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Diane Duarte Hartmann

# FIBRAS MUSCULARES OXIDATIVAS E GLICOLÍTICAS APRESENTAM RESPOSTA INFLAMATÓRIA E ALTERAÇÕES BIOENERGÉTICAS PECULIARES APÓS UMA CONTUSÃO MUSCULOESQUELÉTICA EM RATOS

Santa Maria, RS
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do grau de Doutora em Ciências Biológicas: Bioquímica Toxicológica.

Orientador: Prof. Dr. Gustavo Orione Puntel

Coorientador: Prof. Dr. Félix Alexandre Antunes Soares

Santa Maria, RS

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## DEDICATÓRIA

Dedico este trabalho aos meus exemplos de vida e amor: meus pais Ivani e Carlos, maiores incentivadores, exemplo de luta e dedicação!

São a minha base mais forte, e sempre estiveram presentes em todos os momentos da minha caminhada!

A vocês todo meu amor!

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Muito obrigada a todos!

## EPÍGRAFE

Ensinar não é transferir conhecimento, mas criar as possibilidades para a sua própria produção ou a sua construção.

Paulo Freire

Ninguém é tão grande que não possa aprender, nem tão pequeno que não possa ensinar.

Voltaire

# APRESENTAÇÃO

No item INTRODUÇÃO consta uma revisão sucinta da literatura sobre os temas envolvidos nesta tese.

O DESENVOLVIMENTO da tese está apresentado sob a forma de dois artigos, os quais se encontram alocados no item ARTIGOS CIENTÍFICOS. As seções Materiais e Métodos, Resultados, Discussão dos Resultados, Conclusão e Referências Bibliográficas, encontram-se nos próprios artigos e representam a íntegra deste estudo.

O item DISCUSSÃO apresenta interpretações e comentários gerais sobre os trabalhos científicos aqui incluídos.

Os itens CONCLUSÕES e PERSPECTIVAS são encontrados no final desta dissertação e apresentam interpretações e comentários gerais sobre a investigação desenvolvida.

As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem no item INTRODUÇÃO e DISCUSSÃO, uma vez que o artigo científico contém as suas próprias referências.

## RESUMO

## FIBRAS MUSCULARES OXIDATIVAS E GLICOLÍTICAS APRESENTAM RESPOSTA INFLAMATÓRIA E ALTERAÇÕES BIOENERGÉTICAS PECULIARES APÓS UMA CONTUSÃO MUSCULOESQUELÉTICA EM RATOS

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A contusão muscular representa um tipo de estresse pois altera a homeostase celular. Caracterizada como uma lesão traumática, que altera a disponibilidade de oxigênio ($O_2$) no tecido, promove o aumento da produção de espécies reativas de oxigênio (EROs), aumento de $Ca^{2+}$ intra mitocondrial, consequente abertura do poro de transição que sinaliza para a morte celular e/ou autofagia. Sabe-se que o aumento de EROs, desencadeia ativação de vias pró-inflamatórias no músculo. No entanto, as respostas especificas dos diferentes tipos de fibras musculares são pouco abordadas na literatura. Além disso, pouco ainda se sabe sobre a relação entre a alteração da bioenergética e funcionalidade mitocondrial desencadeadas nos diferentes tipos de fibra muscular pela lesão, como a do tipo contusão, e como essa disfunção mitocondrial irá repercutir na resposta inflamatória imediata no musculo. Assim, o objetivo deste estudo foi elucidar os possíveis mecanismos mitocondriais após uma lesão muscular na forma de contusão nas fibras de metabolismo oxidativo, misto e glicolítico, e as vias de sinalização de resposta inflamatória e morte celular relacionadas a disfunção mitocôndria e o estresse oxidativo imediatamente após a lesão nos diferentes tipos de fibras musculares. Os resultados apresentados no artigo 1, mostraram que a contusão levou a disfunção mitocondrial de formas diferentes em cada tipo de fibras muscular: aumentou o fluxo de oxigênio nas fibras oxidativas quando estimulada por substratos complexos I + II. Por outro lado, aumentou a produção de peróxido de hidrogênio ($H_2O_2$) quando comparada às fibras controle e reduziu a atividade da citrato sintase. Surpreendentemente, a lesão aumentou nas fibras mistas o consumo de oxigênio em relação a OXPHOS, síntese de ATP e a produção de $H_2O_2$, mas reduziu a captação de $Ca^{2+}$. A lesão nas fibras glicolíticas não afetou o fluxo de oxigênio acoplado à síntese de ATP, nem a atividade da citrato sintase e lactato desidrogenase, mas reduziu a captação de $Ca^{2+}$. Já no manuscrito 1, no nosso estudo envolveu a resposta ao longo de duas horas da contusão nos diferentes tipos de fibras musculares observamos um aumento da rota inflamatória através do receptor TLR4, passando por um aumento do escape de elétrons (LEAK state nas fibras oxidativas e mistas; e ROX nas fibras glicolíticas), por um aumento da expressão gênica de citocinas pró-inflamatórias e marcadores importantes de disfunção mitocondrial (BAX e Caspase 3), culminando no aumento da expressão gênica e síntese do próprio TLR4. Portanto, demonstramos respostas mitocondriais distintas entre as diferentes fibras musculares, indicando que a dinâmica mitocondrial está relacionada às flexibilidades no metabolismo e que espécies reativas de oxigênio afetam diretamente a fisiologia e a função normal das fibras musculares. E a disfunção da bioenergética mitocondrial possivelmente interfere na ativação da via TRL4, aumentando a presença de citocinas inflamatórias nas fibras oxidativas e glicolíticas / oxidativas.



Palavras-chaves: fibras musculares, metabolismo, respirometria de alta resolução, disfunção mitocondrial, inflamação.

## ABSTRACT

## OXIDATIVE AND GLYCOLYTIC MUSCLE FIBERS PRESENTS INFLAMMATORY RESPONSES AND BIOENERGETIC ALTERATION PECULIARYS AFTER MUSCLE SKELETAL CONTUSION IN RAT



AUTHOR: Diane Duarte Hartmann
ADVISOR: Gustavo Orione Puntel



Muscle contusion represents a type of stress that disrupts cell homeostasis. Characterized by traumatic lesion, that impair the availability of oxygen (O2) in the tissue, promotes an increase in the production of reactive oxygen species (ROS), an increase in Ca2 + intra mitochondrial, consequent opening of the transition pore of the mitochondrial membrane and signaling for cell death and / or mitophagy. Nowadays, it is known that the increase in ROS triggers activation of pro-inflammatory pathways and proteins. However, the specific responses of different types of muscle fibers are little addressed in the literature. Considering the important role of mitochondrial in muscle tissue function. Little is known about the relation between the bioenergetics alters and mitochondrial functionality triggered in different types of muscle fiber by injury, such as the contusion, and how this mitochondrial dysfunction will affect the immediate inflammatory response developed by muscle. Thus, the objective of this study was to elucidate the possible mitochondrial mechanisms after a muscle contusion injury in the fibers of oxidative, mixed and glycolytic metabolism (Article 1), and the signaling pathways of inflammatory response and cell death related to mitochondria dysfunction and oxidative stress immediately after musculoskeletal injury in different types of muscle fibers (Manuscript 1).The results in article 1, demonstrate that the contusion led to different forms of mitochondrial dysfunction in each type of muscle fibers: increased $O_2$ flux of oxidative fibers when stimulated by complex substrates I + II. On the other hand, increased the production of hydrogen peroxide ($H_2O_2$) when compared to control fibers and reduced citrate synthase activity. Surprisingly, the injury increased oxygen consumption in the mixed fibers in the synthesis of OXPHOS and ATP, and $H_2O_2$ production, however, reduced $Ca^{2+}$ uptake. Glycolytic fibers did not affect the oxygen flux coupled to the synthesis of ATP, and citrate synthase and lactate dehydrogenase activity, but reduced $Ca^{2+}$ uptake. In manuscript 1, in our study it involved the response over two hours of the contusion in the different types of muscle fibers (Manuscript 1) we showed an increase in the inflammatory pathway through the TLR4 receptor, going through an increase in the electron escape (LEAK state in oxidative and mixed fibers; and ROX in glycolytic fibers), due to an increase in pro-inflammatory cytokine gene expression and important markers of mitochondrial dysfunction (BAX and Caspase 3), culminating in increase of gene expression and TLR4 protein. Therefore, we demonstrate distinct mitochondrial responses between different muscle fibers, indicating that mitochondrial dynamics are related to flexibilities in metabolism and that as reactive oxygen species directly affect physiology and normal function. And the dysfunction of mitochondrial bioenergetics possibly interferes with the activation of the TRL4 pathway, increasing the presence of cytokines in oxidative and glycolytic / oxidative fibers.



Key words: muscle fibers, metabolism, high resolution respirometry, mitochondrial dysfunction, inflammation.

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# 1. INTRODUÇÃO

O tecido muscular é um tecido contrátil, altamente dinâmico, eu sofre remodelações contínuas em resposta a várias demandas metabólicas e funcionais (BLAAUW; SCHIAFFINO; REGGIANI, 2013). É composto por fibras musculoesqueléticas que apresentam diferentes isoformas das cadeias pesadas de miosina as quais são utilizadas como critério para sua classificação (LARSSON; MOSS, 1993). Os diferentes tipos de fibras musculares apresentam outras propriedades que as diferem, tais como, diferenças metabólicas e bioquímicas (SCHIAFFINO; REGGIANI, 2011), sendo que fibras do tipo IIb são ricas em enzimas glicolíticas (hexoquinase, fosfofrutoquinase (FFK), lactato desidrogenase (LDH), entre outras) (SCHIAFFINO; REGGIANI, 2011); as fibras do tipo IIa apresentam propriedade intermediarias quanto ao número de mitocôndrias e enzimas glicolíticas e oxidativas. Já as fibras do tipo I apresentam maior atividade de enzimas oxidativas (succinato desidrogenase (SDH) e citrato sintase (CS)) e uma maior densidade mitocondrial (QAISAR; BHASKARAN, 2016).

Macaluso et al. (2012) sugere que as fibras do tipo II apresentam maior suscetibilidade a danos decorrentes do exercício aeróbico em comparação com as fibras do tipo I, devido às diferenças ultra-estruturais e das propriedades metabólicas dos dois tipos de fibras. Foi observado que após um protocolo de exercício de pliometria as fibras do Tipo II apresentaram maior dano no sarcolema e perda da distrofina em comparação as fibras do Tipo I (MACALUSO; ISAACS; MYBURGH, 2012). Martins et al. (2018) utilizou dois protocolos: treino intervalado de alta intensidade e de exercício aeróbico, e observou também nas fibras do tipo IIb a maior estresse oxidativo e alterações mitocondriais.

No metabolismo energético as mitocôndrias são essenciais, além de mediar vias de sinalização celular, processos adaptativos e protetores à célula (KUNZ et al., 1993). Desempenham papel fundamental na fisiologia da fibra musculoesquelética em repouso e durante a atividade contrátil, onde a cadeia transportadora de elétrons é a principal responsável pela produção de energia na forma de adenosina trifosfato (ATP), assim como, é a maior produtora de espécies reativas de oxigênio (EROs) intracelular (PARADIS et al., 2016). Bem como, o Ca2+ que atua na sinalização celular como segundo mensageiro altamente versátil controlando respostas celulares críticas (CLAPHAM, 2007), tais como crescimento celular, miogeneses, processos de remodelação e regeneração muscular (TU et al., 2016).

Além disso, durante a contração, em resposta a um potencial de ação nos túbulos T, os canais de cálcio nos receptores de rianodina se abrem e o cálcio (Ca2+) se difunde para o interior

da fibra, ligando-se à troponina C permitindo a ligação cruzada entre actina e miosina, que é depende de ATP (KOUBASSOVA; TSATURYAN, 2011). O $Ca^{2+}$ é removido por uma bomba ativa de cálcio presente nas vesículas longitudinais do retículo sarcoplasmático (SR), chamadas Cálcio-ATPase (GREIG; JONES, 2016). Quando em concentrações elevadas no citosol, as mitocôndrias auxiliam no seu tamponamento (WILLIAMS et al., 2013). Esse pode atuar promovendo o aumento da respiração mitocondrial e assim o aumento da síntese de ATP (GLANCY et al., 2013), e também mediar a abertura dos Poros de Transição da Membrana Mitocondrial (mPTP), e assim eventos que culminam com o inchaço mitocondrial (LEMASTERS et al., 2010).

As lesões musculoesqueléticas por contusão muscular estão entre os principais tipos de lesões corporais, com uma frequência que varia de 10% a 55% com relação às demais lesões esportivas, em modalidades coletivas (JÄRVINEN et al., 2005), sendo as lesões desportivas mais frequentes (JÄRVINEN et al., 2005; SOUZA; GOTTFRIED, 2013). Caracterizadas pela compressão do tecido muscular em decorrência do impacto causado por alguma força na superfície do musculoesquelético (SOUZA; GOTTFRIED, 2013), essa lesão por contusão leva à descontinuidade de fibras musculares e capilares sanguíneos (MARTINS et al., 2016). O comprometimento da funcionalidade mitocondrial e sua relação com o aumento do dano oxidativo, diminuição do potencial de membrana e inchaço mitocondrial foram observados após modelo de contusão muscular (PUNTEL et al., 2011).

A disfunção mitocondrial após a lesão muscular pode estar relacionada às mudanças na disponibilidade de oxigênio ($O_2$) no tecido danificado em resposta à lesão isquemia / reperfusão (I/R) (PUNTEL et al., 2011). Uma vez que a contusão do músculo esquelético é caracterizada como lesão traumatológica (BEINER; JOKL, 2001), é possível que fatores como episódios de I/R sejam eventos presente na lesão (PUNTEL et al., 2011), promovendo assim mudanças na dinâmica mitocondrial, modificando a morfologia e funções mitocondriais (CALO et al., 2013; ONG; HALL; HAUSENLOY, 2013). A I/R aumenta a geração de EROs e a concentração de $Ca^{2+}$ na mitocôndria, levando a abertura do mPTP (PARADIS et al., 2016).

## 1.1. PROBLEMA

Lesões musculares são amplamente estudadas, porém com um o enfoque principal para o processo inflamatório, mecanismos de regeneração e terapias que possam acelerar/melhorar a recuperação tecidual. No entanto, estudos que analisem a respiração mitocondrial e o metabolismo, além das vias inflamatórias ativadas nos diferentes tipos de fibras após lesões

musculares por trauma são pouco explorados. Estudos anteriores do grupo já demonstraram que a contusão muscular promove a alterações no funcionamento normal da mitocondrial, bem como, sabe-se que os diferentes tipos de fibras musculares apresentam diferentes respostas ao dano oxidativo e celular.

Desta forma, as respostas metabólicas, inflamatórias e bioenergéticas específicas dos diferentes tipos de fibras musculares, subsequentes a um modelo de contusão muscular em ratos podem contribuir significativamente para o desenvolvimento de estratégias de tratamento a serem usadas na clínica.

## 1.2. OBJETIVOS

### 1.2.1. Objetivo geral

Avaliar os eventos agudos decorrentes da lesão muscular por contusão na bioenergética mitocondrial e no perfil inflamatório dos diferentes tipos de fibras musculares (tipo I, IIa e IIb).

### 1.2.2. Objetivos específicos

- Avaliar a resposta da bioenergética mitocondrial aguda, quanto ao consumo de oxigênio e a capacidade respiratória através da respirometria de alta resolução, o metabolismo celular de $Ca^{2+}$, e a geração de EROs em após modelo de contusão muscular em fibras de metabolismo glicolítico, glicolítico/ oxidativo e predominantemente oxidativo.
- Analisar os efeitos da contusão muscular na bioenergética mitocondrial e verificar o perfil inflamatório em 30 min e 2 horas após o modelo de trauma muscular em fibras de metabolismo glicolítico, glicolítico/ oxidativo e predominantemente oxidativo.

## 1.3. JUSTIFICATIVA

As estratégias adequadas ao efetivo tratamento das lesões musculoesqueléticas devem estar relacionadas aos mecanismos pelos quais estas comprometem o funcionamento do tecido muscular. Além disso, o conhecimento das possíveis alterações bioquímicas, bioenergéticas envolvidas na origem e na evolução destas lesões é de fundamental importância para o seu adequado e efetivo tratamento. No entanto, até o momento poucos estudos demonstram a existência, e a magnitude, das alterações mitocondriais; e como estas promovem alterações no perfil inflamatório subsequentes a um modelo de contusão muscular em ratos. Por sua vez, a elucidação destes mecanismos proporciona base para estratégias de tratamentos terapêuticos efetivos para uso na clínica.

Desta forma, neste trabalho serão investigados os efeitos de lesão por contusão na função mitocondrial dos diferentes tipos de fibras musculares (Tipos I, IIa e IIb). Serão analisadas a regulação de enzimas envolvidas na glicólise e ciclo de Krebs (que são vias metabólicas diretamente relacionadas com o consumo de oxigênio mitocondrial), e também a produção da espécie reativa de oxigênio (ERO) peróxido de hidrogênio ($H_2O_2$) em cada sitio mitocondrial (complexos mitocondriais I, II, III), além de correlacionar esses achados com as alterações no perfil inflamatório. Assim, buscar-se-á entender as a resposta dos diferentes tipos de fibras musculares à um mecanismo agudo de lesão muscular, e, desse modo, contribuir na construção de condutas clínicas de treinamento, reabilitação e recuperação tecidual.

# 2. REFERENCIAL TEORICO

## 2.1. TECIDO MUSCULAR

O tecido muscular é um tecido contrátil, altamente dinâmico sofrendo remodelações contínuas em resposta a várias demandas metabólicas e funcionais (BLAAUW; SCHIAFFINO; REGGIANI, 2013), é composto por fibras musculoesqueléticas que apresentam diferentes isoformas das cadeias pesadas de miosina as quais são utilizadas como critério para sua classificação (LARSSON; MOSS, 1993).

Características contráteis, mudanças qualitativas e quantitativas são consideradas marcadores moleculares dos tipos de fibras musculares (BALAGOPAL; LJUNGQVIST; NAIR, 1997). Mamíferos possuem três principais tipos de fibras musculares (BLAAUW; SCHIAFFINO; REGGIANI, 2013; FERRARO et al., 2014; SCHIAFFINO; REGGIANI, 2011), denominadas:

- Tipo I: chamadas de fibras de contração lenta, resistente a fadiga com metabolismo predominantemente oxidativo;
- Tipo IIa: fibra com velocidade rápida de contração, maior resistência a fadiga, com metabolismo misto (glicolítico / oxidativo);
- Tipo IIb: fibras de contração rápida, fadigáveis, e metabolismo predominantemente glicolítico.

Nos anos 1990 foi descoberta a terceira isoforma das fibras do tipo II, chamada de IIx que apresenta características intermediarias entre IIa e IIb (SCHIAFFINO; REGGIANI, 2011).

Além disso, a miosina de cadeia pesadas dessas fibras musculares podem ser expressa como isoforma única (isto é, fibra pura) ou coexpressas (isto é, fibra híbrida) (BALAGOPAL; LJUNGQVIST; NAIR, 1997; QAISAR et al., 2013). Essas terão sua expressão regulada por

vários fatores como alto grau de plasticidade muscular, sendo que o exercício e o desuso são determinantes primários para sua expressão (CASTRO et al., 1999; KUNRATH et al., 2017). Os diferentes tipos de fibras musculares têm outras propriedades metabólicas e bioquímicas que as diferenciam, como os níveis enzimáticos e o número de mitocôndrias (SCHIAFFINO; REGGIANI, 2011). Fibras glicolíticas são ricas em enzimas, tais como hexoquinase, fosfofrutoquinase (FFK), lactato desidrogenase (LDH), entre outras; já as fibras oxidativas apresentam maior atividade da succinato desidrogenase (SDH), citrato sintase (CS) e uma maior densidade mitocondrial (QAISAR; BHASKARAN, 2016).

Como já relatado anteriormente, a literatura aponta uma maior susceptibilidade a danos promovido pelo exercício. Martins et al. (2018) utilizou dois protocolos: treino intervalado de alta intensidade e de exercício aeróbico, e observou o aumento na geração de peróxido de hidrogênio ($H_2O_2$) mitocondrial, e uma redução da capacidade antioxidante nas fibras do tipo IIb do músculo tibial anterior, nos animais submetidos a uma única sessão de exercício aeróbico, demonstrando que as mitocôndrias, assim como as defesas antioxidantes das diferentes fibras, apresentam resposta diferente ao dano celular.

## 2.2. LESÕES MUSCULARES

O músculo esquelético é um dos tecidos mais abundantes no corpo humano e é constantemente exposto a vários tipos de lesões. Nos esportes, as lesões musculares compreendem de 10 a 55% de todas as lesões (JÄRVINEN et al., 2007) a causa mais comum de lesão é um trauma mecânico (HUARD; LI; FU, 2002; MEHALLO; DREZNER; BYTOMSKI, 2006). Porém, estas lesões são frequentes tanto em atletas quanto em não atletas, sendo determinantes para o surgimento de limitações no desempenho das atividades profissionais e da vida diária destes indivíduos (SOUZA; GOTTFRIED, 2013). Os fatores principais relacionados à origem das limitações impostas pelas lesões são a intensa sensibilidade dolorosa e significativa perda das propriedades funcionais musculares (TAYLOR et al., 1993).

