: mouse Antibody (Col-1) (sc-59772, Santa Cruz Biotechnology, Inc.), Collagen III: rabbit Antibody (S-17)-R (sc-8780 R, Santa Cruz Biotechnology, Inc.). After washing in PBS (6 times, 5 min), they were incubated with secondary antibody, agreement with primary antibody used, for 30 min. After washing in PBS (6 × 5 min washes), a coloring reaction was carried out with diaminobenzidine (Wako Pure Chemical Industries, Osaka, Japan) and nuclei were counterstained with hematoxylin. Areas of positive staining for each of the tags.was observed under a light microscope (E200, Nikon, Japan), and images were captured by a microcomputer equipped with IC Capture 2.2 software (The Imaging Source, Germany). From each sample, 4 images were recorded of different fields of view, including each part of the stained, using a 10× objective so

that the length was captured. Once captured, the images were analyzed using a software-based image analysis system (Image-Pro Plus® 4.5, Media Cybernetics, MD, USA).

2.7. Gene expression quantification

Total RNA was extracted from skin wound samples and RT-PCR assay was performed for mRNA quantification. Thawed tissues were homogenized in 1 mL of TRIzol® reagent (Gibco BRL, Gaithersburg, MD) and total RNA was isolated according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis and RT-PCR gene expression analysis. Initially, contaminant DNA was removed using DNase I (Invitrogen) at a concentration of 1 unit/μg RNA in the presence of 20 mM Tris-HCl, pH 8.4, containing 2 mM $MgCl_2$ for 15 min at 37 °C, followed by incubation at 95 °C for 5 min for enzyme inactivation. Then, the reverse transcription (RT) reaction was carried out in a 200 μl reaction volume in the presence of 50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs, and 50 ng of random primers with 200 units of Moloney murine leukemia virus-reverse transcriptase (Invitrogen, CA, USA). The reaction conditions were 20 °C for 10 min, then 42 °C for 45 min, and finally 95 °C for 5 min.

The reaction product was amplified by real time PCR on the 7500 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA, USA) using the SYBR Green® core reaction kit (Applied Biosystems). The thermal cycling conditions were: 50 °C for 2 min, then 95 °C for 10 min, followed by 40 cycles, each at 95 °C for 15 s and then 60 °C for 1 min. Experiments were performed in triplicates for each data point. Target gene mRNA abundance was quantified as a relative value compared with an internal reference, GAPDH, whose abundance was believed not to change between

the varying experimental conditions. Primers used for RT-PCR were: IL-6; GenBank accession number E02522, forward primer 5′-TCCTACCCCAACTTCCAATGCTC-3′ and reverse primer 5′-TTGGATGGTCTTGGTCCTTAGCC-3′. One microliter of RT reaction mix was used for RT-PCR.

2.8. Evaluation of the inflammatory mediators CINC -1

The amount of CINC-1 in skin wounds was quantified by ELISA (R&D Systems, USA) as per the manufacturer's instructions. For this purpose, 96-well plates were coated with 100 mL of monoclonal antibody for each cytokine: anti-CINC-1 diluted in sodium carbonate buffer (0.1 M, pH 9.6). The plates were incubated at 4 °C for 18 h. For blocking, the plates were washed 4 times with PBST (PBS containing 0.05% Tween®-20) and then filled with 300 μL/well of blocking solution (3% gelatin in PBST, Sigma) at 37 °C for 3 h before being subjected to a new cycle of washes. Next, 100 μL/well of diluted samples or recombinant cytokine standards were added to the plate and incubated for 18 h at 4 °C. After washing, 100 μL/well of the respective biotinylated antibody specific for the detection of each cytokine was added and left for 1 h at room temperature (RT). After washing the plates, 100 μL/well of streptavidin–peroxidase was added and incubated for 1 h at room temperature (22 °C) followed by further washes. The reaction was visualized by adding 100 μL/well of 3,3′5,5′-tetramethylbenzidine solution and stopped by adding 50 μL/well of sulfuric acid (2 N). The absorbance of each well was read using a SpectraMax® Plus 384 spectrophotometer (Sunnyvale, CA, USA) at a wavelength of 450 nm with correction at 570 nm. The sample concentrations were calculated from standard curves obtained with recombinant cytokines.

