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Andrezza Bond Vieira Furtado

EFEITOS DA IMERSÃO EM ÁGUA GELADA SOBRE AS ALTERAÇÕES BIOQUÍMICAS INDUZIDAS PELO EXERCÍCIO FÍSICO EXAUSTIVO EM CAMUNDONGOS

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

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Aprovado em 7 de julho de 2017:

Gustavo Orione Puntel, Dr. (UFSM)
(Presidente/orientador)

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Rômulo Pilon Barcelos, Dr. (UPF)

Santa Maria, RS
2017

DEDICATÓRIA

Ao meu irmão, Mauro Bond Vieira Furtado, meu exemplo maior de grandeza, que mesmo não estando presente fisicamente, esteve e está comigo em todas as decisões e conquistas.

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A Deus, por ter me guiado e permitido encontrar pessoas tão boas em meu caminho.

À minha família: meus pais, por não medirem esforços para proporcionar meu crescimento pessoal e profissional e por me apoiarem em todas as decisões que tomei. Ao meu irmão mais novo e minha sobrinha, por partilharem comigo suas vidas e serem o motivo de eu querer ser melhor a cada dia. Aos meus avós, por me apoiarem e serem presentes, mesmo que de longe, em todos os momentos da minha vida. Aos meus tios e tias e primos, por ser meu segundo lar. À Mônica, por todos os cuidados que sempre dedicou a mim e a nossa família. Aos demais familiares, que sempre estiveram torcendo pelas minhas conquistas. Vocês sabem que o motivo de tudo isso são vocês.

As pessoas que estiveram ao meu lado durante esses dois anos e de maneiras diferentes me apoiaram até aqui, principalmente: Tiago, Cleiton, Daiana, Érica, Gabrielle e Lívia. Com a presença de vocês o caminho não deixou de ser difícil, mas com certeza se tornou mais leve e feliz. Obrigada por estarem ao meu lado nos momentos bons e serem companheiros nos momentos que eu não conseguia seguir só.

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Aos demais professores, funcionários e colegas do Programa de Pós-Graduação em Reabilitação Funcional por terem contribuído de alguma forma para que esse trabalho fosse concluído.

"Eu sou, porque nós somos!" Obrigada!

“Reze e trabalhe, fazendo de conta que esta vida é um dia de capina com sol quente, que às vezes custa muito a passar, mas sempre passa. E você ainda pode ter muito pedaço bom de alegria... Cada um tem a sua hora e a sua vez: você há de ter a sua."

Guimarães Rosa

RESUMO

EFEITOS DA IMERSÃO EM ÁGUA GELADA SOBRE AS ALTERAÇÕES BIOQUÍMICAS INDUZIDAS PELO EXERCÍCIO FÍSICO EXAUSTIVO EM CAMUNDONGOS

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ORIENTADOR: Gustavo Orione Puntel

Quando feito de modo intenso ou exaustivo, o exercício físico gera uma resposta inflamatória excessiva, o que pode levar ao dano muscular. A crioterapia, quando usada na forma de imersão em água gelada, tem propriedades anti-inflamatórias e analgésicas e é amplamente utilizada após a prática esportiva, com o objetivo de acelerar a recuperação muscular. O presente estudo objetivou estabelecer os efeitos do tratamento contínuo de imersão em água gelada em camundongos submetidos à exaustão induzida por exercício nos dias posteriores ao dano muscular. Camundongos *Swiss albino* foram divididos em quatro grupos de tratamento: I) Controle; II) Imersão em água gelada (Cold-water immersion - CWI); III) protocolo de exaustão por nado (Swimming Exhaustive Protocol - SEP); IV) SEP+CWI. Os grupos foram subdivididos de acordo com os tempos de análise: 0, 1, 3 e 5 dias. Os grupos SEP foram submetidos a uma única sessão de nado exaustivo e os grupos CWI foram submetidos a uma sessão de imersão em água gelada (12 minutos a 12°C) a cada 24h. A produção de espécies reativas, biomarcadores de dano e inflamação, viabilidade celular e status antioxidante foram mensurados. O grupo SEP+CWI apresentou menor dano e produção de espécies reativas e aumento na viabilidade celular quando comparado com o grupo SEP. Além disso, fortes correlações entre grupos tióis não proteicos e diclorofluoresceína e creatina quinase foram encontradas. Ademais, CWI aumentou a atividade da acetilcolinesterase nas primeiras duas sessões. O diferencial deste estudo foi monitorar as mudanças bioquímicas causadas pelo exercício exaustivo e o tratamento com imersão em água gelada no decorrer do tempo. Pode-se concluir que a imersão em água gelada é um tratamento eficaz após o dano muscular induzido pelo exercício físico, capaz de aumentar a resposta anti-inflamatória, reduzir a produção de espécies reativas, aumentar viabilidade celular e reestabelecer o equilíbrio redox, o que torna o processo de recuperação mais rápido.