Em geral, as lesões musculoesqueléticas podem ser classificadas em lesões entre leve, moderada e grave de acordo com a sua origem e aspectos clínicos (FERNANDES; PEDRINELLI; HERNANDEZ, 2011). Este tecido tem potencial plástico com moderada capacidade de regeneração e remodelação, determinado pela sua característica fenotípica e/ou intervenções, tais como o exercício físico (QAISAR; BHASKARAN, 2016). Recuperação muscular após uma lesão mecânica ocorre sequencialmente, exibindo um conjunto de eventos

celulares e moleculares, são conhecidos como processos inflamatórios e reparo tecidual (BARTLETT et al., 2012; CAROSIO et al., 2011). As lesões mais frequentes são de origem mecânica e são classificadas como contusões, distensão ou lacerações (SOUZA; GOTTFRIED, 2013). Contusões e distensões contabilizam aproximadamente 90% das lesões musculares nos esportes (JÄRVINEN et al., 2000). As lesões mecânicas são descritas como aquelas que envolvem tecido conjuntivo rompido e levam à necrose da miofibra, hematomas e inflamação (JÄRVINEN et al., 2007; QUINTERO et al., 2009).

### 2.1.1. Contusão muscular

A contusão muscular é uma lesão resultante de um trauma direto sobre a superfície do músculo, é caracterizada pelo esmagamento da estrutura musculoesquelética (SOUZA; GOTTFRIED, 2013). Os componentes da estrutura muscular ao serem esmagados sofrem um prejuízo em suas propriedades funcionais tais como a elasticidade, a extensibilidade e a contratilidade (BEINER; JOKL, 2001). Além do tecido muscular, as contusões podem determinar o esmagamento e a ruptura de vasos sanguíneos na região lesada. Como consequência, quantidades variáveis de componentes do sangue podem extravasar por entre os tecidos do parênquima musculoesquelético (JÄRVINEN et al., 2007). Além disso, é importante considerar que o comprometimento dos vasos sanguíneos resulta no prejuízo do fornecimento de oxigênio aos tecidos lesados (WILKIN et al., 2004).

Após o trauma muscular (dano primário), células inflamatórias são atraídas ao local da lesão a fim de promover a limpeza e iniciar o processo de reparo tecidual (LI; FENG; WANG, 2005). Associadas, a resposta inflamatória e a hipóxia imposta aos tecidos lesados podem contribuir para uma maximização do comprometimento funcional decorrente de uma contusão muscular causando um dano secundário ao tecido, com alterações enzimáticas (PUNTEL et al., 2011).

Levando o tecido a um dano secundário, que promove alterações enzimáticas, redução no fornecimento de oxigênio e nutrientes aos tecidos lesados (WILKIN et al., 2004). De modo geral, as alterações enzimáticas estão relacionadas ao aumento na liberação de enzimas pró-inflamatórias (JÄRVINEN et al., 2005; WILKIN et al., 2004), já o prejuízo no fornecimento de oxigênio aos tecidos caracteriza uma condição de hipóxia tecidual (WILKIN et al., 2004).

A hipóxia ativa fator induzível de hipóxia 1α (HIF-1 α) que leva a redução da atividade do complexo Piruvato Desidrogenase (PDH), garantindo que a glicose seja desviada da produção de acetil-Coa oxidada pelo ciclo de Krebs para a produção de lactato (BURNS;

MANDA, 2017). Estudos recentes sugerem que a geração de espécies reativas mitocondriais de oxigênio (ERO), especialmente $H_2O_2$, é necessária para a ativação e estabilização hipóxicas do HIF-1 (CHANDEL et al., 2000). Bonello e colaboradores (2007) demonstraram que o $H_2O_2$ exógeno ativa o promotor HIF-1 α por meio de um local NF κB funcional nas células do músculo liso.

Estas observações indicam que a mitocôndria pode iniciar rearranjos na organização do sistema de transferência de elétrons para liberar moléculas sinalizadoras, como $H_2O_2$, que irão ativar vias envolvidas na biogênese mitocondrial nos diferentes tipos de fibras. Já que o HIF-1α induzido por $H_2O_2$ pode ser suficiente para iniciar a regulação positiva de genes protetores (LI et al., 2019).

## 2.3. MITOCÔNDRIA

As mitocôndrias são organelas intracelulares com dupla membrana que desempenham papéis importantes no metabolismo energético celular, tem como função predominante a geração de ATP através da fosforilação oxidativa, sendo a fonte primária de compostos altamente energéticos na célula (LETTS; SAZANOV, 2017). Além disso, elas desempenham outras funções importantes como a biossíntese de aminoácidos e esteroides, a beta oxidação de ácidos graxos, produção e detoxificação de espécies reativas de oxigênio, regulação do cálcio intramitocondrial e citoplasmático e transdução de sinais nas vias de sinalização intracelular de apoptose (DJAFARZADEH; JAKOB, 2017; MILANE et al., 2015).

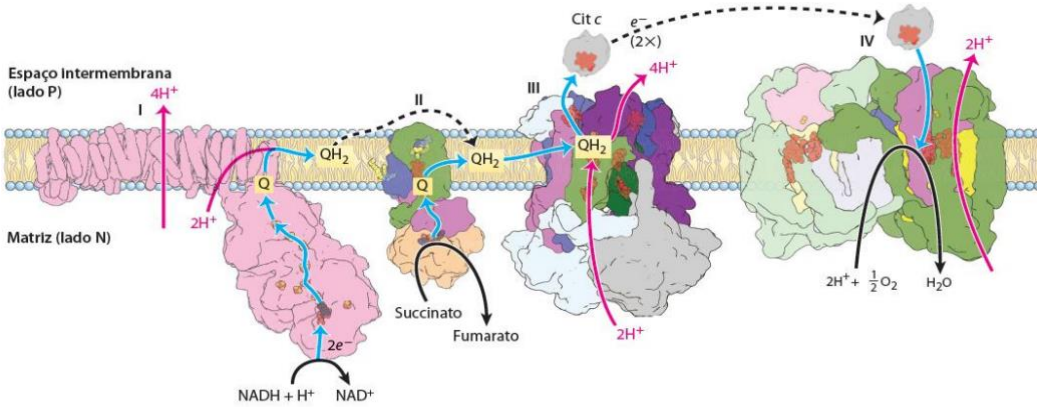
A fosforilação oxidativa utiliza a energia armazenada dos alimentos para gerar um gradiente de prótons através da membrana mitocondrial interna. Durante a respiração celular, os elétrons passam através da cadeia transportadora de elétrons para o oxigênio molecular, produzindo água (PAPA et al., 2012). A energia do gradiente de prótons conduz a síntese de ATP pela ATP-sintase. Ao longo dos anos, muitas desordens têm sido associadas a defeitos na fosforilação oxidativa (CHAUDHURY; DENDI; MIRZA, 2016). Os tecidos requerem energia produzida pela fosforilação oxidativa e isso reflete sua dependência em relação à mitocôndria (CHAUDHURY; DENDI; MIRZA, 2016), exemplo o tecido muscular. Sendo assim, as fibras musculares são bastante dependentes das mitocôndrias, possuindo uma alta densidade destas e, consequentemente, apresentam-se vulneráveis ao comprometimento do metabolismo energético e à disfunção mitocôndrial.

### 2.3.1. Bioenergética mitocondrial, função mitocondrial e estresse oxidativo

O ATP produzido pelas mitocôndrias através do ciclo de Krebs e da fosforilação oxidativa da cadeia transportadora de elétrons, corresponde a > 90% da energia celular corporal (BHATTI; BHATTI; REDDY, 2017). A acetil-CoA formada pela oxidação de combustíveis orgânicos entra no ciclo de krebs e tem seus grupamentos acetil oxidados a gás carbônico ($CO_2$). A energia liberada no processo é armazenada pelas coenzimas derivadas respectivamente de nicotinamida (NADH) e flavina ($FADH_2$). Ambas atuam como transportadores de elétrons em suas formas reduzidas até alcançarem a cadeia respiratória mitocondrial (MILANE et al., 2015). A qual é composta por cinco complexos multi enzimáticos localizados na membrana mitocondrial interna(complexos I, II, III, IV e V), e pelos transportadores de elétrons: coenzima Q e citocromo c e ainda as proteínas ferro-enxofre (NICHOLLS, 2008).

Na cadeia respiratória, elétrons provenientes de diferentes substratos são colecionados na forma de NADH e transferidos para o átomo de ferro central da NADH desidrogenase. O complexo I então transfere elétrons para a forma oxidada da coenzima Q (UQ), levando a sua redução (UQH2) (FORMOSA; RYAN, 2018). Elétrons originários do succinato são transferidos para a flavina adenosina dinucleotídeo (FAD) e UQ pelo complexo II, resultando também em sua redução (LETTS; SAZANOV, 2017). A UQH2 é então desprotonada, resultando na formação do ânion semiquinona ($UQH^{\cdot-}$), responsável pela doação de elétrons ao citocromo b-c1 e posteriormente ao citocromo c (HEKIMI; WANG; NOË, 2016). Existem dois pools distintos de $UQH^{\cdot-}$ um localizado na face citoplasmática da membrana mitocondrial interna, e outro na face matricial da membrana (AMIGO; TRABA; RUEDA, 2016). As duas formas de $UQH^{\cdot-}$ são combinadas quando oxidadas, regenerando UQ e doando seus elétrons (HEKIMI; WANG; NOË, 2016). O citocromo c então transporta elétrons para a citocromo c oxidase, responsável pela transferência de elétrons para o oxigênio, que resulta na formação de água. Esta passagem de elétrons se dá em quatro passos consecutivos de um elétron, devido à característica triplete do oxigênio (NICHOLLS, 2008). A passagem de elétrons é acompanhada pelo bombeamento de prótons da matriz mitocondrial para o espaço intermembranas. O gradiente de prótons gerado favorece a re-entrada destes para a matriz mitocondrial através da ATP sintase, que utiliza a energia próton motriz para promover a síntese de ATP (CERQUEIRA; LAURINDO; KOWALTOWSKI, 2011; PAPA et al., 2012).

Figura 1: Movimentação dos elétrons através da cadeia respiratória.




Fonte: (Lehninger, 2014).

O transporte de elétrons na cadeia respiratória e a fosforilação oxidativa devem ser regulados para a manutenção da funcionalidade e viabilidade celular. A falta de síntese mitocondrial de ATP pode levar a falência energética celular e morte necrótica, provocar acúmulo de EROs mitocondriais (KOWALTOWSKI et al., 2009; SIGNES; FERNANDEZ-VIZARRA, 2018).

As mitocôndrias funcionam continuamente para formação de ATP, através do consumo de oxigênio, o que pode gerar EROs. Entretanto, o fluxo de elétrons através da cadeia respiratória não é um processo perfeito, pois, 0,4 a 4% do oxigênio consumido pela mitocôndria, não é reduzido e leva à produção de EROs, como por exemplo o ânion superóxido ($O_2^{\cdot -}$) designado como ERO “primária” (DEBATTISTI et al., 2017). A geração excessiva de ânion superóxido pode interagir com outros compostos e gerar EROs “secundárias” (TURRENS, 2003). Além disso, a superprodução de EROs danifica as proteínas mitocondriais / enzimas, membranas e o ácido desoxirribonucleico (DNA), acarretando a interrupção da produção de ATP e outras funções essenciais nas mitocôndrias (BHATTI; BHATTI; REDDY, 2017).

O $O_2^{\cdot -}$ originado pela cadeia respiratória pode ser gerado pela NADH desidrogenase (complexo I), pela coenzima Q (complexo III) ou, possivelmente, por desidrogenases matriciais (DRÖSE; BRANDT, 2012). A geração de $O_2^{\cdot -}$ ao nível do átomo central de ferro da NADH desidrogenase é de grande importância fisiopatológica (MAILLOUX, 2015). A presença de rotenona, um inibidor da transferência de elétrons entre o complexo I e a UQ, também estimula sensivelmente a geração de $O_2^{\cdot -}$ pela NADH desidrogenase (HEKIMI; WANG; NOË, 2016). Os radicais livres são fundamentais para qualquer processo bioquímico e são continuamente produzidos no corpo. As células têm muitas maneiras de combater os efeitos do dano oxidativo induzido por EROs, seja diminuindo diretamente a geração de radicais livres ou capturando os

radicais livres por uma série de antioxidantes, mecanismos enzimáticos e não enzimáticos (BHATTI; BHATTI; REDDY, 2017).

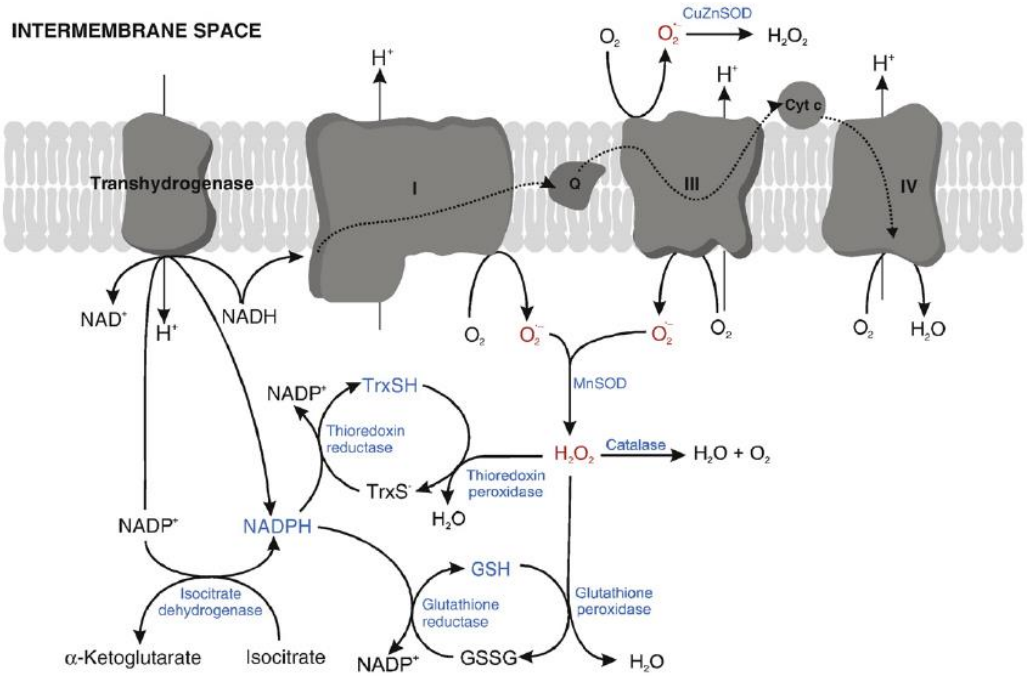
A superóxido dismutase (SOD) catalisa a rápida conversão do radical ânion superóxido em peróxido de hidrogênio ($H_2O_2$) e oxigênio molecular ($O_2$). A isoforma da SOD presente na matriz mitocondrial é dependente de manganês (MnSOD), enquanto a isoforma da SOD presente no citosol e no espaço intermembrana é dependente de cobre e zinco (CuZnSOD) (CARDOSO; SEIÇA; MOREIRA, 2017; LI; FENG; WANG, 2005). O $H_2O_2$ formado, além de ser uma espécie química mais estável, é permeável pelas membranas e pode ser transportado por aquaporinas. Assim, o $H_2O_2$ difunde-se dentro da célula, podendo ser removido tanto por sistemas antioxidantes mitocondriais como citosólicos, como a catalase (BUETTNER, 2011), tioredoxina peroxidase (TPx), e glutationa peroxidase (GPx). Esta última possibilita a conversão do $H_2O_2$ formado em água, ao mesmo tempo em que oxida a glutationa, que é prontamente reduzida pela ação da glutationa redutase (GR) (APOSTOLOVA; VICTOR, 2015).

A glutationa é importante também para manter os grupamentos sulfidril de proteínas em seu estado reduzido, impedindo de sofrerem os efeitos deletérios do estresse oxidativo (MARÍ et al., 2009). ($TrxS^-$) são reduzidas, utilizando o NADPH como fonte de elétrons. O NADP pode ser mantido reduzido pela atividade da transidrogenase NAD / NADP, com transporte de prótons para a matriz, fornecendo uma ligação entre o potencial de membrana interna e a capacidade redox mitocondrial. Alternativamente, o $NADP^+$ é reduzido pela isocitrato desidrogenase (KOWALTOWSKI et al., 2009).

Quando as espécies não são metabolizadas pelos sistemas antioxidantes, elas podem gerar o radical hidroxil ($OH^{\cdot}$), altamente reativo e citotóxico, por meio da reação de Fenton, na qual o $H_2O_2$ reage com íons cobre ($Cu^+$) ou ferro ($Fe^{2+}$). Não há nenhum sistema enzimático de defesa contra o $OH^{\cdot}$ (VENDITTI; WILSON; DE MATTEIS, 2014). Assim, em condições em que ocorre um aumento da produção de $O_2^{\cdot -}$ ou uma falha no sistema antioxidante, pode ocorrer uma maior produção de $OH^{\cdot}$ com lesões oxidativas em biomoléculas (PRAUCHNER, 2017). A mitocôndria é creditada ainda como uma possível fonte de espécies reativas de nitrogênio derivadas do óxido nítrico ($NO^{\cdot}$), produzido pela óxido nítrico sintase mitocondrial (mtNOS) (CARDOSO; SEIÇA; MOREIRA, 2017).

Figura 2: Metabolismo mitocondrial de EROs. O radical ânion superóxido ($O_2^{\cdot -}$) é formado pela redução monoeletrônica de $O_2$, principalmente nos Complexos I e III da cadeia respiratória. $O_2^{\cdot -}$ é dismutado em

$H_2O_2$ pela CuZnSOD, no espaço intermembrana, e MnSOD na matriz. $H_2O_2$ pode ser removido pela catalase mitocondrial ou por peroxidases, tal como glutationa e tiorredoxina peroxidase, utilizando a glutationa reduzida (GSH) e tiorredoxina (TrxSH) como substratos, respectivamente.




Fonte: (KOWALTOWSKI et al., 2009).

### 2.3.1. Disfunção mitocondrial como mecanismo ativado após a contusão

A disfunção mitocondrial induzida pela contusão muscular demonstra-se como um processo complexo causando danos direta e indiretamente a fibra muscular. O desequilíbrio redox do tecido muscular é frequentemente acompanhado pela produção excessiva de EROs, a qual acarreta sérias consequências para a viabilidade da membrana celular e mitocondrial, comprometendo ainda mais a homeostase bioenergética do tecido muscular. Durante esta condição fisiopatológica o dano musculoesquelético pode ser instalado através do prejuízo funcional da fisiologia mitocondrial, devido a limitada capacidade de tamponamento redox (HARTMANN et al., 2020; PUNTEL et al., 2011).

A manutenção do potencial de membrana mitocondrial ($\Delta\psi m$) está associada com a capacidade dos complexos da cadeia respiratória bombear os prótons para o espaço intermembrana, este processo ocorre através da liberação de energia proveniente da oxidação de coenzimas reduzidas (NADH e $FADH_2$) as quais são constantemente produzidas principalmente pela metabolização de substratos energéticos via Ciclo do Ácido Cítrico (MILLS; O'NEILL, 2016). Neste contexto, a lesão muscular por modelo de contusão já

demonstrou o aumento do inchaço e desencadeado pelo comprometimento no potencial de membrana mitocondrial (Δψ), além do desequilíbrio das defesas antioxidante já foram observados em mitocôndrias de músculos lesados (PUNTEL et al., 2011).

Também foi demonstrado que a contusão muscular inibe a enzima citrato sintase, a qual demonstra-se sensível a variações acentuadas no estado redox mitocondrial (HARTMANN et al., 2020). Levando em consideração isso, já foi observado que a reduzida proteção mitocondrial é seguida por uma redução significativa da capacidade de produção energética celular através do rompimento do Δψm e inibição da fosforilação oxidativa (OXPHOS), prejudicando o tecido muscular que é altamente dependente do metabolismo aeróbico(QAISAR; BHASKARAN, 2016). A lesão muscular por contusão causa uma redução da disponibilidade das coenzimas reduzidas e reduzir a atividade dos complexos da cadeia respiratória mitocondrial, o que proporciona uma redução da capacidade bombeadora de prótons para o espaço intermembrana, o que pode prejudicar a produção de ATP e Δψm (BRAND, 2016).

Frequentemente quando analisamos estes processos patológicos a nível mitocondrial, eles estão associados com a abertura do mPTP (LEMASTERS et al., 2010). O poro de transição de permeabilidade consiste em um canal complexo, apresentando em sua composição o canal de ânion voltagem dependente na membrana mitocondrial externa, translocador de nucleotídeo de adenina na membrana mitocondrial interna e ciclofilina D na matriz (KOWALTOWSKI et al., 2009). Desta forma, fatores como uma redução da razão GSH/GSSG, NAD(P)H/NAD(P)+ e a dissipação do gradiente de prótons e a perda do Δψm além de modificações de grupos tiois das proteínas da membrana interna são fatores que podem proporcionar um ciclo vicioso e desencadear abertura do mPTP (CARVALHO et al., 2010; PUNTEL et al., 2013). Assim, a modulação da abertura do poro de transição de permeabilidade mitocondrial é um elemento chave no controle da viabilidade celular independentemente do fenótipo de morte celular.

