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EFEITOS DA CRIOTERAPIA EM MODELOS DE CONTUSÃO E ISQUEMIA/REPERFUSÃO SANGUÍNEA EM MÚSCULO DE RATOS

TESE DE DOUTORADO

Gustavo Orione Puntel

Santa Maria, RS, Brasil
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EFEITOS DA CRIOTERAPIA EM MODELOS DE CONTUSÃO E ISQUEMIA/REPERFUSÃO SANGUÍNEA EM MÚSCULO DE RATOS

Gustavo Orione Puntel

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
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Orientador: Félix Alexandre Antunes Soares

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Universidade Federal de Santa Maria
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A comissão Examinadora, abaixo assinada,
Aprova a Tese de Doutorado

EFEITOS DA CRIOTERAPIA EM MODELOS DE CONTUSÃO E ISQUEMIA/REPERFUSÃO SANGUÍNEA EM MÚSCULO DE RATOS

elaborada por
Gustavo Orione Puntel

como requisito parcial para obtenção do grau de
Doutor em Ciências Biológicas: Bioquímica toxicológica

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
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EFEITOS DA CRIOTERAPIA EM MODELOS DE CONTUSÃO E ISQUEMIA/REPERFUSÃO SANGUÍNEA EM MÚSCULO DE RATOS
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As lesões musculoesqueléticas estão entre as maiores causas de lesões observadas em indivíduos nas áreas de primeiros socorros, na saúde ocupacional e na medicina do esporte. Dentre estas, a contusão é descrita como uma lesão traumática direta que compromete o funcionamento do sistema musculoesquelético. Um evento agudo de isquemia e reperfusão (I/R), por sua vez, pode ser considerado como um dos fatores fundamentais envolvidos na fisiopatologia de uma lesão musculoesquelética. Dentre as principais estratégias empregadas no tratamento de uma lesão está a redução na temperatura dos tecidos com o objetivo terapêutico, mecanismo este definido como crioterapia. Apesar da eficácia clínica os mecanismos pelos quais a crioterapia exerce os seus efeitos terapêuticos são pouco elucidados. O objetivo deste estudo foi analisar os efeitos da crioterapia no tratamento de uma contusão e de um evento agudo de I/R sanguínea no músculo gastrocnêmio de ratos. Desta forma, investigamos os efeitos da crioterapia sobre as alterações bioquímicas e morfológicas relacionadas a uma contusão (**Artigo 1**) e a um evento agudo de I/R (**Manuscrito 1**), bem como os mecanismos envolvidos na origem de seus efeitos terapêuticos. O tratamento com a crioterapia determinou uma redução significativa no dano oxidativo ao limitar a peroxidação lipídica e a formação de espécies reativas de oxigênio (EROs), e também por limitar a perda da viabilidade celular no tecido muscular lesado após uma contusão (**Artigo 1**) e após um evento agudo de I/R (**Manuscrito 1**). Neste contexto, os níveis de antioxidantes não-enzimáticos, tais como os níveis de tióis não-protéicos (-SH), e enzimáticos, tais como a enzima catalase (CAT), também foram mantidos semelhantes aos observados em músculos não lesados. O tratamento com a crioterapia foi efetivo em manter as atividades de enzima sensíveis ao estresse oxidativo, tais como a lactato desidrogenase (LDH) e as enzimas sódio/potássio (Na^+/K^+) e cálcio (Ca^{2+}) ATPases, semelhantes as observadas nos tecidos não lesados tanto após uma contusão (**Artigo 1**), quanto após um evento agudo de I/R (**Manuscrito 1**). De acordo com as análises histopatológicas o tratamento com a crioterapia reduziu as alterações na estrutura morfológica e também a presença de células sanguíneas indicativas de processo hemorrágico ou inflamatório do tecido muscular lesado tanto após uma contusão (**Artigo 1**), quanto após um evento agudo de I/R (**Manuscrito 1**). Em geral os resultados observados neste estudo indicam que um importante mecanismo pelo qual a crioterapia exerce os seus efeitos terapêuticos está relacionado à redução na intensidade da resposta inflamatória no local da lesão. Este resultado foi indicado pela limitada quantidade de células inflamatórias observada nas análises histopatológicas e corroborado pela reduzida atividade da enzima mieloperoxidase (MPO) no tecido muscular lesado e submetido ao tratamento com a crioterapia. Além de reduzir a intensidade da resposta inflamatória, a crioterapia limitou as alterações mitocondriais no tecido muscular lesado ao diminuir a formação de espécies reativas e ao manter a funcionalidade da membrana mitocondrial tanto

após uma contusão (**Artigo 1**), quanto após um evento agudo de I/R (**Manuscrito 1**). Este resultado foi indicado pelo reduzido inchaço e pelo limitado comprometimento no potencial de membrana mitocondrial ($\Delta\psi$), além da manutenção dos níveis de antioxidante semelhantes aos observados em mitocôndrias de músculos não-lesados. Por fim, os resultados deste estudo indicam que um evento agudo de I/R pode ser considerado como um importante mecanismo envolvido na fisiopatologia de uma lesão musculoesquelética uma vez que determinou alterações bioquímicas e morfológicas semelhantes às observadas após uma contusão muscular

Palavras-chaves: crioterapia, contusão muscular, evento agudo de isquemia e reperfusão, dano oxidativo, resposta inflamatória, alterações mitocondriais.

ABSTRACT

Thesis of Doctor's Degree
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EFFECTS OF CRYOTHERAPY IN MODELS OF CONTUSION AND BLOOD ISCHEMIA/REPERFUSION IN SKELETAL MUSCLE OF RATS

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The musculoskeletal disorders are in the most common injuries observed in individuals in the primary care, occupational health, and in sports medicine. Among these disorders, the contusion is described as a direct traumatic lesion that impairs the functioning of the skeletal muscle system. An acute event of ischemia and reperfusion (I/R), on the other hand, could be considered as one of the main issue involved in the pathophysiology of a musculoskeletal disorder. Among the main strategies employed in the treatment of a lesion is the reduction of the temperature of the tissues with the therapeutic aim, this mechanism is defined as cryotherapy. Although the clinical efficacy of the cryotherapy is well established in the literature, the mechanisms involved in its therapeutic effects are unclear. The aim of this study was to analyze the effects of the cryotherapy in the treatment of a contusion and of an acute event of blood I/R in the *gastrocnemius* muscle of rats. Thus, we investigated the effects of cryotherapy under the biochemical and morphological changes related with a contusion (**Article 1**) and with an acute event of I/R (**Manuscript 1**), as well as the mechanisms involved in the genesis of its therapeutic effects. The treatment with cryotherapy determined a significant reduction in the oxidative damage since that limited the lipid peroxidation and the reactive oxygen species (ROS) formation, and also limited the lost of the cellular viability in the skeletal muscle tissue injured after a contusion (**Article 1**) and after an acute event of I/R (**Manuscript 1**). In this context, the levels of non enzymatic antioxidants, such as the levels of non protein thiols (-SH), and enzymatic antioxidants, such as the catalase enzyme (CAT), were also maintained similar to the observed in non injured muscles. The treatment with cryotherapy was effective in maintain the activities of enzymes sensitive to the oxidative stress, such as the lactate dehydrogenase (LDH) and the sodium/potassium (Na^+/K^+) and calcium (Ca^{2+}) ATPases enzymes, similar to the observed in the tissues non injured both after a contusion (**Article 1**) and after as acute event of I/R (**Manuscript 1**). According to the histopathological analysis the cryotherapy treatment reduced the morphologic structure changes an also the presence of blood cells indicatives of hemorrhagic or inflammatory process in the skeletal muscle injured both after a contusion (**Article 1**) and after an acute event of I/R (**Manuscript 1**). In general, the results observed in this study indicate that an important mechanism by which the cryotherapy exerts its therapeutic effects is related with the reduction in the inflammatory response intensity in the site of the lesion. These results are indicated by the limited amount of inflammatory cells observed in the histopathological analysis and corroborated by the reduced activity of the myeloperoxidase (MPO) enzyme activity in the injured skeletal muscle tissue that was treated with cryotherapy. Furthermore,

the cryotherapy limited the mitochondrial changes in the injured skeletal muscle tissue since that decreased the reactive species formation and maintained the mitochondrial membrane functionality both after a contusion (**Article 1**) and after an acute event of I/R (**Manuscript 1**). This result was indicated by the reduced swelling and limited impairment in the membrane potential ($\Delta\psi$) in mitochondria of the injured skeletal muscle, and by the maintenance of the antioxidant levels similar to the observed in mitochondria of non injured skeletal muscle. Finally, the results of this study indicate that an acute event of I/R could be considered as an important mechanism involved in the pathophysiology of a musculoskeletal disorder since that determined biochemical and morphological changes similar to the observed after a skeletal muscle contusion.

Key Words: cryotherapy, skeletal muscle contusion, acute event of ischemia and reperfusion, oxidative damage, inflammatory response, mitochondrial changes.

LISTA DE ABREVIATURAS

AINES – Aintiinflamatórios não-esteroidais
ATPases – Adenosina trifostatases
Ca^{2+} ATPase – Cálcio adenosina trifostatase
CAT – Catalase
Cl^- - Íons de cloro
CQ – Creatina quinase
Cu/ZnSOD – Cobre/Zinco superóxido dismutase
DAPs – Doenças arteriais periféricas
DCFA-DA – Diclorofluoresceína-diacetato
DCF-RS – Diclorofluoreceina oxidada por espécies reativas
EROs – Espécies Reativas de Oxigênio
GSSG – Glutationa oxidada
GSH – Glutationa reduzida
HOCl – Ácido hipocloroso
H_2O – Água
H_2O_2 – Peróxido de hidrogênio
I/R – Isquemia e reperfusão
LDH – Lactato desidrogenase
MDA – Malondialdeído
MMI – Membrana mitocondrial interna
MnSOD – Manganês superóxido dismutase
MPO – Mieloperoxidase
MTT – Metiltetrazólio
nm – nanômetros
Na^+/K^+ ATPase – Sódio/Potássio adenosina trifosfatase
$O^-_2{}^{\bullet}$ – Ânion superóxido
SNC – Sistema Nervoso Central
- SH – Grupos tióis
TBA – Ácido tiobarbitúrico
TBARS - Espécies Reativas ao Ácido Tiobarbitúrico
$\Delta\psi$ – potencial de membrana

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APRESENTAÇÃO

No item **INTRODUÇÃO**, está descrita uma sucinta revisão bibliográfica sobre os temas trabalhados nesta tese.

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo e manuscrito científico, os quais se encontram alocados no item **ARTIGO E MANUSCRITO CIENTÍFICO**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos respectivos artigo e manuscrito científico e representam a íntegra deste estudo.

Os itens, **DISCUSSÃO E CONCLUSÕES**, no final desta tese, apresentam interpretações e comentários gerais sobre os resultados contidos neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** se referem somente às citações que aparecem nos itens **INTRODUÇÃO** e **DISCUSSÃO** desta tese.

1. INTRODUÇÃO

O corpo humano é dotado de uma impressionante capacidade de movimentação a qual é proporcionada por um elaborado sistema musculoesquelético. O desenho anatômico corporal, a disposição das articulações e dos segmentos articulados, e o trajeto das fibras musculares proporcionam o desenvolvimento de uma atividade corporal sincronizada e harmônica. Como resultado tem-se a exuberância de um corpo móvel e ao mesmo tempo estável para suportar toda a descarga de peso e para proteger os órgãos responsáveis pela manutenção de nossa vida. De modo geral, o corpo humano comporta-se como uma verdadeira "máquina" onde os músculos poderiam ser comparados aos "motores", capazes de converter energia química em mecânica, e as articulações as "engrenagens", que garantem a mobilidade e também a estabilidade aos segmentos corporais articulados.

As disfunções do sistema músculo-esquelético, tais como as decorrentes de lesões musculares, dificultam a realização de tarefas básicas na vida das pessoas. As atividades como vestir-se, alimentar-se, locomover-se e manter a higiene de modo satisfatório e independente acabam tornando-se difíceis, o que determina uma perda na qualidade de vida das pessoas. As atividades realizadas nos momentos de lazer também podem ser dificultadas resultando em um aspecto negativo no bem estar psíquico e social dos indivíduos. Do ponto de vista econômico as lesões musculares podem suscitar um aumento nos gastos públicos em decorrência do afastamento das pessoas economicamente ativas de seus setores de trabalho, além dos elevados custos relacionados ao tratamento e reabilitação das mesmas. Assim, as lesões musculoesqueléticas constituem uma das causas mais frequentes de limitações e incapacidades funcionais de trabalhadores observadas nas áreas da medicina ocupacional e desportiva (RAHUSEN e cols., 2004).

Uma das lesões que mais acomete o sistema musculoesquelético é a contusão muscular. A contusão é classificada como uma lesão traumática não invasiva que se caracteriza pelo esmagamento da estrutura musculoesquelética em decorrência de uma carga impactante na superfície do músculo (BEINER e JOKL, 2001). Como resultado deste esmagamento muitos dos elementos contráteis componentes da estrutura muscular podem ser danificados (BEINER e JOKL 2001; RAHUSEN e cols., 2004). Nestas circunstâncias as propriedades de elasticidade, extensibilidade e contratilidade das células musculares podem ser comprometidas. Além disso, as contusões musculares podem determinar o esmagamento e/ou a ruptura de vasos sanguíneos com o consequente extravasamento de componentes do

sangue na região lesada (JÄRVINEN e cols., 2005). Como resposta a este dano, uma quantidade variável de células inflamatórias pode ser atraída ao local da lesão a fim de iniciar o processo de reparo dos tecidos musculares lesados (LI e cols., 2005).

A resposta inflamatória seguinte a uma lesão é fundamental para que o processo de cicatrização e reestruturação tecidual aconteça. No entanto, esta é acompanhada por um excessivo aumento na produção de espécies reativas de oxigênio (EROs) sempre que aconteça de modo demasiado (SPITELLER, 2006; SUPINSKI e CALLAHAN, 2007). O equilíbrio entre a geração de EROs e a capacidade dos sistemas de defesa antioxidante celular é essencial para que se mantenha a homeostase endógena necessária às funções intracelulares (GUTTERIDGE e HALLIWELL, 1994). A geração excessiva de EROs ou a diminuição da capacidade dos sistemas de defesa antioxidantes pode culminar com o comprometimento de biomoléculas celulares e desencadear a sua disfunção num mecanismo definido como dano oxidativo (HALLIWELL, 2006). Contudo, o estudo das alterações oxidativas teciduais em modelos de lesão muscular é recente. Da mesma forma, o mecanismo pelo qual os métodos mais utilizados no tratamento destas lesões musculares exercem os seus efeitos terapêuticos é, até o momento, pouco elucidado (CARVALHO e cols, 2010).

Além da excessiva resposta inflamatória, é importante considerar que outros mecanismos podem estar envolvidos na origem do dano oxidativo seguinte a uma lesão muscular. Tendo em vista a origem traumática de algumas lesões musculares, tais como a contusão, é possível compreender as alterações circulatórias subsequentes ao rompimento dos vasos sanguíneos do local lesado. Neste contexto, a ausência do fluxo sanguíneo arterial nas áreas adjacentes ao local lesado caracteriza um evento circulatório conhecido como isquemia. Por sua vez, o retorno do fluxo sanguíneo a estas áreas caracteriza um evento circulatório conhecido como reperfusão (WELBOURN e cols., 1991; GRACE, 1994). Coletivamente, estes eventos circulatórios são conhecidos como isquemia e reperfusão (I/R) e podem ser classificados quanto a sua origem e evolução em crônicos ou agudos. Os eventos de I/R crônicos estão geralmente associados ao desenvolvimento de doenças arteriais periféricas (DAPs) e caracterizam-se por uma evolução insidiosa e lenta. As DAPs podem ser acompanhadas por inúmeras manifestações clínicas que comprometem a independência funcional dos indivíduos, constituindo-se um importante problema de saúde pública (NORGREN e cols., 2007; PIPINOS e cols., 2008a). Os eventos de I/R agudos, por sua vez, são comuns após lesões musculares relacionadas à prática esportiva e apresentam uma evolução repentina e rápida (McEWEN & INKPEN, 2004; HAMMERS e cols., 2008).

Em geral, os eventos circulatórios de I/R podem ser acompanhados por significativas oscilações dos níveis de oxigênio e nutrientes disponíveis aos tecidos envolvidos, (FERRARI, 1994; GRACE, 1994). O adequado suprimento de oxigênio é fundamental para o ideal desenvolvimento das funções mitocondriais de uma célula (KEMP, 2004; MAKRIS e cols., 2007; PIPINOS e cols., 2007; PIPINOS e cols., 2008b). Tendo em vista que a funcionalidade mitocondrial é fundamental para a apropriada realização das reações metabólicas oxidativas e também para a regulação dos níveis de EROs intracelulares, é possível entender os comprometimentos funcionais seguintes a um evento de I/R (HARRIS e cols., 1986; CHOUDHURY e cols., 1991; CARDEN e cols., 2000; COLLARD e cols., 2001). Neste contexto, apesar das evidentes disfunções mitocondriais observadas após eventos crônicos de I/R (KEMP, 2004; MAKRIS e cols., 2007; PIPINOS e cols., 2007; PIPINOS e cols., 2008b), não há estudos enfocando o envolvimento dos mesmos na fisiopatologia dos eventos agudos de I/R.

O desenvolvimento de estratégias terapêuticas que sejam efetivas na prevenção e na reabilitação de lesões musculares é fundamental na prática esportiva (KUJALA e cols., 1997; CLANTON e COUPE, 1998). As medidas terapêuticas no período de reabilitação após uma lesão muscular estão relacionadas à aceleração do processo de reabilitação e ao aumento da resistência corporal a novas lesões. A escolha do método ou técnica de tratamento mais adequado depende do local, da intensidade e do estágio evolutivo da lesão (JÄRVINEN e cols., 2005). Além disso, a disponibilidade dos recursos materiais influencia diretamente na escolha do método terapêutico a ser empregado. Neste contexto, a crioterapia é geralmente utilizada uma vez que os recursos terapêuticos que causam a diminuição da temperatura dos tecidos tratados são facilmente disponíveis, e, em geral, apresentam um relativo baixo custo de aquisição (BLEAKLEY e cols., 2004; JÄRVINEN e cols., 2005). A crioterapia no local da lesão tem por finalidade a diminuição da intensidade dos sinais cardinais característicos da resposta inflamatória seguinte a uma lesão muscular. Além disso, a crioterapia pode determinar uma redução no extravasamento de componentes sanguíneos pelo local lesado, e desta forma reduzir o dano oxidativo ao promover uma diminuição na atividade metabólica do local tratado (SCHASER e cols., 2007).

1.1. JUSTIFICATIVA

As estratégias adequadas ao efetivo tratamento das lesões musculoesqueléticas devem estar relacionadas aos mecanismos pelos quais estas comprometem o funcionamento dos tecidos envolvidos. Além disso, o conhecimento das possíveis alterações bioquímicas e morfológicas envolvidas na origem e na evolução destas lesões é de fundamental importância para o seu adequado e efetivo tratamento. No entanto, até o momento não existem estudos que demonstrem a existência, bem como a magnitude, do dano oxidativo e das alterações morfológicas subsequentes a uma contusão e/ou a um evento agudo de I/R sanguínea em músculo de ratos. Por sua vez, a elucidação dos mecanismos pelos quais as estratégias de tratamento exercem os seus efeitos terapêuticos é de suma importância clínica. Neste contexto, a redução na intensidade da resposta inflamatória e das alterações circulatórias decorrentes de uma contusão muscular e/ou de um evento agudo de I/R sanguínea podem ser mecanismos importantes na origem dos efeitos terapêuticos da crioterapia.

2. REVISÃO BIBLIOGRÁFICA

2.1 As lesões musculoesqueléticas

As lesões musculoesqueléticas estão entre as maiores causas de lesões observadas nas áreas de primeiros socorros, na saúde ocupacional e na medicina do esporte (RAHUSEN e cols., 2004). Estas lesões são bastante freqüentes tanto em atletas quanto em não atletas, sendo determinantes para o surgimento de limitações no desempenho das atividades profissionais e também da vida diária destes indivíduos (PAGE, 1995). Os fatores principais relacionados à origem das limitações impostas pelas lesões são a intensa sensibilidade dolorosa e significativa perda das propriedades funcionais musculares (TAYLOR e cols., 1993).

Em geral, as lesões musculoesqueléticas podem ser classificadas em agudas ou crônicas de acordo com a sua origem e evolução. As lesões agudas geralmente apresentam uma origem traumática seguida de uma evolução rápida dos sintomas, podendo ser subdivididas em traumáticas diretas, tais como as contusões musculares, ou indiretas, tais como as distensões musculares (PAGE, 1995). Estas lesões agudas são acompanhadas por uma evolução imediata dos sintomas, advindas de um único trauma de maior magnitude, os macro-traumas (GARRET, e cols., 1988). As lesões crônicas, por sua vez, apresentam uma origem insidiosa e de evolução lenta, as quais resultam de traumas repetitivos e de menor magnitude, os micro-traumas (PAGE, 1995; MERRICK, 2002).

Como resultado de uma lesão musculoesquelética os tecidos podem ser diretamente lesados em diferentes magnitudes, de acordo com o tipo de lesão, caracterizando uma condição descrita como “teoria do dano primário” (MERRICK, 2002). Esta condição é acompanhada pelo comprometimento estrutural imediato dos tecidos envolvidos, o qual pode resultar na ruptura das membranas e dos componentes celulares e assim na eventual morte das células por necrose (ARMSTRONG, 1990; FISCHER e cols., 1990). O mecanismo de dano primário é marcado pelo comprometimento de diferentes estruturas anatômicas, tais como os músculos, os ossos, os nervos e os vasos sanguíneos próximos ao local da lesão (MERRICK, 2002).