2.9. Statistical analysis

The data were tabulated using Excel 2007 software (Microsoft Corporation, WA, USA) and initially assessed for normality using the Shapiro–Wilk test. Since a normal distribution was observed, ANOVA with Tukey's post hoc test was used for comparisons between experimental groups. All of the data were expressed as mean and standard deviation values. Prism® 5 software (GraphPad, CA, USA) was used. Differences from the null hypothesis were considered to be significant when $p < 0.05$.

3. Results

3.1. Quantification of collagen I

Significant differences in the percentage of collagen I deposition at the wound site were observed among groups at 3 days post-wounding. There was a significant difference in the means of the percentage of collagen deposition at the wound site between the control young and aged (control and LLLT) groups. At 7 days, groups had similar behavior at 3 days, with significant differences between groups. At 14 days, the control young group showed significantly higher expression that the control aged and LLLT aged groups. There was also a significant difference between the control aged and LLLT aged groups, showing the biomodulatory effect of laser in deposition of collagen type I (Figure 1).

3.2. Quantification of collagen III

At 3 days post-wounding was found significant lower percentage of collagen III deposition at the wound site in the aged (control and LLLT) groups compared with control young group, however the LLLT aged group had higher percentage than the control aged group. At 7 days aged (control and LLLT) groups also had percentage of collagen III lower than the control young group and the LLLT aged group obtained

higher percentage than the control aged group. However, at 14 days the control young group had lower amount of collagen than the control aged and LLLT aged groups, and the LLLT aged group had a lower percentage than the control aged group (Figure 2).

3.7. Effect of LLLT on CINC -1 protein expression

The control young group showed a significant decrease in the concentration of IL-8 (CINC-1) compared with the control aged and LLLT aged groups at 3 and 7 days post-wounding. At 14 days showed a significant decrease compared to aged (control and LLLT) groups, however, LLLT administration led to a significant decrease in expression of IL-8 protein when comparing the aged (control and LLLT) groups, in 3, 7 and 14 days after injury (Figure 3).

3.8. Effect of LLLT on IL-6 mRNA expression in wound healing

Figure 4 shows a statistically significant increase of IL-6 mRNA expression between aged control group and control young group in 3, 7 and 14 days after injury. When compared the LLLT aged and control young groups, also occurred a significant increase on day 3 and on days 7 and 14. Although the aged groups showed higher expression compared the control young group, LLLT promoted a significant decrease expression in LLLT aged group on days 3, 7 and 14 days.

4. Discussion

The present study was designed to seek possible explanations as to whether the behavior of the healing process in elderly animals may be similar to that of the young animals already studied in previous work by our research group [16]. We observed that the response to LLLT was different in aged rats compared with young rats. The present

study was conducted to analyze the gene expressions of pro-inflammatory cytokine (IL-6) protein expression and a cytokine-induced neutrophil chemoattractant (CINC-1), a chemokine that belongs to the interleukin 8 family, and immunohistochemical expression of VEGF, MMP-3, MMP-9, TIMP-2, collagen type I and III.

The main results of this study included the following: The behavior of the repair process occurred differently between young and aged rats, with an obvious delay in the aged groups, as was verified by analysis of collagen and the expression of inflammatory mediators. The aged group treated with LLLT showed an increased rate of wound repair process, compared with their respective control group; however, the LLLT aged group still showed a lag in repair compared with the control young group, mainly regarding the inflammatory phase and proliferation, angiogenesis, and fibrogenesis.