## 2.4. VIA DOS TLRS E CASCATA INFLAMATÓRIA

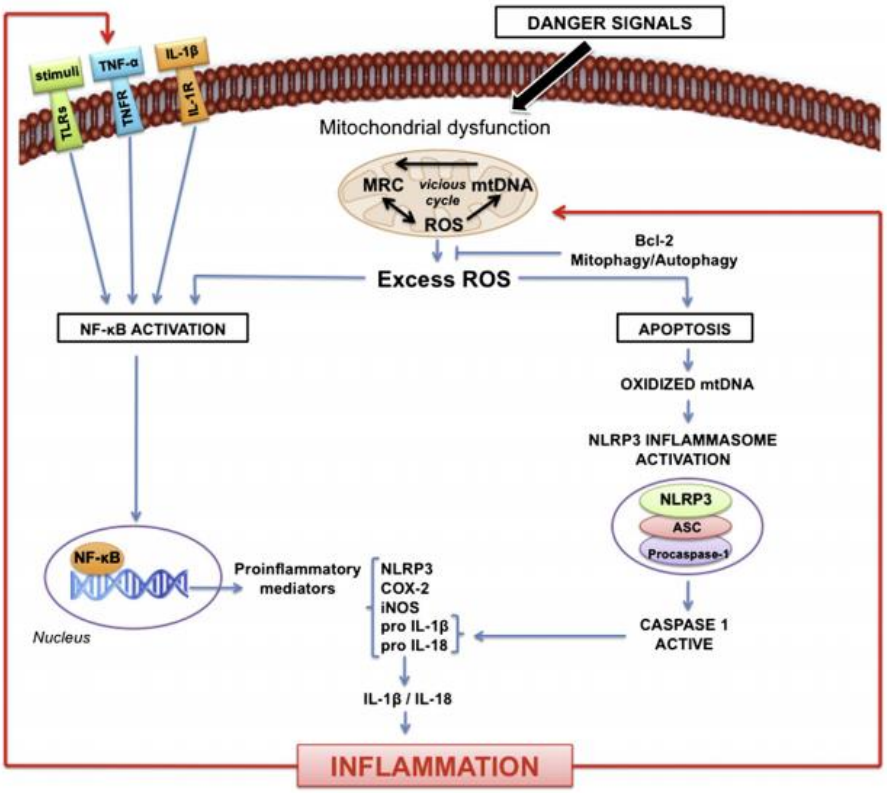
Dados da literatura já comprovam que além de disfunções mitocondriais, a geração excessiva de EROs, principalmente o $H_2O_2$ promove o aumento significativo da expressão dos receptores imuno metabólicos TLR2 e TLR4 em PBMC (AKHTER et al., 2019). Sabe-se também que a estimulação com TLR4 leva à geração de ERO mitocondrial através do complexo I, um mecanismo ainda pouco explorado (WEST; KOBLANSKY; GHOSH, 2006; WEST; SHADEL; GHOSH, 2011). Além do mais, macrófagos após a ativação de TLR4 redirecionam

suas mitocôndrias da produção de ATP para a geração de EROs dependentes de succinato, com a glicólise assumindo o papel de geração de ATP, permitindo que as mitocôndrias sustentem um alto potencial de membrana (LÓPEZ-ARMADA et al., 2013; SMITH et al., 2015). Assim, a produção aumentada de succinato é um regulador crítico da resposta pró-inflamatória, tanto através da geração de EROs após oxidação pela cadeia transportadora de elétrons, quanto através de uma via estabilização de HIF-1α (MILLS; O'NEILL, 2016).

Os TLRs são expressos em vários tecidos (MUZIO; MANTOVANI, 2000) e possuem papéis importantes no reconhecimento e indução de respostas tanto do sistema imune inato quanto do adquirido (AKIRA; TAKEDA, 2004; TAKEDA; AKIRA, 2005). Onze membros da família TLRs foram identificados em humanos e 13 membros dessa família em camundongos (DU et al., 1999). O TLR2 e o TLR4 são encontrados em vários tipos celulares incluindo adipócitos, hepatócitos e miócitos (LANG; MANSELL, 2007).

Os TLRs são glicoproteínas transmembranas do tipo I expressas em diferentes tipos celulares de resposta imune inata e em fibroblastos e células epiteliais (AKIRA; UEMATSU; TAKEUCHI, 2006). Os TLRs mediam o reconhecimento de padrões moleculares associados à patógenos e possuem um papel importante nas respostas imune e anti-inflamatória (GLEESON; MCFARLIN; FLYNN, 2006). Os TLRs de monócitos, macrófagos, e células dentríticas contribuem significantemente para o desenvolvimento da resposta imune adaptativa (PASARE; MEDZHITOV, 2004), sendo que a produção de citocinas IL-1β, IL-6, IL-8, e TNF-α (IWASAKI; MEDZHITOV, 2015; PASARE; MEDZHITOV, 2004) também levam a geração de uma resposta imune adaptativa (ALEXOPOULOU et al., 2001).

Figura 3. Modelo teórico da inter-relação entre disfunção mitocondrial e resposta inflamatória. Inúmeras doenças inflamatórias agudas quanto as crônicas, bem como o processo de envelhecimento, têm sido associadas a um excesso de ROS, que poderia ser a principal fonte de instabilidade genômica mitocondrial levando à disfunção da cadeia respiratória. Disfunção mitocondrial derivada de vários sinais de perigo ou dano celular poderia ativar a via do fator redox-sensível NF-κB através do estresse oxidativo ou / e induzir a via apoptótica. Na presença de estímulos pró-inflamatórios que ativam NF-κB, a via apoptótica poderia induzir a ativação do inflamassoma de NLRP3. O fator desencadeante dessa ativação é a ligação entre o NLRP3 e o mtDNA oxidado liberado durante apoptose. O inflamassoma de NLRP3 e a via de NF-κB podem trabalhar juntos para estimular a produção de citocinas inflamatórias, levando à superestimulação da resposta inflamatória. Finalmente, mediadores inflamatórios gerados podem induzir disfunção mitocondrial, perpetuando e exacerbando um ciclo vicioso de inflamação. A autofagia / mitofagia e a expressão de Bcl-2 podem remover mitocôndrias danificadas e podem mitigar reações inflamatórias.




Fonte: (LÓPEZ-ARMADA et al., 2013)

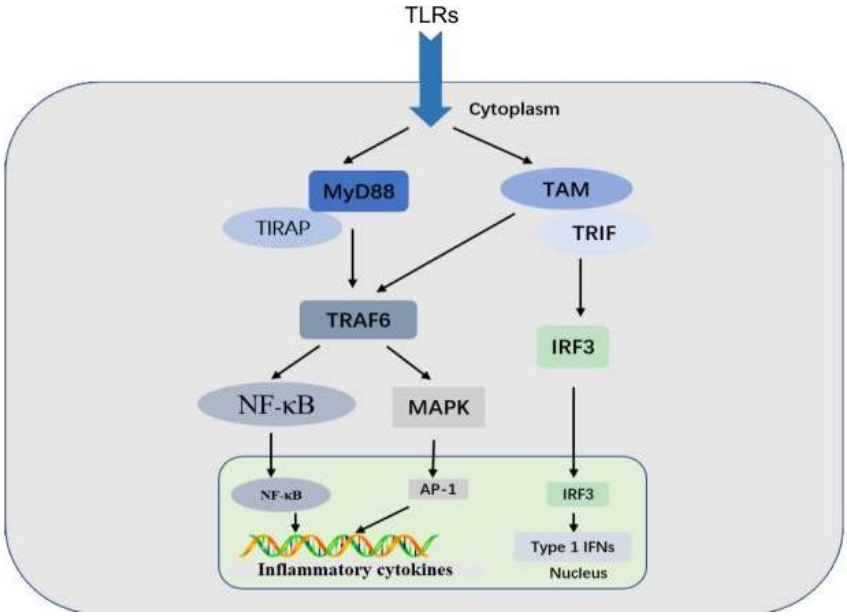
Há vários ligantes endógenos de TLRs que podem ter um papel na regulação da expressão desses receptores ou permitirem o sistema imune responder ao dano ou a sinais de dano (KILMARTIN; REEN, 2004). O reconhecimento de antígenos pelos TLRs dispara uma sinalização intracelular (TAKEUCHI; AKIRA, 2001) que resulta na indução de um conservado programa de defesa que inclui a produção de citocinas anti-inflamatórias (ALEXOPOULOU et al., 2001). A família dos TLRs utiliza motivos proteicos de grande quantidade de leucina para a ligação e um domínio citoplasmático que é homólogo ao do receptor da IL-1. Frente a um estímulo, o TLR recruta adaptadores como o "myeloid differentiation primary response gene 88" (MyD88) e o "Toll/interleukin-1 receptor domain containing adapter protein" (TIR-AP) (CHEN et al., 2018; WEST; KOBLANSKY; GHOSH, 2006). A cascata de sinalização leva a ativação de "mitogen- activated protein kinase" (MAPKs), proteína quinase C (PKC), ativação de fatores de transcrição como o NFκB, PGC-1 e de fatores pró-inflamatórios (AKIRA; SATO, 2003). Além disso, a ativação de TLR-4 está relacionada com a estabilização de HIF-1α e IL-1B (ALVES-FILHO; PÅLSSON-MCDERMOTT, 2016).

O TLR4 utiliza: MyD88, TIRAP, TRIF (TIR domain-containing adaptor inducing IFN-b) e TRAM (TRIF-related adaptor molecule) (O'NEILL; BOWIE, 2007). Juntamente com o

CD14, TLR4 orquestra vários processos na cascata inflamatória (AKIRA; UEMATSU; TAKEUCHI, 2006) e pode ativar diferentes vias de sinalização que aumentam ou inibem a atividade de muitos fatores de transcrição. Três fatores de transcrição principais são controlados, pelo menos em grande parte, pela via de sinalização do TLR4: o fator de transcrição κB (NFκB), a proteína de ativação-1 (AP-1), e o fator de transcrição regulatório interferon 3 (IRF3) (ZENG et al., 2020).

De fato, os TLRs induzem um aumento da expressão de citocinas através da via do NFκB, o qual está envolvido na modulação da expressão dos próprios TLRs (MIETTINEN et al., 2008; SEIBL et al., 2003). Frente a uma estimulação, estes receptores induzem o recrutamento de adaptadores MyD88 e TRIF, que resultam na ativação de uma série de sinalização, incluindo o NFκB e o MAPK (AKIRA; SATO, 2003). Os quais controlam respostas inflamatórias e imunológicas pela indução da expressão de várias citocinas pró-inflamatórias tais como o TNF-α e o interferon tipo I (IFN)(DEJBAN et al., 2020).

Figura 4. Vias de sinalização TLR ativa vias dependentes de MyD88 e/ ou TAM/ TRIF. A sinalização por meio de TLRs ativa cascatas de sinalização intracelular que levam à translocação nuclear de AP-1 e NF-κB ou IRF3, que regulam a resposta inflamatória.




Fonte: (CHEN et al., 2018).

As subunidades de NFκB, p50 e p65, formam o mais frequente heterodímero de NFκB (JIMÉNEZ-JIMÉNEZ et al., 2008). A ativação de NFκB, tanto pela via dependente ou independente de MyD88, leva o recrutamento da sua subunidade p50 no citoplasma e a

translocação da subunidade p65 para o núcleo (KAWAI; AKIRA, 2006). Este fator de transcrição faz um link entre múltiplas vias de sinalização que orquestram diversos processos em virtualmente todos os tipos celulares. A translocação da subunidade p65 do NFκB ao núcleo altera diretamente a produção de muitos genes, incluindo aqueles que geram citocinas, reguladores do status redox, proteínas de resposta de fase aguda, apoptose e atrofia (PAHL, 1999). Muitos dos genes ativados por NFκB são conhecidos por serem pró-inflamatórios (DEJBAN et al., 2020; JIMÉNEZ-JIMÉNEZ et al., 2008).

No entanto, os dados na literatura com relação a expressão dos TLR depois de lesões musculares são bastante escassos.

## 3. DESENVOLVIMENTO

O desenvolvimento que faz parte desta tese está apresentado sob a forma de dois artigos científicos. Os itens Introdução, Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos.

O artigo 1 foi publicado na revista *Free Radical Research* encontra-se no formato da mesma.

O artigo 2 está apresentado em forma de manuscrito,

### 3.1. ARTIGO CIENTÍFICO I

**A single muscle contusion promotes an immediate alteration in mitochondrial bioenergetics response in skeletal muscle fibres with different metabolism**

Diane D. Hartmann, Débora F. Gonçalves, Pamela C. Da Rosa, Rodrigo P. Martins, Aline A. Courtes, Jeferson L. Franco, Félix A. A. Soares & Gustavo O. Puntel

Free Radical Research

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ARTICLE

# A single muscle contusion promotes an immediate alteration in mitochondrial bioenergetics response in skeletal muscle fibres with different metabolism

Diane D. Hartmann[a], Débora F. Gonçalves[a], Pamela C. Da Rosa[a], Rodrigo P. Martins[a], Aline A. Courtes[a], Jeferson L. Franco[b], Félix A. A. Soares[a] and Gustavo O. Puntel[c]

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**ABSTRACT**

Skeletal muscle is the most abundant tissue in the human body and mechanical injuries are common; these are frequently of mechanical origins, such as contusion. However, the immediate mitochondrial response to injury and energetic substrate utilisation is still unclear. We evaluated the acute response in mitochondrial function after a single muscle contusion, either in fast twitch fibres (glycolytic metabolism), fast and slow twitch (oxidative and glycolytic metabolism), or slow twitch fibres (oxidative metabolism). Rats were assigned to two groups: control and Lesion (muscle contusion). We collected the gastrocnemius and soleus muscles. The fibres were analysed for mitochondrial respiration, lactate dehydrogenase (LDH), citrate synthase (CS) activity, $Ca^{2+}$ uptake, and $H_2O_2$ production. We found that muscle injury was able to increase ATP synthesis-dependent and OXPHOS oxygen flux in the oxidative fibres when stimulated by complex I+II substrates. On the other hand, the muscle injury increased hydrogen peroxide ($H_2O_2$) production when compared to control fibres, and reduced citrate synthase activity; however, it did not change $Ca^{2+}$ uptake. Surprisingly, injury in mixed fibres increased the OXPHOS and ATP synthesis oxygen consumption, and $H_2O_2$ production, but it reduced $Ca^{2+}$ uptake. The injury in glycolytic fibres did not affect oxygen flux coupled to ATP synthesis, citrate synthase, and lactate dehydrogenase activity, but did reduce $Ca^{2+}$ uptake. Finally, we demonstrated distinct mitochondrial responses between the different muscle fibres, indicating that the mitochondrial dynamics is related to flexibilities in metabolism, and that reactive oxygen species directly affect physiology and normal function.

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Bioenergetics; contusion injury; high-resolution respirometry; mitochondrial function; ROS production; skeletal muscle fibres

## Introduction

Skeletal muscle is the most abundant tissue in the human body [1] and is responsible for the majority of energy expenditure at rest (20–30%) [2]. Muscle tissue is highly dynamic, and composed of fibres that have different myosin heavy chain isoforms. Different types of muscle fibres have other metabolic and biochemical properties that differentiate them, such as oxidative or glycolytic metabolism, calcium dynamics, enzyme levels and the number of mitochondria [3].

The majority of muscle injuries (more than 90%) are caused by contusion or excessive strain of the muscle [4]. Contusion is considered in the literature to be the most common mechanical injury [5,6], especially when playing sports [1]. It occurs from a rapid and strong compressive force, typically from a direct blow or fall [1]. Such compression leads to the breakage of muscle fibres and blood capillaries, resulting in an overflow of blood components to the injured region [7,8].

In sports, contusion are among the main type of injury, with a frequency varying from 10–55% of all collective sports injuries [7], with sports injuries being more frequent [1,7]. This type of injury has implications to an athlete's functional disability, increasing lesion recurrence; in many cases the loss of muscular function can persist for 30–40 days until complete rehabilitation [9].

Fibre death is a relatively rare event in muscle; muscle injuries [8,10] that can be classified by their

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magnitude are of higher frequency, for example: mild (first degree), characterised by damage of a small number to muscle fibres with little swelling and no restriction of movement; moderate (second degree), characterised by partial loss of function with slight haematoma; and severe (third degree), characterised by a complete loss of function, a pronounced haematoma, and consequently, a longer time required for muscle recovery [7,11]. As well as inducing a marked impairment of muscle function, such as greater loss of strength [12], there is a model of mitochondrial functionality impairment after muscle contusion, which is related to increased oxidative damage, decreased membrane potential and mitochondrial swelling [11], as well as the induction of inflammatory processes, and pathways responsible for initiating tissue regeneration or cell damage [7,8].

While there has been much focus in physiotherapy on strategies to accelerate the rehabilitation process, it is essential to identify the events that occur in muscle fibres after contusion. In fact, previous studies have already shown that muscle contusion promotes changes in normal mitochondrial function [11], alters sarcoplasmic reticulum calcium release, and increases several markers of muscle damage (i.e. protein carbonyls) [12]. It has also been shown that different types of muscle fibres present different responses to oxidative and cellular damage [13].

Mitochondria, beyond their role as the central organelle in the metabolism of catabolic substrates and in the anabolic conversion of Krebs' cycle intermediates into different biomolecules [14], is also primarily responsible for the cellular generation of reactive oxygen species (ROS), which can establish an oxidative stress condition when in imbalance with antioxidant defences [13]. However, ROS play dual crucial roles as molecules in cellular redox metabolism [15], because they can determine what the organelle uses as available substrates, maintaining the redox homeostasis and adenosine triphosphate (ATP) turnover in different muscle fibres with oxidative contraction (slow contraction) or glycolytic (rapid contraction) metabolism [13]. Another role for mitochondria is to assist with calcium buffering when there are high concentrations in the cytosol [16]. This influx can act to promote an increase in mitochondrial respiration and thus an increase in ATP synthesis, and can also generate irreversible organelle damage when at high concentrations, triggering the opening of mitochondrial membrane transition pores, thus inducing mitochondrial swelling [17].

Although different muscle fibres types have different metabolic defences and antioxidants [18], studies that analyse the mitochondrial response and metabolism of these different types of fibres have not really explored the role of these processes in muscular injuries. While muscle contusion induces redox signalling and oxidative stress in after the injury, there are no data regarding whether such responses are due to changes in mitochondrial bioenergetics. Our hypothesis is that skeletal muscle contusion promotes a different mitochondrial response dependent on fibre types. While the genesis of oxidative damage and the metabolic changes following muscle contusion are not fully understood, finding these mechanisms of response to a single contusion, will enable thought in treatment more effectively and earlier. As such, the aim of the present work was to evaluate mitochondrial function after a single muscle contusion in fast twitch (Type IIB- glycolytic metabolism), fast and slow twitch (Type IIA- oxidative and glycolytic metabolism), and slow twitch fibres (Type I- oxidative metabolism), as well as to evaluate the regulation of enzymes involved in glycolysis and the Krebs cycle, and the metabolic pathways directly related to mitochondrial oxygen consumption.

## Materials and methods

### *Animals*

One hundred fifty male adult Wistar rats weighing 250–300 g (4 months y.o.) were obtained from the Animal Breeding Unit of the Federal University of Santa Maria (UFSM). Animals were allocated in cages with food and water available ad libitum and maintained under standard conditions of temperature (22 ± 1 °C) and illumination (12-h light/dark cycle). The study protocol followed the ethical rules established by the Guide for Care and Use of Experimental Animals published by the National Institutes of Health (NIH Publication no. 85–23, revised in 1996). All procedures outlined in this study were approved by the Ethics and Research Committee UFSM 4638020916.

### *Contusion injury protocol*

The animals were subjected to an experimental contusion injury protocol, anaesthetised by isoflurane were injured by dropping a known weight (0.2 kg) from a fixed height (0.3 m) onto a rounded impactor bar contact with the medial gastrocnemius muscle. The kinetic energy delivered during each impact was 0.484 J and one impact was performed, while the hindlimb was held with the knee in full extension. This method is considered more similar to human muscle contusion injuries than other blunt trauma models, such as crush

injury [1]. The specific protocol used here is derived from previous work [10]. Contralateral muscles nonexposed were used for the Control.

### Tissue collection and preparation of permeabilized fibres

The animals were killed by decapitation 30 min after the contusion, previous studies showing a restabilization of the intramuscular lactate concentration and pH after 1 h of reperfusion, we determined 30 min after the injury [19]. Two types of skeletal muscle from the same hindlimbs were analysed: gastrocnemius and soleus. Muscle fibres types were determined according to Raja et al. [20], Martins et al. [13], and Bloemberg et al. [21], in the white portion of gastrocnemius fast fibres – glycolytic metabolism (92% type IIb fast twitch fibres) and the intermediate portion between red and white of gastrocnemius mixed fibres – glycolytic and oxidative metabolism (50–60% of type II fibres, fast contraction and 40–50% type I fibres, slow-contraction) and soleus slow fibres – oxidative metabolism (approximately 84% type I slow twitch fibres). Extracted and maintained in BIOPS buffer (10 mM EGTA; 5.77 mM ATP; 6.56 mM $MgCl_2$; 20 mM taurine; 15 mM Phosphocreatine; 20 mM imidazole; 0.5 mM DTT; 50 mM K-MES; pH 7.1) for analysis of high resolution respirometry and hydrogen peroxide production. The same proportion of fibres were extracted from each respective muscle.