Além do dano primário, outro importante fator relacionado ao comprometimento tecidual é a alteração secundária que acompanha as lesões musculoesqueléticas (MERRICK, 2002). Estas alterações, em geral, potencializam o dano primário e estendem o comprometimento aos tecidos próximos ao local da lesão, caracterizando a “teoria do dano

secundário” (KNIGHT, 1995; MERRICK, 2002). Estudos sobre os mecanismos envolvidos na gênese do dano secundário sugerem o envolvimento de alterações enzimáticas (FISCHER e cols., 1990) e também da redução no fornecimento de oxigênio e nutrientes aos tecidos lesados (KNIGHT, 1995; MERRICK, 2002). De modo geral, as alterações enzimáticas estão relacionadas ao aumento na liberação de enzimas pró-inflamatórias, tais como as hidrolase ácidas, as fosfolipases e as proteases, especialmente pelos neutrófilos atraídos ao local da lesão a fim de iniciar o processo de reparo tecidual (FISCHER e cols., 1990; MERRICK, 2002; JÄRVINEN e cols., 2005). O prejuízo no fornecimento de oxigênio aos tecidos, por sua vez, caracteriza uma condição de hipóxia tecidual a qual decorre especialmente do comprometimento circulatório determinado pelas lesões musculoesqueléticas (KNIGHT, 1995; MERRICK, 2002).

É importante considerar que as estratégias terapêuticas são ineficazes no tratamento das alterações estruturais e funcionais relacionadas ao dano primário aos tecidos uma vez que não há como revertê-las. No entanto, é plausível considerar que o dano secundário decorrente das alterações enzimáticas e da hipóxia tecidual podem ser minimizados a partir de estratégias terapêuticas apropriadas. Neste contexto, a redução da resposta inflamatória e do comprometimento circulatório é crucial para o efetivo tratamento das lesões musculoesqueléticas.

2.1.1. A contusão muscular

A contusão muscular é uma das lesões que mais acomete o sistema musculoesquelético, sendo comum entre os praticantes de esportes de maior contato (JARVINEN, 1976; MAEHLUM & DALJORD, 1984; KIBLER, 1993; BEINER e JOKL, 2001). Por ser uma lesão resultante de um trauma direto sobre a superfície do músculo, a contusão é caracterizada pelo esmagamento da estrutura musculoesquelética (BEINER e JOKL, 2001). Os elementos contráteis componentes da estrutura muscular ao serem esmagados sofrem um prejuízo em suas propriedades funcionais tais como a elasticidade, a extensibilidade e a contratilidade (BEINER e JOKL 2001; RAHUSEN e cols., 2004). 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 e cols., 2005).

O esmagamento do tecido muscular é descrito como o dano primário subsequente a uma contusão (MERRICK, 2002). Após o dano primário, uma quantidade variável de células

inflamatórias deve ser atraída ao local da lesão a fim de promover a limpeza e iniciar o processo de reparo tecidual (LI e cols., 2005). Apesar de fundamental para o reparo dos tecidos acometidos pela contusão, a resposta inflamatória exagerada pode resultar em um comprometimento ainda maior destes tecidos (FISCHER e cols., 1990; MERRICK, 2002; JÄRVINEN e cols., 2005). Além disso, é importante considerar que o comprometimento dos vasos sanguíneos resulta no prejuízo do fornecimento de oxigênio aos tecidos lesados (KNIGHT, 1995; MERRICK, 2002). Assim, tanto a excessiva resposta inflamatória quanto a hipóxia imposta aos tecidos lesados pode contribuir para uma maximização do comprometimento funcional decorrente de uma contusão muscular.

Apesar da semelhante fisiopatologia das contusões musculares existem diferenças clínicas significativas que devem ser consideradas na elaboração do protocolo de tratamento a ser executado. Neste contexto, quanto maior for o impacto acarretado pela contusão muscular sobre a superfície do segmento corporal maior será a extensão dos tecidos diretamente e também secundariamente comprometidos.

2.2. A resposta inflamatória

As lesões teciduais causadas por um agente agressor desencadeiam uma série de manifestações no local lesado que são denominadas de sinais cardinais da resposta inflamatória. Estes sinais clínicos foram primeiramente descritos pelo enciclopedista romano Aulus Cornelius Celsus (25 a.C. – 50 d.C) como "*rubor et tumor cum calore et dolore*", ou seja, rubor e tumor com calor e dor. O médico grego Cláudio Galeno (131 – 200 d.C), por sua vez, definiu como "functio laesa", ou seja, perda da função o que viria a ser conhecido como o quinto sinal cardinal da resposta inflamatória a uma lesão tecidual (WJHITING & ZERNICKE, 2001).

Os sinais cardinais rubor, tumor, e calor são decorrentes da vasodilatação capilar no local da lesão. O aumento no aporte sanguíneo determina o aumento na pressão hidrostática intra-capilar e o consequente extravasamento de líquido do meio intravascular para o espaço intersticial no local da lesão. Além disso, nestas condições ocorre também um aumento na permeabilidade capilar e assim na passagem de líquido para o espaço intersticial. Desta forma, ocorre a formação do edema no local da lesão. A sensação de dor, por sua vez, pode estar associada aos componentes mecânicos e bioquímicos da resposta inflamatória. A hiperalgia de origem mecânica está relacionada ao estiramento e conseqüente estimulação de terminações nervosas nociceptivas em decorrência do acúmulo de líquido no local da lesão. Além disso, substâncias químicas capazes de estimular as terminações nervosas nociceptivas

também são liberadas durante a resposta inflamatória e também podem determinar o surgimento de uma hiperalgia no local da lesão.

O processo de reparo do tecido muscular lesado pode ser dividido em três fases segundo Järvinen e cols., (2005):

1ª Fase de Destruição-Inflamação: caracterizada pela formação do hematoma, necrose de miofibrilas, e reações celulares inflamatórias;

2ª Fase de Reparo: caracterizada pela fagocitose do tecido muscular necrotizado, regeneração de miofibrilas, produção de tecido conjuntivo cicatricial, e proliferação de capilares;

3ª Fase de Remodelamento: Caracterizada pela maturação das miofibrilas regeneradas, contração e reorganização do tecido conjuntivo cicatricial, e restauração da funcionalidade capilar do tecido muscular reparado.

É importante salientar que a duração de cada uma das fases da resposta inflamatória depende da intensidade da lesão musculoesquelética, sendo que as fases de reparo e de remodelamento em geral ocorrem simultaneamente (JÄRVINEN e cols., 2005). Ao final de todas as fases características do processo de reparo tecidual, o tecido reconstruído deve apresentar características funcionais similares ao parênquima tecidual de origem não lesado.

2.2.1. O reparo tecidual após uma contusão muscular

O surgimento de uma resposta inflamatória após uma lesão musculoesquelética é fundamental para que o processo de cicatrização tecidual possa acontecer. No entanto, uma resposta inflamatória demasiada pode determinar uma deposição exagerada de tecido cicatricial no local da lesão (JÄRVINEN e cols., 2005; TIDBALL, 2005). Esta situação resulta em disfunções de todo o sistema musculoesquelético uma vez que o tecido de reparo apresenta características funcionais diferentes do parênquima dos tecidos lesados (TIDBALL, 2005; JÄRVINEN e cols., 2005). Por outro lado, a inexistência ou a intensidade insuficiente de uma resposta inflamatória também inviabiliza o processo de reparo tecidual. Desta forma, é necessário que após uma lesão ocorra uma resposta inflamatória controlada.

Um dos objetivos principais no tratamento das contusões musculares é modular a resposta inflamatória que ocorre subsequente ao comprometimento dos tecidos lesados. No entanto, o tratamento ideal destas lesões traumáticas diretas permanece incerto (RAHUSEN e cols., 2004). Apesar de seu freqüente emprego como alternativa de tratamento, os fármacos antiinflamatórios não-esteroidais (AINES) podem comprometer o processo de reparo tecidual ao inibir de modo exacerbado a resposta inflamatória (BEINER & JOLK, 1992; JARVINEN e

cols., 1992). Além disso, estudos sugerem que os fármacos AINES sejam pouco eficazes no tratamento de processos inflamatórios periféricos localizados, tais como os determinados por contusões (RAHUSEN e cols., 2004). Uma forma alternativa de tratamento das contusões envolve o emprego de agentes físicos como o frio terapêutico, o qual é utilizado com o objetivo principal de reduzir a atividade metabólica dos tecidos lesados (KNIGHT, 1976; MERRICK, 1999).

2.3. Os eventos de isquemia e reperfusão (I/R) sanguínea

A ausência do fluxo de sangue em um determinado tecido, causada pela obstrução ou ruptura de um vaso sanguíneo, caracteriza um evento circulatório conhecido como isquemia (WELBOURN e cols., 1991; GRACE, 1994). Nestas condições, o fornecimento de oxigênio e nutrientes aos tecidos situados após o ponto de obstrução/ruptura pode ser comprometido parcialmente (hipóxia) ou até mesmo totalmente (anóxia) (FERRARI, 1994; GRACE, 1994). Como resultado, podem ocorrer alterações na funcionalidade das mitocôndrias, as quais necessitam de oxigênio e substratos energéticos para a geração de energia na forma de adenosina trifosfato (ATP) através do mecanismo de fosforilação oxidativa (FREDERIKS e cols., 1984; BELKIN e cols., 1988; KIM e cols., 1999).

Por outro lado, o retorno do fluxo sanguíneo após o período de isquemia caracteriza um evento circulatório conhecido como reperfusão, o qual é acompanhado do aumento no fornecimento de oxigênio e nutrientes às áreas até então isquêmicas (FERRARI, 1994; GRACE, 1994). Embora o período de isquemia resulte no comprometimento do funcionamento mitocondrial (FREDERIKS e cols., 1984; BELKIN e cols., 1988; KIM e cols., 1999), existe consenso no fato de que o maior comprometimento ocorre durante o período de reperfusão de oxigênio aos tecidos (DONATI e cols., 1990; PIPER e cols., 1994). Estudos sugerem que durante a reperfusão sanguínea ocorre um aumento na geração de EROs além da capacidade de neutralização pelos tecidos envolvidos (DONATI e cols., 1990; PIPER e cols., 1994; KIM e cols., 1999; HONDA, 2005). Tendo em vista que as mitocondrias são fundamentais para a regulação dos níveis de EROs intracelulares, é possível entender o comprometimento mitocondrial seguinte a um evento de reperfusão sanguínea aos tecidos isquêmicos (HARRIS e cols., 1986; CHOUDHURY e cols., 1991; CARDEN e cols., 2000; COLLARD e cols., 2001).

Em geral, os eventos circulatórios descritos coletivamente como isquemia e reperfusão (I/R) sanguínea podem ser classificados quanto a sua origem e evolução em crônicos ou agudos. Os eventos crônicos de I/R estão associados ao desenvolvimento de patologias

crônicas, tais como as DAPs, e caracterizam-se por uma evolução insidiosa e lenta dos sintomas (NORGREN e cols., 2007; PIPINOS e cols., 2008a). As DAPs podem ser acompanhadas por inúmeras manifestações clínicas que comprometem a independência funcional dos indivíduos, constituindo-se um importante problema de saúde pública (PIPINOS e cols., 2008a; NORGREN e cols., 2007). Além disso, é importante considerar que os eventos crônicos de I/R são reconhecidamente acompanhados de significativos comprometimentos funcionais mitocondriais (KEMP, 2004; MAKRIS e cols., 2007; PIPINOS e cols., 2007; PIPINOS e cols., 2008b). Nestas circunstâncias, é possível entender que os eventos crônicos de I/R são acompanhados pelo aumento no dano oxidativo aos tecidos envolvidos (ROBIN e cols., 1996; GUTE e cols., 1998).

Os eventos agudos de I/R, por sua vez, são caracterizados como alterações transitórias no fluxo sanguíneo aos tecidos. Estas alterações são evidentes como resultados da utilização terapêutica da I/R na prática clínica (McEWEN & INKPEN, 2004; HAMMERS e cols., 2008). Como resultado, o aumento no dano oxidativo e das alterações funcionais dos tecidos submetidos ao evento agudo de I/R aumenta proporcionalmente com o tempo, sendo que o padronizado é não exceder um máximo de 2 horas de isquemia (BLAISDELl, 2002). Neste contexto, é possível estabelecer uma semelhança entre a fisiopatologia dos eventos agudos de I/R induzidos na prática clínica com os decorrentes de lesões musculares relacionadas à prática esportiva.

2.3.1. O evento agudo de I/R após uma contusão muscular

As alterações circulatórias subsequentes a uma lesão musculoesquelética são caracterizadas como eventos agudos de I/R sanguínea (ROBIN e cols., 1996; GUTE e cols., 1998). O prejuízo no fornecimento de oxigênio e nutrientes aos tecidos constitui um dos mecanismos principais de dano secundário às contusões musculares (KNIGHT, 1995; MERRICK, 2002). Assim, o comprometimento na função mitocondrial dos tecidos envolvidos nas lesões musculares é uma condição provável considerando-se às alterações funcionais aos tecidos isquêmicos/reperfundidos. No entanto, até o momento não existem estudos demonstrando as alterações funcionais dos tecidos após eventos agudos de I/R tais como os observados após uma contusão muscular.

A resposta inflamatória também constitui uma condição freqüente após a ocorrência de eventos agudos de I/R (ROBIN e cols., 1996; GUTE e cols., 1998). Os tecidos envolvidos podem sofrer alterações morfológicas estruturais as quais necessitam de reparo, o qual é iniciado a partir da resposta inflamatória local (CARVALHO e cols., 1995; WALTER e cols,

2008; VIGNAUD e cols., 2010). As alterações enzimáticas relacionadas à resposta inflamatória estão envolvidas no mecanismo de dano secundário observado após a ocorrência de lesões musculoesqueléticas (FISCHER e cols., 1990; MERRICK, 2002; JÄRVINEN e cols., 2005). Assim, é importante considerar que, de modo semelhante à fisiopatologia das lesões musculoesqueléticas, os eventos agudos de I/R podem resultar no dano secundário dos tecidos envolvidos a partir da excessiva resposta inflamatória subsequente ao comprometimento circulatório.

2. 4. O estresse oxidativo

As células aeróbias estão continuamente produzindo espécies reativas de oxigênio (EROs) como parte de seu processo metabólico (HALLIWELL, 2006). Quando geradas em concentrações adequadas estas cumprem um importante papel fisiológico relacionado à manutenção da "homeostase redox" envolvida nos processos de sinalização intracelular (DROGE, 2002; PACHER e cols., 2007). Além disso, as EROs participam dos mecanismos de defesa orgânica contra processos infecciosos e no desenvolvimento da resposta inflamatória subsequente a um dano tecidual (HALLIWELL, 2006). No entanto, um desequilíbrio entre a geração de EROs e a capacidade em neutralizá-las determina o estabelecimento de um estado funcional de estresse oxidativo celular (GUTTERIDGE & HALLIWELL, 1994).

O dano oxidativo decorrente do desequilíbrio entre a geração de EROs e a atividade dos sistemas de defesa antioxidantes resulta no comprometimento do funcionamento de importantes sistemas biológicos celulares (AUGUSTO e cols., 2002; HALLIWELL, 2006). A peroxidação lipídica imposta às membranas biológicas prejudica a manutenção da homeostase intracelular, uma vez que favorece a entrada e saída indiscriminada de metabólitos e detritos da célula (JOSEPHY, 1997; TIMBRELL, 2000). Além disso, importantes sistemas biológicos cuja funcionalidade depende da integridade dos grupos tióis (-SH) podem ser comprometidos (HUSCHENBET e cols., 1998; SUN e cols., 2001).

É importante considerar que as mitocôndrias são as principais organelas responsáveis pela modulação na geração de EROs intracelulares (VALKO, 2007; STARCOV, 2008; MURPHY, 2009). Desta forma, é plausível considerar que o dano oxidativo subsequente a uma lesão musculoesquelética esteja relacionado ao comprometimento no funcionamento das mitocôndrias dos tecidos lesados.

2.4.1. O dano oxidativo após uma contusão muscular

O dano oxidativo é considerado um dos principais responsáveis pelo comprometimento secundário após uma contusão muscular (MERRICK, 2002). Os mecanismos envolvidos na gênese do dano oxidativo estão relacionados à resposta inflamatória (FISCHER e cols., 1990; MERRICK, 2002; JÄRVINEN e cols., 2005) e ao comprometimento circulatório (ROBIN e cols., 1996; GUTE e cols., 1998) subsequentes a contusão.

O aumento na liberação de enzimas pró-inflamatórias pelos neutrófilos atraídos aos tecidos lesados pela contusão muscular contribui para a excessiva geração de EROs (FORMIGLI e cols., 1992; GUTE e cols., 1998; TIIDUS, 1998). Os componentes biológicos podem ser comprometidos em tais condições, dentre eles as mitocôndrias, as quais estão diretamente envolvidas na modulação dos níveis de EROs (BUTTERFIELD e cols., 2006). As alterações no fornecimento de oxigênio e nutrientes aos tecidos lesados também contribui diretamente para a geração de EROs ao comprometerem o mecanismo de geração de energia pelas mitocôndrias (DONATI e cols., 1990; PIPER e cols., 1994; KIM e cols., 1999; HONDA, 2005). Apesar disso, o comprometimento da funcionalidade mitocondrial nos tecidos lesados por uma contusão muscular não é conhecido.

2. 5. Os agentes físicos terapêuticos

A utilização de agentes físicos com fins terapêuticos representa a base na qual está fundamentada a fisioterapia. Os agentes físicos mais utilizados no tratamento de lesões musculoesqueléticas são classificados em térmicos (frio e calor), elétricos (correntes elétricas terapêuticas) e cinéticos (movimento).

Os agentes térmicos, como o frio terapêutico, são geralmente empregados no tratamento de lesões musculares devido a sua fácil disponibilidade e custo relativamente pequeno (BLEAKLEY e cols., 2004; THORSSON, 2001).

2. 5. 1. A crioterapia no tratamento da contusão muscular

A crioterapia consiste em toda e qualquer forma de aplicação de objetos que provoquem o resfriamento dos tecidos com finalidades terapêuticas (KNIGHT, 1976). A utilização do frio constitui a principal estratégia de tratamento nas fases iniciais após as lesões musculoesqueléticas, tais como as contusões musculares (MERRICK e cols., 1993; MERRICK e cols., 1999).

A vasoconstrição capilar induzida pelo frio determina uma redução do fluxo sanguíneo e consequentemente no rubor, no calor e no tumor dos tecidos tratados (SCHASER e cols., 2007). Ao ser aplicado sobre o local da lesão, o frio terapêutico tem como objetivo principal atuar como um modulador da resposta inflamatória. O frio pode efetivamente reduzir o dano secundário decorrente das alterações enzimáticas que acompanham a resposta inflamatória seguinte a uma contusão. Ao reduzir o fluxo de sangue no local da lesão, o frio proporciona uma diminuição na atividade metabólica nos tecidos lesados (KNIGHT, 1976; MERRICK e cols., 1999; SCHASER e cols., 2007). Assim, o dano secundário ao comprometimento circulatório observado após uma contusão muscular pode ser adequadamente modulado pela aplicação do frio terapêutico. Contudo, os mecanismos fisiológicos envolvidos nos benefícios do frio são até o momento pouco conhecidos (CARVALHO e cols., 2010).

Um dos mais importantes efeitos do frio terapêutico é o seu efeito analgésico. A aplicação do frio é acompanhada de uma série de sensações que passam pelo formigamento, cócegas, frio, ardência, queimação, diminuição da sensação tátil, e por fim a anestesia do local tratado. A analgesia produzida pela aplicação do frio terapêutico tem sua explicação baseada na “teoria das comportas” descrita inicialmente por Ronald Melzack e Patrick David Wall. A teoria das comportas sugere que a estimulação de vias aferentes sensoriais térmicas pode interferir na condução dos impulsos nervosos pelas vias aferentes nociceptivas. A sensação produzida pelo frio terapêutico é conduzida especialmente por fibras nervosas mielínicas de grande calibre (tipo Aβ), enquanto a sensação álgica é conduzida por fibras nervosas amielínicas (tipo C) ou mielínicas de pequeno calibre (tipo Aδ). Desta forma, os impulsos nervosos produzidos pela estimulação dos receptores térmicos são conduzidos ao sistema nervoso central (SNC) em uma velocidade maior do que os impulsos dolorosos. Ao ingressar no SNC por intermédio da raiz dorsal dos nervos espinais, a informação térmica promove o bloqueio (“fecha a comporta”) da entrada das informações dolorosas num mecanismo descrito como teoria das comportas (WALL E MELZACK, 1965).

3. OBJETIVOS

3.1. Objetivo Geral

Analisar os efeitos da crioterapia no tratamento de uma contusão e de um evento agudo de I/R sanguínea no músculo gastrocnêmio de ratos.

3.2. Objetivos Específicos

1. Analisar os efeitos da crioterapia sobre o dano oxidativo e as alterações morfológicas no tecido muscular esquelético após uma contusão em ratos;
2. Investigar o envolvimento da redução na resposta inflamatória e nas alterações na funcionalidade mitocondrial como mecanismos importantes na origem dos efeitos da crioterapia empregada no tratamento de uma contusão muscular;
3. Avaliar o possível envolvimento de um evento agudo de I/R sanguínea na fisiopatologia de uma lesão muscular em ratos;
4. Analisar os efeitos da crioterapia sobre o dano oxidativo e as alterações morfológicas no tecido muscular esquelético após um evento agudo de I/R em ratos;
5. Investigar o envolvimento da redução na resposta inflamatória e nas alterações na funcionalidade mitocondrial como mecanismos importantes na origem dos efeitos da crioterapia empregada no tratamento de um evento agudo de I/R no músculo esquelético.

4. RESULTADOS

Os resultados que fazem parte desta tese serão apresentados sob a forma de um artigo científico e um manuscrito, os quais se encontram aqui organizado. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no artigo científico e no manuscrito.