The early response to wounding also results in the release of chemokines by keratinocytes, which act as chemoattractants for the migration of immune cells to the site of injury. Neutrophils arrive at the wound within minutes of wounding and become the predominant cells in the wound site for the first 2 days after the injury occurs. Neutrophils and platelets entrapped and aggregated in the blood clot release a wide variety of factors that amplify the aggregation response, initiate the coagulation cascade, and/or act as chemoattractants for cells involved in the inflammatory phase. Among other proinflammatory cytokines, IL-6 is produced by neutrophils and has been shown to be important in initiating the healing response; IL-6 has mitogenic and proliferative effects on keratinocytes and, at the same time, acts as a chemoattractant for neutrophils [18]. CINC-1 (a homolog of human IL8) expression increased the migration and proliferation of keratinocytes, induces the expression of MMPs and it is a strong chemoattractant for neutrophils [7].

According to Ershler and Keller (2000) [21], IL-6 is normally expressed at low levels except during infection, trauma or other stress factors. Among the many factors that regulate the expression of the IL-6 gene are estrogen and testosterone. After menopause or andropause, levels of IL-6 are elevated in the absence of infection, trauma or stress.

After tissue injury, IL-6 expression since increases in the 15 to 20 minutes of injury, with peak levels in 24-48 hours, gradually reducing in 7 days [18]. CINC-1 shows increased expression in the first hour after injury, down to 24 hours of follow-up, when it starts an increase up to 48 hours and from 7 days ago decreased expression [19]. The three groups of our study showed higher expression of IL-6 and CINC-1 in the initial phase of the repair process, to decrease over time, however, the aged groups showed high values IL-6 and CINC-1 and that LLLT was effective in decreasing the expression of these inflammatory mediators, throughout the experimental period. However, it is observed that although LLLT have attenuated the levels of IL-6 and CINC-1 in the irradiate group, the expression was higher than the control young group. This finding suggests a new proposal for LLLT dosimetry, which may be used in aged animals to achieve better results. This finding reinforces the results achieved by Rambo et al. [16] similar to ours in that compared the levels of pro inflammatory cytokines (IL-1 and TNF) in young and old animals, and these suggest a differentiated dosimetry of LLLT for treatment of young and aged.

The presence of elevated levels of IL-6 and IL-8 in the older age groups was also found by Sansoni et al. [20] showing a chronic inflammatory state.

The potential of LLLT in modulating inflammatory cytokines among them the IL-6 and CINC -1 is clear in the study. Alves et al. [21] found that LLLT reduces IL-6 expression in joint inflammation in rats. On human cell cultures, the red laser increased

the migration and proliferation of keratinocytes and the granulation tissue, by modulating IL-8 expression promoting more rapid re-epithelialization [22]. Lima et al [23] reported that LLLT (GaAlAs, continuous, 9 mW, 670 nm, 0.031 W / cm2) significantly decreased IL-6 levels, strongly suggesting an inflammatory biomodulation of LLLT.

We observed that in the control young group, the collagen I and III showed a normal course in the repair process, with greater deposition of collagen III and consequently less deposition of collagen I at the early phase, and increased deposition of collagen I and lower collagen III in the late phase of the process. In aged groups, we found that there was a delay in the deposition of collagen type I and III when compared to the control young group, however LLLT was able to reduce this delay in the LLLT aged group, especially in collagen III in the early stages of repair and collagen I at 14 days after injury.

The less collagen deposition in aged groups may be related to the small number of fibroblasts found in aged skin [36]. The improvement in collagen deposition in the LLLT aged group can be explained by the fact that the laser helps the proliferation of fibroblasts [16], so there will be more collagen deposition.

Increased collagen deposition at the beginning of the healing process with LLLT was also found by Biondo-Simões and Busnardo [37], with increased deposition of collagen III in the 3rd postoperative day and by Silva et al. [15] in wounds of diabetic rats with an increase in the total percentage of type III collagen.