The muscles fibres obtained were transferred to 24-well plates containing 1 mL BIOPS with 50 µg/mL saponin on ice. After 30 min, the fibres were transferred and held for 10 min in another well containing 1 mL of the mitochondrial respiration medium 05 – MIR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-MES, 20 mM taurine, 10 mM $KH_2PO_4$, 20 mM HEPES, 110 mM sucrose, BSA fat free 1 g/L, pH 7.1) for washing the detergent and the completion of the permeabilization process Kuznetsov [22]. The permeabilized fibres were used to perform high-resolution respirometry and the hydrogen peroxide production assay.

### High-resolution respirometry

For the analysis of oxygen consumption, 2.5–3.5 mg of permeabilized fibres were used. The analyses were performed on O2k-system high-resolution Oxygraph (Oroboros Instruments, Innsbruck, Austria) with MIR05 at 37 °C. The protocol consisted of sequential titration of multiple substrates, uncouplers and inhibitors (SUIT protocol, Adapted from Pesta and Gnaiger [23]: pyruvate (5 mM), malate (2.5 mM) and glutamate (10 mM); succinate (10 mM); adenosine diphosphate (ADP) (1 mM); cytochrome c (10 µM); oligomycin (2 µg/mL); carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP – titres of 0.25 µM until reaching the maximum oxygen consumption); rotenone (0.5 µM); malonate (5 mM) and potassium cyanide (2 mM KCN). During the measure of mitochondrial respiratory capacity in skeletal muscle in permeabilized fibres, oxygen levels were maintained above air saturation to avoid intracellular "hypoxia" due to limited oxygen diffusion. Alteration the addition order of substrates of the SUIT protocol above mentioned (addition of ADP after CI + CII substrates), aimed to optimise the inspection of the Oxidative phosphorylation (OXPHOS) modulation. This saturation manoeuvre of the Q junction in SUIT protocol facilitates the analysis of OXPHOS at its maximum threshold, both for the leak state and for phosphorylation state. These changes would be less likely in isolated CI or CII analysis where the OXPHOS threshold is lower.

Respirometry nomenclature was defined based on the blue book and the MitoPedia network from Gnaiger [24]. The oxygen flux relative to complex I and the complexes I + II was calculated in the nonphosphorylative state (in the presence of pyruvate, malate, and glutamate or in the presence of succinate, beside those substrates previously mentioned, respectively). The ATP synthesis was calculated by oxygen consumption in the presence of ADP + Pi, minus consumption in the presence of oligomycin. The contribution of each complex in the maximal respiratory capacity (ETS) was estimated by the maximum oxygen consumption in the presence of FCCP minus rotenone for the calculation of complex I, and respiration in the presence of rotenone minus malonate for the calculation of complex II.

### Mitochondrial content and enzymes activities

#### Citrate synthase

For the analysis of mitochondrial content, the activity of the citrate synthase enzyme was used as described previously [25] by monitoring the reduction of 5,5-dithiobis 2-nitrobenzoic acid (DTNB) in a spectrophotometer at 412 nm (SpectraMax® i3x, Molecular Devices, CA, USA). The reaction was initiated with 5 mM oxaloacetate. Data were expressed as $nmol.min^{-1}.mg^{-1}$.

#### Lactate dehydrogenase

In order to evaluate the metabolic status after the experimental procedure, the enzyme lactate dehydrogenase (LDH) was determined in muscle tissue using commercially available assay kits (Labtest®, Lagoa

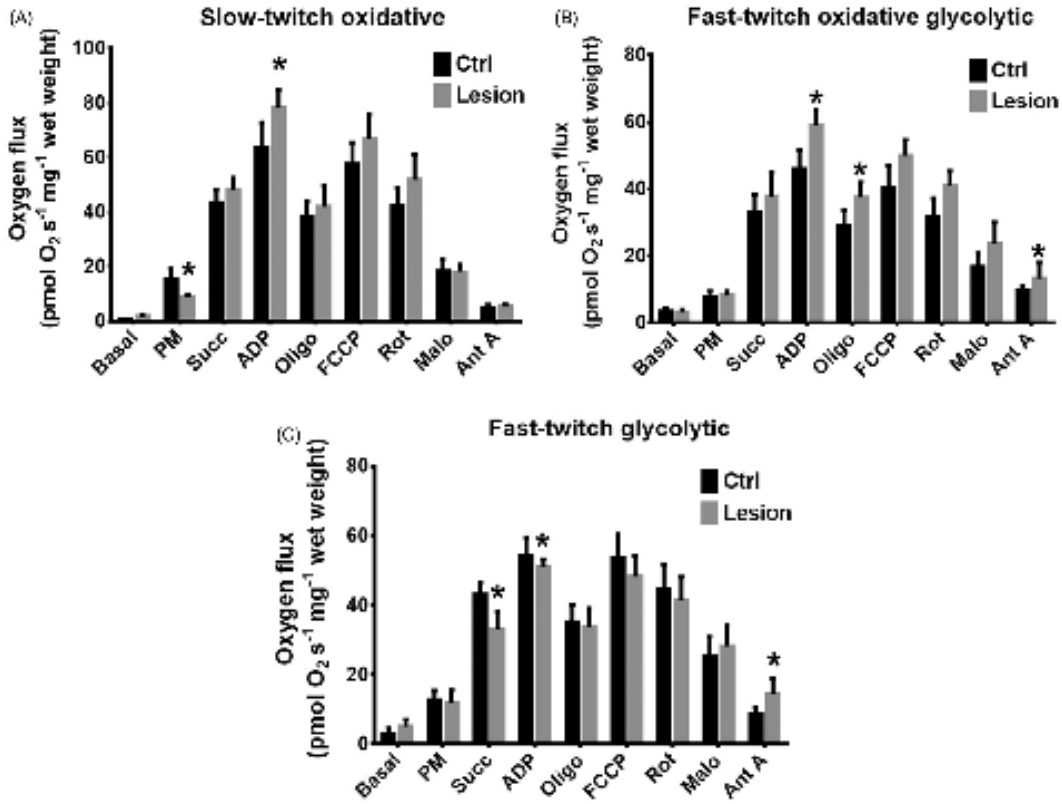


**Figure 1.** Oxygen flux in high-resolution respirometry experiments per fibre mass. **Gastrocnemius**, Oxidative fibres (Slow-twitch) (A), Oxidative/glycolytic fibres (Fast-twitch), Glycolytic fibres (Fast-twitch). The black line represents the oxygen concentration (nmol/mL) and the grey line represents the flux of oxygen normalised by mass in milligrams [(pmol/(s·mg)]. PMG (5:5:10 mM); Succ (10 mM); ADP (2.5 mM); Oligo (2 μg/mL); FCCP (1 μM); ROT (1.0 μM); MALO 10 mM); AmA (5 mM). Black (Ctrl); grey (Lesion). The data are expressed as the mean ± standard error. $^{*}p < 0.05$ vs. control ($n = 8$–9).

Santa, Brazil), the kinetic method according to the manufacturer's instructions. The data expressed as U/mL.

### *$Ca^{2+}$ uptake capacity*

Extramitochondrial free $Ca^{2+}$ was monitored in the presence of saponin-permeabilized (0.5 mg/mL) skeletal muscle fibres using the Calcium green-5N (Molecular Probes) [26]. Muscle fibres (1.0–0.7 mg) were suspended in KCl buffer (125 mM KCl, 20 mM HEPES, 1 mM $K_2HPO_4$, 2 mM $MgCl_2$, 40 μM EGTA, pH 7.2) containing 0.1 mM calcium green-5N and either 1 M pyruvate and 500 mM malate. Fluorescence was continuously monitored at 37 °C using a SpectraMax® i3x (Molecular Devices, CA, USA) excitation/emission of 506/532 nm.

The fluorescence values were calibrated by successive addition of known amounts of $CaCl_2$ to medium containing permeabilized fibres. $Ca^{2+}$ uptake was observed by the titration of calcium in medium and monitored the subsequent changes in the fluorescence intensity. This fluorescence reflects mitochondrial $Ca^{2+}$ overload and mitochondrial permeability transition pore (mPTP) opening, the total amount of $Ca^{2+}$ taken up by mitochondria prior to mPTP activation.

### *Hydrogen peroxide ($H_2O_2$) production*

$H_2O_2$ flux was measured in the O2k-Fluorometer using the $H_2O_2$-sensitive probe Amplex® Ultra. A total of 4–5 mg of permeabilized fibre was placed in the cuvette containing 2 mL of MiR05, 2 u/mL of peroxidase (HRP) and 5.5 μM of Amplex red (AmR) (Invitrogen, Life Technologies SAS, Brazil – A22188), the reaction product between AmR and $H_2O_2$, catalysed by HRP, is fluorescent, similar to resorufin. All assays were performed at 37 °C [27]. The process of muscle fibres permeabilization creates pores in the sarcolemma membrane that leads to extravasation of cytosolic enzymes and metabolites. The apparent rate of mitochondrial $H_2O_2$ production is the result of electron leakage and matrix

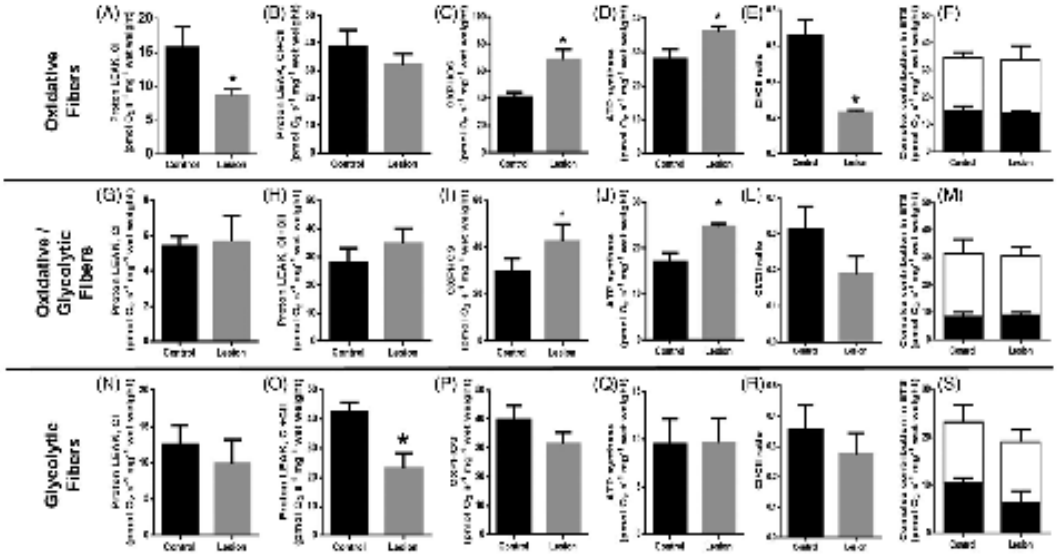


**Figure 2.** $O_2$ consumption rates of complex I, complex I+II, OXPHOS and oxygen flux coupled to ATP synthesis in different fibres types after skeletal muscle contusion. (A, G and N): Oxygen consumption rate in the nonphosphorylative state with complex I linked substrates. (B, H, and O): Oxygen consumption in the nonphosphorylative state with complex I+II−linked substrates. (C, I and P): Oxygen consumption in the phosphorylative state (OXPHOS) with complex I+II and ADP substrates. (D, J, and Q): Oxygen flux coupled to ATP synthesis. (E, L, and R): Ratio of oxygen flux between complex I and complex II. (F, M, and S): Complex I (black bars) or complex II (white bars) contribution in maximal respiratory capacity (ETS). The oxygen flux was derived taking in account the fraction that is an inhibitor-sensitive contribution to total ETS uncoupled respiration to each correspondent inhibitor specific complex. Black (Ctrl); grey (injury). Data are expressed as the mean ± standard error ($n = 8–9$). *$p < 0.05$ vs. control.

mitochondrial antioxidant system once the HRP does not enter the mitochondrial matrix.

Two experiments were performed with sequential additions of the following substrates and inhibitors:

1. Pyruvate (5 mM), malate (2.5 mM) and glutamate (10 mM); succinate (10 mM); ADP (500 µM); oligomycin (2 µg/mL) and FCCP (0.25 µM) for determination of $H_2O_2$ production at different states of mitochondrial respiration.
2. Pyruvate (5 mM), malate (2.5 mM) and glutamate (10 mM); succinate (10 mM) and rotenone (0.5 µM) to calculate specific $H_2O_2$ production by reverse flow at ubiquinone site in complex I ($CI_Q$), it was added rotenone to inhibits CI in the specific complex site. The other mitochondrial sites such as, complex III (site $III_{Qo}$), mG3PDH (reverse flow), electron transfer Flavoprotein (ETF, reverse flow), Flavin site in CI (IF), Flavin site in CII (IIF) and matrix dehydrogenases (Brand [28]; Wong et al. [29]) were assumed to be responsible for the remaining rate in $H_2O_2$ production. $H_2O_2$ production by reverse flow in $CI_Q$ was calculated from the rate of $H_2O_2$ production with pyruvate, malate, glutamate and succinate (PMGS) – the rate of $H_2O_2$ production after rotenone addition.

The data generated were treated in the Origin Pro-8 programme (Origin Lab Corporation, Northampton, USA). The rates of $H_2O_2$ production generated by the programme in arbitrary fluorescence units were then converted to pmol/mg/min by normalisation by titration curve of $H_2O_2$. The $H_2O_2$ calibration curve was performed in several experiments both in the presence and absence of permeabilized fibres and no changes were detected in the slope of the curves or suppression of resorufin fluorescence by the fibres.

### *Statistics*

Kolmogorov-Smirnov and Bartlett's tests were performed for analysed normality of data and homogeneity of variances, respectively. The effects on mitochondrial function were analysed by unpaired Student's t test by the GraphPad Prism programme (GraphPad Software, CA, USA). Data were expressed as means ± standard error of the mean (SEM), considering $p \leq 0.05$ as significant.

**Table 1.** Coupled, inhibited and uncoupled parameters of oxygen flux.

| | Oxidative fibres | | Oxidative/Glycolytic fibres | | Glycolytic fibres | |
|---|---|---|---|---|---|---|
| | Ctrl ($n = 7$–9) | Lesion ($n = 7$–9) | Ctrl ($n = 6$–7) | Lesion ($n = 8$) | Ctrl ($n = 7$) | Lesion ($n = 7$) |
| Respiratory control ratio (RCR) | 1.78 ± 0.07 | 2.31 ± 0.21* | 1.63 ± 0.08 | 1.92 ± 0.13 | 1.65 ± 0.15 | 1.80 ± 0.21 |
| Maximal respiratory capacity (ETS) | 49.60 ± 6.36 | 49.75 ± 8.26 | 38.94 ± 5.10 | 37.86 ± 6.15 | 41.09 ± 6.85 | 36.20 ± 8.76 |
| LEAK state | 30.83 ± 5.23 | 30.44 ± 4.30 | 19.11 ± 4.65 | 41.13 ± 4.85* | 41.64 ± 6.09 | 37.10 ± 5.80 |
| ROX | 3.22 ± 0.96 | 4.20 ± 0.67 | 7.19 ± 1.10 | 11.08 ± 1.33* | 7.84 ± 1.50 | 7.01 ± 1.64 |
| LEAK / ETS | 0.57 ± 0.03 | 0.62 ± 0.06 | 0.61 ± 0.03 | 0.63 ± 0.05 | 0.64 ± 0.08 | 0.63 ± 0.05 |

Data were expressed in [pmol $O_2$/(s × mg)] as mean ± SEM. *$p < 0.05$ vs. control.

## Results

### A single induced muscle injury differentially alters oxygen flux in different muscle fibre types

The effects of contusion on HRR were analysed in different muscle fibres with oxidative, mixed, or glycolytic metabolism to investigate how these fibres are affected by mechanical muscle injury. Figure 1 illustrates the SUIT protocol. Oxygen flux rates in the basal state and after the addition of mitochondrial substrates, inhibitors and decouplers were evaluated in oxidative fibres (Figure 1(A)), mixed fibres (Figure 1(B)) and glycolytic fibres (Figure 1(C)).

The multisubstrate respirometry experiments demonstrated that $O_2$ flux per mass in oxidative fibres decreases with the substrate of CI (PM) 30 min after the injury in comparison to the control ($t_{(0.05;\ 11)} = 2.483$; $p = 0.0304$). However, the addition of ADP increased $O_2$ flux in the oxidative ($t_{(0.05;\ 12)} = 2.949$, $p = 0.0122$) and mixed ($t_{(0.05;\ 12)} = 2.300$, $p = 0.0402$) muscle fibres in comparison with the respective controls, while the injury also caused an increase in $O_2$ flux in glycolytic/oxidative metabolism fibres in the presence of oligomycin ($t_{(0.05;\ 10)} = 2.216$, $p = 0.0487$) and antimycin A ($t_{(0.05;\ 10)} = 2.501$, $p = 0.0314$) (Figure 1(A,B)).

Furthermore, the lesion reduces succinate and ADP-stimulated respiration in glycolytic fibres compared to the control [($t_{(0.05;9)} = 2.506$, $p = 0.0216$), ($t_{(0.05;9)} = 2.805$, $p = 0.0205$), respectively], while it significantly increases it in the presence of antimycin A ($t_{(0.05;9)} = 2.805$, $p = 0.0205$) (Figure 1(C)).

The rates extracted from the experiment model protocols demonstrate that oxidative fibres significantly reduce oxygen flux in the nonphosphorylated Leak state, using substrates linked to complex I (CI), when compared to the control ($t_{(0.05;10)} = 2.264$, $p = 0.0470$). Specifically, the lesion oxygen flux decreased by −0.45-fold when stimulated by CI substrates compared to the control (Figure 2(A)). Recovery to the control levels of oxygen flux occurred when succinate, the substrate for complex II, was added (Figure 2(B)). Indeed, ratios of oxygen flux between complex I and complex II (CI/CII ratio) were calculated (Figure 1(D)); contusion was able to decrease the ratio between CI and CII in the non-phosphorylated state compared to the control ($t_{(0.05;9)} = 5.235$, $p = 0.0005$) (Figure 2(E)).

Oxygen flux in a nonphosphorylated state did not change in comparison to the control when using both substrates linked to complex I (CI) or complex I plus II (CI + CII) mixed fibres (Figure 1(G,H)). In contrast, in the glycolytic fibres, injury related to the control decreased oxygen flux by −0.42-fold when stimulated by CI + CII substrates ($t_{(0.05;9)} = 2.216$, $p = 0.0106$) (Figure 2(O)).

Similarly, oxidative and oxidative/glycolytic fibres showed an increase in coupled oxygen flux (OXPHOS) after injury compared to the respective controls. ADP/Mg-induced oxygen consumption was 0.68-fold ($t_{(0.05;10)} = 3.209$; $p = 0.0094$) and 0.44-fold ($t_{(0.05;10)} = 2.796$; $p = 0.0209$) higher after injury than in the control in oxidative and oxidative/glycolytic fibres, respectively (Figure 2(C,I)). Similar results were observed with oxygen flux coupled to ATP synthesis in oxidative ($t_{(0.05;10)} = 2.430$; $p = 0.0354$) and mixed fibres ($t_{(0.05;10)} = 4.027$; $p = 0.0030$) in comparison to the respective control (Figure 2(E,L)), but no change was observed in glycolytic fibres (Figure 2(Q,R)).

Other respirometric parameters that were altered in comparison with the control following muscle injury included the respiratory control ratio (RCR), which increased in oxidative fibres ($t_{(0.05;10)} = 4.027$; $p = 0.0030$), while mixed fibres showed an increase in LEAK state and residual oxygen flux (ROX) compared to the control group as seen in Table 1.