4.1. – ARTIGO CIENTÍFICO 1:

FRIO TERAPÊUTICO: UMA FERRAMENTA EFETIVA NA MODULAÇÃO DO DANO OXIDATIVO SUBSEQUENTE A UMA CONTUSÃO MUSCULAR

Therapeutic cold: An effective kind to modulate the oxidative damage resulting of a skeletal muscle contusion

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Therapeutic cold: An effective kind to modulate the oxidative damage resulting of a skeletal muscle contusion

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Abstract

Muscular contusions affect the function of the skeletal muscle system. This study investigated the oxidative damage as well as the main morphological changes related to a skeletal muscle contusion in the gastrocnemius muscle of rats and also the capacity of therapeutic cold to modulate these parameters. The therapeutic cold modulated the increase of oxidative stress markers and also modulated the reduction in the antioxidants levels in the injured muscle. In enzyme assays, therapeutic cold was also effective in normalizing the muscle Na^+/K^+ and Ca^{2+} ATPases, lactate dehydrogenase and myeloperoxidase activities. Similarly, the lesioned non-treated animals presented evident impairments in the mitochondrial functions and in the muscle morphology which were diminished by the cold treatment. The therapeutic cold was able to modulate the oxidative damage possibly by its capacity to limit the inflammatory response intensity, to attenuate the impairment of the mitochondrial function and also to preserve the skeletal muscle morphology.

Keywords: *Contusion, therapeutic cold, oxidative damage, mitochondria*

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Introduction

Skeletal muscle lesions are responsible for the majority of the functional limitations of workers observed in sportive and occupational medicine [1]. One of the most common lesions which affect the function of the skeletal muscle system is the muscular contusion [2]. These lesions are characterized by the compression of the skeletal muscle cells due to an impacting weight under the muscle surface [2]. As a result, the contracting elements of the muscle structure could be damaged and become dysfunctional, leading to an impairment of some of the normal skeletal muscle functional properties such as elasticity, extensibility and contractility [1,2]. In response to a skeletal muscle contusion, the compression and consequently the rupture of some blood capillaries as well as the overflow of blood components in the injured region may occur [3]. Thus, inflammatory cells could be attracted to injured regions in order to promote the clearance, starting the rehabilitation and the restructuration of the tissues [4].

Currently, it is well established that an inflammatory response is needed to the structural and functional rehabilitation of the damaged tissues [3]. However, an excessive inflammatory response could be accompanied by an uncontrolled reactive species (RS) generation [5,6]. An imbalance between the antioxidant defense systems and the generated RS

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may determine the impairment of the normal cell functions [7]. Among the biological molecules that could be impaired are those that depend on the sulphydryl groups (-SH) for their normal functioning. Some enzymes, such as the lactate dehydrogenase (LDH) [8–10] and the delta aminolevunilate dehydratase (δ-ALA-D) [11–13], as well as the non-enzymatic antioxidant three-peptide glutathione (GSH) may be affected in these conditions [14,15]. Besides, oxidant agents may interact with the thiol groups located at the active site of other important enzymes as the Na^+/K^+ and the Ca^{2+} ATPases, which are needed for the preservation of the adequate ionic gradient across the cellular membranes [16–19].

Many studies have indicated a central role of the oxidative damage in the development of several acute and chronic human disorders [20]. However, up to now, there are few data depicting the existence of such alterations in models of skeletal muscle tissue lesions, for example due to a skeletal muscle contusion [4]. The therapeutic cold has been considered one of the most efficient physical agents to treat different skeletal muscle lesions [21,22], but the biochemical mechanisms involved in its protective action are still unclear. In view of the potential oxidative damage induced by a contusion lesion, cold therapy, at least in part, is likely to have an important role in modulating this oxidative damage [23].

Thus, considering that data are scarce in the literature regarding the biochemical phenomena that underlie the therapeutic effects of cold in skeletal muscle lesions, we examined the possible role of the oxidative stress related to a skeletal muscle contusion induced in gastrocnemius muscle of rats. Subsequently, we also analysed the involvement of the inflammatory response intensity as well as the mitochondrial function impairment as possible mechanisms involved in the genesis of the oxidative damage in response to a skeletal muscle contusion. Besides, the benefits of the cold therapy under these parameters were investigated in order to improve the knowledge regarding its possible mechanism of action.

Materials and methods

Chemical reagents

The reagents thiobarbituric acid (TBA), dicloroflouresceine diacetate (DCFH-DA), methyltetrazolium (MTT), ethylene glycol tetraacetic acid (EGTA), Ellman's reagent (DTNB), N,N,N',N'-tetramethylbenzidine and ouabaine were supplied by Sigma–Aldrich Chemical Co. (St. Louis, MO). The other used reagents were obtained from local suppliers.

Animals

Adult male Wistar rats weighing 270–320 g from our own breeding colony were kept in cages of five animals each, with food and water *ad libitum* in a room with controlled temperature (22 ± 3°C) and on a 12-h light/dark cycle with lights on at 7:00 am. The animals were maintained and used in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil. The animals were divided into four main groups:

1) Control non-treated and non-lesioned animals—animals not submitted to the standard skeletal muscle contusion;
2) Control cold treated and non-lesioned animals—animals not submitted to the standard skeletal muscle contusion and treated with the therapeutic cold;
3) Lesioned non-treated animals—animals submitted to the standard skeletal muscle contusion without any treatment; and
4) Lesioned and cold treated animals—animals submitted to the standard skeletal muscle contusion and treated with the therapeutic cold.

Skeletal muscle contusion

The skeletal muscle contusion was developed according to the method proposed by Crisco et al. [24], with few modifications. First, the animals were anaesthetized with ketamine (50 mg/kg; i.p.) and xilazine (10 mg/kg; i.p.). The fully anaesthetized animals were placed in a prone position and the right hind limb was placed to perform the skeletal muscle contusion. A mass of 100 g fell through a polyvinyl chloride tube used as a guide from a height of 100 cm onto the top of the impactor (radius of 6.0 mm) placed in direct contact with the skin covering the mid-belly of the right gastrocnemius muscle. After the contusion, the rats were allowed to recover from anaesthesia and returned to the cage. The animals of the lesioned and cold treated animals were also submitted to the first treatment section for 5 min immediately after the skeletal muscle contusion.

Therapeutic cold treatment

The treatment of the animals with the therapeutic cold was performed by the application of ice cubes directly under the contused muscle [23]. The treatment sections were developed twice a day for 5 min each section. The first application was performed immediately after and the second application 6 h after the skeletal muscle contusion.

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The protocol of cold treatment used in this study was based in the previous data of our research group [23]. We observed that the cold treatment produced by the ice cubes application directly under the site of the lesion for 5 min immediately after the lesion and repeated 6 hours after the lesion is able to modulate significantly the oxidative damage induced by a strain muscle lesion [23].

Biochemical analysis

Biochemical analyses were performed in two distinct sets of time. The first set of biochemical analysis was carried out 30 min after the skeletal muscle contusion in order to check the immediate biochemical changes indicative of oxidative damage, as well as the effects of a single therapeutic cold treatment section under these conditions. The second set of biochemical analysis was carried out in the day following the skeletal muscle contusion in order to investigate the long-term biochemical changes indicative of the oxidative damage, as well as the effects of two therapeutic cold treatment sections under these conditions.

Tissue preparation

Whole blood and blood components. Rats were euthanized and the whole blood was collected (cardiac puncture) in previously heparinized tubes and kept under refrigeration. Whole blood samples were precipitated with TCA 40% (1:1) and centrifuged (4000 × *g* at 4°C for 10 min) in order to obtain the supernatant fraction that was used for TBARS determination. Other heparinized blood samples were centrifuged at 1000 × *g* at 4°C for 10 min in order to obtain plasma and cellular blood fractions which were used for DCF-RS measurement. In addition, plasma aliquots were kept at −20°C for posterior creatine kinase activity measurement.

Skeletal muscle homogenates. For the determination of some of the oxidative damage markers and also the enzyme activity measurement, the right gastrocnemius muscle was removed, quickly homogenized in NaCl (150 mM), and kept in ice. After the homogenization, the skeletal muscle samples were centrifuged at 4000 × *g* at 4°C for 10 min to yield a low speed supernatant fraction (S1). For the MPO enzyme activity measurement, the muscle samples were homogenized in potassium phosphate buffer (20 mM, pH 7.4) containing EDTA (0.1 mM). After the homogenization, the skeletal muscle samples were centrifuged at 2000 × *g* at 4°C for 10 min to yield a low speed supernatant fraction (S1). Then, the S1 fraction was centrifuged again at 20 000 × *g* at 4°C for 15 min to yield a final pellet that was re-suspended in potassium phosphate buffer (50 mM, pH 6.0) containing hexadecyltrimethylammonium bromide (0.5%). The samples were finally freeze–thawed twice for the posterior enzymatic MPO assay. Besides, aliquots of skeletal muscle preparations were frozen (-20°C) for posterior analysis.

Isolation of skeletal muscle mitochondria. Rat skeletal muscle mitochondria were isolated as described by Tonkonogi and Salhin [25], with some modifications. First, the right gastrocnemius muscle was quickly removed and homogenized in a buffer containing mannitol (225 mM), sucrose (75 mM), EGTA (1 mM), bovine serum albumin (BSA) (0.1%) and HEPES (10 mM, pH 7.2). After the homogenization, the resulted suspension was centrifuged for 7 min at 2000 *g* in order to obtain a low speed supernatant fraction (S1). Then, S1 was re-centrifuged for 10 min at 12 000 *g*. The obtained pellet was re-suspended in a buffer containing mannitol (225 mM), sucrose (75 mM), EGTA (1 mM) and HEPES (10 mM, pH 7.2) and re-centrifuged at 12 000 *g* for 10 min. The supernatant was decanted and the final pellet re-suspended in a buffer containing KCl (65 mM), sucrose (100 mM), EGTA (0.05 mM), BSA (0.2%) and HEPES (10 mM, pH 7.2), to yield a protein concentration of 30–40 mg/mL.

Oxidative stress markers and cell viability determination

Thiobarbituric acid reactive substances (TBARS) levels. Analyses were performed in whole blood and in skeletal muscle S1 samples according to the method described by Ohkawa et al. [26]. Aliquots of 500 μL of supernatant fraction obtained after blood sample precipitation or 200 μL of skeletal muscle S1 were added to colour reaction. TBARS levels were measured at 532 nm using a standard curve of MDA and corrected by the protein content [26].

Oxidized diclorofluoresceine (DCF-RS) levels. DCF-RS levels were determined as an index of the peroxide production by the cellular components [27]. Aliquots of plasma (200 μL), cellular blood fraction (10 μL) or skeletal muscle S1 (50 μL) were added to a medium containing Tris-HCl buffer (0.01 mM; pH 7.4) and DCFH-DA (7 μM). After DCFH-DA addition, the medium was incubated in the dark for 1 h until fluorescence measurement procedure (excitation at 488 nm and emission at 525 nm and both slit widths used were at 5 nm). DCF-RS levels were determined using a standard curve of DCF and the results were corrected by the protein content [28].

Non-protein thiol (-SH) levels. Levels of non-protein –SH were determined in skeletal muscle S1 samples according to the method proposed by Ellman [29]

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with some modifications. Briefly, the samples of the skeletal muscle S1 (0.5 mL) were precipitated with TCA (5%) (1 mL) and subsequently centrifuged at 4000 *g* for 10 min. After the centrifugation, the supernatant fraction (500 μL) was added to a reaction medium containing K^+-phosphate (0.25 mM and pH = 7.4) and DTNB (1 mM). Non-protein –SH levels were measured spectrophotometrically at 412 nm. Results were calculated in relation to a standard curve constructed with GSH at known concentrations and also corrected by the protein content [29].

Methyl-tetrazolium (MTT) reduction levels. MTT reduction levels were determined as an index of the dehydrogenase enzymes functions, which are involved in the cellular viability [30]. Aliquots of skeletal muscle S1 (500 μL) were added to a medium containing 0.5 mg/mL of MTT and were incubated in the dark for 1 h at 37°C. The MTT reduction reaction was stopped by the addition of 1 mL of dimethylsulphoxide (DMSO). The formed formazan levels were determined spectrophotometrically at 570 nm and the results were corrected by the protein content [31].

Enzymes activity determination

Creatine kinase (CK). The CK enzyme activity was measured spectrophotometrically in plasma samples as an index of the damage caused by the skeletal muscle contusion using diagnosis kits (CK-NAC Liquiform, Labtest, MG, Brazil).

Lactate dehydrogenase (LDH). The LDH enzyme activity was determined spectrophotometrically in skeletal muscle S1 samples as an index of the oxidative damage to this tissue using diagnosis kits (LDH Liquiform, Labtest, MG, Brazil).

Sodium potassium (Na^+/K^+) ATPase. The Na^+/K^+ ATPase enzyme activity was determined in skeletal muscle S1 samples according to the method proposed by Musbeck et al. [32], with some modifications. Briefly, the aliquots of skeletal muscle S1 (20 μL) were added to a reaction medium containing NaCl (115 mM), $MgCl_2$ (2.5 mM), KCl (18 mM) and Tris-HCl buffer (45 mM and pH 7.4), with or without the Na^+/K^+ ATPase enzyme inhibitor ouabaine (5 μM). The method for ATPase activity measurement was based on the determination of the inorganic phosphate (Pi) released to the reaction medium by the hydrolysis of the ATP according to the method proposed by Atkinson et al. [33]. The reaction was initiated with the addition of the substrate ATP (1.5 mM) to the reaction medium and was finished by the addition of the colour reagent (1 mL) containing ammonium molibdate (2%), triton-100X (5%) and H_2SO_4 1.8 M (10%) after 15 min of incubation at 37°C. The formed molibdate-Pi complexes were measured spectrophotometrically at 405 nm. Values were calculated in relation to a standard curve constructed with Pi at known concentrations and also corrected by the protein content.

Calcium (Ca^{2+}) ATPase. The Ca^{2+} ATPase enzyme activity was determined in skeletal muscle S1 samples according to the method proposed by Zaidi and Michaelis [34], with some modifications. Briefly, the aliquots of skeletal muscle S1 (20 μL) were added to a reaction medium containing $MgCl_2$ (1 mM), KCl (50 mM), EGTA (0.2 mM) and Tris-HCl buffer (25 mM and pH 7.4), with or without the $CaCl_2$ (150 μM) in order to ensure a final concentration of 1 μM of Ca^{2+} ions in the medium. The experimental procedures were similar to those used for the determination of the Na^+/K^+ ATPase enzyme activity, which were described above.

Superoxide dismutase (SOD). The SOD enzyme activity was determined in skeletal muscle S1 according to the method proposed by Misra and Fridovich [35]. This method is based on the capacity of SOD in inhibiting auto-oxidation of adrenaline to adrenochrome. Briefly, different S1 aliquots (10–50 μL) were added to a medium containing glycine buffer (50 mM; pH 10.5) and adrenaline (1 mM). The kinetic analysis of SOD was started after adrenaline addition and the colour reaction was measured at 480 nm.

Catalase (CAT). The CAT enzyme activity was determined in skeletal muscle S1 according to the method proposed by Aebi [36]. Briefly, S1 aliquot (50 μL) was added to a medium containing potassium phosphate buffer (50 mM; pH 7.4) and H_2O_2 (1 mM). The kinetic analysis of CAT was started after H_2O_2 addition and the colour reaction was measured at 240 nm.

Myeloperoxidase (MPO). The MPO enzyme activity was determined in skeletal muscle S1 according to the method proposed by Grisham et al. [37], with some modifications. Briefly, a sample of the skeletal muscle preparation (20 μL) was added to a medium containing potassium phosphate buffer (50 mM; pH 6.0), hexadecyltrimethylammonium bromide (0.5%) and N,N,N',N'-tetramethylbenzidine (1.5 mM). The kinetic analysis of MPO was started after H_2O_2 (0.01%) addition and the colour reaction was measured at 655 nm at 37°C.

Indicators of the skeletal muscle mitochondria function

Mitochondrial DCF-RS level determination. The mitochondrial DCF-RS generation was assayed according

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to Garcia-Ruiz et al. [38]. Briefly, the mitochondria samples (150 µg of protein per mL) were incubated in a medium containing KCl (65 mM), sucrose (100 mM), EGTA (0.05 mM), bovines serum albumin (BSA) (0.2%), HEPES (10 mM, pH 7.2) and the respiratory substrates glutamate (5 mM) and succinate (5 mM). The reaction was started with the DCFA-DA (1 µM) addition and the medium was kept at constant stirring during the assay period. The fluorescence analysis was performed at 488 nm for excitation and 525 nm for emission, with slit widths of 5 nm.

Mitochondrial membrane potential ($\Delta\Psi$) determination. The mitochondrial $\Delta\Psi$ determination was assayed according to Akerman and Wikstron [39]. Briefly, the mitochondria samples (150 µg protein/mL) were incubated in a medium containing KCl (65 mM), sucrose (100 mM), EGTA (0.05 mM), BSA (0.2%), HEPES (10 mM, pH 7.2), safranine O (10 µM) and the respiratory substrates glutamate (5 mM) and succinate (5 mM). The reaction was started with the mitochondria addition and the medium was kept at constant stirring during the assay period. The fluorescence analysis was performed at 495 nm for excitation and 586 nm for emission, with slit widths of 5 nm.

Mitochondrial swelling. The mitochondrial swelling was assayed according to Velho et al. [40]. Briefly, the mitochondria samples (150 µg of protein per mL) were incubated in a medium containing KCl (65 mM), sucrose (100 mM), EGTA (0.05 mM), BSA (0.2%), HEPES (10 mM, pH 7.2), $CaCl_2$ (0.2 mM), Pi (1 mM), as well as the respiratory substrates glutamate (5 mM) and succinate (5 mM). The reaction was started with the mitochondria addition and the medium was kept at constant stirring during the assay period. The fluorescence analysis was performed at 600 nm (slit 1.5 nm) for both excitation and emission wavelengths.

Protein determination. The protein content was determined according to Lowry et al. [41] using bovine serum albumin (BSA) as standard.

Histopathological analysis

One sample of the skeletal muscle tissue was used for the histopathological analysis in order to investigate microscopic changes in the normal tissue structure. We investigated the loss of skeletal muscle transverse striations and nucleus degeneration as well as the presence of necrotic skeletal muscle cells. Besides, the presence of neutrophils was examined as an index of the acute inflammatory infiltration extension. After being excised, the skeletal muscle was maintained in buffered formaldehyde solution (10%) until the microscopic preparation and colourization. The muscle samples were sectioned longitudinally along its proximal and distal origins. The histological slides were stained with hematoxylin and eosin and then submitted to the histopathological analysis.

Statistical analysis

Data were analysed by one-way and two-way ANOVA followed by Tukey test. Differences between groups were considered significant when $p < 0.05$.

Results

Effects of the cold treatment under markers of the oxidative damage and cell viability in the site of the lesion

Figures 1A–D depict the potential of the therapeutic cold in modulating the increased levels of some oxidative stress markers in the skeletal muscle tissue submitted to the contusion lesion. The increased DCF-RS and TBARS levels in the lesioned non-treated animals were significantly abolished by the therapeutic cold treatment (Figures 1A and B, respectively). Besides, the decreased MTT reduction levels in the lesioned non-treated animals were completely restored by the therapeutic cold treatment (Figure 1C).

Figures 2A and B show the role of the therapeutic cold treatment under the levels of some enzymatic and non-enzymatic antioxidant defense systems. Our data show that the cold treatment maintained the non protein –SH levels at control non-treated and non-lesioned animals values, which were significantly decreased in the lesioned non-treated animals (Figure 2A). Besides, the cold treatment counteracted the increased CAT enzyme activity depicted in the lesioned non-treated animals (Figure 2B). However, the SOD enzyme activity was not significantly changed by the cold treatment nor by the muscle contusion (data not shown).

Effects of the cold treatment under enzyme activities in the site of the lesion

Data presented in Figure 3 revealed that the therapeutic cold treatment was able to reduce the impairment in Na^+/K^+ ATPase and Ca^{2+} ATPase enzyme activities which were observed in the lesioned non-treated animals (Figures 3A and B, respectively). The LDH activity was altered 24 h after the lesion, and this alteration was modulated by the therapeutic cold treatment (Figure 3C). Furthermore, Figure 3D shows the power of the therapeutic cold to modulate the MPO enzyme

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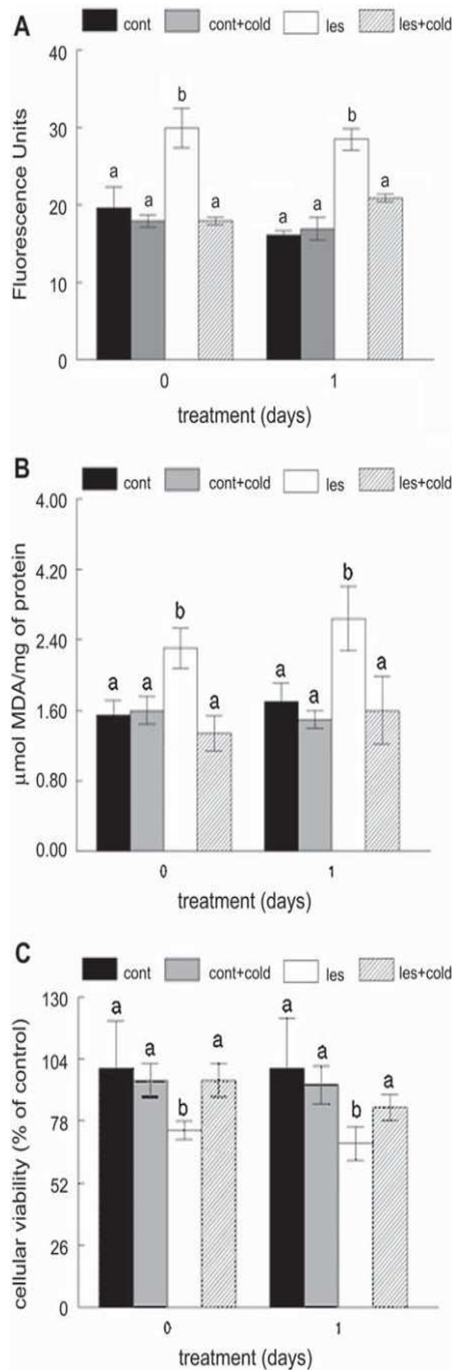

Figure 1. Effects of the cold treatment under oxidative stress markers and cell viability in the skeletal muscle tissue: (A) DCF-RS levels; (B) TBARS levels; (C) MTT reduction levels. In (A) the DCF-RS levels are expressed in fluorescence units/mg of protein; in (B) the TBARS levels are expressed in µmol of MDA/mg of protein; and in (C) the MTT reduction levels are expressed as a percentage of the control non-treated and non-lesioned animals value. Data are expressed as mean ± SE (n = 5–6) and were analysed by ANOVA, followed by Tukey test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [b]when compared to control non-treated and non-lesioned animals.