Other studies have found increased collagen deposition with laser use. Fiorio et al [38] found increase collagen deposition in third-degree burns in rats. Carvalho et al. [39] demonstrated that the application of low intensity He-Ne laser (λ632.8 nm) promoted increase in the percentage of collagen in skin of diabetic rats wounds by

increasing the amount of collagen fibers similar to process observed in animals non-diabetic efficacy, indicating of LLLT effects in the healing process.

The healing process involves the coordinated efforts of several cell types such as cytokines, chemokines, metalloproteinases and their inhibitors, growth factors and fibroblast, that will deposit collagen in the wound. According to the results of this study, we conclude that even in aged tissue there is delayed healing, this tissue satisfactorily responded to LLLT with modulation of inflammatory mediators IL-6, CINC-1, as well as increased collagen production in aged animals during different phases of the tissue regeneration process.

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Author Disclosure Statement

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Figure 1- Comparison of the mean and standard deviation of immuno-labeled areas collagen I in mm^2 by immunohistochemistry in (A) Representative stained with immuno-labeled for collagen Iin at 3, 7 and 14 days after experimental wounds in rats aged in groups control, aged groups and LLLT aged. ×20. *Scale bar* = 20 μm. Panel (B) Represents areas immuno-labeled for collagen I in 3, 7 and 14, days after experimental induced tendinopathy in rats aged in groups control, aged tendinopathy groups and tendinopathy LLLT aged. *Denotes $p<0.05$; Denotes **$p<0.01$and***denotes $p<0.001$ using Turkey test with comparisons against the control group; #Denotes $p<0.05$, ## Denotes $p<0.01$and##denotes $p<0.001$ using Turkey test with comparisons against the aged tendinopathy group .Data are presented as mean ± SD.

Figure 2- Comparison of the mean and standard deviation of immuno-labeled areas collagen III in mm^2 by immunohistochemistry in (A) Representative stained with immuno-labeled for collagen I in at 3, 7 and 14 days after experimental wounds in rats aged in groups control, aged groups and LLLT aged. ×20. *Scale bar* = 20 μm. Panel (B) Represents areas immuno-labeled for collagen III in 3, 7 and 14, days after experimental induced tendinopathy in rats aged in groups control, aged tendinopathy groups and tendinopathy LLLT aged. *Denotes $p<0.05$; Denotes **$p<0.01$and***denotes $p<0.001$ using Turkey test with comparisons against the control group; #Denotes $p<0.05$, ## Denotes $p<0.01$and##denotes $p<0.001$ using Bonferroni test with comparisons against the aged tendinopathy group .Data are presented as mean ± SD.

Figure 3. Comparison of the mean and standard deviation levels of mRNA expression for interleukin-6 (IL-6) extracted from skin wound tissue and quantified by real-time polymerase chain reaction. Panel A represents concentrations in wounds 3 days after injury; panel B represents concentrations in wounds 7 days after injury; and panel C represents concentrations in wounds 14 days after injury. * denotes $p < 0.05$ and ** denotes $p < 0.001$ using Tukey's test with comparisons against the young rat control group; # denotes $p < 0.05$ and ## denotes $p < 0.001$ using Tukey's test with comparisons against the aged rat control group; and ϕ denotes $p < 0.05$ using Tukey's

Figure 3. Comparison of the mean and standard deviation levels of CINC-1 (a homolog of human IL-8) in skin wound tissue obtained by the enzyme-linked immunosorbent assay method. Panel A represents concentrations in wounds 3 days after injury; panel B represents concentrations in wounds 7 days after injury; and panel C represents concentrations in wounds 14 days after injury. * denotes $p < 0.05$ and ** denotes $p < 0.001$ using Tukey's test with comparisons against the young rat control group; # denotes $p < 0.05$ and ## denotes $p < 0.001$ using Tukey's test with comparisons against the aged rat control group; and ϕ denotes $p < 0.05$ using Tukey's test comparing the young rat group administered low-level laser therapy (LLLT) with the aged rat group administered LLLT.