Since we detected different effects of the lesion on different fibres, we evaluated the electron transfer system (ETS); this parameter measures maximal respiratory capacity and the contribution of electrons from CI + CII substrates for respiration. The uncoupled maximal flux was not altered in relation to controls in all of the tested fibres (Table 1). Similarly, we did not detect a difference in ETS oxygen flux in the CI (R) and CII (MNA) among all fibre types tested compared to the controls (Figure 2(F,M and S)). Although the nonphosphorylated state showed a decrease in $O_2$ flux related to CI after injury in oxidative fibres compared to the control, these results suggest that there was no alteration in the

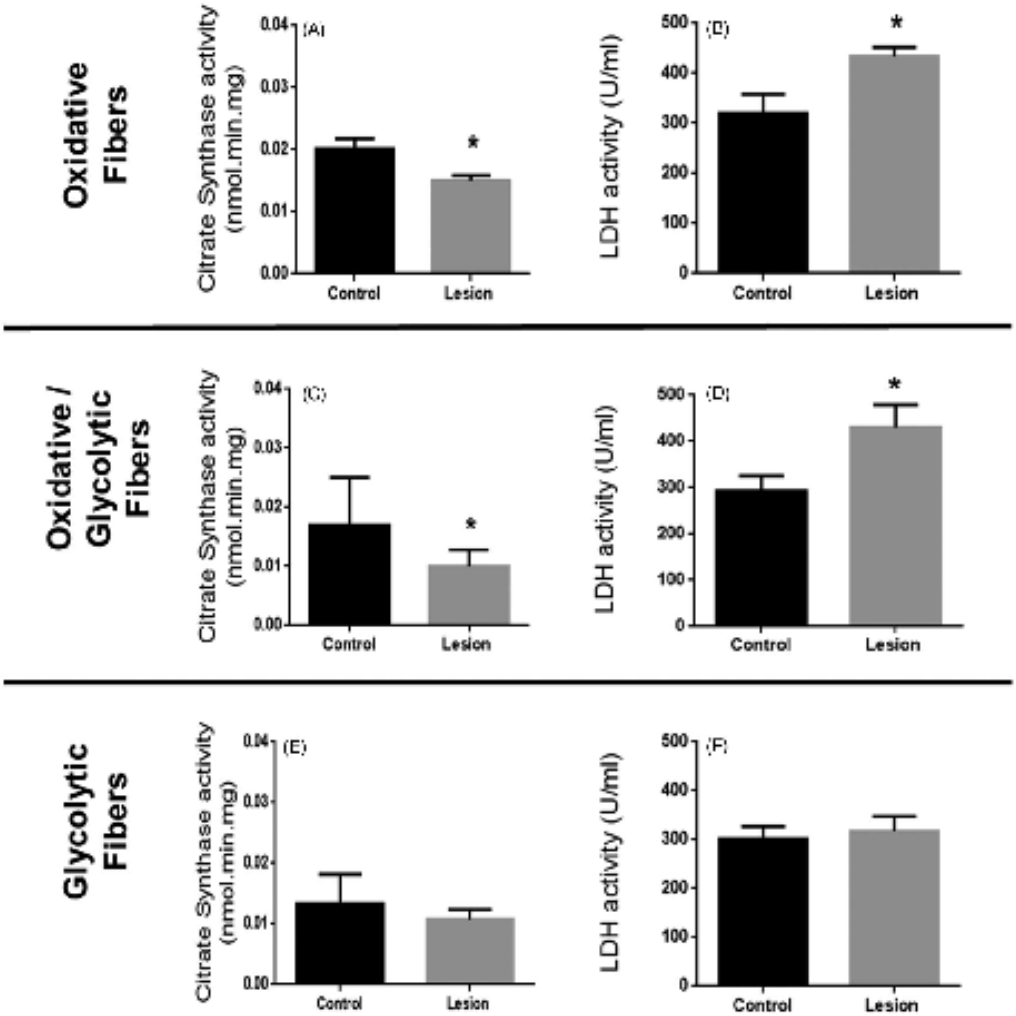


**Figure 3.** Contusion-induced muscle injury changes the enzymatic activity. Lactate dehydrogenase (LDH) activity in oxidative (A), oxidative/glycolytic (C) and glycolytic fibres (E); citrate synthase (CS) in oxidative (B), oxidative/glycolytic (D) and glycolytic fibres (F), after injury in different types of muscle fibres. Black (Ctrl); grey (injury). Data are expressed as the mean ± standard error ($n = 5–8$). *$p < 0.05$ vs. control.

capacity of ETS to transfer electrons for the time period and conditions tested. In addition, muscle injury did not change CIV activity (Supplementary Figure 1S).

### *Changes in mitochondrial content and metabolic markers after a contusion injury*

We investigated whether CS activity, which is an enzyme present in the mitochondrial matrix that is used as a marker of mitochondrial content, was altered in skeletal muscle after contusion injury. We detected differences in CS activity between the lesion and control groups immediately after the muscle insult. The oxidative fibres showed a significant 0.25-fold reduction in CS activity compared to the control ($t_{(0.05;10)} = 3.188$; $p = 0.0243$) (Figure 3(A)), while mixed fibre activity decreased −0.44-fold in comparison to the control ($t_{(0.05;10)} = 2.355$; $p = 0.0382$) (Figure 3(C)).

We also observed that LDH, a metabolic activity marker, increased significantly after a single muscle contusion in the oxidative and mixed fibres by 0.35-fold ($t_{(0.05;12)} = 2.342$; $p = 0.0377$) and 0.47-fold ($t_{(0.05;12)} = 2.368$; $p = 0.0355$) compared to controls, respectively (Figure 3(B,C)). However, there were no changes in the

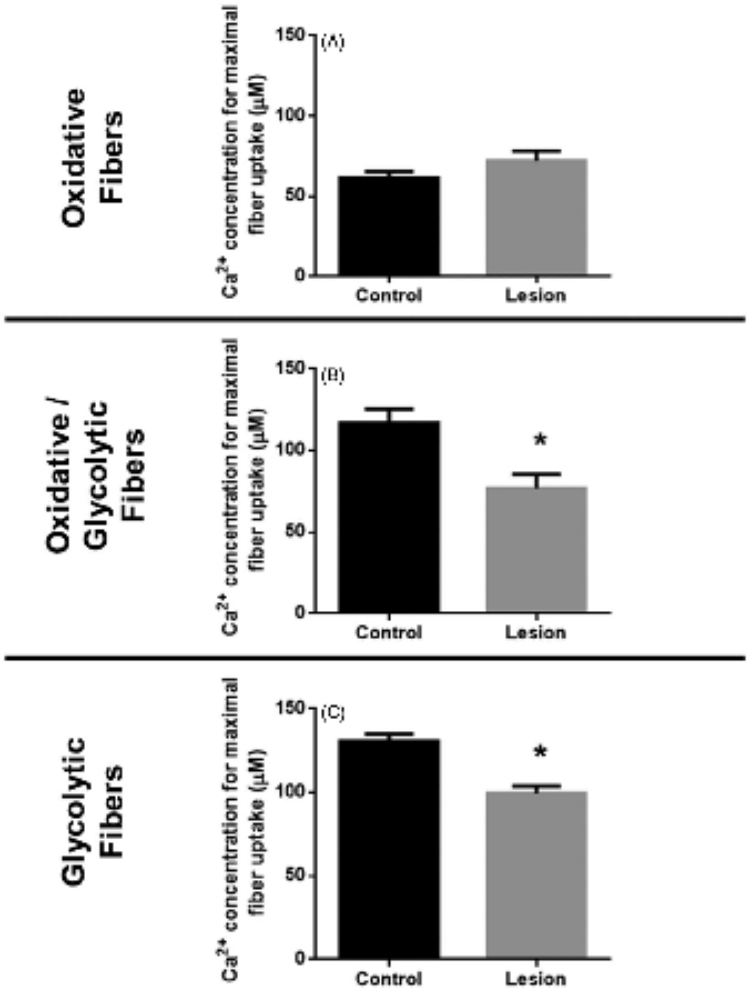


**Figure 4.** Fluorescent measurement of $Ca^{2+}$ uptake using permeabilized skeletal muscle fibres. Maximal $Ca^{2+}$ uptake by different muscle fibres types in the presence of 1 M pyruvate and 500 mM malate was measured as described using calcium green-5N as an indicator of the free $Ca^{2+}$ concentration in the medium (506-nm excitation, 532-nm emission). Equivalent pulses of $CaCl_2$ (5 µL of 0.6 mM) were added to every 2 min until there was no longer evidence of uptake of the added $Ca^{2+}$. For measurement of maximal $Ca^{2+}$ uptake using saponin (5 mg/mL) permeabilized fibres, intact fibres were previous permeabilized in optimum medium (BIOPS) and added to the KCl buffer containing pyruvate and malate. Black (Ctrl); grey (injury). Data are expressed as the mean ± standard error ($n = 5$–6). $^{*}p < 0.05$ vs. control.

mitochondrial activity levels of citrate synthase or LDH in glycolytic fibres (Figure 3(E,F)).

### *Muscle contusion alters $Ca^{2+}$ uptake*

Given the observed changes in mitochondrial oxygen consumption induced by muscular contusion in the different types of fibres, we next investigated whether calcium homeostasis would also be affected. Immediately after contusion, we observed no significant changes in $Ca^{2+}$ uptake by oxidative fibres (Figure 4(A)). We observed consistent calcium concentrations < 60 µmol, which is the concentration required to induce the formation of mPTP, in both control and injured fibres (Figure 4(A,B)), suggesting a long-term resistance to the formation of mPTP. On the other hand, we observed a reduction of $Ca^{2+}$ uptake in injured tissue in the other fibre types, noting the formation of mPTP in oxidative/glycolytic fibres when they were exposed to < 90 µM of $Ca^{2+}$, with a decrease of −0.37-fold compared to the control ($t_{(0.05;11)} = 4.400$; $p = 0.0011$) (Figure 4(C,D)). For the glycolytic fibres, the formation of mPTP was

**Table 2.** Hydrogen peroxide production rates after muscle contusion.

| | Oxidative fibres | | Oxidative/Glycolytic fibres | | Glycolytic fibres | |
|---|---|---|---|---|---|---|
| | Ctrl ($n=8$) | Lesion ($n=8$) | Ctrl ($n=5$) | Lesion ($n=5$) | Ctrl ($n=5$) | Lesion ($n=5$) |
| PMG | 0.0074 ± 0.0030 | 0.0196 ± 0.013* | 0.0140 ± 0.0097 | 0.0886 ± 0.055* | 0.0231 ± 0.0214 | 0.0415 ± 0.0371 |
| S | 0.0010 ± 0.0011 | 0.0029 ± 0.0039 | 0.0018 ± 0.0012 | 0.0108 ± 0.0162 | 0.0080 ± 0.0078 | 0.0150 ± 0.0156 |
| D | 0.0016 ± 0.0019 | 0.0026 ± 0.0024 | 0.0030 ± 0.0025 | 0.0052 ± 0.0085 | 0.0033 ± 0.0037 | 0.0049 ± 0.0059 |
| Omy | 0.0013 ± 0.0018 | 0.0034 ± 0.0041 | 0.0018 ± 0.0041 | 0.0072 ± 0.0120 | 0.0035 ± 0.0061 | 0.0064 ± 0.0095 |
| FCCP | 0.0695 ± 0.0565 | 0.1348 ± 0.1110 | 0.1130 ± 0.1000 | 0.1038 ± 0.1339 | 0.0841 ± 0.0536 | 0.1694 ± 0.1128 |

Data were expressed in (pmol $H_2O_2$/mg/min) as mean ± SEM. *$p < 0.05$ vs. control.

observed when exposure was < 100 μM of $Ca^{2+}$, which was 0.20-fold less in relation to the control ($t_{(0.05;11)} = 5.213$; $p = 0.0003$) (Figure 4(D,F)); before this we observed the maintenance of $Ca^{2+}$ uptake by fibres.

### *Muscle contusion alters $H_2O_2$ production rates at different release and electron leakage sites*

In order to determine which events, occur in cells due to changes in mitochondrial respiration, we specifically measured $H_2O_2$ production in relation to electron leakage, and the relative contribution of the reversal of electron flux in the $CI_Q$, and other sites of the mitochondria.

Muscle contusion was able to change the $H_2O_2$ production rates generated by mitochondrial complex I in the nonphosphorylated state, as oxidative fibres showed increases of 1.6-fold ($t_{(0.05;10)} = 3.184$; $p = 0.0077$) compared to the control group. Also, the $H_2O_2$ production of injured mixed fibres when the electron transport chain is fed by CI substrates was 1.9-fold higher in comparison to the control ($t_{(0.05;10)} = 2.450$; $p = 0.0019$) (Table 2).

In addition, a detailed analysis of the rate of $H_2O_2$ production sites was similar to $H_2O_2$ production by electron leakage. Oxidative fibres increased the $H_2O_2$ production rate by 2.3-fold for reverse flow in $CI_Q$ ($t_{(0.05;10)} = 3.384$; $p = 0.0070$) (Figure 5(A)) and 1.2-fold in other sites of the mitochondria ($t_{(0.05;\ 9)} = 3.334$; $p = 0.0280$) compared to control oxidative fibres (Figure 5(B)). The contusion in mixed fibres promoted a 2.3-fold higher generation and affected only the rate of $H_2O_2$ production generated by $CI_Q$ compared to control fibres ($t_{(0.05;9)} = 5.684$; $p = 0.0003$) (Figure 5(C,D)). The effect of injury in the glycolytic fibres increased the $H_2O_2$ production rate by 1.7-fold for reverse flow in $CI_Q$ ($t_{(0.05;\ 10)} = 2.266$; $p = 0.0469$) (Figure 5(E)) and by almost 1-fold in other sites of mitochondria in relation to glycolytic fibres control ($t_{(0.05;\ 10)} = 2.450$; $p = 0.0280$) (Figure 5(F)). These experiments examining specific $H_2O_2$ leakage (the ratio between $H_2O_2$ production and its respective $O_2$ flux), suggest that contusion causes an increase in ROS production by CI in the fibres.

## Discussion

Muscle contusion is a significant cause of an athlete's inability to train or compete for at least several weeks [1], and often occurs in team sports that have greater contact the between athletes, such as football, basketball, and handball [7]. This injury is caused by a direct and nonpenetrating hit to the muscle belly, causing a strong compressive force [1]. Here we showed for the first time that muscle contusion injury modulates mitochondrial respiratory physiology in a fibre-dependent manner. Oxidative, mixed, and glycolytic fibres change different forms of mitochondrial oxygen consumption after contusion damage. It is not a paradox in respiration as these fibres types have different kinds of metabolism, as well as structures and physiology [30].

Mitochondria are central regulators of energy and cell homeostasis and evidences support the critical role of mitochondrial dysfunction [31], however, the effects of contusion injury on mitochondrial bioenergetics in muscle fibres with different metabolism was never investigated before. In this work, our results indicate a change to the contribution of slow twitch fibres (oxidative metabolism) in relation to CI and CII coupled to ATP synthesis, where muscle injury promotes an important apparent depression in $O_2$ flux sustained by the CI and CI/CII ratio compared to the control oxidative fibre (Figure 2(A,B)). These data suggest a more significant contribution from CII than CI in the mitochondria respiration pathway of these fibres when exposed to injury.

In contrast in the fast/slow fibres (glycolytic and oxidative metabolism), muscular injury leads to an exacerbation of mitochondrial respiration in the presence of specific substrates (ADP), increasing respirometric parameters, such as OXPHOS and ATP synthase (Figure 2(J,L)). A high ratio of $NAD^+$/NADH, ADP/ATP, and $Ca^{2+}$ can induce the dehydrogenase activity of the tricarboxylic acid cycle, leading to enhanced oxygen flux [13]. However, an increase in oxygen consumption by the electron transport chain may favour the escape of electrons by CI and other sites of the

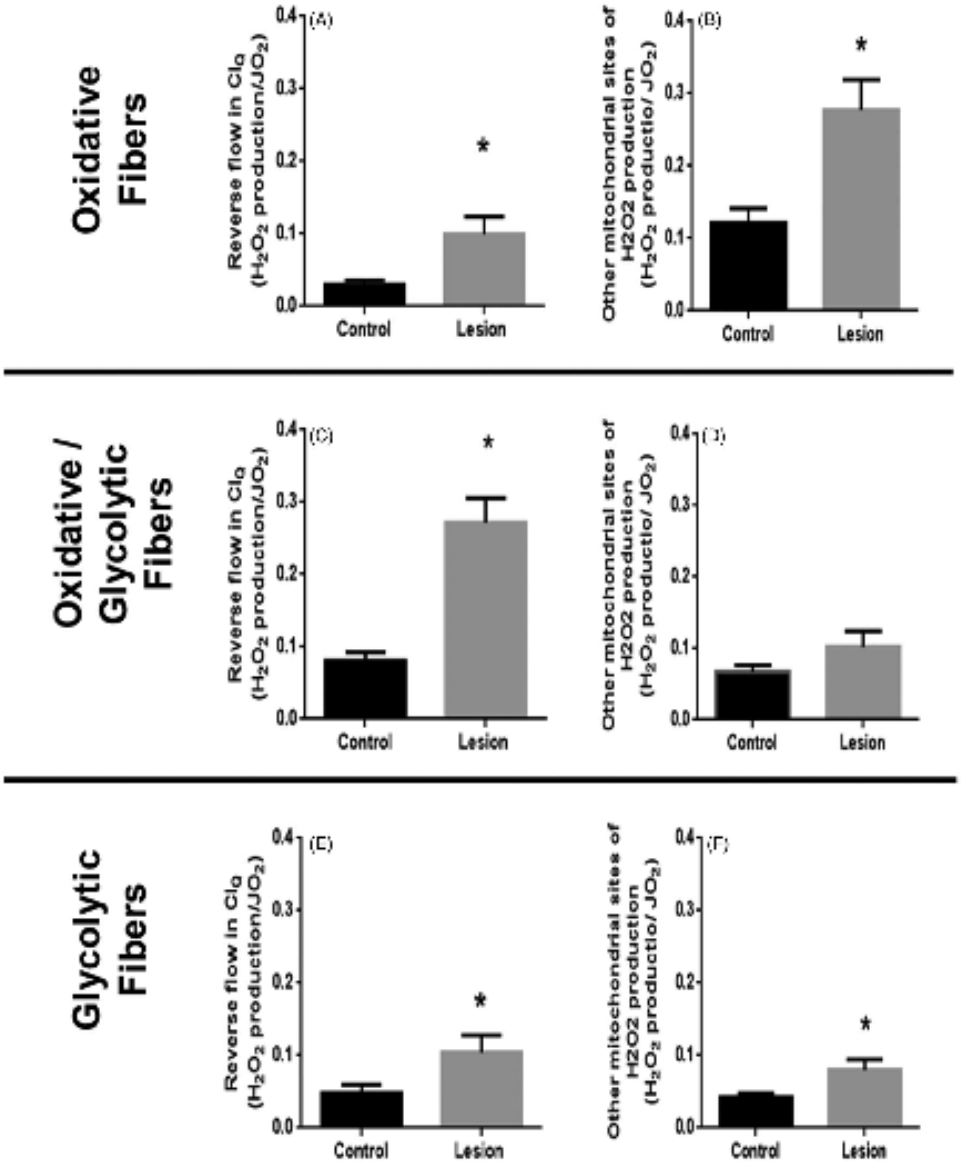


**Figure 5.** Hydrogen peroxide production rates after contusion. Derivation of $H_2O_2$ production rates in the different sites of mitochondrial electron escape; by reverse flow in the complex $I_Q$ ($CI_Q$), oxidative fibres (A), oxidative/glycolytic (C) and glycolytic fibres (E); or by other mitochondrial sites of $H_2O_2$ production, which are defined as being the contribution of complex III (site $III_{Qo}$), mG3PDH, ETF, flavin site in CI (IF), flavin site in CII (IIF) and matrix dehydrogenases, oxidative fibres (B), oxidative/glycolytic (D) and glycolytic fibres (F). Black (Ctrl); grey (injury). Data are expressed as the mean ± standard error ($n = 5–7$). $^*p < 0.05$ vs. control.

ETS, as well as the formation of ROS, such as $H_2O_2$ [28,29]. Indeed, when we measured ROS production in *ex vivo* conditions, our results presented altered values due to contusion injury in oxidative and mixed fibres (Table 2).

In skeletal muscle mitochondria in media, mimicking muscle cytosol at rest, the sites of complex I, II and III dominate $H_2O_2$ production [28]. In addition, recent studies had demonstrated that succinate accumulation occurs from the Krebs cycle occurs during ischaemia

and reperfusion, leading to extensive ROS production by the reversal of electron flow through complex I [32].

Nevertheless, we also observed different rates of generation in slow and fast twitch fibres from other mitochondrial sites, such as complex III (site $III_{Qo}$) and other sites assumed to be responsible for the remaining $H_2O_2$ production rate [28,29] after muscle injury (Figure 5(B,F)), although contusion in the fast twitch fibres (oxidative/glycolytic metabolism) did not show different responses in the other mitochondrial sites (Figure 5(D)). Mitochondrial ROS have a physiological role to promote cellular adaptations to stress conditions by the regulation of oxidative metabolism, supporting cell survival [33]. The diverse characteristics of muscle fibres, such as high oxidative capacity and mitochondrial density of the slow twitch fibre (oxidative metabolism) [18], are related to an increase in peroxide production in oxidative and mixed fibres when compared to the control groups.

According to previous studies, superoxide/hydrogen peroxide production by complex III can signal hypoxia to the hypoxia-inducible factor-1α (HIF-1α) signalling system in cells [33,34]. This event can be caused by the direct effects of decreased oxygen tension [35], and indirectly by changes in metabolite concentrations that then deliver more electrons to the electron transport chain and lead to higher electron leakage to oxygen [28]. HIF plays a major role in the source of metabolic energy by exchanging glucose metabolites from mitochondrial respiration to cytosolic glycolysis; this metabolic exchange leads to increased lactate levels [36].

In accordance, this regulation affects mitochondrial metabolism and may explain the reduction in CS activity and the increase in LDH activity upon contusion (Figure 3(B,D)), indicating a metabolic change towards anaerobic glycolysis in oxidative and mixed fibres. This promotes a metabolic environment with the flux of lactate and oxygen to sustain cellular activity and tissue recovery, as demonstrated by the increase in oxygen flux and the exchange to other energy sources, such as lactate, as demonstrated by increased LDH activity. CS is used as a marker of mitochondrial content as this enzyme is present in the mitochondrial matrix, and a reduction in its activity indicates a loss of mitochondrial content that could be at least in part explained by altered mitochondrial biogenesis and/or increased elimination by mitophagy [37].