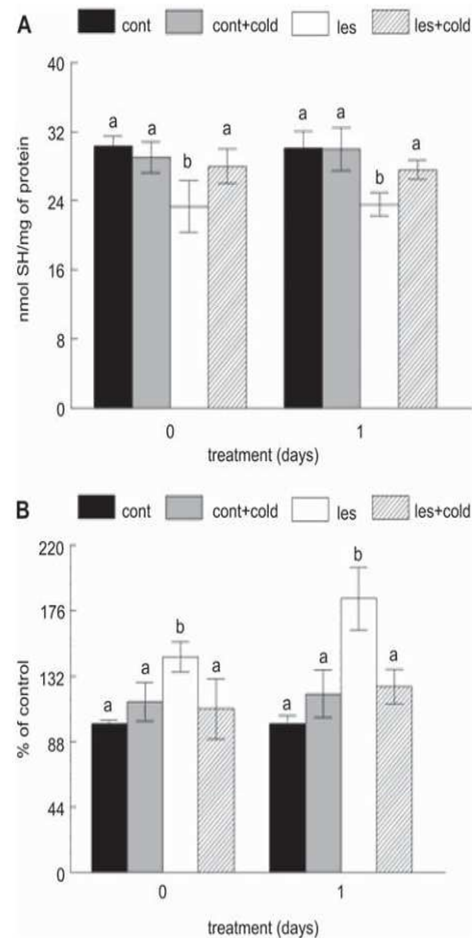

Figure 2. Effects of the cold treatment under antioxidant defense systems in the skeletal muscle tissue: (A) non-protein –SH levels; (B) CAT activity. In (A) the non-protein –SH levels are expressed in nmol of SH/mg of protein; in (B) the CAT activity is expressed as a percentage of the control non-treated and non-lesioned animals value (the control CAT activity was 135.7 ± 8.7 Units/mg of protein. Data are expressed as mean ± SE (n = 5–6) and were analysed by ANOVA, followed by Tukey test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [b]when compared to control non-treated and non-lesioned animals.

activity which was strongly increased only in the day following the skeletal muscle contusion.

Effects of the cold treatment under markers of the oxidative damage in the blood

Figures 4A–C show the capacity of the therapeutic cold treatment in modulating the increased levels of some oxidative stress markers in the whole blood and in blood components samples. The animals of the lesioned non-treated animals exhibited augmented DCF-RS levels both in plasma and in cellular blood fraction, which were significantly abolished by the cold treatment (Figures 4A and B, respectively). In the same way,

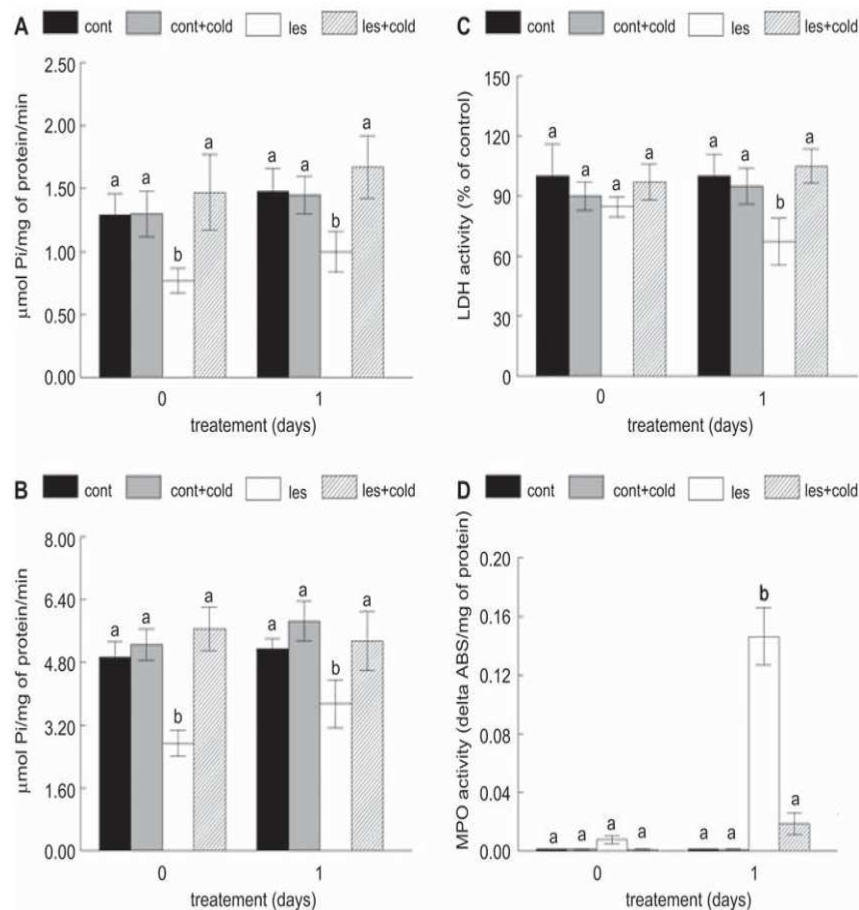

Figure 3. Effects of the cold treatment under enzymes activities in the skeletal muscle tissue: (A) Na^+/K^+ ATPase activity; (B) Ca^{2+} ATPase activity; (C) LDH activity; (D) MPO activity. In (A) and (B) the ATPases activities are expressed in µmol of Pi/mg of protein/minute of reaction; in (C) the LDH activity is expressed as a percentage of the control non-treated and non-lesioned animals value (the control LDH activity was 37.5 ± 3.2 Units/mg of protein); in (D) the MPO activity is expressed in absorbance variation unites (delta ABS) per mg of protein. Data are expressed as mean ± SE (n = 5–6) and were analysed by ANOVA, followed by Tukey test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [b] when compared to control non-treated and non-lesioned animals.

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the increased TBARS levels in the whole blood were also reduced by the cold treatment (Figure 4C).

Effects of the cold treatment under CK enzyme activity

The cold treatment effectively modulated the CK enzyme activity which was highly increased in the lesioned non-treated animals (Figure 5).

Effects of the cold treatment under morphological changes in the site of the lesion

The histopathological analysis depicted the capacity of the therapeutic cold treatment to minimize the morphological changes induced by the muscle contusion (Figure 6). The effect of the cold was more evident at short-time (30 min after the lesion) since neither neutrophils infiltration nor loss of skeletal muscle transverse striation was observed (Figure 6C). At moderated-time (1 day after the lesion) the cold decreased the neutrophils infiltration, but localized sites of changes in skeletal muscle transverse striation were observed (Figure 6E). In general, the skeletal muscle contusion was accompanied by an accentuated neutrophils infiltration in the site of the lesion (Figures 6B and D). Besides, we observed some localized sites of necrosis in the muscle cells and also the impairment of the cell structures characterized by the loss of skeletal muscle transverse striations and nucleus degeneration mainly on the day after the lesion (Figure 6D).

Effects of the cold treatment under skeletal muscle mitochondria function

Mitochondrial DCF-RS generation. Figure 7A shows that the cold treatment was effective in diminishing the mitochondrial DCF-RS generation depicted by

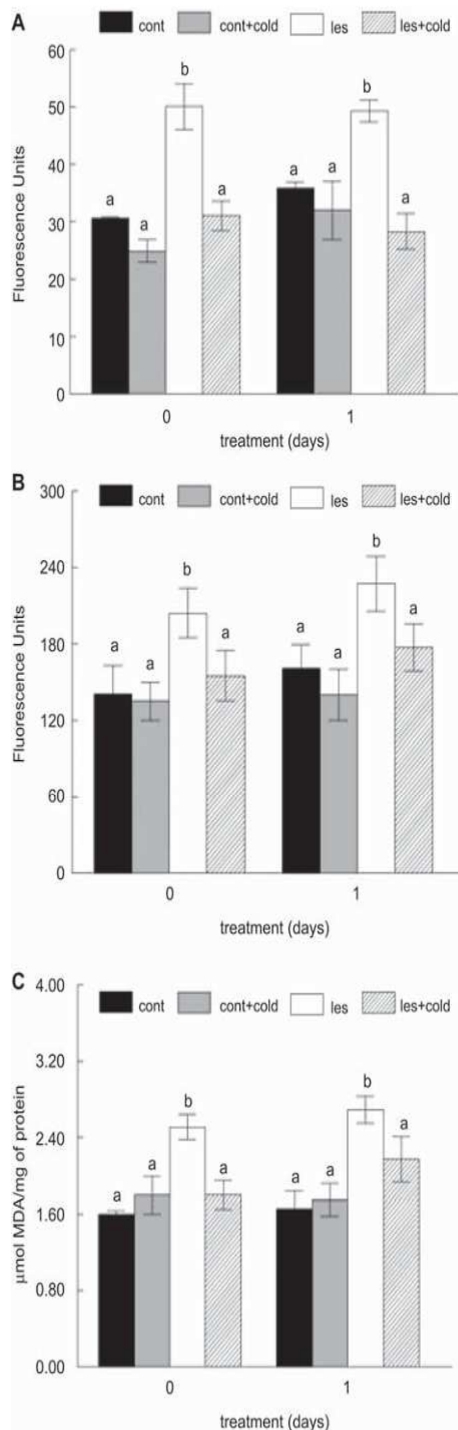

Figure 4. Effects of the cold treatment under oxidative stress markers in the whole blood and in blood components samples: (A) DCF-RS levels in plasma; (B) DCF-RS levels in cellular blood fractions; (C) TBARS levels in whole blood; (D) CK activity. In (A) and (B) the DCF-RS levels are expressed in fluorescence units/mg of protein; in (C) the TBARS levels are expressed in µmol of MDA/mg of protein; and in (D) the CK activity is expressed as a percentage of the control non-treated and non-lesioned animals value (the control CK activity was 540.3 ± 45.8 Units/L). Data are expressed as mean ± SE (n = 5–6) and were analysed by ANOVA, followed by Tukey test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [b] when compared to control non-treated and non-lesioned animals.

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the skeletal muscle contusion. However, this effect was more pronounced 30 min after the lesion.

Mitochondrial $\Delta\psi$. Likewise to DCF-RS generation, the mitochondrial $\Delta\Psi$ in lesioned and cold treated animals was maintained similar to that observed in control non-treated and non-lesioned animals. The effect of cold treatment was also more pronounced at short-time (30 min after the lesion) (Figures 7B I–III). As illustrated in Figure 7B, the levels of fluorescence were more elevated in the mitochondrial samples of the lesioned non-treated animals, indicating that the contusion process promoted changes in the mitochondrial $\Delta\Psi$.

Mitochondrial swelling. Figure 7C (I–III) shows that the mitochondrial swelling was significantly diminished in response to the cold treatment (Figure 7C) in both sets of time analysed. The increase in mitochondrial swelling was more pronounced at short-time (30 min after the lesion) than in the long-time (24 h after the lesion) as depicted in Figure 7C parts II and III, respectively.

Discussion

The purpose of our study was to verify if the benefits of therapeutic cold could be associated with the modulation of the oxidative damage induced by a muscle contusion. In this way, the results of the present work clearly indicated that the skeletal muscle contusion increased the oxidative damage in both muscular and blood tissue and that the therapeutic cold was able to modulate these alterations. We believe that this similar variation in skeletal muscle and in blood could be related to the inflammatory response intensity that follows a common skeletal muscle lesion [3] such as strain [23] and muscle contusion [1].

Considering that an uncontrolled inflammatory response to a muscle damage determines an excessive RS generation [5,6], we suggest that the oxidative damage could extrapolate the site of the lesion and thus propagate to the blood. In agreement with this, our results showed a significant increase in the MPO enzyme activity in the site of the lesion in the day following the skeletal muscle contusion (Figure 3D). Besides, the presence of a pronounced neutrophils infiltration was also observed in the histopathological analysis of the contused skeletal muscle in this period (Figure 6D). Thus, we propose that the higher DCF-RS (Figures 1A and 4A and B) and TBARS

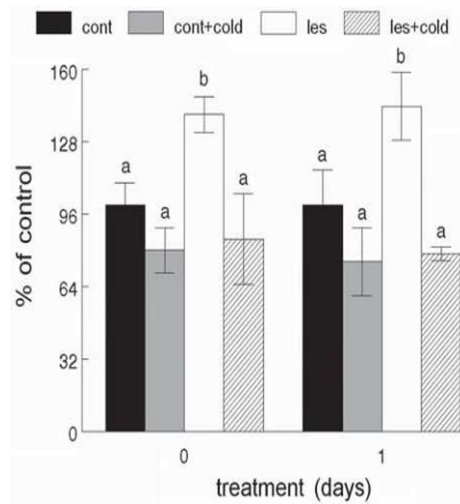

Figure 5. Effects of the cold treatment under CK activity in plasma: Figure shows the CK activity expressed as a percentage of the control non-treated and non-lesioned animals value (the control CK activity was 540.3 ± 45.8 Units/L). Data are expressed as mean ± SE (n = 5–6) and were analysed by ANOVA, followed by Tukey test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [b]when compared to control non-treated and non-lesioned animals.

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(Figures 1B and 4C) levels observed in the site as well as in the blood of the lesioned non-treated animals could be related to the excessive RS production. Although we did not measure specifically the RS formation, it is well known that an excessive RS could supply not only the DCF-RS formation but also contribute to start a complex cascade of reactions, which culminates with the lipid peroxidation (increase in TBARS levels).

In addition, excessive RS are able to cause alterations in structure and function of enzymes and deficits in the antioxidant defense systems. In line with this, our results show the impairment of some important functional systems caused by the skeletal muscle contusion and possibly related to the excessive RS production. In fact, we observed a significant decrease in the LDH activity in the day following the skeletal muscle contusion (Figure 3C). The LDH is an enzyme which classically becomes functionally impaired due to the oxidation of critical –SH groups located in its active site [8–10]. Moreover, we observed a significant decrease in the Na^+/K^+ ATPase (Figure 2A) and Ca^{2+} ATPase (Figure 2B) activities in the contused skeletal muscle. These enzymes are also reported to depend on the –SH groups integrity to be functionally active [10,16]. Besides, the involvement of the –SH groups oxidation in the genesis of these functional impairments was improved in our results which show a significant decrease in the non-protein –SH levels (glutathione as major compound) in the site of the lesion (Figure 2A). It is interesting to note also that the lesion caused a significant increase in the CAT activity in the site of the lesion (Figure 2B). This response may be related to a compensatory response of tissue to a previous oxidative insult, as for example an increased H_2O_2 production. These corroborate with the highest production of RS and the lipid peroxidation in the lesioned non-treated animals when compared to the therapeutic cold group as demonstrated in the manuscript.

Regarding treatment, our results showed a significant capacity of the therapeutic cold to limit all parameters linked to oxidative stress. In fact, cold therapy was effective in reducing the increase of the DCF-RS (Figure 1A) and the TBARS (Figure 1B) levels. Furthermore, the therapeutic cold limited the oxidation of the non-protein –SH groups (Figure 2A) and, consequently, the functional impairment of the LDH (Figure 3C), Na^+/K^+ ATPase (Figure 3A) and Ca^{2+} ATPase (Figure 3B) enzyme activities, which were depicted by the skeletal muscle contusion. In this way, the increase in CAT activity observed in lesioned non-treated animals was effectively changed by the cold treatment (Figure 2B). It is important to observe that our results are in accordance with those observed in previous studies that show the benefits of the repeated and short-term cold exposure in the improvement of the antioxidant defense systems in humans and in rats [42–44]. Overall, we believe that the benefits of therapeutic cold are likely to be linked to its potential to modulate the intensity of the inflammatory response that follows the skeletal muscle contusion. This hypothesis is supported by our results which depict that the therapeutic cold treatment limited the significant increase in MPO enzyme activity in the day following the skeletal muscle contusion (Figure 3D). Besides, the histopathological assay revealed low levels of neutrophils infiltration in lesioned and cold-treated animals (Figures 6C and E). Since the cold treatment is well reported to modulate the intensity of the inflammatory response due to its ability to reduce the blood flow intensity to the treated areas [21,22], we understand that this could be an important factor to explain its capacity to limit the oxidative damage determined by the skeletal muscle contusion. The reduction in the blood flow in the cold-treated areas could also depict a decrease in the oxygen availability and consumption for these tissues. This condition could result in a decrease of the reactive oxygen species formation (ROS).

More than the oxidative insult, our results lead us to put forward that the skeletal muscle contusion determined a significant damage to the structure of the muscle cells, and then compromised their viability. We observed that the lesioned non-treated animals presented a significant increase in the plasma CK enzyme activity (Figure 5A). The CK is a cytosolic enzyme known to flow to the extra-cellular

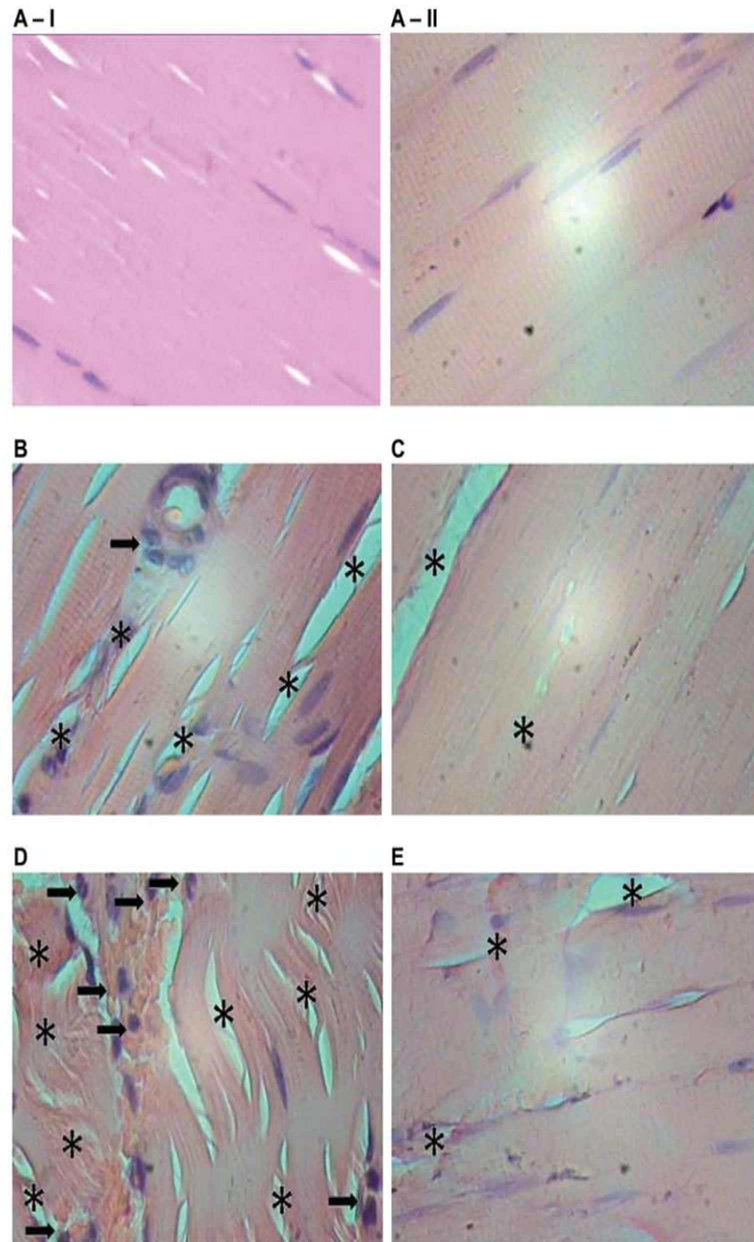

Figure 6. Histopathologic changes in skeletal muscle: The neutrophils infiltrations areas (arrow), as well as the impaired skeletal muscle cells striations areas (sharp) were identified: (A-I) control non-treated and non-lesioned muscle; (A-II) control cold treated and non-lesioned muscle; (B) lesioned and non-treated muscle after 30 min; (C) lesioned and cold treated muscle after 30 min; (D) lesioned and non-treated muscle after 24 h; (E) lesioned and cold treated muscle after 24 h. In all cases (A–E) the images were 400-times increased.