Table 1

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Tables

Table 1 : LLLT parameters

Wavelength: nm	Frequency	Power density: W/cm²	Power output: mW	Spot size: cm²	Energy density: J/cm²	Total energy delivered : Joules	Irradiation time per treatment: seconds
660	Continuous	1,07	30	0,028	72	2	67

Figure 1.

Click here to download Figure Figure 1_Collagen I.tif

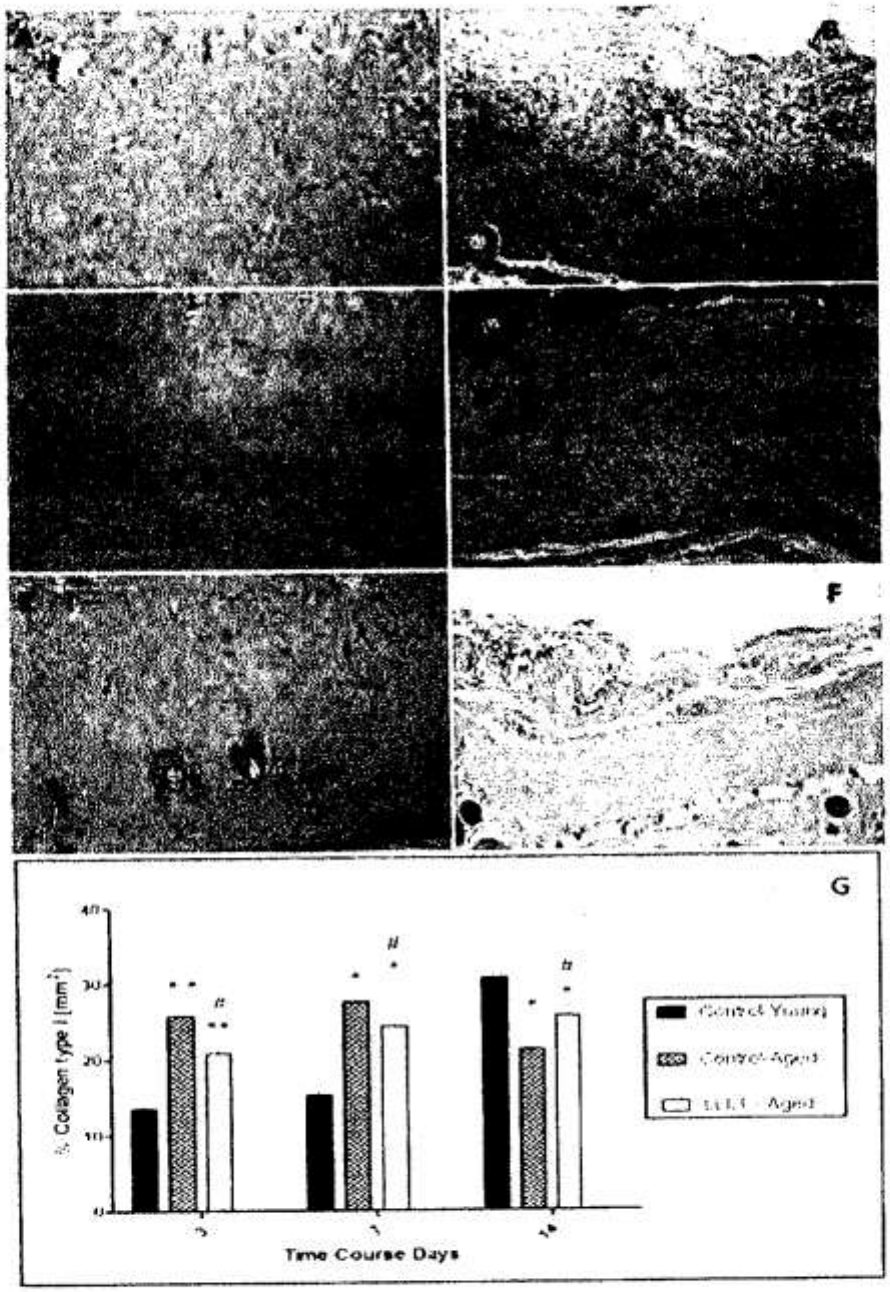

Figure 2.