On the other hand, the oxygen consumption sustained by CI + CII substrates decreased in the nonphosphorylated state, specifically in fast twitch fibres (glycolytic metabolism) after a contusion (Figure 2(P)). However, a decrease in $O_2$ flux by CI + II did not affect the $O_2$-coupled consumption of OXPHOS and ATP synthase (Figure 2(Q,R)). This demonstrates that significant changes in mitochondrial functionality and metabolism did not occur in the glycolytic fibres after muscle injury model; supporting this idea, glycolytic fibres did not show alterations in LDH and CS activity during the tested time frame (Figure 3(E,F)). Moreover, in our data, the contusion model induced an increase in $H_2O_2$ generation by a reverse flow in $CI_Q$ and mitochondrial sites; however, this was less pronounced in fast twitch fibres compared to other (Figure 5(E)).

Based on our evidence, we believe that glycolytic fibres are more adaptable to muscle insults because, as they present less pronounced peroxide production at all of the mitochondrial sites, they did not exchange energy sources as demonstrated by maintaining an LDH activity that is similar to control levels; also, they did not change mitochondrial functionality and content after muscle contusion, as oxygen flux in the OXPHOS and CS activity were not altered.

Regarding the mitochondrial membrane permeability transition, Puntel and colleagues [11] demonstrated a significant increase in mitochondrial swelling, this effect in mitochondria occurred in a more pronounced manner in a short-time frame (30 min) than 24 h after the lesion, indicating that mPTP opening occurs after contusion. Here, we analysed this response in different fibres, and observed the increased resistance to mPTP formation and increased calcium buffering in oxidative metabolism fibres immediately after the muscle insult (Figure 4(A,B)). This demonstrates the importance of analysing the muscle fibres separately, especially since the mitochondrial population in different fibres is not homogeneous and this apparent divergence between results may be dependent on differences between metabolism subtypes as well as influenced by differing storage capacities and the uptake of $Ca^{2+}$ in each fibre.

Previous studies have shown that mPTP formation has a physiological effect, and that the acute opening of mPTP has a critical physiological role in regulating mitochondrial $Ca^{2+}$ handling and metabolism [38,39]. Hausenloy et al. [40] previously demonstrated that modest mitochondrial uncoupling and mPTP opening are a critical to inducing protection in the infarct, and showed that this protection can be abolished by inhibiting mPTP opening. Our data, however, show that with a muscle injury in slow twitch fibres (oxidative metabolism), increased resistance to $Ca^{2+}$ and the opening of mPTP might contribute to sustained mitochondrial matrix $Ca^{2+}$ overload, leading to mitochondrial dysfunction and cell death [38]. Wang and colleagues [41] demonstrated an association in striated muscle mitochondria between

transient mPTP opening and superoxide flashes, acting to maintain the mitochondrial homeostasis of $Ca^{2+}$ and provide the possible regulation of physiological processes. In agreement with the physiological role of MPTP, we found significant changes in $Ca^{2+}$ uptake in mixed and glycolytic fibres (Figure 4(C,D,E and F)), and injury caused a decrease in resistance to $Ca^{2+}$ and opening of the mPTP.

In addition, dysregulated $Ca^{2+}$ homeostasis and ROS are able to stimulate Toll-like receptors-9 (TLR9) to induce nuclear factor kappa B (NF-κB) activation, which causes transcriptional activation of the multiple inflammatory cytokines tumour necrosis factor-α (TNF-α) and interleukin (IL-1β, and IL-6) [42]. A recent review by Hiroyuki Nakayama1 and Kinya Otsu [43] proposes that the activation of nuclear factor kappa B (NF-κB) and cytokine regulation, such as TNF-α and IL-1β, mediate the down-regulation of $Ca^{2+}$-cycling-associated genes such as sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA2), thus leading to contractility reduction through alterations in intracellular $Ca^{2+}$ homeostasis in cardiac myocytes. In this context, this inflammatory response triggered by a disturbance in $Ca^{2+}$ homeostasis is involved in the remodelling process. However, in the chronic state, these alterations may reflect the impairment of muscle recovery and normal function, TNF-α can trigger apoptosis and IL-6 has been reported to increase cardiomyocyte stiffness through the reduction of titin phosphorylation [43].

## Conclusion

In conclusion, our study demonstrated distinct mitochondrial responses among the different muscle fibres, and variations in specific flux and oxygen metabolism, beyond $Ca^{2+}$ flux, indicating that the mitochondrial dynamics are related to the flexibilities of metabolism, and that reactive oxygen species directly affect physiology and normal function immediately after muscle contusion. The findings of this work expand the available knowledge regarding the role of mitochondria in muscle injury, demonstrating how mitochondrial functions in the different skeletal muscle fibres are affected by the contusion model, and enabling new strategies for better treatments based on the injury and muscle fibres type rather than severity alone.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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3.2. ARTIGO CIENTÍFICO II

**3.2.1. Manuscript 1**

**Full Title: Mitochondrial dysfunction promotes TLR 4-mediated inflammatory reactions in injured skeletal muscle of rats**

**Run Title: The role of mitochondrial dysfunction and TLR signaling in skeletal muscle contusion**

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Abstract

Contusion is a mechanical trauma in skeletal muscle and the most common cause of injury. Mitochondrial reactive oxygen species (ROS) production is a primary event of the muscle contusion and immediately alters mitochondrial bioenergetics response. Nonetheless, it is still unclear whether oxidative stress modulates the expression of Toll-like receptors-4 (TLR4) and which may be related to pro-inflammatory cytokines production. Therefore, this study analyzed the influence of muscle injury on the TLR4 pathway in the skeletal muscle of rats. We evaluated the acute mitochondrial function response after a single muscle contusion in fast-twitch fibers (glycolytic metabolism), fast and slow twitch (oxidative and glycolytic metabolism), and slow-twitch fibers (oxidative metabolism). The rats were divided into two groups (Control or Lesion) and studied in 30 minutes and 2 hours after the protocol beginning. The gastrocnemius and soleus muscles were used to obtain the tested muscle fibers, that were analyzed for mitochondrial respiration capacity, BAX, caspase-3 IL-1β, TNF-α, and TLR 4 mRNA levels. Data obtained showed that a single contusion promotes mitochondrial dysfunction and increases mRNA TLR 4 levels at 30 min and 2 hours. Similarly, mRNA levels of TNF-α, inflammation-related genes also increased, moreover, mRNA of BAX, caspase-3, IL-1β increased only oxidative and mixed fibers. The current study identifies a novel mechanism mediating injury and mitochondrial dysfunction in muscle contusion model in rats. These findings suggest that mitochondrial dysfunction possibly interferes with the TRL-4 pathway activation, upregulating the inflammation response in injured muscle fibers. We suggest that TLR 4 upregulation may represent an additional target for ROS-suppressive/antioxidant strategies to mediate inflammation.

**Keywords:** contusion, inflammation, cytokine, IL-1β, TNF-α.

**Introduction**

The skeletal muscle is continuously exposed to several kinds of injuries, comprising 10–55% of all sports activity injuries (SOUZA; GOTTFRIED, 2013b). Muscle contusion is the most common sports-related damage, not just for athletes but also for recreational players (OYAIZU et al., 2018), often resulting in pain or muscle disruption leading to the incapacity to train or compete for several weeks. Previous studies have shown that mitochondrial reactive oxygen species (ROS) production is a primary muscle contusion event (PUNTEL et al., 2011), promoting an immediate alteration in mitochondrial bioenergetics response in skeletal muscle fibers (HARTMANN et al., 2020). Muscle recovery after mechanical damage then begins by triggering a set of well-known molecular events (CAROSIO et al., 2011).

In addition to being the primary source of adenosine triphosphate (ATP), mitochondria play several roles in the cell and other cell signaling events, including regulation of calcium ($Ca^{2+}$) homeostasis, orchestration of apoptosis, and ROS production (LÓPEZ-ARMADA et al., 2013). Moreover, mitochondria have also been identified as principal source of inflammation mediators (TSCHOPP, 2011). These organelles modulate innate immunity via redox-sensitive inflammatory pathways or inflammasome activation in dysregulated processes (CAUWELS et al., 2014), leading to the immediate activation of caspase-1 and the inactive precursors' cytokines, which is likely via mitochondrial $Ca^{2+}$ exchange, ROS generation, as demonstrated in our previous study (HARTMANN et al., 2020), and NF-κB activation. Together, these pathways may overstimulate the inflammatory response (ESCAMES, LÓPEZ, GARCÍA, 2012; LÓPEZ-ARMADA et al., 2013).

Furthermore, mitochondria have been recently reported as critical sources of damage-associated molecular patterns (mito-DAMPs); since that they activate the same receptors that detect pathogen-associated molecular patterns (PAMPs) (KRYSKO et al., 2011), such as Toll-like membrane receptors (TLRs) family. The danger signals may interact with members of the TLR family resulting in impairments in mitochondrial function, increases of mitochondrial ROS generation, and overstimulation of the inflammatory pathways (LÓPEZ-ARMADA et al., 2013), perpetuating and exacerbating a vicious cycle of inflammation. In previous studies we have shown that mitochondrial bioenergetics responds differently in the types of muscle fibers to contusion insult. In additions, in view of studies few studies that investigate the role of TLR4 (a well-known pathway by induce inflammatory responses) in the muscle injury, its involvement in inflammation / mitochondrial dysfunction in different types of muscle fibers, and a possible influence of the bioenergetics response on inflammatory markers after muscle

injury in different types of fibers. The present study aimed to determine the significance of mitochondrial dysfunction and its contribution to the inflammatory response on the contusion model in the skeletal muscle of rats in different fibers types.

## Materials and Methods

### Animals

Sixty male adult Wistar rats weighing 250 to 300 g (4 months y.o.) were obtained from the Animal Breeding Unit of the Federal University of Santa Maria (UFSM). Animals were allocated in cages with food and water available ad libitum and maintained under standard conditions of temperature (22 ± 1° C) and illumination (12-h light/dark cycle). The study protocol followed the ethical rules established by the Guide for Care and Use of Experimental Animals published by the National Institutes of Health (NIH publication no. 85-23, revised in 1996). All procedures outlined in this study were approved by the Ethics and Research Committee UFSM 4638020916.

### Contusion injury protocol

The animals were subjected to an experimental contusion injury protocol, anesthetized by isoflurane were injured by dropping a known weight (0.2 kg) from a fixed height (0.3m) onto a rounded impactor bar contact with the medial gastrocnemius muscle. The kinetic energy delivered during each impact was 0.484 J and one impact was performed, while the hindlimb was held with the knee in full extension. This method is considered more similar to human muscle contusion injuries than other blunt trauma models, such as crush injury (SOUZA; GOTTFRIED, 2013a). The specific protocol used here is derived from previous work (MARTINS et al., 2016). Contralateral muscles non-exposed were used as control.

### Tissue collection and preparation of permeabilized fibers

The animals were killed by decapitation 30 min and 2 hours after the contusion. Two types of skeletal muscle from the same hindlimbs were analyzed: gastrocnemius and soleus. Muscle fibers types were determined according to Raja et al. (RAJA et al., 2003) , Martins et al. (MARTINS et al., 2018), and Bloemberg et. al. (BLOEMBERG; QUADRILATERO, 2012), in the white portion of Gastrocnemius fast fibers – glycolytic metabolism (92% type IIb fast-twitch fibers) and the intermediate portion between red and white of Gastrocnemius mixed fibers – glycolytic and oxidative metabolism (50 to 60% of type II fibers, fast contraction and

40 to 50% type I fibers, slow-contraction) and Soleus slow fibers – oxidative metabolism (approximately 84% type I slow-twitch fibers). Extracted and maintained in BIOPS buffer (10 mM EGTA; 5.77 mM ATP; 6.56 mM $MgCl_2$; 20 mM Taurine; 15 mM Phosphocreatine; 20 mM Imidazole; 0.5 mM DTT; 50 mM K-MES; pH 7.1) for analysis of high resolution respirometry and hydrogen peroxide production. The same proportion of fibers were extracted from each respective muscle.

The muscle fibers obtained were transferred to 24-well plates containing 1 mL BIOPS with 50 µg/mL saponin on ice. After 30 min, the fibers were transferred and held for 10 min in another well containing 1 mL of the mitochondrial respiration medium 05 - MIR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-MES, 20 mM Taurine, 10 mM $KH_2PO_4$, 20 mM HEPES, 110 mM Sucrose, BSA fat-free 1 g/L, pH 7.1) for washing the detergent and the completion of the permeabilization process Kuznetsov (2008). The permeabilized fibers were used to perform high-resolution respirometry and the hydrogen peroxide production assay.

**High-resolution respirometry**

For the analysis of oxygen consumption, 2.5 to 3.5 mg of permeabilized fibers were used. The analyses were performed on O2k-system high-resolution oxygraph (Oroboros Instruments, Innsbruck, Austria) with MIR05 at 37 °C. The protocol consisted of sequential titration of multiple substrates, uncouplers and inhibitors (SUIT protocol, adapted from Pesta and Gnaiger (2012): pyruvate (5 mM), malate (2.5 mM) and glutamate (10 mM); succinate (10 mM); adenosine diphosphate (ADP) (1 mM); Cytochrome c (10 µM); oligomycin (2 µg/mL); carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP - titres of 0.25 µM until reaching the maximum oxygen consumption); rotenone (0.5 µM); malonate (5 mM) and potassium cyanide (2 mM KCN). During the measure of mitochondrial respiratory capacity in permeabilized muscle fibers, oxygen levels were maintained above air saturation to avoid intracellular ‘hypoxia’ due to limited oxygen diffusion. Alteration the addition order of substrates of the SUIT protocol above mentioned (addition of ADP after CI +CII substrates), aimed to optimize the inspection of the Oxidative phosphorylation (OXPHOS) modulation. This saturation maneuver of the Q junction in SUIT protocol facilitates the analysis of OXPHOS at its maximum threshold, both for the leak state and for the phosphorylation state. These changes would be less likely in isolated CI or CII analysis where the OXPHOS threshold is lower.

Respirometry nomenclature was defined based on the MitoPedia network from Gnaiger (2014). The oxygen flux relative to complex I and the complexes I+ II was calculated in the non-phosphorylative state (in the presence of pyruvate, malate, and glutamate or in the presence of succinate, besides those substrates previously mentioned, respectively). The ATP synthesis was calculated by oxygen consumption in the presence of ADP + Pi, minus consumption in the presence of oligomycin. The contribution of each complex in the maximal respiratory capacity (ETS) was estimated by the maximum oxygen consumption in the presence of FCCP minus rotenone for the calculation of complex I, and respiration in the presence of rotenone minus malonate for the calculation of complex II.

**Real-time quantitative RT-PCR**

Total RNA was obtained by using a Trizol reagent (Life Technologies, Carlsbad, CA) and quantified by spectrophotometry (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA). Residual genomic DNA was removed by incubating RNA with RQ1 RNase-free DNase (Promega, Madison, WI). The first-standard cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems, Weiterstadt, Germany). The negative control (no transcriptase control) was performed in parallel. First-standard complementary DNA (cDNA) was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems®, Paisley, UK), and then, it was amplified using TaqMan® Universal PCR Master Mix (Applied Biosystems®) on a StepOnePlus™ Real-Time PCR Systems (Applied Biosystems®)(FILIPPIN et al., 2011). TaqMan primers and probes for BAX (GenBank accession no. U32098.1), Caspase 3 (GenBank accession no. RAZU01000234.1), IL-1B (GenBank accession no. NM_031512.2, TNF-a (GenBank accession no. AJ002278.1), TRL-4 (GenBank accession no. D12520.1) and Beta-actin (GenBank accession no. KJ696744.1). Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method as described previously (GARCÍA-MEDIAVILLA et al., 2005). The cycle number at which the transcripts were detectable (CT) was normalized to the cycle number of Beta-actin detection, referred to as ΔCT.

**Western Blot**

Western blot analysis was performed according to Severo et al. (SEVERO et al., 2020) with some modifications. Samples of skeletal muscle were lysed on ice in RIPA (radio-immunoprecipitation assay) and centrifuged for 20 min at 12.700×g and 4 °C. The protein concentration of each sample was determined by the bicinchoninic acid protein assay (Thermo

Fisher Scientific). Samples (40 μg protein) were then subjected to a 4-12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using Trans-Blot® Turbo™ Transfer System and equal protein loading was confirmed by Ponceau S solution (Sigma Aldrich - P7170). After specific blocking, the blots were incubated overnight at 4°C with a correspondent antibody. Antibodies against TLR4 (95 KDa), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-β-actin (42 kDa, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was stained as additional control of the protein loading. After primary antibody incubation, membranes were washed with TBS-T (TBS plus 0.1% Tween 20) two times at room temperature for 10 min and incubated with anti-rabbit (Sigma Aldrich – A6154) or anti-mouse (Santa Cruz Biotechnology – sc-2005) secondary antibodies conjugated with horseradish peroxidase (1:5000), for 2h at room temperature. Bands were visualized by enhanced chemiluminescence using ECL Western Blotting Substrate (Pierce ECL, BioRad) and the signals were captured with photo-documenter (ChemiDoc XRS+ (BioRad). Then the bands were quantified by using Image Lab software (Bio-Rad).

**Statistics**

Kolmogorov-Smirnov and Bartlett's tests were performed for analyzed the normality of data and homogeneity of variances, respectively. The effects on mitochondrial function and qPCR and western blot were analyzed by Two-way ANOVA in the GraphPad Prism program (GraphPad Software, California, USA). Data were expressed as means ± standard error of the mean (SEM), considering $p \leq 0.05$ as significant.

**Results**

**Muscle contusion effects on mitochondrial oxygen flux in different muscle fiber types**

The two-way analysis of variance (ANOVA) of $O_2$ consumption rates coupled to ATP synthesis revealed significant Fibers × Lesion interaction ($F_{2,8} = 11.89$; $P<0.05$). Thirty minutes after contusion, the oxidative fibers showed an increased in $O_2$ consumption rates coupled to ATP synthesis compared to the respective controls and other injury fibers ($p <0.05$; Fig 1A). Nevertheless, respiratory control ratio (RCR) did not change compared to the control and other fibers (Fig. 1B). The rates extracted from the LEAK State by two-way ANOVA showed significant main of effects of Lesion ($F_{2, 10} = 6.505$; $p<0.05$). Oxidative and oxidative/glycolytic had significantly higher oxygen flux in the LEAK state than respective controls ($p<0.05$) and glycolytic fibers ($p<0.05$) (Fig. 2A).

Neither the fiber types nor contusion altered the ETS coupling efficiency in 30 min, as illustrated in Figure 2 (B). The two–way ANOVA of the ROX revealed the significant main effects of Fibers ($F_{2,\ 12}$ = 5.206; p<0.05). The muscle injury increased residual oxygen consumption compared to the control group (p<0.05) and oxidative contusion fibers (p<0.05) (Fig. 2C).

Two hours after the lesion, $O_2$ flux coupled to ATP synthesis did not demonstrate any difference between the tested fibers (Fig 3 A). Surprisingly, two-way ANOVA of the RCR revealed a significant main effect of Fibers ($F_{2,10}$ = 7.764; p<0,05). The post-hoc analysis showed that contusion decreased the RCR of oxidative fibers when compared to the control group (P<0.05), while glycolytic fibers increased compared to the other fibers types (p <0.05; Fig 3B).

The two–way ANOVA of the LEAK state after two hours revealed a significant effect of Fibers ($_{F2,}$10= 7.764; p<0.05). In oxidative fiber, electron leakage continued to increase compared to the control (p<0.05), and other fibers, as observed in the post-hoc analyses (p <0.05; Fig. 4 A). In addition, two–way ANOVA of ETS coupling efficiency showed a significant main effects of Fibers ($F_{2,\ 6}$ = 6.963; p<0.05) and Lesion ($F_{1,\ 13}$ = 11,09; p<0.05). The contusion decreased ETS coupling efficiency in oxidative fibers compared to the control (p <0.05; Fig. 4B).

On the other hand, tow-way ANOVA of ROX after 2 hours indicated a significant main effect of Fibers ($F_{2,10}$ = 4.4004; p<0.05) and Lesion ($F_{1,5}$ = 38.60). All experimental groups had higher Residual oxygen consumption when compared to controls (p <0.05; Fig. 4C).

**Muscle contusion effect on ATP content in different muscle fiber types**

Two–way ANOVA of ATP levels revealed a significant Fibers × Lesion interaction ($F_{2,8}$ = 5.577; P<0.05), Fibers ($F_{1,4}$ = 17.86; p<0.05) and Lesion ($F_{2,8}$ = 7.349; p<0.05) 30 minutes after contusion. Mixed fibers increased the ATP level in comparison to oxidative fibers (p<0.05). And glycolytic injury fibers increased the ATP levels when compared to those of the others injury fibers (p<0.05) and control (p<0.05) (Fig. 5A).

After two hours, two–way ANOVA showed a significant Fibers × Lesion interaction ($F_{2,6}$ = 9.144; P<0.05), Lesion ($F_{1,3}$ = 0.4788; p<0,05). The ATP content continued to increase in glycolytic fibers (p <0.05; Fig 5A), while glycolytic/oxidative fibers had better ATP levels than the control (p <0.05) and oxidative fibers (p<0.05) (Fig. 5B).