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space when the cell structure is impaired [45,46]. Moreover, the impairment of the skeletal muscle cell structure becomes evident in the histopathological analysis of the lesioned non-treated animals (Figures 6B and C). Since the integrity of the cell membrane is important to the maintenance of the cell survival, the damage induced by the skeletal muscle contusion could also depict a reduction in the skeletal muscle cell viability [45,46]. This hypothesis is in accordance with our results that showed a significant decrease in muscle MTT reduction levels in the lesioned non-treated animals (Figure 1D). The MTT reduction depends on the adequate functionality of the oxidoreductase enzyme family, such as the dehydrogenase enzymes [30]. Since the majority of these enzymes are located in the mitochondria [30,47], their functional impairment could be related to the mitochondria functional impairment. The lesioned and cold treated animals, however, presented a preservation of the skeletal muscle cell structure. Our

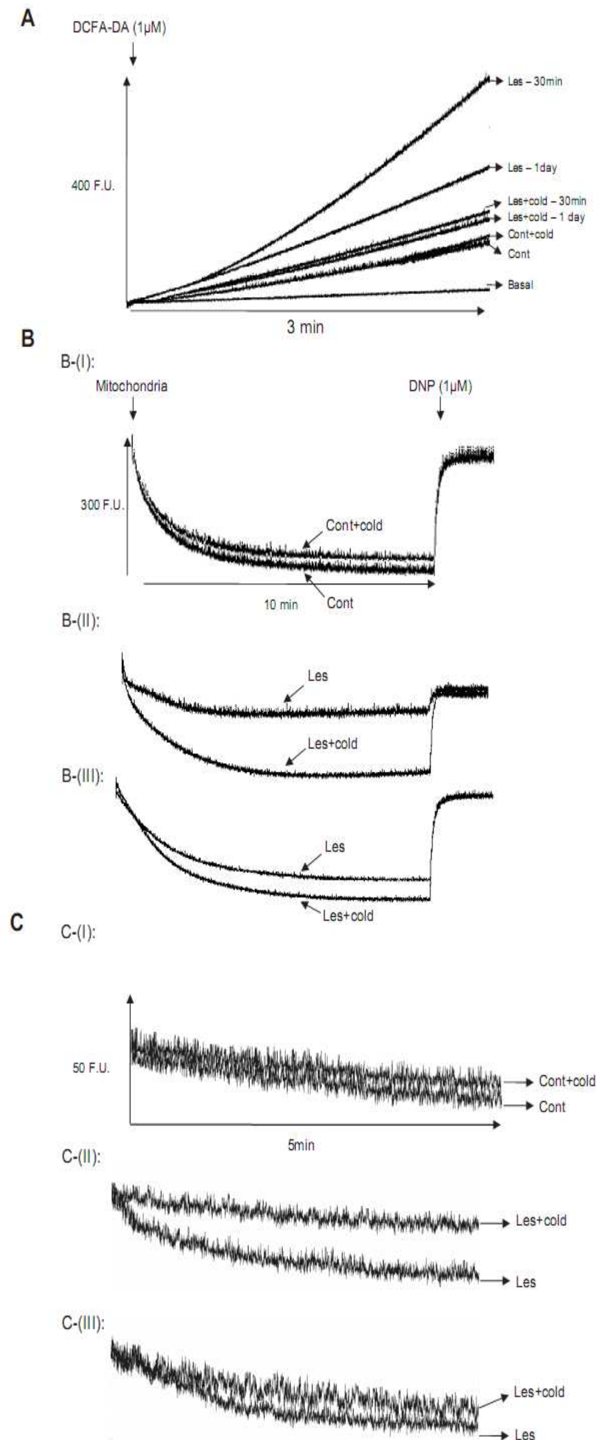

Figure 7. Indicators of the rat skeletal muscle mitochondria functioning: (A) mitochondrial DCF-RS generation; (B) mitochondrial $\Delta\psi$; (C) mitochondrial swelling. The values are presented in fluorescence units (F.U.) as described in the Materials and methods. In (B–D) panel (I) indicates control non-treated and non-lesioned animals mitochondria, panel (II) indicates the mitochondria 30 min after lesion and panel (III) indicates the mitochondria 1 day after lesion. Similar results were obtained with at least three different independent mitochondrial preparations.

results support this hypothesis since the lesioned and cold-treated animals showed a plama CK activity close to the control non-treated and non-lesioned animals (Figure 5A) and did not reveal considerable differences in the histopathological analysis from the control non-treated and non-lesioned animals (Figures 6C and E). Besides, the lesioned and cold-treated animals presented the muscle MTT reduction levels similar to

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the control non-treated and non-lesioned animals (Figure 1C). We believe that this result could also be due to the capacity of the cold to limit the mitochondrial functioning impairment.

Interestingly, while the inflammatory response seems to increase significantly only in the day following the skeletal muscle contusion (Figures 3D and 6C), the oxidative damage and the impairment in the skeletal muscle cell structure was more pronounced a short-time (30 min) after the lesion. In order to understand these results we investigated the involvement of the mitochondrial function in the genesis of these short-time alterations. Moreover, the significant decrease in muscle MTT reduction levels observed in lesioned non-treated animals lead us to suppose the possible involvement of the mitochondrial dysfunction in the genesis of the short-time oxidative damage.

We observed that the mitochondrial DCF-RS generation as well as the mitochondrial swelling were increased in the lesioned non-treated animals and that these effects were higher in the initial moments after the skeletal muscle contusion (Figure 7A). Besides, the mitochondrial $\Delta\Psi$ was decreased at this moment (Figure 7A). According to these results we are able to suppose that the impairment of the mitochondrial membrane integrity and the high mitochondrial RS generation in the lesioned non-treated animals group in comparison to the control non-treated and non-lesioned animals and the lesioned and cold treated animals. Thus, taken together these results corroborate the previous data regarding the lost of skeletal muscle cells integrity (plama CK activity) and the RS formation (DCF-RS and TBARS levels) in analysis developed with skeletal muscle S1. Moreover, we observed that the mitochondrial functioning impairment could be a primary issue responsible for the oxidative damage in the early stages after a skeletal muscle contusion while the inflammatory response following a contusion injury is a secondary issue responsible for the oxidative damage. Regarding the effects of the cold treatment we observed that the mitochondrial swelling, mitochondrial DCF-RS formation and mitochondrial $\Delta\Psi$ of the lesioned and cold-treated animals were similar to those observed in control non-treated and non-lesioned animals. Thus, we believe that the benefits of the cold treatment could be related also to its capacity to modulate the mitochondrial functioning impairment depicted by the skeletal muscle contusion.

It is important to highlight that other mechanisms, beyond the inflammatory response and the mitochondrial impairment, could be involved in the genesis of the oxidative damage that follows a skeletal muscle contusion. Since the skeletal muscle contusion is characterized as a traumatological lesion [2] it is possible to hypothesize that factors such as the ischemia/reperfusion injury could be involved in the oxidative damage genesis. The ischemia/reperfusion injury is an event well known to depict an increase in the reactive oxygen species (ROS) in the injured tissue [48,49]. Furthermore, the mitochondrial functioning is directly involved in the oxidative stress resulting from a ischemia/reperfusion injury [50]. In this context, we believe that our results, which point to the impairment in the mitochondrial functioning as a result of the skeletal muscle contusion, could be related to the changes in oxygen availability to the damaged tissue in response to the ischemia/reperfusion injury. However, more studies are necessary to improve the knowledge regarding the involvement of the ischemia/reperfusion insult in the genesis of the oxidative damage that follows a skeletal muscle contusion.

Concluding, our results depict that the skeletal muscle contusion was followed by significant oxidative damage in the skeletal muscle and in the blood tissues. These oxidative impairments were accompanied by morphological changes in the skeletal muscle cell structure and related to the mitochondrial functioning impairment in the early stage and to the inflammatory response intensity in the late stage after the lesion. The absence of significant differences in the results obtained in the different moments after the lesion could be related with the different mechanisms involved in the genesis of the oxidative damage after the skeletal muscle contusion. Besides, the cold treatment was able to modulate the oxidative damage that follows the contusion injury, possibly by its capacity to limit the inflammatory response and the mitochondrial dysfunction. Furthermore, the benefits of the therapeutic cold could also be linked to its ability to preserve the muscle cell structure against the damage induced by the skeletal muscle contusion. Finally, our results contribute to improve the knowledge regarding the benefits and the mechanisms related with the use of the therapeutic cold as a kind to treat skeletal muscle contusions.

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4.2. MANUSCRITO CIENTÍFICO 1:
BENEFÍCIOS DO FRIO TERAPÊUTICO EM UM EVENTO AGUDO DE ISQUEMIA E REPERFUSÃO (I/R) NO MÚSCULO ESQUELÉTICO DE RATOS

Benefits of therapeutic cold in an acute event of ischemia and reperfusion (I/R) injury in skeletal muscle of rats

Gustavo O. Puntel, Nélson R. Carvalho, Fernando Dobrachinski, Antônio Carlos Galarça, Nilda B. V. Barbosa, Luis F. F. Royes, João B. T. Rocha, Félix A. A. Soares

Submetido para publicação

Benefits of therapeutic cold in an acute event of ischemia and reperfusion (I/R) injury in skeletal muscle of rats

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Abstract

The ischemia and reperfusion (I/R) injury is a condition characterized by an impairment in the arterial blood flow in the injured areas. This injury could be a crucial factor involved in the genesis of the oxidative damage that follows skeletal muscle lesions such as those related to the sportive practice. The major goal of our study was to investigate the potential of the therapeutic cold to preserve the morphological structure and also to modulate the oxidative damage depicted by an acute event of I/R injury in the skeletal muscle tissue of rats. We observed that two major phenomena are directly involved in the genesis of the oxidative metabolism impairment in the skeletal muscle tissue following as acute event of I/R injury, such as the high inflammatory response intensity and the mitochondrial functioning impairment. Moreover, we demonstrated the capacity of the therapeutic cold to preserve the morphological structure and also to modulate the oxidative damage following an acute event of I/R injury. Finally, our results contribute to improve the knowledge regarding the benefits and the mechanisms related with the use of the therapeutic cold as a way to treat skeletal muscle lesions.

Key Words: sportive lesion, oxidative damage, inflammatory response, mitochondrial impairment;

1. Introduction

The ischemia and reperfusion (I/R) injury is a common pathological event characterized by a reduction in the blood flow through the arterial vessels of the injured areas that could affect the skeletal muscle tissue. Among the factors that could result in an I/R injury in the skeletal muscle tissues are the peripheral artery diseases (PADs), which constitute a group of important public health problem [1,2]. Together, the PADs are conditions characterized as chronic circulatory insults that could be accompanied by many consequences to the population, such as the impairment of the ability to walk (claudication) and, in more advanced stages, claudication worsens, nonhealing foot ulcers and gangrene [1,2]. Many published studies have reported the mitochondrial functioning impairment as an important event related with the pathophysiolgy of the PADs [1]. These conditions could result in the compromised performance of the skeletal muscle tissue mitochondria as primary energy producers and regulators of reactive oxygen species (ROS), consequently contributing to a progressive deterioration in muscle function and morphology [3-6].

It is important to note that the I/R injury could also be a crucial factor involved in the genesis of the oxidative damage that follows skeletal muscle lesions such as those related to the sportive practice. Since sportive lesions are generally characterized as traumatological injuries [7], factors such as the capillary rupture and the impairment of the blood flow through the site of the lesion could probably be involved in the pathophysiology of the injury in the skeletal muscle tissues. Therefore, the circulatory insult that follows a skeletal muscle lesion could be characterized as an acute I/R injury that could lead to morphological and oxidative impairments in the skeletal muscle tissue. However, no studies regarding the phenomena involved in the genesis of the oxidative damage that follows an acute event of I/R injury such as those related to common skeletal muscle lesions have been performed until now. On the other hand, common skeletal muscle lesions, such as strain muscle injury [8] and skeletal

muscle contusion [9] were recently reported to depict a significant oxidative damage in the site of the lesion and also in the blood components.

The pathophysiology of the I/R injury is well acknowledged by the impairment in the oxidative metabolism of the involved tissues [10,11]. As a result, an excessive ROS generation could be observed in I/R injury [12,13]. In these conditions the elevated ROS levels could exceed the cellular antioxidant defense system capacity to scavenge these molecules resulting in a condition known as oxidative stress. Moreover, important biological systems, such as those which depend on the sulfhydryl group (-SH) integrity for their normal functioning, could be impaired [14,15]. Some important enzyme activities, such as the lactate dehydrogenase (LDH) [16-18], the delta aminolevunilate dehydratase (Δ-ALA-D) activities [19-21], and the non enzymatic antioxidant glutathione (GSH) levels could also be affected in these conditions [22,23].

The development of therapies that could effectively modulate the oxidative damage resulting from a skeletal muscle damage is of interest. In this context, the use of the therapeutic cold has already been reported to be effective to modulate the oxidative damage that follows a skeletal muscle strain [8] and contusion lesions [9]. Furthermore, the use of the therapeutic cold to minimize the impairment of the oxidative metabolism in the skeletal muscle tissues after an I/R injury has already been described in the literature [24]. Therapeutic cold has been considered one of the most efficient physical agents to treat different skeletal muscle lesions [25,26], but the biochemical mechanisms involved in its protective action have not been enough elucidated yet [8,9]. At least in part, the benefits of the treatment with therapeutic cold could be related to its capacity to limit the inflammatory response intensity, to attenuate the impairment of the mitochondrial function, and also to preserve the skeletal muscle morphology [8,9].

Therefore, the major goal of our study was to investigate the potential of the therapeutic cold to preserve the morphological structure and also to modulate the oxidative damage depicted in the skeletal muscle tissue by an experimental model of an acute event of I/R injury in the hind limb of rats. In this context, we approximate to a pathophysiological condition that follows a common skeletal muscle lesion. In order to construct a body of data that effectively answer this central question we firstly investigated some parameters that could effectively demonstrate the oxidative damage showed by the I/R injury. Subsequently, we investigated the main morphological changes depicted by the I/R injury in the skeletal muscle tissue. Furthermore, we analyzed some mechanisms that could be related with the genesis of the oxidative damage that follows an acute event of I/R injury, such as the inflammatory response intensity and also the mitochondrial functioning impairment. It is important to note that in all the steps of this study, we searched for the benefits of the therapeutic cold.

2. Material and Methods

2.1. Chemical reagents

The reagents thiobarbituric acid (TBA), dicloroflouresceine diacetate (DCFH-DA), methyltetrazolium (MTT), ethylene glycol tetraacetic acid (EGTA), Ellman's reagent (DTNB), and N,N,N',N'-tetramethylbenzidine were supplied by Sigma–Aldrich Chemical Co. (St. Louis, MO). The other used reagents were obtained from local suppliers.

2.2. Animals

Adult male wistar rats weighing 270–320 g from our own breeding colony were kept in cages of 5 animals each, with food and water ad libitum in a room with controlled temperature (22 ± 3°C), and on a 12-h light/dark cycle with lights on at 7:00 am. The animals were maintained and used in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil. The animals were divided into four main groups:

1 – Control non treated and non lesioned animals – animals not submitted to the standard I/R injury;

2 – Control cold treated and non lesioned animals - animals not submitted to the standard I/R injury and treated with the therapeutic cold.

3 – Lesioned non treated animals – animals submitted to the standard I/R injury without any treatment;

4 – Lesioned and cold treated animals – animals submitted to the standard I/R injury and treated with the therapeutic cold;

2.3. Ischemia and reperfusion (I/R) injury

The skeletal muscle I/R injury was developed according to the method proposed by Strock and Majno (1969) [27], with few modifications. Firstly, the animals were anesthetized with ketamine (50 mg/kg; i.p.) and xilazine (10 mg/kg; i.p.). The fully anesthetized animals

were placed in a prone position and the left hind limb was placed to perform the standard I/R injury. The ischemia was performed as an external tourniquet model. Briefly, we used an elastic rubber band that was tensioned in the proximal portion of the thigh near the hip junction of the rat in order to obliterate completely the blood flow distally to the tourniquet. The obliteration of the blood flow was observed by the clinical signs in the hind limb, such as the absence of the arterial pulse and the cyanosis. The ischemia was maintained for 3 hours. During all the ischemia period the animals were maintained in fully anesthetized condition and under treatment with the cold (to see the therapeutic cold treatment section). Thereafter, the tourniquet was removed in order to start the reperfusion period and the cold treatment was finished. The reperfusion period was maintained for 2 hours and then the animals were euthanized in order to remove the muscle to perform the histophatological and the biochemical analysis.

2.4. Therapeutic cold treatment

The treatment of the animals with therapeutic cold was performed by the application of ice pieces placed into a malleable bag of ice in order to cover the entire hind limb that was submitted to the standard I/R injury [24]. The treatment section was developed for 3 hours during all the ischemia period.

2.5. Biochemical analysis

Biochemical analysis were performed immediately after the animal euthanasia in order to check the biochemical changes indicative of oxidative damage depicted by the I/R injury, as well as the effects of the treatment with therapeutic cold under these conditions.

2.5.1. Tissue preparation

Skeletal muscle homogenates

For the determination of some of the oxidative damage markers and also the enzyme activity measurement, the left gastrocnemius muscle was removed, quickly homogenized in

sodium chloride solution (NaCl 150 mM), and kept in ice. After the homogenization, skeletal muscle samples were centrifuged at 4,000 x *g* at 4^oC for 10 min to yield a low speed supernatant fraction (S1). The obtained S1 was used to analyze some oxidative stress and cell viability indexes, such as thiobarbituric acid reactive substances (TBA-RS), dichlorofluorescein oxidized by reactive substances (DCF-RS), non-protein -SH, and MTT level determination, as well as for catalase (CAT), superoxide dismutase (SOD), calcium (Ca^{2+}) ATPase, and LDH enzyme activity determination.

For myeloperoxidase (MPO) enzyme activity measurement, the muscle samples were homogenized in potassium phosphate buffer (20mM, pH 7.4) containing EDTA (0.1mM). After the homogenization, the skeletal muscle samples were centrifuged at 2,000 x *g* at 4^oC for 10 min to yield a low speed supernatant fraction (S1). Then, the S1 fraction was centrifuged again at 20,000 x *g* at 4^oC for 15 min to yield a final pellet that was ressuspended in potassium phosphate buffer (50mM, pH 6.0) containing hexadecyltrimethylammonium bromide (0.5%). The samples were finally freeze-thawed two times for the posterior enzymatic MPO assay. Moreover, aliquots of skeletal muscle preparations were frozen (-20°C) for posterior analysis.

Isolation of skeletal muscle mitochondria

Rat skeletal muscle mitochondria were isolated as described by Tonkonogi e Salhin (1997) [28] with some modifications. Firstly, the left gastrocnemius muscle was quickly removed and homogenized in a buffer containing mannitol (225mM), sucrose (75mM), ethylene glycol tetraacetic acid (EGTA 1mM), bovine serum albumin (BSA) (0.1%), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES 10mM, pH 7.2). After the homogenization, the resulted suspension was centrifuged for 7 min at 2,000 *g* in order to obtain a low speed supernatant fraction (S1). Then, S1 was re-centrifuged for 10 min at 12,000 *g*. The obtained pellet was re-suspended in a buffer containing mannitol (225mM),

sucrose (75mM), EGTA (1mM) and HEPES (10mM, pH 7.2), and re-centrifuged at 12,000 *g* for 10 min. The supernatant was decanted and the final pellet re-suspended in a buffer containing KCl (65mM), sucrose (100mM), EGTA (0.05mM), BSA (0.2%) and HEPES (10mM, pH 7.2) to yield a protein concentration of 30-40 mg/mL. The isolated mitochondria were used to perform the analysis of some indicators of the skeletal muscle mitochondrial function, such as the mitochondrial DCF-RS, the mitochondrial membrane potential ($\Delta\Psi$) determination, the manganese superoxide dismutase (MnSOD) enzyme activity, and the mitochondrial reduced glutathione and oxidized glutathione ratio (GSH/GSSG) levels.

2.5.2 Oxidative stress markers and cell viability determination

TBA-RS levels

Analyses were performed in skeletal muscle S1 samples according to the method described by Ohkawa et al. (1979) [29]. Aliquots of 200 µL of skeletal muscle S1 was added to color reaction. TBA-RS levels were measured at 532 nm using a standard curve of malondialdehyde (MDA) and corrected by the protein content.

DCF-RS levels

DCF-RS levels were determined as an index of the peroxide production by the cellular components [30]. Aliquots of skeletal muscle S1 (50 µL) were added to a medium containing Tris-HCl buffer (0.01 mM; pH 7.4) and 2',7'-dichlorofluorescein diacetate (DCFH-DA 7 µM). After DCFH-DA addition, the medium was incubated in the dark for 1 h until fluorescence measurement procedure (Excitation at 488 nm and Emission at 525 nm, and both slit widths used were at 5 nm). DCF-RS levels were determined using a standard curve of oxidized dichlorofluorescein (DCF) and the results were corrected by the protein content [31].

Non protein -SH levels

Levels of non protein –SH were determined in skeletal muscle S1 samples according to the method proposed by Ellman (1952) [32] with some modifications. Briefly, the samples

of the skeletal muscle S1 (1mL) were precipitated with trichloroacetic acid (TCA 5%) (0.5mL) and subsequently centrifuged at 4,000 *g*. for 10 min. After the centrifugation, the supernatant fraction (500µL) was added to a reaction medium containing potassium ion (K^+) phosphate (0.25mM and pH=7.4) and DTNB (1mM). Non protein –SH levels were measured spectrophotometrically at 412 nm. Results were calculated in relation to a standard curve constructed with reduced glutathione (GSH) at known concentrations and also corrected by the protein content [32].

MTT reduction levels

MTT reduction levels were determined as an index of the dehydrogenase enzyme functions, which are involved in the cellular viability [33]. Aliquots of skeletal muscle S1 (500µL) were added to a medium containing 0.5mg/mL of MTT and were incubated in the dark for 1 h at 37ºC. The MTT reduction reaction was stopped by the addition of 1mL of dimethylsulfoxide (DMSO). The formed formazan levels were determined spectrophotometrically at 570 nm and the results were corrected by the protein content [34].

2.5.3. Enzyme activity determination

CAT activity

The CAT enzyme activity was determined in skeletal muscle S1 according to the method proposed by Aebi (1984) [35]. Briefly, S1 aliquot (50 µL) was added to a medium containing potassium phosphate buffer (50 mM; pH 7.4) and hydrogen peroxide (H_2O_2 1 mM). The kinetic analysis of CAT was started after H_2O_2 addition, and the color reaction was measured at 240 nm.

SOD activity

The SOD enzyme activity was determined in skeletal muscle S1 according to the method proposed by Misra and Fridovich (1972) [36]. This method is based on the capacity of SOD in inhibiting autoxidation of adrenaline to adrenochrome. Briefly, different S1 aliquots

(10-50μL) were added to a medium containing glycine buffer (50 mM; pH 10.5) and adrenaline (1mM). The kinetic analysis of SOD was started after adrenaline addition, and the color reaction was measured at 480 nm.