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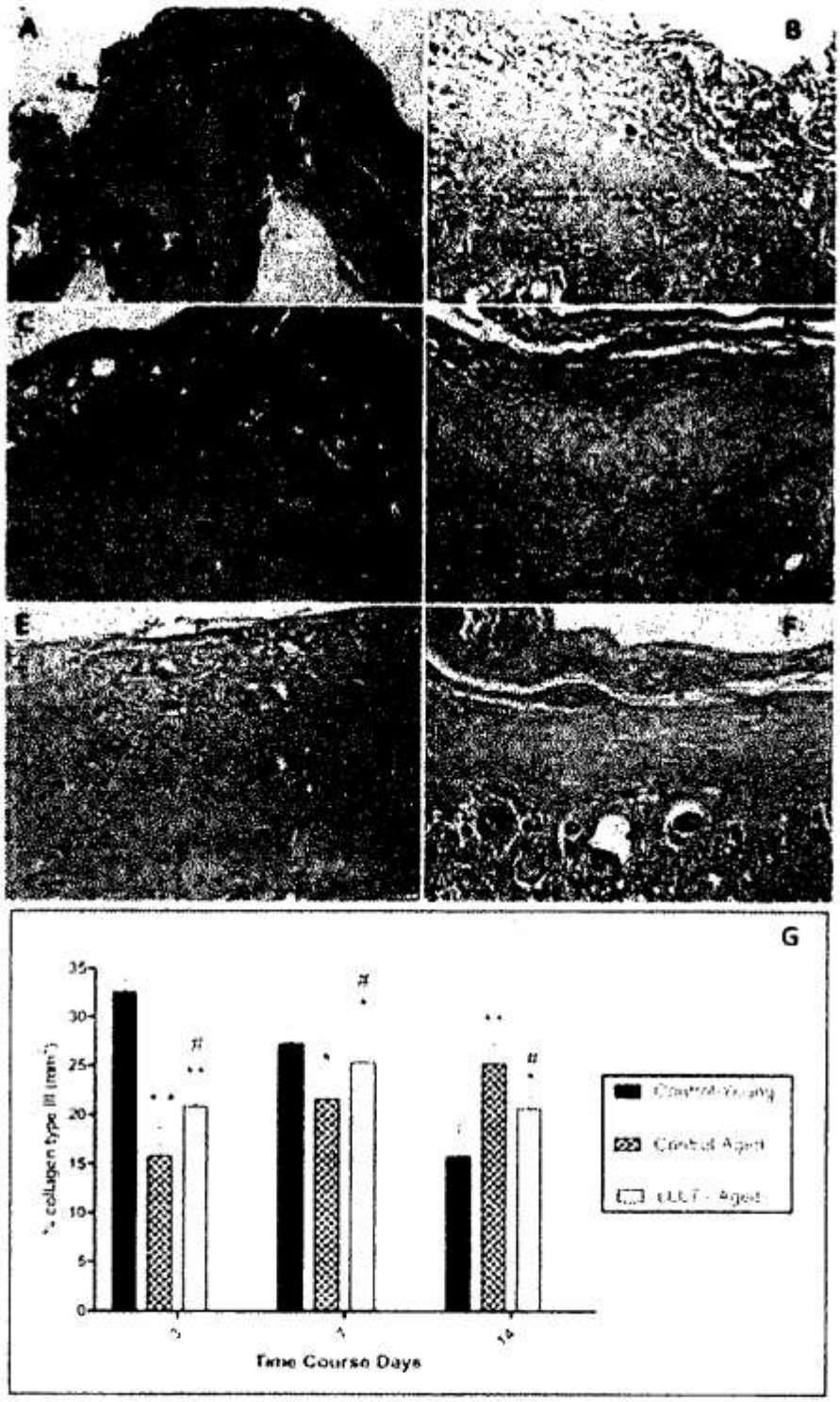

Figure 3.