**mRNA expression and protein content of inflammation-related genes after muscle-skeletal contusion in different muscle fiber types.**

To determine the effects of a single contusion on mRNA and protein expression of different inflammation-related genes we quantified qPCR genes involved in mitochondrial dysfunction marker (BAX), apoptosis (caspase 3), pro-inflammatory cytokines (TNF- α and IL-1β.) and membrane receptors involved in pro-inflammatory response (TLR 4). Two–way ANOVA of TNF-α revealed a significant Lesion ($F_{1, 8}$ = 42,39; p<0.05). Thirty minutes after the contusion, all experimental groups had higher mRNA levels (P<0.05) (Fig. 6A). IL-1β showed a significant Fibers × Lesion interaction ($F_{2,16}$ = 4.08; p<0.05) and Lesion ($F_{1,8}$ = 30.31; p<0,05). Transcriptional upregulation of IL-1β in oxidative (p<0.05) and glycolytic/oxidative fibers (p<0.05) was observed after the muscle injury compared to those of the controls group, as per post-hoc analysis (Fig. 6B). Moreover, glycolytic fibers demonstrated significate difference in compared to others lesion fibers after the muscle damage (p<0.05)

Two–way ANOVA of BAX levels showed a significant Fibers × Lesion interaction ($F_{2,20}$ = 4.644; p<0.05), Fibers ($F_{2,20}$ = 9.797; p<0.05) and Lesion ($F_{1,10}$ = 0,013; p<0,05). Moreover, post–hoc analysis demonstrated a transcriptional upregulation of BAX in oxidative (p<0.05) and glycolytic/oxidative fibers (p<0.05) after muscle injury compared to the controls group (Fig. 7A).

Moreover, two–way ANOVA Caspase 3 (apoptosis signaling) indicated a substantial Fibers × Lesion interaction ($F_{2,20}$ = 3.938; p<0.05), Fibers ($F_{2,20}$ = 9.630; p<0.05) and Lesion ($F_{1,10}$ = 13.62; p<0,05). Similarly, there wastranscriptional upregulation of BAX in oxidative (p<0.05) and glycolytic/oxidative fibers (p<0.05) after the muscle injury when compared to the controls group, as determined by post-hoc analysis (Fig. 7B).

Two–way ANOVA analysis of TLR4 revealed a significant Fibers ($F_{2, 8}$ = 106.8; p<0.05) and Lesion ($F_{1,4}$ = 14.01; p<0,05) vallues. All experimental groups had higher mRNA levels (P<0.05), although the post–hoc analysis demonstrated a notable difference compared to glycolytic fibers after the muscle damage (P<0.05) (Fig. 6C).

When measured 2 hours after injury, two-way ANOVA of BAX underlined considerable effects of Fibers ($F_{2,12}$ = 12.28; p<0.05) and Lesion ($F_{1,4}$ = 16.13; p<0,05). The post–hoc analysis revealed increased BAX transcription in oxidative and glycolytic/oxidative fibers after the muscle injury compared to the controls group and glycolytic fibers (Fig. 8A).

Two-way ANOVA of Caspase 3 indicated a significant effect of Lesion ($F_{1,6}$ = 7.163; p<0,05). Nonetheless, none of the excremental groups altered mRNA Caspase 3 in the muscle

(Fig. 8B). Furthermore, two–way ANOVA of TLR4 mRNA demonstrated a significant main effect of Lesion ($F_{1,8}$ = 33.42; p<0,05), while TLR4 in oxidative and glycolytic fibers continued to increase compared to controls and glycolytic/oxidative fibers (P<0.05) (Fig. 8C).

In two hours, two-way ANOVA of TLR4 protein revealed a high Lesion ($F_{1,4}$ = 35.63; p<0.05). The post–hoc analysis demonstrated increased TLR4 protein in oxidative (p<0.05) and glycolytic/oxidative fibers (p<0.05) after the muscle injury compared to the controls group (Fig. 8A).

**Discussion**

The present study determined the significance of mitochondrial dysfunction and its contribution to triggering the inflammatory response in skeletal muscle after the contusion model. Our finding showed a specific modulation of key inflammatory genes expression according to fibers types possible due to a decreased activation of nuclear transcription factors. Corroborating the hypothesis that mitochondrial dysfunction is crucial to developing the inflammatory phenotype, we observed an increased in Leak state in 30 min (oxidative and Oxidative/Glycolytic fibers), causing all the fibers to an increase in ROX and reduce ETS coupling efficiency 2 hours after the contusion. Moreover, this evidence further validates the premise that mitochondrial bioenergetics dysfunction greatly affects the inflammatory response (TRL4-MyD88-NF-κB pathway) in the contusion model of rat skeletal muscle of different fiber types.

Mitochondria play an important role in pro-inflammatory signaling, and pro-inflammatory mediators may also alter mitochondrial function; these processes can increase mitochondrial oxidative stress, promoting a vicious inflammatory cycle (LÓPEZ-ARMADA et al., 2013). Excess ROS affects the mitochondrial integrity and causes a decline in the respiratory chain compromising the mitochondrial efficiency (ETS coupling efficiency) and decreasing de oxidative phosphorylation (OXPHOS) (LÓPEZ-ARMADA et al., 2013).

In this sense, changes in mitochondria functionality (increase in LEAK state in oxidative fibers) were mainly observed 30 minutes after injury in a dependent fiber type manner, which is in accordance with the mitochondrial $H_2O_2$ generation observed in our previous study (HARTMANN et al., 2020). Mitochondrial ROS has a crucial role in cellular adaptations to stress conditions by regulation of oxidative metabolism, supporting cell survival (MAKRECKA-KUKA; KRUMSCHNABEL; GNAIGER, 2015). The diverse characteristics of muscle fibers, such as high oxidative capacity and mitochondrial density of the slow-twitch

fibers (oxidative metabolism), may have increased in peroxide production in oxidative and mixed fibers compared to the control groups (HARTMANN et al., 2020).

The $O_2$ consumption coupled to ATP synthase was measured after 30 min or 2 hours after a contusion injury to determine the extension of different mitochondrial dysfunction in different skeletal muscle fibers. A change in oxygen flux coupled to ATP synthase of slow-twitch fibers (oxidative metabolism) was observed 30 minutes after contusion (Fig 1A). When considered independently, this result suggests that mitochondria attempt to maintain ATP levels, although this did not occur, as demonstrated by RCR (Fig 1B), and ATP analysis (Fig 4A). No changes were observed in mitochondrial respiration coupled to ATP syntheses in glycolytic/oxidative and glycolytic fibers, according to RCR (Fig 1A and 1B, respectively). However, ATP content increased after 30 min and 2 hours in glycolytic fibers, and at 2 hours in mixed fibers, implying that they are exchanging glucose metabolites from mitochondrial respiration to cytosolic glycolysis. This metabolic exchange leads to increased lactate levels that are similar to observed in the Warburg effect (LU; FORBES; VERMA, 2002).

Skeletal muscle injuries, especially involving contusion, are known to trigger several inflammatory-related events (HOWARD et al., 2020; SMITH et al., 2008). Upregulation of pro-inflammatory cytokine production is expected in the acute phase and, consequently, increased inflammatory cells mobilization and migration to the muscle tissue (HOWARD et al., 2020; YANG; HU, 2018). Mitochondrial dysfunction is present in cellular damage during tissue injuries (BRAND, 2016; MARTINS et al., 2018; MILANE et al., 2015), and is often associated with the release of mitochondria-derived components (TESTRO; VISVANATHAN, 2009). Hence, endogenous TLR4 ligands will be released as components of dysfunctional mitochondria and will likely be recognized by TLR4 in skeletal muscle (ZBINDEN-FONCEA et al., 2012). In this regard, mitochondria are sources of DAMPs, and these molecules activate the Toll-like membrane receptors (TLRs) and other receptors that detect pathogen-associated molecular patterns (PAMPs) (KRYSKO et al., 2011), thus underlining the role of mitochondria play a role in DAMP-modulated inflammation (AKHTER et al., 2019).

Increased oxygen consumption by the electron transport chain showen by LEAK state and ROX may favor the escape of electrons, resulting in excessive mitochondrial production of superoxide ($O_2$•-) and hydrogen peroxide ($H_2O_2$) (CHANDEL et al., 2000), promoting inflammasome and NF-κB activation (LÓPEZ-ARMADA et al., 2013). The inflammatory stimulus also can activate TLRs and their signaling mRNA/protein expression of proinflammatory mediators by activating inflammatory cascade (AKHTER et al., 2019).

TLR 4 are inflammation receptors that induce pro-inflammation mediators such as TNF-α, IL-6, and IL-1β (BARCELOS et al., 2016; SMITH et al., 2015). Therefore, our experiments clearly demonstrated that dysfunctional mitochondria may be strongly linked to TLR4 expressed by skeletal muscle fibers (Fig. 7C and 8C), as confirmed by an increased by LEAK state-linked an upregulation of mRNA BAX (fig. 7A and 8A) and caspase 3 (Fig. 7B and 8B). Mitochondrial oxidative stress triggers the opening of voltage-dependent anion channels (VDACs), which is essential for inflammasome activation, and the BAX pathway (apoptosis induction) (YAMAGATA et al., 2009). In addition, contusion increases the expression of the pro-inflammatory cytokines IL-1β and TNF-α in 30 minutes after the muscle injury (Figs. 6A and B).

Moreover, TLR4 mediates the initial inflammatory response through MyD88(BARCELOS et al., 2016; JIANG et al., 2010), which is crucial to stimulate proinflammatory cytokine production (KAWAI; AKIRA, 2006). Upon stimulation, TLR4 recruits IL-1 receptor type 1-associated protein kinases via adaptors, such as MyD88(BARCELOS et al., 2016), leading to modifications in IκB kinase (IKK) proteins, following in recruitment of p50 in cytoplasm and translocation of p65 to the nucleus (KAWAI; AKIRA, 2006). Consequently, TNF-α, IL-1β, and other cytokines are overexpressed, increasing tissue inflammation. Cytokines were also elevated after contusion injury in oxidative and glycolytic/oxidative, and fibers with predominantly glycolytic metabolism showed lower levels in IL-1B and TNF-α (Fig 6 A and B). Despite appearing to be a simple effect, these consequences must be studied given the vital role that cytokines play in skeletal muscle inflammatory modulation and regeneration (MARKWORTH et al., 2014).

In conclusion, the evidence presented herein suggests that the mitochondrial bioenergetic dysfunction may interferes in the TRL4-MyD88-NF-κB pathway activation, thus increasing cytokine production in oxidative and glycolytic/oxidative fibers. The current study identifies a novel mechanism mediating injury and mitochondrial dysfunction in the muscle contusion model in rats. Hence, we suggest that TLR4 upregulation may be an additional target for ROS-suppressive/antioxidant strategies to mediate inflammation.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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**Figures**

**Figure 1.** Effects of muscle contusion after 30 minutes on mitochondrial bioenergetics function. $O_2$ consumption rates coupled to ATP synthesis (A); and (B) Respiratory control ratio (RCR) in different fibers types after 30 min skeletal muscle contusion. Soleus, Oxidative fibers (Slow-twitch); Gastrocnemius, Oxidative/Glycolytic fibers (Fast-twitch); Gastrocnemius, Glycolytic fibers (Fast-twitch). The flux of oxygen normalized by mass in milligrams [(pmol/(smg)]. Black (Ctrl); White (Lesion). Different letters indicate significant differences between groups (mean ± SEM for n = 6-8 in each group, two-way test (ANOVA) followed by Tukey's test, $p < 0.05$).

**Figure 2**. Contusion-induced muscle injury changes in LEAK state after 30 min. (A) LEAK State, (B) ETS coupling efficiency, and (C) ROX. Soleus, Oxidative fibers (Slow-twitch); Gastrocnemius, Oxidative/Glycolytic fibers (Fast-twitch); Gastrocnemius, Glycolytic fibers (Fast-twitch). The flux of oxygen normalized by mass in milligrams [(pmol/(smg)]. Different letters indicate significant differences between groups (mean ± SEM for n = 6-8 in each group, two-way test (ANOVA) followed by Tukey's test, $p < 0.05$).

**Figure 3.** Effects of muscle contusion after 2 hours on mitochondrial bioenergetics function. **(**A)$O_2$ consumption rates coupled to ATP synthesis; and (B) Respiratory control ratio (RCR) in different fibers types after 2 hours skeletal muscle contusion. Soleus, Oxidative fibers (Slow-twitch); Gastrocnemius, Oxidative/Glycolytic fibers (Fast-twitch); Gastrocnemius, Glycolytic fibers (Fast-twitch). The flux of oxygen normalized by mass in milligrams [(pmol/(smg)]. Black (Ctrl); White (Lesion). Different letters indicate significant differences between groups (mean ± SEM for n = 6-8 in each group, two-way test (ANOVA) followed by Tukey's test, $p < 0.05$).

**Figure 4.** Contusion-induced muscle injury changes in LEAK state after 2 hours. (A) LEAK State, (B) ETS coupling efficiency, and (C) ROX. Soleus, Oxidative fibers (Slow-twitch); Gastrocnemius, Oxidative/Glycolytic fibers (Fast-twitch); Gastrocnemius, Glycolytic fibers (Fast-twitch). The flux of oxygen normalized by mass in milligrams [(pmol/(smg)]. Different letters indicate significant differences between groups (mean ± SEM for n = 6-8 in each group, two-way test (ANOVA) followed by Tukey's test, $p < 0.05$).

**Figure 5.** Effects of muscle contusion on ATP levels in skeletal muscle fibers after 30 minutes contusion (A), or after 2 hours (B). Data are presented as the mean± SEM, for seven animals

per group. Different letters indicate significant differences between groups (mean ± SD for n = 4–5 in each group, two-way test (ANOVA) followed by Tukey's test, $p < 0.05$)

**Figure 6.** Effects of single muscle contusion of mRNA expression for TNF-a (A) and Il-1B (B) after 30 minutes. Levels of mRNA were analyzed by quantitative q-PCR assays and were normalized to CypA. Different letters indicate significant differences between groups (mean ± SEM for n = 5–6 in each group, two-way test (ANOVA) followed by Tukey's test, $p < 0.05$)

**Figure 7.** Effects of single muscle contusion of mRNA expression for BAX (A), Caspase 3(B), and TLR 4 (C) in rat muscle after 30 minutes. Levels of mRNA were analyzed by quantitative q-PCR assays and were normalized to CypA. Different letters indicate significant differences between groups (mean ± SEM for n = 6 in each group, two-way test (ANOVA) followed by Tukey's test, $p < 0.05$)

**Figure 8.** Effects of single muscle contusion of mRNA expression for BAX (A), Caspase 3(B), and TLR 4 (D) in rat muscle after 2 hours. Levels of mRNA were analyzed by quantitative q-PCR assays and were normalized to CypA. Different letters indicate significant differences between groups (mean ± SEM for n = 6 in each group, two-way test (ANOVA) followed by Tukey's test, $p < 0.05$).

**Figure 9.** Single muscle contusion increase TLR4 protein after 2 hours, in rat muscle. Different letters indicate significant differences between groups (mean ± SEM for n = 4 in each group, two-way test (ANOVA) followed by Tukey's test, $p < 0.05$).

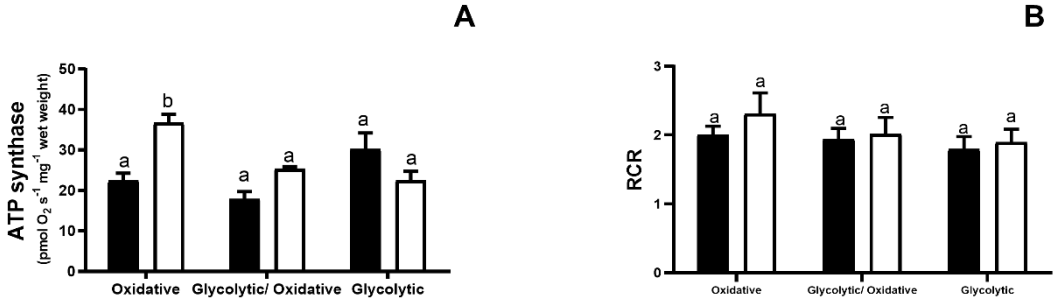
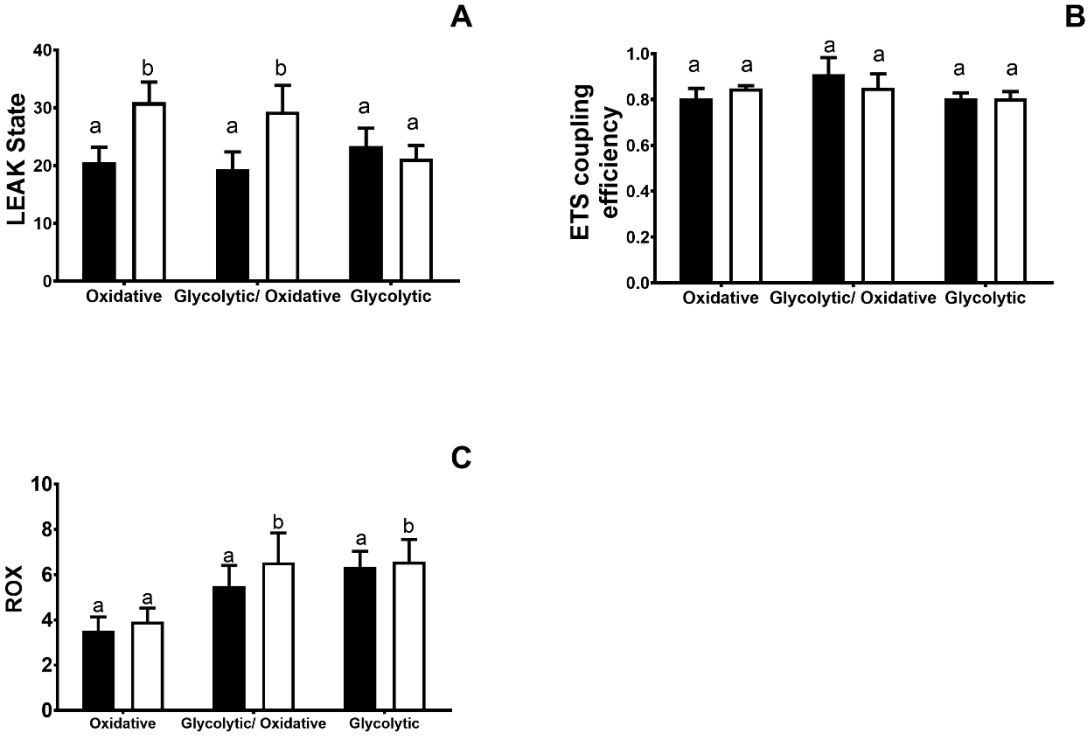



**Figure 1.**

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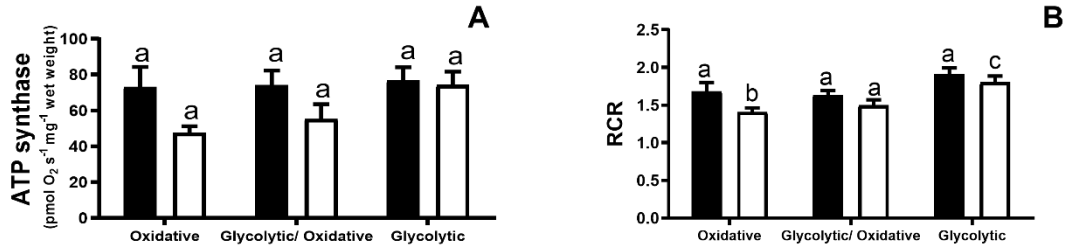


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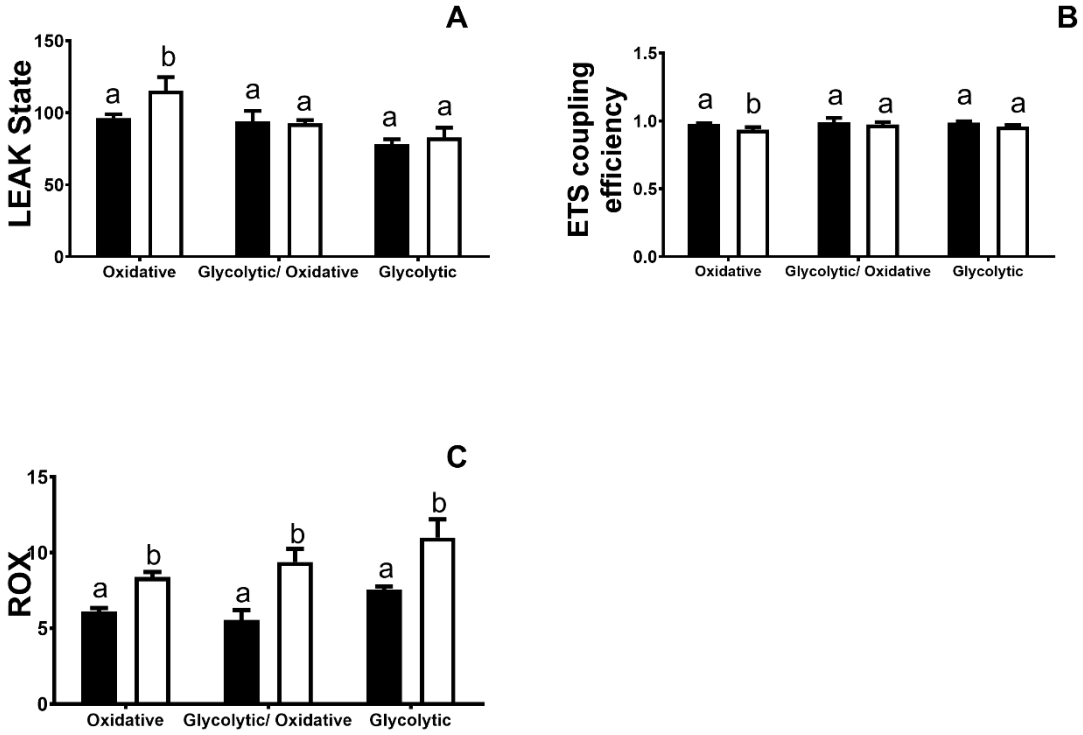