Ca^{2+} ATPase activity

The Ca^{2+} ATPase enzyme activity was determined in skeletal muscle S1 samples according to the method proposed by Zaidi and Michaelis (1999) [37], with some modifications. Briefly, the aliquots of skeletal muscle S1 (20μL) were added to a reaction medium containing magnesium chloride ($MgCl_2$ 1mM), potassium chloride (KCl 50mM), EGTA (0.2mM) and Tris-HCl buffer (25mM and pH 7.4), with or without the calcium chloride ($CaCl_2$ 150μM) in order to ensure a final concentration of 1μM of calcium ion (Ca^{2+}) in the medium. The method for ATPase activity measurement was based on the determination of the inorganic phosphate (Pi) released to the reaction medium by the hydrolysis of the adenosine triphosphate (ATP) according to the method proposed by Atkinson et al. (1973) [38]. The reaction was initiated with the addition of the substrate ATP (1.5mM) to the reaction medium and was finished by the addition of the color reagent (1mL) containing ammonium molibdate (2%), triton-100X (5%) and sulfuric acid (H_2SO_4 1.8M - 10%) after 15 min of incubation at 37ºC. The formed molibdate-Pi complexes were measured spectrophotometrically at 405nm. Values were calculated in relation to a standard curve constructed with Pi at known concentrations and also corrected by the protein content.

LDH activity

The LDH enzyme activity was determined spectrophotometrically in skeletal muscle S1 samples as an index of the oxidative damage to this tissue using diagnosis kits (LDH Liquiform, Labtest, MG, Brazil).

MPO activity

The MPO enzyme activity was determined in skeletal muscle samples obtained as described previously in the section tissue preparations in the subheading skeletal muscle homogenates, according to the method proposed by Grisham et al. (1986) [39], with some modifications. Briefly, a sample of the skeletal muscle preparation (20 µL) was added to a medium containing potassium phosphate buffer (50 mM; pH 6.0), hexadecyltrimethylammonium bromide (0.5%), and N,N,N',N'-tetramethylbenzidine (1.5mM). The kinetic analysis of MPO was started after H_2O_2 (0.01%) addition, and the color reaction was measured at 655nm at 37ºC.

2.5.4. Indicators of the skeletal muscle mitochondria function

Mitochondrial DCF-RS level determination

The mitochondrial DCF-RS generation was assayed according to Garcia-Ruiz et al. (1997) [40]. Briefly, the skeletal muscle mitochondria samples (150µg of protein per mL) were incubated in a medium containing KCl (65mM), sucrose (100mM), EGTA (0.05mM), bovines serum albumin (BSA) (0.2%), HEPES (10mM, pH 7.2), and the respiratory substrate glutamate (5mM) and succinate (5mM). The reaction was started with the DCFA-DA (1µM) addition, and the medium was kept at constant stirring during the assay period. The fluorescence analysis was performed at 488 nm for excitation and 525 nm for emission, with slit widths of 5nm.

Mitochondrial $\Delta\Psi$ determination

The mitochondrial $\Delta\Psi$ determination was assayed according to Akerman and Wikstron (1976) [41]. Briefly, the skeletal muscle mitochondria samples (150µg protein / mL) were incubated in a medium containing KCl (65mM), sucrose (100mM), EGTA (0.05mM), BSA (0.2%), HEPES (10mM, pH 7.2), safranine O (10µM), and the respiratory substrates glutamate (5mM) and succinate (5mM). The reaction was started with the mitochondria addition and the medium was kept at constant stirring during the assay period. The

fluorescence analysis was performed at 495 nm for excitation and 586 nm for emission, with slit widths of 5nm.

Mitochondrial MnSOD activity

The mitochondrial MnSOD enzyme activity was determined in skeletal muscle isolated mitochondria according to the method proposed by Misra and Fridovich (1972) [36]. Briefly, aliquots of 100µL of isolated mitochondria were added to a medium containing sodium bicarbonate-carbonate buffer (50mM; pH 10.2), EDTA (2mM) and adrenaline (0.4mM). The kinetic analysis of SOD was started after adrenaline addition and the color reaction was measured at 480nm.

Mitochondrial GSH and GSSG levels

Mitochondrial GSH and GSSG levels were determined according to Hissin and Hilf [42] with some modifications. Briefly, skeletal muscle isolated mitochondria (150µg protein/mL) were resuspended in 1.5ml sodium-phosphate buffer (100mM NaH_2PO_4, 5mM EDTA, pH 8.0) and 500µl of phosphoric acid (H_3PO_4) 4.5%, and were centrifuged at 100.000*g* for 30 min. For GSH determination, 100µl of the supernatant resulting from the centrifugation was added to 1.8ml phosphate buffer and 100µl *o*-phthalaldehyde (OPT). After 15 min, the solution was transferred to a quartz cuvette and the fluorescence was measured at 420nm for emission and 350nm for excitation, with slit widths of 3nm. For oxidized glutathione (GSSG) determination, 250µl of the supernatant resulting from the centrifugation was added to 100µl of N-ethylmaleymide and incubated at room temperature for 30 min. After the incubation, 140µl of the mixture was added to 1.76ml sodium hydroxide (NaOH 100mM) solution and 100µl OPT. After 15 min, the solution was transferred to a quartz cuvette and the fluorescence was measured at 420nm for emission and 350nm for excitation, with slit widths of 3nm. GSH and GSSG levels were determined from comparisons with a linear GSH or GSSG standard curve, respectively.

2.5.5. Protein determination

The protein content was determined according to Lowry et al. (1951) [43] using bovine serum albumin (BSA) as standard.

2.6. Histopathological analysis

One sample of skeletal muscle tissue was used for the histopathological analysis in order to investigate microscopic changes in the normal tissue structure. We investigated the loss of the normal skeletal muscle cell architecture. Moreover, the presence of neutrophils was examined as an indicator of the acute inflammatory infiltration extension. After excised, the skeletal muscle was maintained in buffered formaldehyde solution (10%) until the microscopic preparation and colorization. The muscle samples were sectioned longitudinally along their proximal and distal origins. The histological slides were stained with hematoxylin and eosin in order to analyze the main morphological changes in the skeletal muscle tissue architecture. Moreover, we performed the Giemsa's staining in order to underline the presence of inflammatory cells infiltrated among the skeletal muscle cells.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan test. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Effects of the cold treatment under markers of the oxidative damage and cell viability in the site of the lesion

Figure 1 (A-C) depicts the potential of the therapeutic cold in modulating the increased levels of some oxidative stress markers in the skeletal muscle tissue submitted to the I/R injury. The increased DCF-RS and TBA-RS levels in the lesioned and non treated animals were significantly abolished by the therapeutic cold treatment (Figure 1A and 1B, respectively) ($p \leq 0.05$). Moreover, the decrease in the cell viability presented by the lesioned and non treated animals, measured through the MTT reduction analysis, was also completely restored by the therapeutic cold treatment (Figure 1C) ($p \leq 0.05$).

Likewise, Figure 2A shows the role of the therapeutic cold treatment under the non protein –SH levels. Our data point to a significant decrease in the non protein –SH group levels in the skeletal muscle tissue of the lesioned and non treated animals (Figure 2A) ($p \leq 0.05$). Furthermore, the cold treatment presented the potential to maintain the non protein –SH levels at control values (Figure 2A).

3.2. Effects of the cold treatment under enzyme activities in the site of the lesion

The analysis of some enzymatic antioxidant systems in the skeletal muscle tissue revealed that the I/R injury depicted a significant decrease in CAT enzyme activity in the lesioned and non treated animals (Figure 2B) ($p \leq 0.05$). On the other hand, the animals submitted to the cold treatment presented levels of CAT enzyme activity similar to the control non lesioned and non treated animals (Figure 2B). The SOD enzyme activity in the skeletal muscle was not significantly changed by the cold treatment or by the I/R injury (Figure 2C).

Data presented in Figure 3 revealed that the therapeutic cold treatment was able to reduce the impairment in Ca^{2+}ATPase enzyme activity which was observed in the lesioned and non treated animals (Figure 3A) ($p \leq 0.05$). Furthermore, the significant increase in the

skeletal muscle LDH enzyme activity presented by the lesioned and non treated animals was appropriately modulated by the therapeutic cold treatment (Figure 3B) ($p \leq 0.05$).

The skeletal muscle MPO enzyme activity was significantly increased in the lesioned and non treated animals and the cold treatment was able to modulate this increase (Figure 4).

3.3. Effects of the cold treatment under skeletal muscle mitochondria function

Figure 5A shows that the cold treatment was able to modulate significantly the increase in the mitochondrial DCF-RS generation which was depicted by the I/R injury (Fig 5A) ($p \leq 0.05$). Likewise, the cold treatment presented also a capacity to maintain the skeletal muscle mitochondrial $\Delta\Psi$, which was significantly impaired in the lesioned and non treated animals, similar to the values observed in control conditions (Figure 5B) ($p \leq 0.05$). Data presented in Figure 5C show the capacity of the therapeutic cold to modulate the increase in mitochondrial MnSOD enzyme activity which was significantly increased in the lesioned and non treated animals ($p \leq 0.05$). Furthermore, both the GSH and GSSG levels and the GSH/GSSG ratio were preserved in the lesioned and cold treated animals in contrast with the significant impairment depicted in the lesioned and non treated animals (Fig 5D) ($p \leq 0.05$).

3.4. Effects of the cold treatment under morphological changes in the site of the lesion

The histopathological analysis depicted the capacity of the therapeutic cold treatment to minimize the morphological changes induced by the I/R injury (Figures 6A-III and 6B-III). A pronounced increase in the neutrophils infiltration was observed after the I/R injury (Figures 6A-III and 6B-III). Moreover, the swelling of the skeletal muscle tissue with several vacuoles space formation among the muscular fasciculus was also evident. It is important to note that both the neutrophils infiltration and the swelling of the skeletal muscle tissue were appropriately counteracted by the cold treatment (Figures 6A-IV and 6B-IV). The therapeutic

cold did not exert any apparent morphological changes in the skeletal muscle tissue structure when applied in control non lesioned animals (Figures 6A-II and 6B-II).

4. Discussion

The central question of our study was to research the therapeutic cold potential to modulate the oxidative damage and also to preserve the morphological changes depicted in the skeletal muscle tissue by an experimental model of an acute event of I/R injury in the hind limb of rats. The major reason to design this study protocol was that these acute events of I/R injury could be directly involved in the phenomena that underline the pathophysiology of the most common skeletal muscle lesions. We observed an evident potential of the therapeutic cold to modulate the increased oxidative damage and also to limit the histopathological changes in the skeletal muscle tissue submitted to an experimental model of I/R injury. In this way, our results are in agreement with our previously published studies that reported the therapeutic cold to be effective to modulate the oxidative damage that follows a skeletal muscle strain lesion [8], as well as the morphological changes and the oxidative damage that follows a skeletal muscle contusion lesion [9].

It is important to point out that previews studies have already reported the impairment in the oxidative metabolism of the skeletal muscle tissue after an acute event of I/R injury [12,13,44]. However, the understanding of the phenomena involved in the genesis of the oxidative damage that follows these acute events of I/R injury is unclear until now. Our results clearly demonstrated the morphological changes in the skeletal muscle structure after the I/R injury such as the excessive inflammatory cell infiltration among the muscular fasciculus (Figure 6B-III). These morphological changes could be linked with the increase observed in skeletal muscle MPO enzyme activity (Figure 4). Thus, it is possible to infer that the inflammatory response intensity is an important phenomenon involved in the genesis of the skeletal muscle functional impairment that follows an acute event of I/R injury. In line with this, we previously depicted the relationship between the presence of a large amount of neutrophil cell infiltration and the increased MPO enzyme activity in the skeletal muscle

tissue after a skeletal muscle contusion [9]. Moreover, we observed the impairment in the striated structure of the skeletal muscle tissue with the consequent formation of many vacuoles among the muscular fibers (Figures 6A-III and 6B-III).

Another important phenomenon that is suitable to be involved in the genesis of the oxidative damage that follows an acute event of I/R injury is the mitochondrial functioning impairment. In fact, there is a considerable number of studies that have already described the mitochondrial functioning impairment involvement in the pathophysiology of chronic events of I/R injury, such as those related with the PADs [1-6]. Nevertheless, the relationship between the mitochondrial functioning impairment and the pathophysiology of an acute event of I/R injury is unclear until now. Here, our results suggest that the mitochondrial functioning impairment is a fundamental phenomenon related to the genesis of the oxidative damage that follows an acute event of I/R injury in the skeletal muscle tissue. We observed a significant increase in the mitochondrial DCF-RS generation (Figure 5A) as well as a significant impairment in the mitochondrial $\Delta\Psi$ (Figure 5B) of the lesioned and non treated skeletal muscle mitochondria. Moreover, our results showed a significant increase in the mitochondrial MnSOD activity (Figure 5C) and also a significant decrease in mitochondria GSH/GSSG ratio in the lesioned and non treated animals (Figure 5D). In this context, it is possible to hypothesize that the increased activity of the mitochondrial MnSOD activity could be understood as a mechanism to counteract the increased superoxide anion (O^-_2) production. As a result of the increased SOD activity an augmented amount of H_2O_2 could be expected. Thus, the increased consumption of GSH as a mechanism to counteract this augmented amount of H_2O_2 via glutathione peroxidase (GPx) enzyme activity and a consequent change in the mitochondrial GSH/GSSG ratio could be expected. These oxidative changes observed in the skeletal muscle mitochondria could be involved in the genesis of the mitochondrial functional impairment such as the increased DCF-RS production and the impairment in the

$\Delta\Psi$. Therefore, the involvement of the mitochondrial functioning impairment in the genesis of an acute event of I/R injury was similar to the results observed by our research group after a skeletal muscle contusion [9]. Thus, we could appropriately hypothesize that an acute event of I/R injury could be a suitable condition involved in the genesis of the functional impairments that go together with a common skeletal muscle lesion.

Therefore, both the uncontrolled inflammatory response and the mitochondrial functioning impairment observed in lesioned and non treated animals could be directly related with the higher DCF-RS and TBA-RS. It is important to note that previous studies have already demonstrated that an uncontrolled inflammatory response [45,46] as well as the mitochondrial functioning impairment [47-50] could result in the excessive ROS formation in the injured tissue. Although we did not measure specifically the ROS formation in the skeletal muscle tissue following an acute I/R injury, it is well known that an excessive amount of ROS could not only supply the DCF-RS formation but also contribute to start a complex cascade of reactions, which culminate with the lipid peroxidation (increase in TBA-RS levels). We have previously reported that an uncontrolled inflammatory response and the mitochondrial functioning impairment are important features and could be appropriately related with the oxidative damage genesis that follows a skeletal muscle contusion [9].

Some important functional impairment was observed in the skeletal muscle tissue following an acute event of I/R injury, which was probably related with the excessive ROS production. In fact, we observed a significant decrease in the Ca^{2+} ATPase enzyme activity, which is an important enzyme reported to depend on the –SH group integrity to be functionally active [17,51]. The involvement of the –SH group oxidation in the genesis of the skeletal muscle tissue functional impairments was corroborated by our results which show a significant decrease in the non protein –SH levels (glutathione as major compound). It is also interesting to note that the lesion caused a significant increase in the skeletal muscle CAT

enzyme activity. This condition may be related to a compensatory response of the skeletal muscle tissue due to an increased H_2O_2 production. More than the oxidative metabolism impairment, our results lead us to put forward that an acute event of I/R injury to the skeletal muscle tissue could determine a significant damage to the structure of the muscle cells, and then affect their viability. Lesioned non treated animals exhibited an evident morphological change in the skeletal muscle structure such as the lost of the muscular fasciculus integrity with a large amount of vacuoles space formation. Since the integrity of the skeletal muscle cell structure is important to the cell survival, the morphological damage induced by the I/R injury could also depict a reduction in the skeletal muscle cell viability [52,53]. This hypothesis is in accordance with our findings that showed a significant decrease in muscle MTT reduction levels in the lesioned non treated animals (Figure 1D). The MTT reduction depends on the adequate functionality of the oxidoreductase enzyme family, such as the dehydrogenase enzymes [54]. Since the majority of these enzymes are located in the mitochondria [33,56], their functional impairment could be related to the mitochondria functional impairment and the MTT reduction levels could be usually used as an index of the cellular viability [55,56]. Taken together, these results are in accordance with the functional impairments observed in the skeletal muscle tissue after a skeletal muscle contusion [9], which improve the hypothesis that an acute event of I/R injury is a suitable condition involved in the genesis of the functional impairments that go together with a common skeletal muscle lesion.

On the other hand, the capacity of the therapeutic cold to minimize the functional impairment of the skeletal muscle tissues after an acute event of I/R injury presented here, is in agreement with previous published studies [24]. In fact, we observed that the therapeutic cold was able to modulate the two main phenomena related to the oxidative damage in the skeletal muscle tissue following an acute event of I/R injury, which were previously described

by us. In this context, we show the potential of the therapeutic cold to modulate the inflammatory response intensity. This fact is evident in lesioned and cold treated animals due to the absence of significant amounts of the neutrophils cell infiltration among the muscular fasciculus and also due to the fact that skeletal muscle MPO enzyme activity is not significantly different from the observed in control non lesioned and non treated animals. Furthermore, the cold therapy was effective in modulating the mitochondrial functioning impairment, which seems to be one of the most important phenomena related to the oxidative damage in the skeletal muscle tissue following an acute event of I/R injury. As a result, the therapeutic cold was able to appropriately modulate the increase of the oxidative damage markers in the skeletal muscle tissue, such as the DCF-RS and the TBA-RS levels. Additionally, the functional impairment observed in the skeletal muscle tissue following an acute event of I/R injury was adequately decreased in the lesioned and cold treated animals. This fact was observed by the maintenance of the skeletal muscle Ca^{2+}ATPase and CAT enzyme activities, as well as the non protein –SH group and MTT reduction levels near the control non lesioned and non treated animal levels. Similar benefits of the therapeutic cold were observed in the treatment of common skeletal muscle lesions such as the skeletal muscle strain [8] and contusion lesion [9]. Therefore, our results lead us to hypothesize that the potential of the therapeutic cold to modulate the oxidative damage that follows an acute event of I/R injury could be directly related to its capacity to limit the oxidative damage that follows a skeletal muscle lesion.

In conclusion, we observed that two major phenomena are directly involved in the genesis of the oxidative metabolism impairment in the skeletal muscle tissue following as acute event of I/R injury. One of the phenomena was high inflammatory response intensity. The other phenomenon was the mitochondrial functioning impairment. It is important to underline that this is the first time that the mitochondrial functioning impairment was reported

to be involved in the genesis of the oxidative damage that follows an acute event of I/R injury. Therefore, according to the results observed in the present study the I/R injury may be a suitable condition involved in the genesis of the functional impairments that go together with a common skeletal muscle lesion. Moreover, we demonstrated the capacity of the therapeutic cold to modulate these two main phenomena involved in the genesis of the oxidative damage that follows an acute event of I/R injury in the skeletal muscle tissue as depicted here. Thus, we believe that the central question of our study was appropriately answered since our findings clearly revealed the potential of the therapeutic cold to preserve the morphological structure and also to modulate the oxidative damage depicted in the skeletal muscle tissue by an experimental model of an acute event of I/R injury in the hind limb of rats. Finally, our results contribute to improve the knowledge regarding the benefits and the mechanisms related with the use of the therapeutic cold as a way to treat skeletal muscle lesions.

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7. List of Abbreviations

ATP – Adenosine thriphosphate;

ANOVA – Analysis of variance;

BSA – Bovine serum albumin;

CAT – Catalase;

Ca^{2+} - Calcium ions;

Ca^{2+} ATPase – Calcium ATPase;

$CaCl_2$ – Calcium chloride;

DCF – Oxidized dichlorofluorescein

DCFA-DA - 2',7'-dichlorofluorescein diacetate;

DCF-RS – dichlorofluorescein oxidized by reactive substances;

DMSO – dimethylsulfoxide;

DTNB – Ellman's reagent;

EDTA – Ethylenediamine tetraacetic acid;

EGTA – ethylene glycol tetraacetic acid;

GSH – Reduced glutathione;

GSH/GSSG – Reduced glutathione and oxidized glutathione ratio;

GSSH – Oxidized glutathione;

HCl – Chloridric acid;

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

H_2O_2 – Hydrogen peroxide;

H_2SO_4 – Sulfuric acid;

H_3PO_4 – phosphoric acid;

I/R – ischemia and reperfusion

K^+ - Potassium ion;

KCl – Potassium chloride;

LDH – Lactate dehydrogenase

MDA – Malondialdehyde;

$MgCl_2$ – Magnesium chloride;

MnSOD –Mangasese superoxide dismutase;

MPO – Myeloperoxidase;

MTT – Methyltetrazolium;

NaCl – Sodium chloride;

NaH_2PO_4 – sodium-phosphate buffer;

NaOH – Sodium hydroxide;

OPT – *o*-phthalaldehyde;

PADs – peripheral artery diseases;

Pi – Inorganic phosphate;

ROS – Reactive oxygen species;

-SH – Sufhydryl group

SOD – superoxide dismutase;

S1 – Low speed supernatant fraction;

TBA – Thiobarbituric acid

TBA-RS – Thiobarbituric acid reactive substances;

TCA – Trichloroacetic acid;

Δ-ALA-D – delta aminolevunilate dehydratase;

$\Delta\psi$ – Membrane potential;

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9. Figures legends

Figure 1: Oxidative damage and cell viability in the site of the lesion: (**A**) DCF-RS levels; (**B**) TBARS levels; (**C**) MTT reduction levels. In 1A the DCF-RS levels are expressed in μmol of DCF-oxidized/mg of protein; in 1B the TBARS levels are expressed in μmol of MDA/mg of protein; in 1C the MTT reduction levels is expressed in % of the control values. Data are expressed as mean ± S.E. (n=6-8) and were analyzed by ANOVA, followed by Dunkan test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as ^{a} or ^{b}.

Figure 2: Non enzymatic and enzymatic systems activities in the site of the lesion: (**A**) non protein –SH levels; (**B**) CAT activity; (**C**) SOD activity. In 2A the non protein –SH levels are expressed in nmol of SH/mg of protein; in 2B the CAT activity is expressed in μmol H_2O_2/minute/mg of protein; and in 2C the SOD activity is expressed in units of absorbance/mg of protein. Data are expressed as mean ± S.E. (n=6-8) and were analyzed by ANOVA, followed by Duncan test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as ^{a} or ^{b}.