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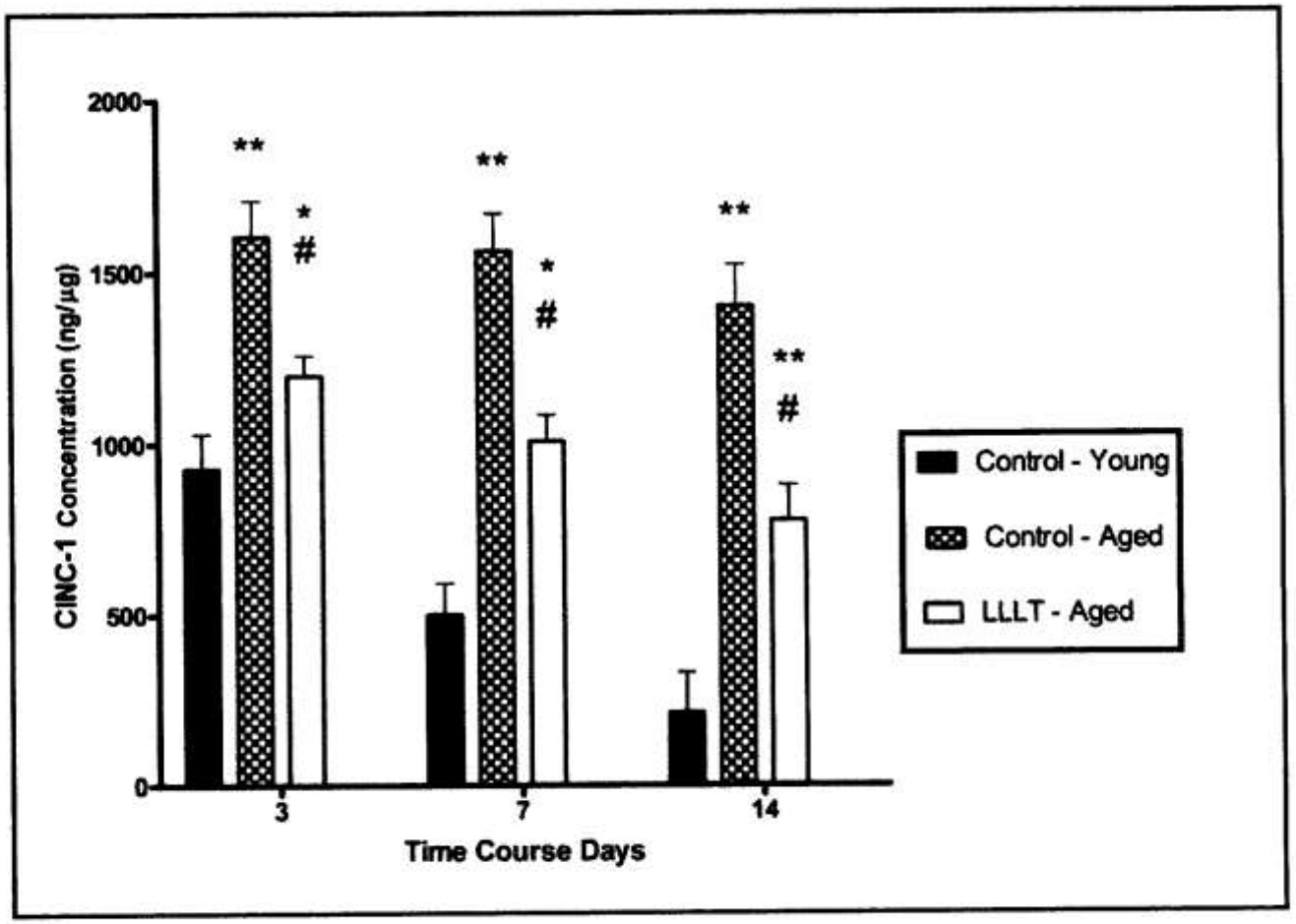