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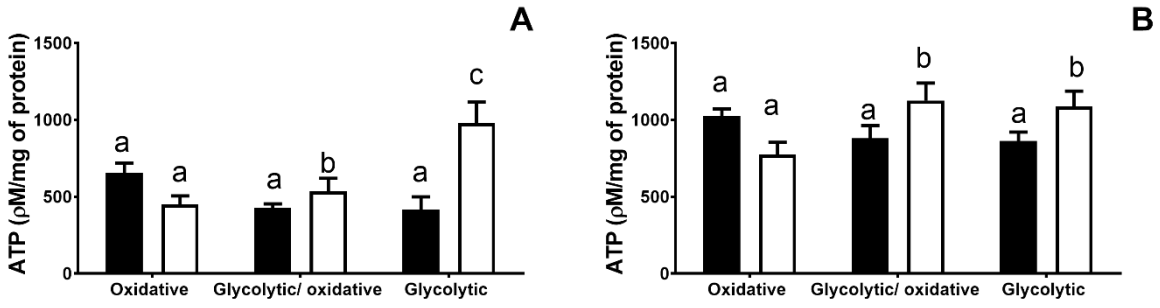


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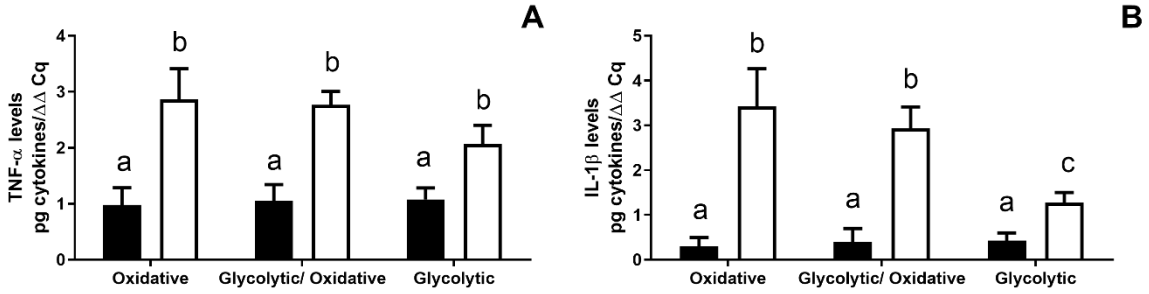


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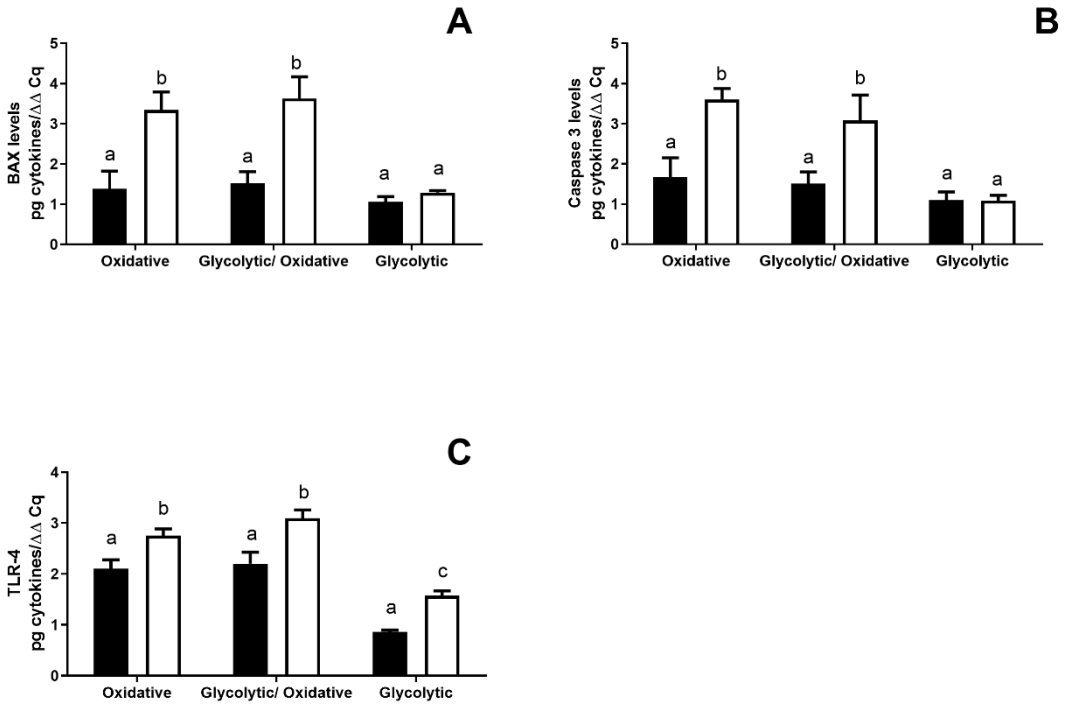


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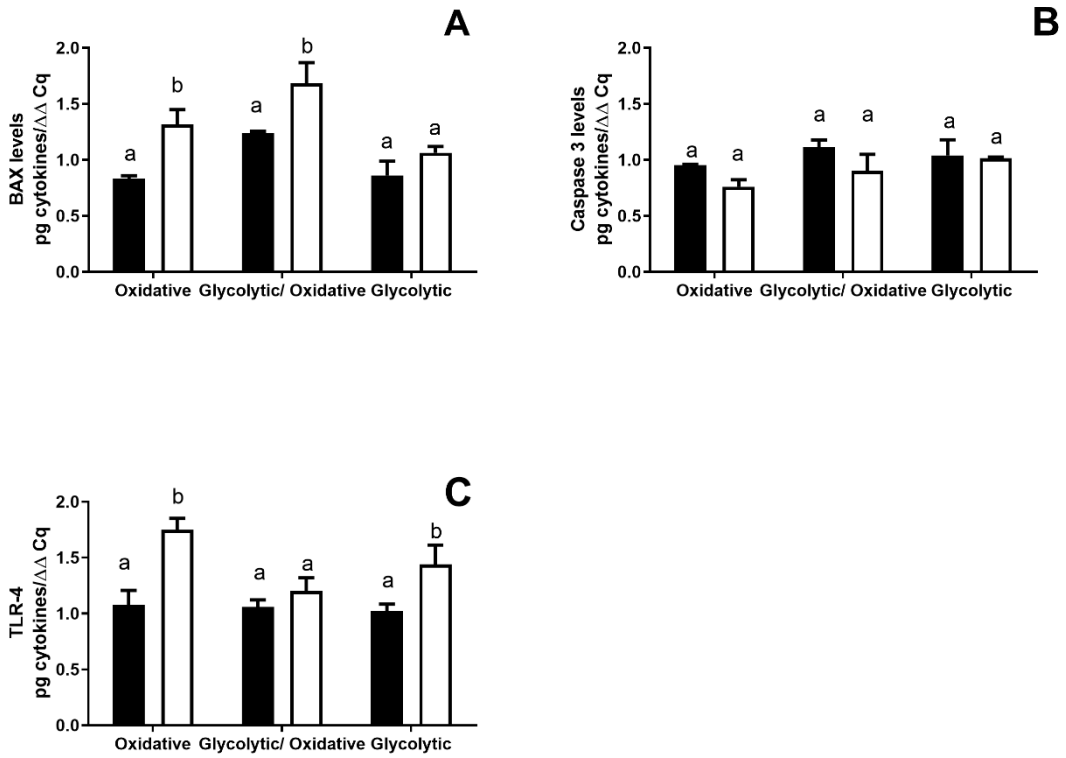


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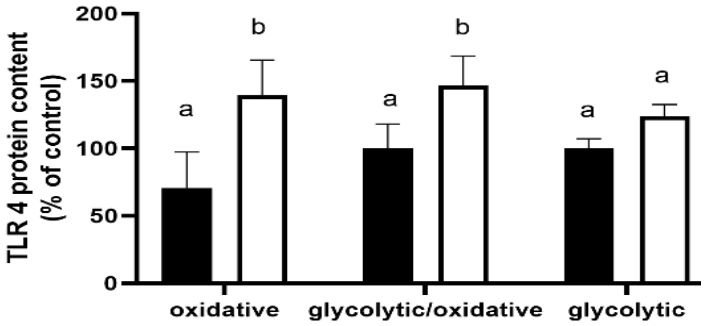


**Figure 9.**

## 4. DISCUSSÃO

Apesar de as alterações de estresse / dano oxidativo e da resposta inflamatória decorrente de lesões musculares por contusão serem conhecidas, as respostas especificas dos diferentes tipos de fibras musculares são pouco abordadas na literatura. O questionamento inicial norteador dessa tese foi investigar as possíveis mudanças do perfil bioenergético dos diferentes tipos de fibras musculares em resposta ao modelo de contusão muscular (Artigo 1).

Pouco ainda se sabe sobre a relação entre a alteração da bioenergética e funcionalidade mitocondrial desencadeadas nos diferentes tipos de fibra muscular pela lesão como a do tipo contusão, e como essa disfunção mitocondrial irá repercutir na resposta inflamatória imediata desenvolvidas no musculo. Assim, o objetivo deste estudo foi elucidar os possíveis mecanismos mitocondriais após uma lesão muscular na forma de contusão nas fibras de metabolismo oxidativo, misto e glicolítico (Artigo 1), e as vias de sinalização de resposta inflamatória e morte celular relacionadas a disfunção mitocôndria e o estresse oxidativo imediatamente após a lesão musculoesquelética nos diferentes tipos de fibras musculares (Manuscrito 1).

A partir dos dados do estudo, constatamos que a contusão apresentou um efeito imediato promovendo alterações no metabolismo mitocondrial, demonstrando uma mudança na contribuição de CI e CII nas fibras de metabolismo oxidativo em relação à síntese de ATP. Esses dados demonstram que a contusão causou uma mudança significativa, a contribuição de CII foi mais expressiva do que CI na respiração mitocondrial dessas fibras quando exposto a lesões. Já as as fibras rápidas / lentas (metabolismo misto), a lesão muscular promoveu a exacerbação da respiração mitocondrial na presença de substratos específicos (ADP), aumentando os parâmetros, como OXPHOS e ATP sintase. Supreendentemente, em fibras de metabolismo rápido o consumo de oxigênio sustentado pelos substratos do CI + CII diminuiu no estado não fosforilado, no entanto, esta diminuição não afetou o consumo de $O_2$ acoplado a síntese de ATP.

Concomitantemente, a contusão altera a homeostase do $Ca^{2+}$, aumentando da resistência da abertura do mPTP, através do aumento no tamponamento de $Ca^{2+}$, além do aumento dos níveis de $H_2O_2$ que ocorrem de forma diferente nos tipos de fibras testado. Demonstrando a importância de analisar as fibras musculares separadamente, especialmente porque a população mitocondrial em diferentes fibras não é homogênea e essa aparente divergência entre os resultados pode ser dependente de diferenças entre subtipos de metabolismo, bem como influenciada por diferentes capacidades de armazenamento e captação de $Ca^{2+}$ em cada fibra.

Sabe-se que as mitocôndrias afetam o metabolismo do $Ca^{2+}$ citoplasmático de maneira

indireta, via ATP, o qual é utilizado por ATPases dependentes de $Ca^{2+}$ para bombear o $Ca^{2+}$ para fora da célula; ou diretamente, através do ΔΨm que impulsiona a absorção do $Ca^{2+}$ liberado pelo retículo sarcoplasmatico pode ser absorvido pela mitocôndria, através do canal uniporter de $Ca^{2+}$ (MNATSAKANYAN et al., 2017). Os principais mecanismos de influxo de $Ca^{2+}$ incluem, o '*mitochondrial* $Ca^{2+}$ *uniporter*' (MCU), modo rápido de captação de $Ca^{2+}$ (RaM) e receptor de rianodina (mRyR) (MAMMUCARI et al., 2016). Esses mecanismos de regulação da captação de $Ca^{2+}$ e abertura aguda de mPTP parece ter um papel fisiológico crítico na lesão muscular imediata. O moderado desacoplamento mitocondrial e a abertura de mPTP induzem proteção durante episódios de infarto cardíaco, essa proteção pode ser suprimida pela inibição da abertura de mPTP (HAUSENLOY et al., 2004). Nossos dados, entretanto, mostram que com uma lesão muscular em fibras de contração lenta (metabolismo oxidativo), o aumento da resistência ao $Ca^{2+}$ e a abertura do mPTP podem contribuir para a sobrecarga da matriz mitocondrial, levando à disfunção mitocondrial, aumento da geração $H_2O_2$ e morte celular (LI et al., 2018).Portanto, demonstrando que o aumento da captação e/ou do volume da matriz mitocondrial contribuem para o estresse, consequentemente o aumento dos níveis de EROs (WANG et al., 2008). O que parece favorecer no modelo de contusão muscular a modulação do metabolismo mitocondrial, apresentando um efeito que sugere uma maior contribuição do CII em relação ao CI na respiração mitocondrial das fibras de metabolismo oxidativo quando expostas a lesões. Já as fibras de metabolismo fibras rápidas / lentas (metabolismo glicolítico e oxidativo), a lesão muscular promove um aumento nos parâmetros, como OXPHOS.

As mitocôndrias são organelas que fornecem energia celular na forma de ATP, que é fornecida em equilíbrio com as demandas de energia colocadas pela célula (BHATTI; BHATTI; REDDY, 2017). Mudanças ambientais no meio celular repercutem na rede mitocondrial, que atua através da regulação de outras funções importantes, como iniciar uma sinalização mitocondrial-nuclear, manter a homeostase redox e de $Ca^{2+}$, bem como determinar o destino da célula (HOOD et al., 2019). A rede mitocondrial existe em um estado de fluxo continuo, para assim permitir a sua influência complexa e multifacetada nas funções celulares e assim combinar a morfologia e a função das mitocôndrias com essas demandas existentes (EISNER; PICARD; HAJNÓCZKY, 2018). Nossos resultados indicam que o modelo de contusão pode perturbar a bioenergética e homeostase mitocondrial, bem como ser acompanhada pelo excessivo aumento da produção de $H_2O_2$.

O estresse oxidativo e a disfunção mitocondrial tem sido correlacionada a uma grande variedade de doenças degenerativas e metabólicas, câncer e envelhecimento, uma vez que todas

essas manifestações clínicas resultam do papel central da bioenergética na biologia celular (BHATTI; BHATTI; REDDY, 2017; JANIKIEWICZ et al., 2018; VALERO, 2015). No entanto, pouco se sabe sobre o seu papel nas disfunções e patologias musculares até o presente momento. Estudos prévios do grupo de pesquisa já demonstraram o aumento do dano oxidativo e prejuízo no $\Delta\Psi m$ das mitocôndrias do tecido muscular nos momentos iniciais após uma contusão (CARVALHO et al., 2010; PUNTEL et al., 2011).

Além do mais, as possíveis alterações estruturais e morfológicas desencadeadas pela lesão muscular impactam de forma diferente na funcionalidade de importantes enzimas nos diferentes tipos de fibra muscular, com a redução significativa na atividade da enzima CS e o aumento da atividade da LDH demonstrada nas fibras de metabolismo oxidativas e glicolítico / oxidativo. Essa mudança na atividade enzimática pode ser associada a condições de alteração metabólica em direção à glicólise anaeróbica nas fibras oxidativas e mistas, o que leva um ambiente metabólico com o fluxo de lactato e oxigênio para sustentar a atividade celular e iniciar a recuperação do tecido (GONÇALVES et al., 2019).

A contusão aumentou a fosforilação oxidativa (OXPHOS) e a produção de ATP relacionada ao consumo de oxigênio, nas fibras de metabolismo oxidativo e glicolítico / oxidativo na comparação com o respectivo grupo controle. Entretanto, quando avaliado na comparação entre os tipos de fibra muscular os níveis de ATP (Manuscrito 1) nas fibras oxidativas não demonstraram aumento significativo. Já as fibras de metabolismo glicolítico / oxidativo e de metabolismo apenas glicolítico apresentaram um aumento em 30 minutos e duas horas após a contusão. Assim, especulamos que este resultado esteja relacionado a uma resposta compensatória à lesão muscular, uma vez que as fibras de metabolismo oxidativo apresentaram um aumento no escape de elétrons (LEAK state) em comparação as outras fibras, e na geração de $H_2O_2$ (Artigo 1)

O $H_2O_2$ mitocondrial apresenta um importante papel fisiológico promovendo adaptações celulares às condições de estresse pela regulação do metabolismo oxidativo, apoiando a sobrevivência celular (BRAND, 2016; CHANDEL et al., 2000). As diversas características das fibras musculares, como alta capacidade oxidativa e densidade mitocondrial da fibra de contração lenta (metabolismo oxidativo) (LUNDBERG et al., 2002), estão relacionadas ao aumento da produção de peróxido nas fibras oxidativas e mistas quando comparadas aos grupos controle (Artigo 1).

A partir desses resultados buscamos compreender, como a disfunção mitocondrial e a produção excessiva de $H_2O_2$ poderia atuar junto na resposta inflamatória já estabelecida na

literatura no modelo de contusão, tais como IL-1β, IL-6 e TNF-α (SMITH et al., 2008). Neste contexto, trabalhos mostram que a exacerbação do estresse oxidativo, principalmente através de $H_2O_2$ tem um papel importante na indução de fator de transcrição de resposta inflamatória através do receptores TLR4 em células mononucleares no sangue periférico (PBMC) (FERNANDEZ-GONZALO et al., 2014). Corroborando com esses dados, em nosso estudo envolvendo a resposta ao longo de duas horas da contusão nos diferentes tipos de fibras musculares (Manuscrito 1) vimos um aumento da rota inflamatória através do receptor TLR4, passando por um aumento do escape de elétrons (LEAK state e ROX), por um aumento da expressão gênica de citocinas pró-inflamatórias e marcadores importantes de disfunção mitocondrial (BAX e Caspase 3), culminando no aumento da expressão gênica e síntese do proteica de TLR4. Apesar de muitos estudos terem demonstrado a ativação dessa rota em PBMC e em outras células, na lesão muscular pouco se sabe sobre essa rota após a contusão muscular.

Um aumento no consumo de oxigênio pela cadeia de transporte de elétrons mostrado pelo estado LEAK e ROX pode favorecer o escape de elétrons, levando ao excesso de produção mitocondrial de superóxido ($O2^{\bullet-}$) e peróxido de hidrogênio ($H_2O_2$) (CHANDEL et al., 2000; LI et al., 2018; WEINBERG et al., 2010)(CHANDEL et al., 2000), ativação do inflamassoma e da via de NF-κB (LÓPEZ-ARMADA et al., 2013). O estímulo inflamatório também pode estimular receptores TLRs e sua sinalização pró-inflamatórios, com a expressão de mRNA / proteína de mediadores (AKHTER et al., 2019), por meio da ativação da cascata pró-inflamatória.

A ativação do TRL4 dispara uma cascata de sinalização intracelular que leva a ativação do NF-kB e à produção de citocinas pró-inflamatórias, TNF-α, IL-6 e IL-1β (BARCELOS et al., 2016; SMITH et al., 2015). Nossos experimentos demonstraram claramente que as mitocôndrias disfuncionais podem estar fortemente ligadas ao TLR4 expresso pelas fibras musculares esqueléticas, confirmado por um aumento no escape de elétrons ligado a uma suprarregulação de mRNA BAX e Caspase 3. O estresse oxidativo mitocondrial pode desencadeiar a abertura dos canais voltagem-dependentes (VDACs) presentes na membrana mitocondrial, que tem importante papel na ativação do inflamassoma, e também a via BAX (indução de apoptose) (YAMAGATA et al., 2009). Além disso, a exposição à contusão causa aumento na expressão das citocinas pró-inflamatórias IL-1β e TNF-α em 30 minutos após a lesão muscular. Sugerindo uma possível ação regulatória a nível celular da mitocondrial, impulsionada principalmente pelos efeitos da disfunção da bioenergética mitocondrial através

dos complexos I e II, e escape de elétrons pela cadeia respiratória, levando a maior geração de $H_2O_2$.

# 5. CONCLUSÕES

## 5.1. CONCLUSÃO GERAL

Por fim, demonstramos respostas mitocondriais distintas entre as diferentes fibras musculares, indicando que a dinâmica mitocondrial está relacionada às flexibilidades no metabolismo e que as espécies reativas de oxigênio afetam diretamente a fisiologia e a função normal. A disfunção da bioenergética mitocondrial possivelmente interfere na ativação da via TRL4-MyD88-NF-κB, consequentemente aumentando a produção de citocinas nas fibras oxidativas e glicolíticas / oxidativas. Portanto, o presente estudo identifica um novo mecanismo mediado pela lesão musculoesquelética e disfunção mitocondrial em modelo de contusão muscular em ratos. Sugerimos que a regulação positiva de TLR4 pode representar um alvo adicional para estratégias de supressão / antioxidante de ROS para mediar a inflamação.

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