Figure 3: Functional enzymes activities in the site of the lesion: (**A**) Ca^{2+}ATPase activity levels; (**B**) LDH activity levels. In 3A Ca^{2+}ATPase activity levels is expressed in nmol of Pi/minute/mg of protein; in 3B the LDH activity is expressed in % of the control values (the mean value of control LDH activity was 46.9±10.7 units/mg of protein). Data are expressed as mean ± S.E. (n=6-8) and were analyzed by ANOVA, followed by Duncan test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as ^{a} or ^{b}.

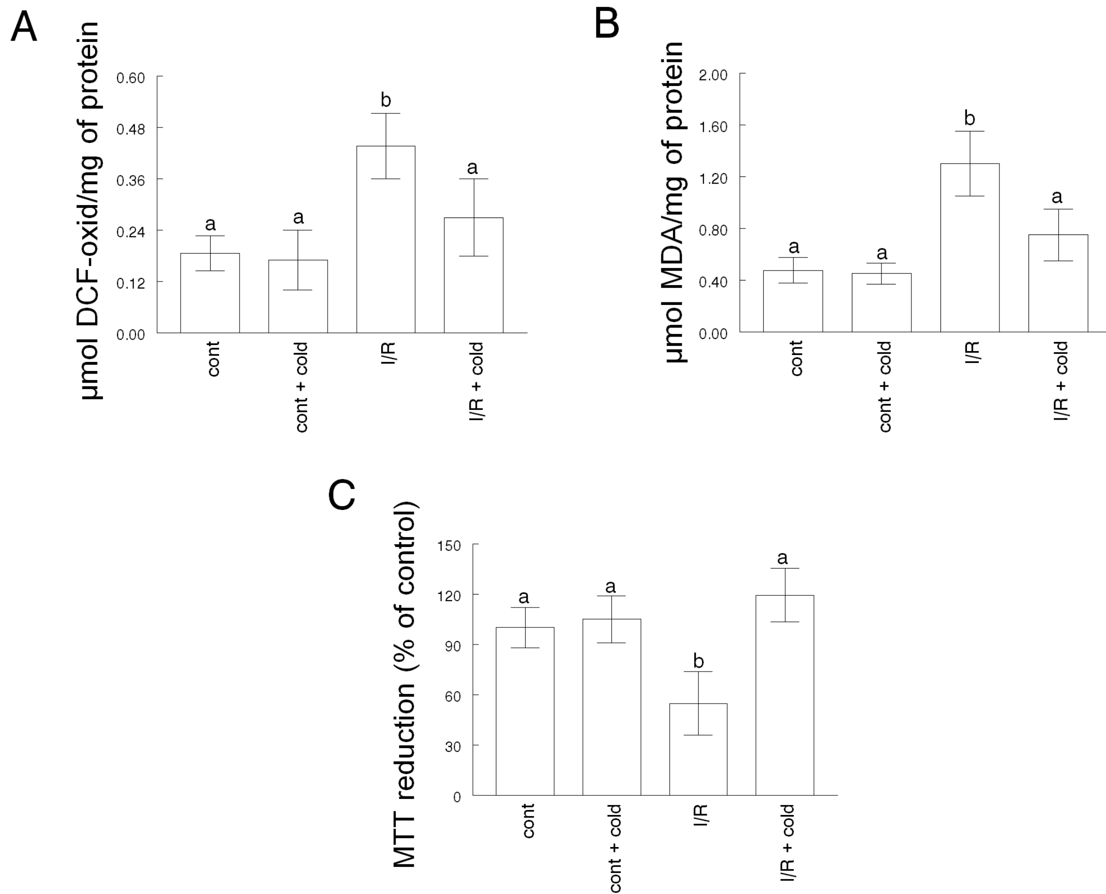
Figure 4: Skeletal muscle MPO activity: The MPO activity is expressed in absorbance variation unites (delta ABS) per mg of protein. Data are expressed as mean ± S.E. (n=6-8) and

were analyzed by ANOVA, followed by Duncan test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [a] or [b].

Figure 5: Indicators of the rat skeletal muscle mitochondria functioning: (A) mitochondrial DCF-RS generation; **(B)** mitochondrial $\Delta\psi$; **(C)** mitochondrial MnSOD activity; **(D)** mitochondrial GSH (I), GSSG(II), and GSH/GSSG ratio (III) levels. In Fig 5A-B the values are presented in fluorescence units (F.U.) according to described in material and methods. In 5A-B a representative graph illustrating a single experimental trial and a graph showing the results of 6-8 independent experiments are presented. In 5C the values are presented in absorbance variation unites (delta ABS) per mg of protein. In 5D the GSH (I) levels were expressed in nmol GSH/mg of protein, the GSSH (II) levels were expressed in nmol GSSG/mg of protein. For each experimental trial an independent and fresh mitochondrial preparation was performed. In Fig 5A-D the data are expressed as mean ± S.E. (n=6-8) and were analyzed by ANOVA, followed by Duncan test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as ^{a} or ^{b}.

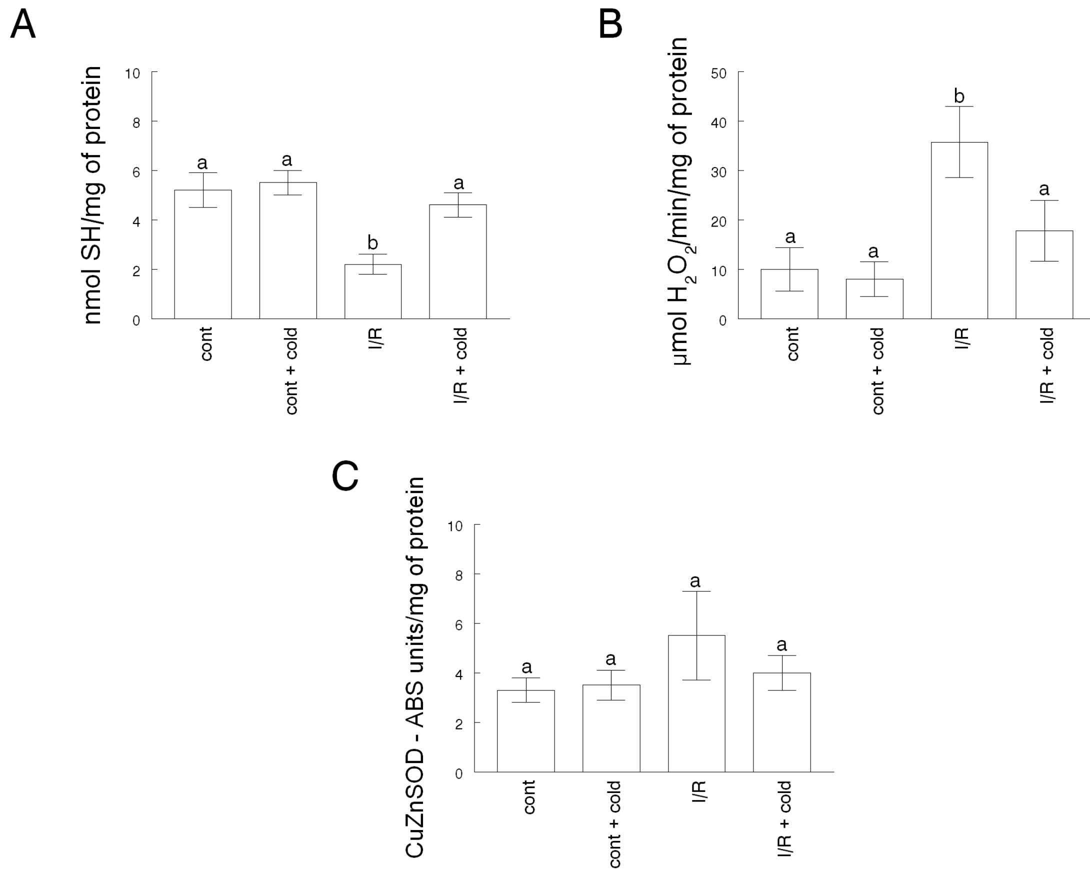
Figure 6: Histopathologic changes in skeletal muscle: The neutrophils infiltrations areas (arrow), as well as the impaired skeletal muscle cells striations areas (sharp) were identified: In 6A the skeletal muscle slides were prepared with the hematoxiline/eosine staining; and in 6B with the Giemsa's staining. In all cases, I – control non treated and non I/R lesioned muscle; II – control cold treated and non I/R lesioned muscle; III – I/R lesioned and non treated skeletal muscle; and III – I/R lesioned and cold treated skeletal muscle. In all cases the images were 400 times increased.

Figure 1:



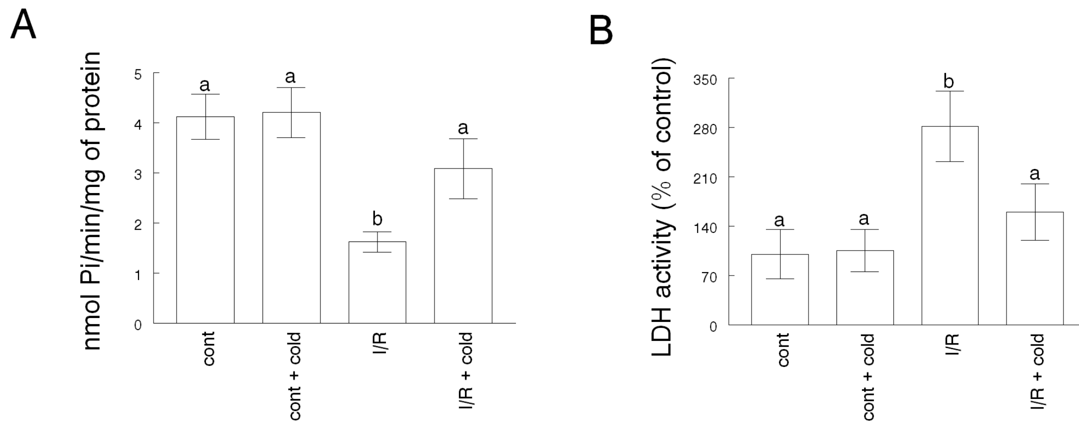
Oxidative damage and cell viability in the site of the lesion: (**A**) DCF-RS levels; (**B**) TBARS levels; (**C**) MTT reduction levels. In 1A the DCF-RS levels are expressed in µmol of DCF-oxidized/mg of protein; in 1B the TBARS levels are expressed in µmol of MDA/mg of protein; in 1C the MTT reduction levels is expressed in % of the control values. Data are expressed as mean ± S.E. (n=6-8) and were analyzed by ANOVA, followed by Dunkan test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [a] or [b].

Figure 2:



Non enzymatic and enzymatic systems activities in the site of the lesion: (**A**) non protein –SH levels; (**B**) CAT activity; (**C**) SOD activity. In 2A the non protein –SH levels are expressed in nmol of SH/mg of protein; in 2B the CAT activity is expressed in µmol H_2O_2/minute/mg of protein; and in 2C the SOD activity is expressed in units of absorbance/mg of protein. Data are expressed as mean ± S.E. (n=6-8) and were analyzed by ANOVA, followed by Duncan test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [a] or [b].

Figure 3:



Functional enzymes activities in the site of the lesion: (**A**) Ca^{2+}ATPase activity levels; (**B**) LDH activity levels. In 3A Ca^{2+}ATPase activity levels is expressed in nmol of Pi/minute/mg of protein; in 3B the LDH activity is expressed in % of the control values (the mean value of control LDH activity was 46.9±10.7 units/mg of protein). Data are expressed as mean ± S.E. (n=6-8) and were analyzed by ANOVA, followed by Duncan test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as ^{a} or ^{b}.

Figure 4:

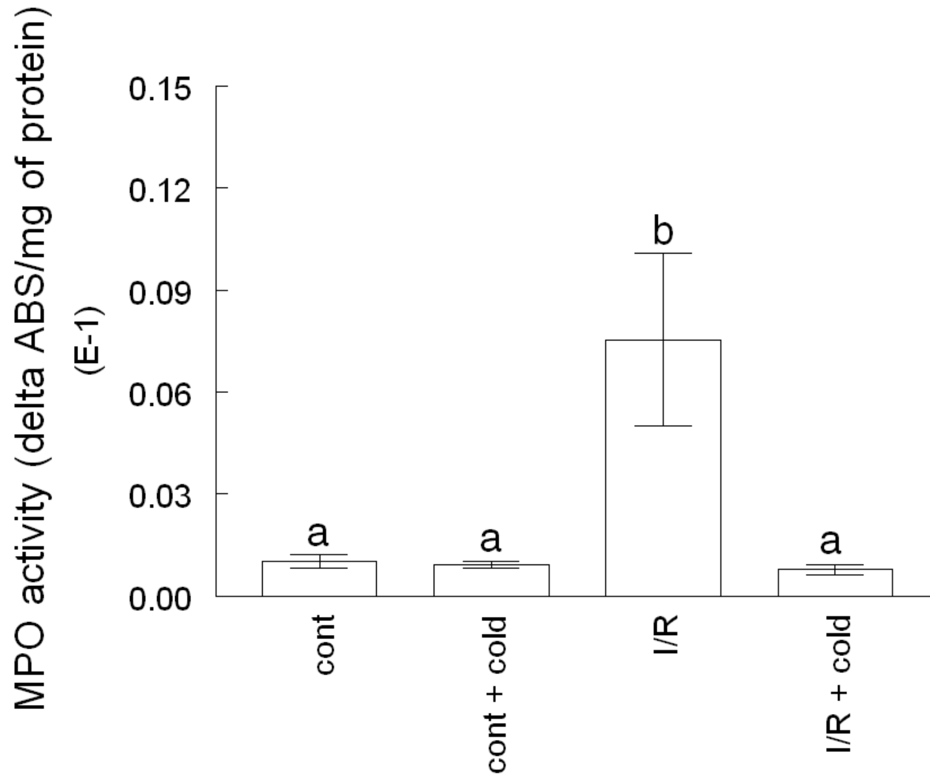


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Figure 5:

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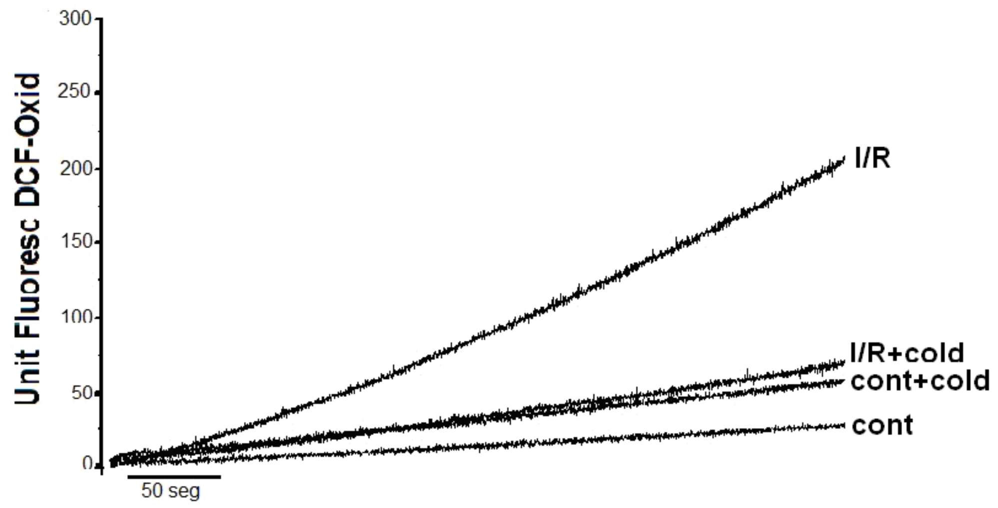

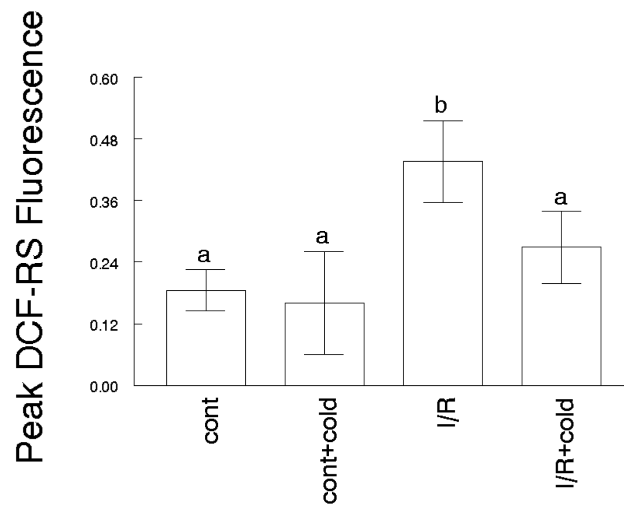

Figure 5:

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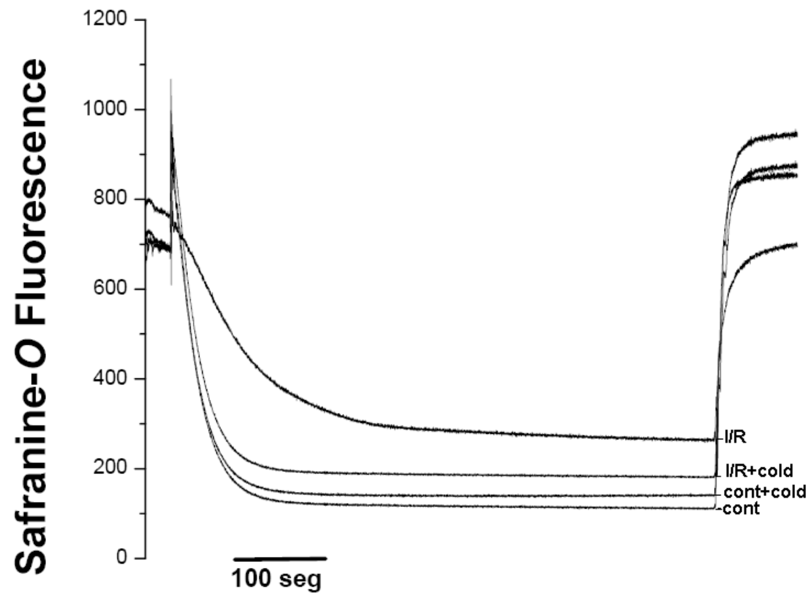

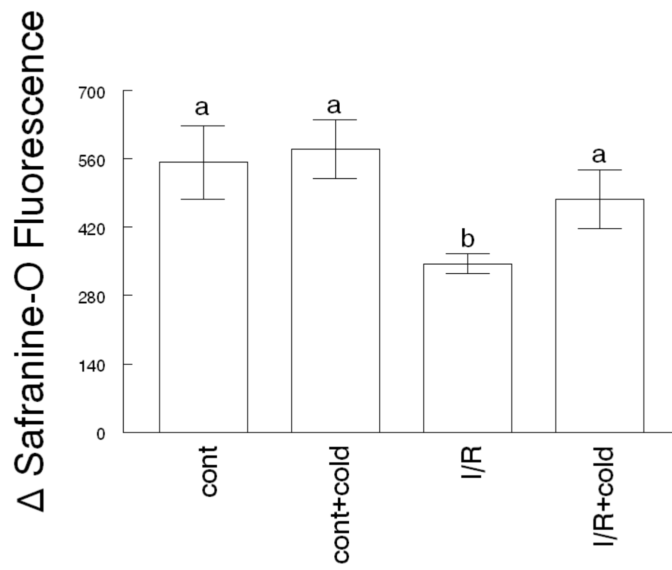

Figure 5:

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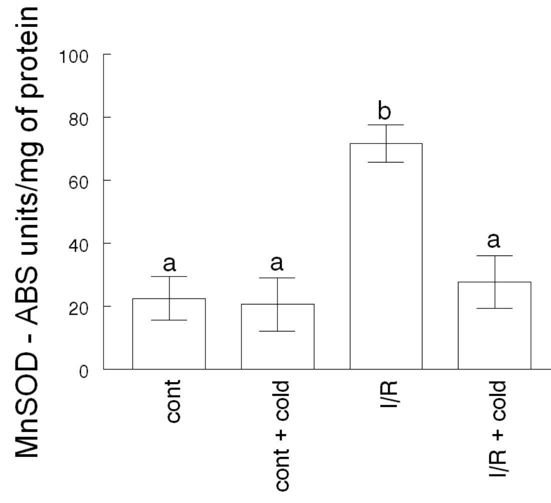
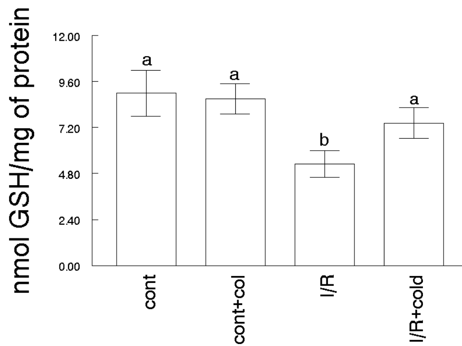


Figure 5:

D

I



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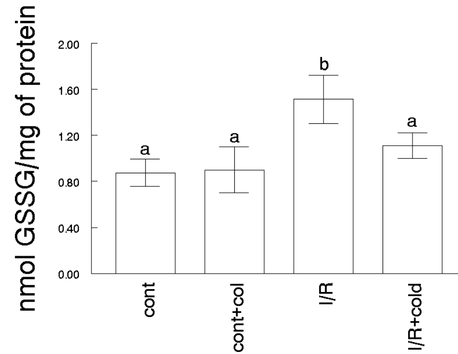

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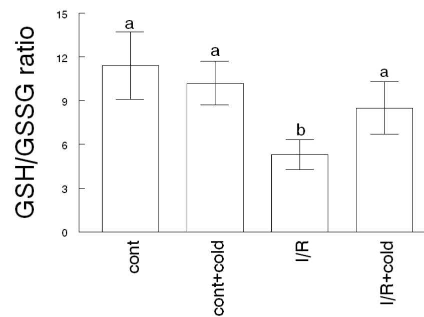
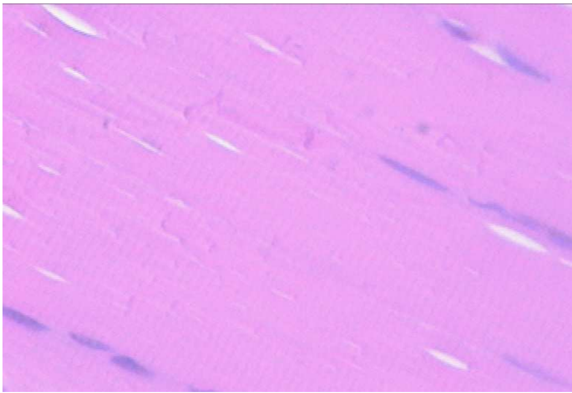


Indicators of the rat skeletal muscle mitochondria functioning: (A) mitochondrial DCF-RS generation; **(B)** mitochondrial $\Delta\psi$; **(C)** mitochondrial MnSOD activity; **(D)** mitochondrial GSH (I), GSSG(II), and GSH/GSSG ratio (III) levels. In Fig 5A-B the values are presented in fluorescence units (F.U.) according to described in material and methods. In 5A-B a representative graph illustrating a single experimental trial and a graph showing the results of 6-8 independent experiments are presented. In 5C the values are presented in absorbance variation unites (delta ABS) per mg of protein. In 5D the GSH (I) levels were expressed in nmol GSH/mg of protein, the GSSH (II) levels were expressed in nmol GSSG/mg of protein. For each experimental trial an independent and fresh mitochondrial preparation was performed. In Fig 5A-D the data are expressed as mean ± S.E. (n=6-8) and were analyzed by ANOVA, followed by Duncan test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [a] or [b].