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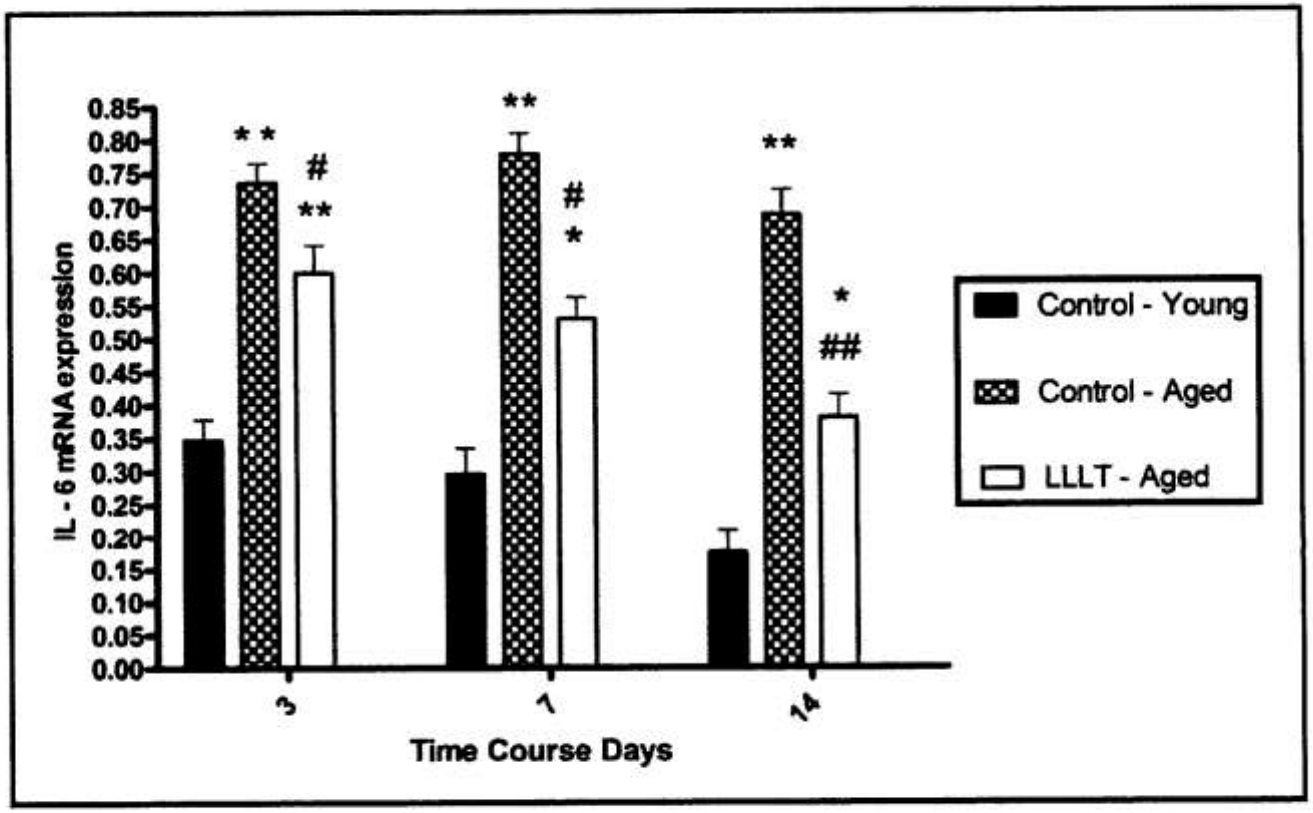
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