Figure 6A:

I



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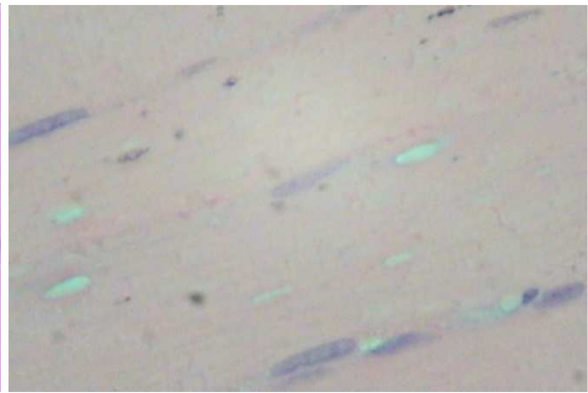

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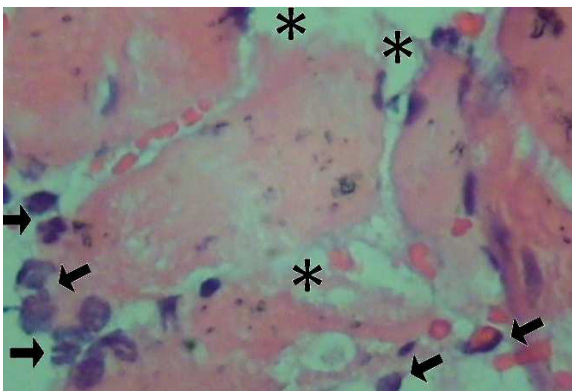

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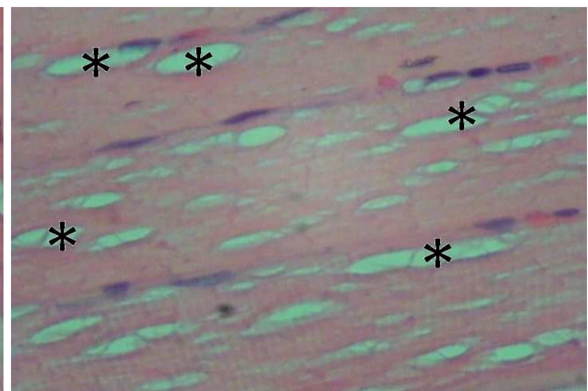
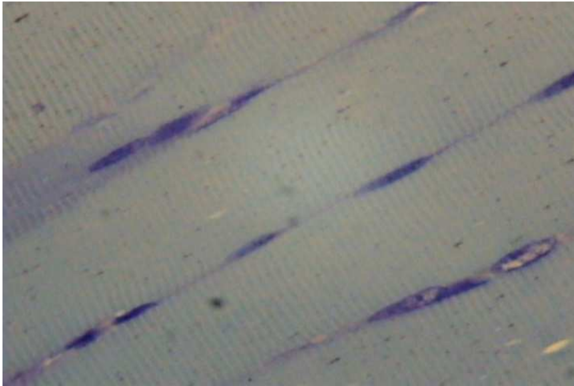
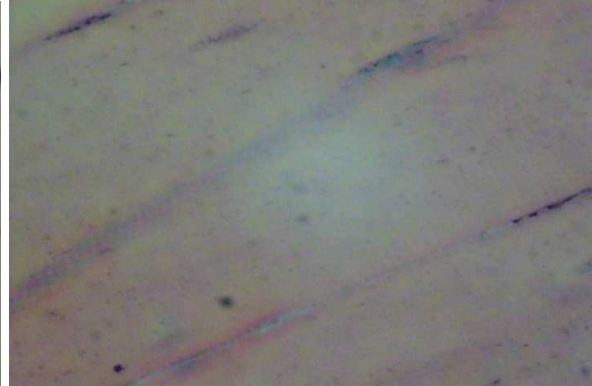


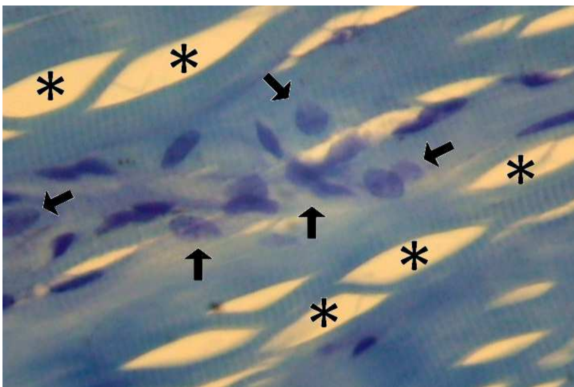
Figure 6B:

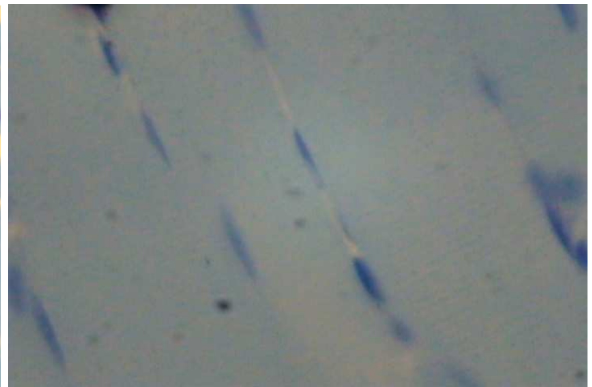
I **II**





III **IV**





Histopathologic changes in skeletal muscle: The neutrophils infiltrations areas (arrow), as well as the impaired skeletal muscle cells striations areas (sharp) were identified: In 6A the skeletal muscle slides were prepared with the hematoxiline/eosine staining; and in 6B with the Giemsa's staining. In all cases, I – control non treated and non I/R lesioned muscle; II – control cold treated and non I/R lesioned muscle; III – I/R lesioned and non treated skeletal muscle; and III – I/R lesioned and cold treated skeletal muscle. In all cases the images were 400 times increased.

5. DISCUSSÃO

O propósito deste estudo foi investigar os benefícios do frio no tratamento de lesões musculares na forma de uma contusão (**Artigo 1**) ou de um evento agudo de I/R sanguínea (**Manuscrito 1**) em ratos.

A fim de responder a esta questão central investigamos inicialmente a magnitude do dano oxidativo determinado por estes modelos de lesão muscular. Observamos um aumento significativo nos níveis de DCF-RS e de TBARS no tecido muscular (Figura 1A e 1B, respectivamente - **Artigo 1**) e no tecido sanguíneo (Figura 4A-B e 4C, respectivamente - **Artigo 1**) dos animais submetidos à contusão, e no tecido muscular (Figura 1A e 1B, respectivamente – **Manuscrito 1**), dos animais submetidos ao evento agudo de I/R. Resultados semelhantes foram encontrados em estudos envolvendo outros modelos de lesão muscular esquelética (RAHUSEN e cols., 2004; JÄRVINEN e cols., 2005; CARVALHO e cols., 2010). Estes resultados indicam que tanto a contusão muscular quanto o evento agudo de I/R podem ser acompanhadas pelo excessivo aumento na geração de EROs. Embora não tenhamos efetuado a análise da formação específica de EROs, é importante considerar que a formação das mesmas pode ser indiretamente analisada pelo método de oxidação da DCFA-DA (MYHRE e cols., 2003). Da mesma forma, a peroxidação lipídica analisada pelo método de formação de TBARS também está relacionada ao aumento na formação de EROs.

As alterações na estrutura e na funcionalidade de importantes enzimas, bem como a depleção dos sistemas de defesa antioxidantes celulares, também são observadas em condições associadas à excessiva geração de EROs (MEISTER, 1983; MEIJER, 1991; SIES, 1997; VERTUANI e cols., 2004). A redução significativa na atividade das enzimas LDH (Figura 3C - **Artigo 1**), Na^{+}/K^{+} ATPase (Figura 3A - **Artigo 1**) e Ca^{2+} ATPase (Figura 3B - **Artigo 1**; Figura 3A - **Manuscrito 1**) no tecido muscular pode ser associada a condição de excessiva geração de EROs. Estas importantes enzimas são reconhecidas por dependerem da integridade dos grupos –SH para serem funcionalmente ativas (ZHENG e cols., 2003; FOLMER e cols., 2004). Neste contexto, observamos também uma significativa diminuição nos níveis de –SH não protéicos após as lesões musculares (Figura 2A - **Artigo 1**; Figura 2A - **Manuscrito 1**). Desta forma, é possível considerar que os fatores envolvidos na oxidação dos grupos –SH foram determinantes para o comprometimento funcional de enzimas dependentes da integridade destes grupos à sua funcionalidade. É importante considerar que os grupos –SH não protéicos são encontrados especialmente na estrutura do tri-peptídeo de função antioxidante não-enzimático glutationa (GSH). Os sistemas de defesa antioxidante

enzimáticos no tecido muscular, por sua vez, também foram alterados em decorrência das lesões musculares. Observamos que a atividade da enzima CAT aumentou significativamente após a contusão (Figura 2B - A**rtigo 1**) e após o evento agudo de I/R **(**Figura 2B - **Manuscrito 1**). Acreditamos que este resultado esteja relacionado a uma resposta compensatória do tecido lesado na tentativa de neutralizar uma maior formação de H_2O_2 condicionada as lesões musculares. Em geral, os resultados obtidos a partir da análise das atividades enzimáticas dependentes da integridade dos grupos –SH e dos sistemas de defesa antioxidantes enzimáticos e não enzimáticos corroboram indiretamente a excessiva geração de EROs subsequente à contusão (A**rtigo 1**) e ao evento agudo de I/R (**Manuscrito 1**) no tecido muscular.

Os resultados apresentados evidenciam os benefícios do frio terapêutico no tratamento das lesões musculares. O tratamento com o frio determinou uma redução significativa nos níveis de DCF-RS (Figura 1A - A**rtigo 1**; Figura 1A - **Manuscrito 1**) e de TBARS (Figura 1B - A**rtigo 1**; Figura 1B - **Manuscrito 1**) no tecido muscular após as lesões musculares. Observamos também, que os níveis de –SH (Figura 2A - A**rtigo 1**; Figura 2A - **Manuscrito 1**) e a atividade das enzimas CAT (Figura 2B - A**rtigo 1**; Figura 2B - **Manuscrito 1**), LDH (Figura 3C - A**rtigo 1**), Na^+/K^+ (Figura 3A - A**rtigo 1**) e Ca^{2+} ATPase (Figura 3B - A**rtigo 1**; Figura 3A - **Manuscrito 1**), no tecido muscular dos animais submetidos ao tratamento com o frio mantiveram-se semelhantes aos observados nos animais não submetidos a lesão. Desta forma, acreditamos que a capacidade do frio em limitar o dano oxidativo no tecido muscular subsequente a um evento agudo de I/R pode ser relacionada ao seu potencial de modular o dano oxidativo seguinte a uma lesão musculoesquelética, tal como uma contusão muscular. Estes resultados estão de acordo com estudos que mostram os benefícios de repetidas e breves exposições terapêuticas ao frio (SPASIC e cols., 1993; SIEMS e cols., 1994 e 1999). Além disso, resultados semelhantes foram observados após o tratamento com o frio terapêutico de lesões musculoesqueléticas por tensão (CARVALHO e cols., 2010). Acreditamos que a capacidade do frio em reduzir o dano oxidativo no tecido muscular lesado esteja relacionada à sua capacidade de limitar o fluxo de sangue pelos tecidos tratados e, consequentemente, a intensidade da resposta inflamatória à lesão tecidual (THORSSON, 2001; BLEAKLEY e cols., 2004). Ao reduzir o fluxo de sangue no local da lesão, o frio também proporciona uma diminuição na atividade metabólica nos tecidos (KNIGHT, 1976; MERRICK e cols., 1999; SCHASER e cols., 2007). Assim, o dano secundário ao comprometimento circulatório observado após uma contusão pode ser adequadamente modulado pela aplicação do frio terapêutico (MEEUSEN e cols., 1986; MERRICK e cols.,

1993).

Além do dano oxidativo, observamos que as lesões musculares foram acompanhadas por um dano significativo à estrutura das células musculares, determinando assim um comprometimento na viabilidade das mesmas. A atividade da enzima CQ aumentou significativamente no plasma dos animais submetidos à contusão muscular (Figura 5 - A**rtigo 1**). A CQ é descrita como uma enzima citosólica a qual pode extravasar para o espaço extracelular, e deste para o plasma, quando a estrutura celular for comprometida (FINK e cols., 1983; BRANCACCIO e cols., 2007). Além disso, a análise histopatológica realizada corroborou a hipótese de dano à estrutura do tecido muscular ao evidenciar a extensão do comprometimento morfológico determinado pela contusão (Figura 6B e 6D - A**rtigo 1**). As alterações na disposição morfológica dos fascículos musculares bem como a formação de vacúolos entre as células do tecido muscular também foram observadas após o evento agudo de I/R (Figura 6A e 6B - **Manuscrito 1**). A perda da integridade da membrana celular pode ser considerada como um dos fatores preponderantes para a diminuição na capacidade de redução do MTT no tecido muscular lesado (Figura 1C - A**rtigo 1**; Figura 1C - **Manuscrito 1**). A funcionalidade celular de enzimas da família das oxirredutases, tais como as desidrogenases, é essencial para a capacidade de redução do MTT (BERNA & DOBRUCKI, 2002). Uma vez que a maioria das enzimas desta família está localizada nas mitocôndrias das células (BERNA & DOBRUCKI, 2002; BERRIDGE & TAN, 2003), a redução na capacidade de redução do MTT pode ser relacionada ao comprometimento no funcionamento mitocondrial das mesmas. Neste contexto, a incapacidade de manter uma adequada produção de energia intracelular associada à prejudicada regulação dos níveis de EROs pode resultar na morte das células musculares. O tratamento com o frio, por sua vez, limitou o aumento na atividade da enzima CQ no plasma (Figura 5 - A**rtigo 1**) e também diminuiu o comprometimento histopatológico no tecido muscular (Figura 6C e 6D - A**rtigo 1**; Figura 6A e 6B - **Manuscrito 1**) após as lesões musculares. O frio terapêutico também manteve os níveis de redução do MTT (Figura 1C - A**rtigo 1**; Figura 1C- **Manuscrito 1**) semelhantes aos animais que não foram submetidos às lesões. Assim, acreditamos que além da capacidade de reduzir o dano à estrutura morfológica das células musculares, os benefícios do tratamento com o frio estejam relacionados à sua capacidade de reduzir o comprometimento funcional mitocondrial dos tecidos lesados.

A análise histopatológica do parênquima muscular após as lesões musculares revelou também a presença de uma grande quantidade de neutrófilos infiltrados (Figura 6B e 6D - A**rtigo 1**; Figura 6A e 6B - **Manuscrito 1**). Este resultado foi coerente com o aumento

observado na atividade da enzima MPO no tecido muscular lesado (Figura 3D - A**rtigo 1**; Figura 4 - **Manuscrito 1**). Neste contexto, estudos indicam que uma exagerada resposta inflamatória pode ser acompanhada pelo aumento excessivo na geração de EROs (SPITELLER., 2006; SUPINSKI & CALLAHAN, 2007). Assim, sugerimos que uma resposta inflamatória intensa pode ser considerada como um importante fenômeno envolvido no comprometimento funcional do tecido muscular após uma contusão (A**rtigo 1**) ou um evento agudo de I/R (**Manuscrito 1**). A semelhança observada no tecido muscular após um evento agudo de I/R com o observado após contusão muscular no que concerne ao significativo aumento na resposta inflamatória nos permite sugerir uma similaridade na fisiopatologia entre estas lesões. Com referência aos efeitos terapêuticos do frio, observamos que o tratamento modulou o aumento na atividade da enzima MPO (Figura 3D - A**rtigo 1**; Figura 4 - **Manuscrito 1**) no tecido muscular após as lesões musculares. Neste mesmo sentido, observamos que a infiltração de neutrófilos pelo parênquima tecidual muscular foi reduzida após o tratamento com o frio (Figuras 6C e 6E - A**rtigo 1**; Figura 6A e 6B - **Manuscrito 1**).

Outro importante fenômeno investigado na tentativa de elucidar os mecanismos envolvidos na origem do dano oxidativo subsequente às lesões musculares foi o comprometimento no funcionamento das mitocôndrias do tecido muscular. Nossos resultados mostram um evidente aumento na formação de DCF-RS nas mitocôndrias do tecido muscular (Figura 7A - A**rtigo 1**; Figura 5A - **Manuscrito 1**), e no inchaço mitocondrial (Figura 7C - A**rtigo 1**). Observamos também o prejuízo no $\Delta\psi$ das mitocôndrias do tecido muscular nos momentos iniciais após uma contusão (Figura 7B - A**rtigo 1**) e após um evento agudo de I/R (Figura 5B - **Manuscrito 1**). Além disso, observamos um aumento significativo na atividade da enzima MnSOD (Figura 5C - **Manuscrito 1**) e uma redução significativa na razão mitocondrial de GSH/GSSG (Figura 5D - **Manuscrito 1**) após um evento agudo de I/R. Neste contexto, acreditamos que o aumento na atividade da enzima MnSOD mitocondrial pode ser entendida como um mecanismo para neutralizar um aumento na formação de ânions superóxido (O^-_2). O aumento na atividade da MnSOD, por sua vez, determinaria uma elevação nos níveis de H_2O_2. Assim, um aumento no consumo de GSH, na tentativa de neutralizar o aumento dos níveis de H_2O_2 via atividade da enzima glutationa peroxidase (GPx), e consequentemente uma redução na razão GSH/GSSG poderia ser explicada. Em geral, estes resultados nos levam a acreditar que o comprometimento no funcionamento mitocondrial é um fenômeno fundamental na origem do dano oxidativo subsequente às lesões musculares.

Considerando-se os efeitos do tratamento, observamos que o frio foi capaz de limitar a formação mitocondrial de DCF-RS (Figura 7A - A**rtigo 1**; Figura 5A - **Manuscrito 1**) observados após as lesões musculares, e o inchaço mitocondrial (Figura 7C - A**rtigo 1**) subsequente a uma contusão muscular. Além disso, o $\Delta\psi$ mitocondrial dos animais tratados com o frio foi semelhante ao observado nas mitocôndrias dos animais não submetidos às lesões musculares (Figura 7B - A**rtigo 1**; Figura 5B - **Manuscrito 1**). A atividade da enzima MnSOD, e os níveis da razão GSH/GSSG mitocondriais observados após um comprometimento circulatório também foram mantidos similares aos observados no tecido muscular não lesado (Figura 5C e 5D, respectivamente - **Manuscrito 1**). Desta forma, os efeitos do tratamento podem ser relacionados à capacidade do frio em limitar a formação de EROs nas mitocôndrias e também em manter a integridade das membranas mitocondriais. Assim, acreditamos que os benefícios do frio terapêutico em modular o dano oxidativo subsequente a uma lesão muscular está relacionado à sua capacidade em limitar o comprometimento funcional mitocondrial dos tecidos lesados.

De modo geral, os resultados deste estudo fundamentam a hipótese de que um evento agudo de I/R sanguínea pode ser considerado como um importante fenômeno envolvido no dano oxidativo ao tecido muscular subsequente a uma lesão musculoesquelética, tal como a contusão muscular. Além disso, evidenciamos a eficácia do frio como estratégia no tratamento de lesões musculares na forma de uma contusão e de um evento agudo de I/R. Neste contexto, sugerimos que os benefícios do frio estão relacionados à sua capacidade de modular a intensidade da resposta inflamatória e o comprometimento no funcionamento mitocondrial dos tecidos lesados.

6. CONCLUSÕES

De acordo com os resultados apresentados nesta tese podemos concluir que:

- O frio terapêutico limitou de modo efetivo o dano oxidativo e as alterações morfológicas no tecido muscular após uma contusão em ratos;
- Os efeitos do frio como estratégia no tratamento de uma contusão estão relacionados à sua capacidade de modular a intensidade da resposta inflamatória e as alterações no funcionamento mitocondrial no tecido muscular;
- Um evento agudo de I/R pode ser considerado como um importante mecanismo envolvido na fisiopatologia de uma lesão muscular uma vez que determinou alterações funcionais semelhantes às observadas após uma contusão muscular;
- O frio terapêutico limitou de modo efetivo o dano oxidativo e as alterações morfológicas no tecido muscular após um evento agudo de I/R sanguínea;
- Os efeitos terapêuticos do frio como estratégia no tratamento de um evento agudo de I/R estão relacionados à sua capacidade de modular a intensidade da resposta inflamatória e as alterações no funcionamento mitocondrial no tecido muscular.

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