Palavras-chave: Crioterapia. Lesão Induzida por Exercício. Dano Muscular. Espécies Reativas.

ABSTRACT

COLD-WATER IMMERSION EFFECTS ON BIOCHEMICAL CHANGES INDUCED BY EXHAUSTIVE PHYSICAL EXERCISE IN MICE

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When exercise is performed intensively or exhaustively, it generates excessive inflammatory response that may lead to muscle damage. Cryotherapy using cold-water immersion is known for its anti-inflammatory and analgesic properties, and it is largely used to accelerate the muscular recovery process. The present study aimed to establish which effects are promoted by continuous cold-water immersion treatment in mice submitted to exercise-induced exhaustion after muscle damage. *Swiss albino* mice were divided into 4 treatment groups: Control, Cold-water Immersion (CWI), Swimming Exhaustive Protocol (SEP), and SEP+CWI. Treatment groups were subdivided in times of analysis: 0, 1, 3 and 5 days. Exhaustion groups were submitted to one SEP session, and the CWI groups submitted to one CWI session every 24 h (12 minutes at 12°C) every 24 h. Reactive species production, inflammatory and damage biomarkers, cell viability and antioxidant status were assessed. SEP+CWI group showed a decrease in the levels of inflammatory and damage biomarkers and reactive species production, and presented increased cell viability when compared to the SEP group. Additionally, strong correlations between non-protein –SH levels and oxidized dichlorofluorescein, and creatine kinase were found. Furthermore, CWI increased acetylcholinesterase activity in the first two sessions. This study aimed to monitor the evolution of biochemical changes caused by exhaustive exercise and CWI treatment in time. The present study reports that CWI is an effective treatment after exercise-induced muscle damage. It is able to enhance anti-inflammatory response, decrease reactive species production, increase cell viability and promote redox balance, which shorten the recovery process.

Key-words: Cryotherapy. Exercise-induced damage. Muscular damage. Reactive species.

LISTA DE SIGLAS E/OU ABREVIATURAS

AChE: acetilcolinesterase
CK: creatina quinase
DCF-RS: diclorofluoresceína oxidada
MTT: metil tetrazólio
ROS: espécies reativas de oxigênio
-SH: grupos tióis

SUMÁRIO

1. INTRODUÇÃO

1.1 O EXERCÍCIO FÍSICO EXTENUANTE COMO INDUTOR DE DANO MUSCULAR

A prática regular de exercício é capaz de gerar adaptações crônicas ao organismo e melhorar o sistema imune através da redução de monócitos circulantes, do menor recrutamento de macrófagos adicionais e menor da expressão de genes inflamatórios, como Interleucina 6 e Fator de Necrose Tumoral Alfa (KAWANISHI et al., 2010). Por outro lado, quando feito em intensidades muito elevadas ou durações extenuantes, seja em séries de exercício intenso intermitente ou em série de exercício moderado contínuo geram aumento da expressão destes genes (Mc.CLEAN et al., 2016) e, consequentemente, a supressão do sistema imune. Se em longo prazo o exercício físico, ao aumentar o consumo de oxigênio e, consequentemente, a produção de radicais livres, gera regulação e resposta antioxidante, em curto prazo ocorre dano tecidual, estresse oxidativo, oxidação de proteínas, peroxidação lipídica e dano no DNA. Em quantidade aumentada, os radicais livres tendem a reduzir as defesas do organismo e induzir dor e lesões musculares, o que marca o início do processo inflamatório (McARDLE; KATCH; KATCH, 2011). Devido a esta capacidade do exercício físico de desequilibrar a homeostase celular, o organismo se torna mais suscetível a danos musculares (SIMPSON et al., 2012).

Segundo Reid (2016), o exercício físico vigoroso estimula a produção de espécies reativas de oxigênio (ROS). Este acúmulo de agentes oxidantes no organismo, principalmente no tecido muscular, diminui a função contrátil miofibrilar e leva, consequentemente, à fadiga induzida por exercício físico. O aumento de ROS, causado por exercício físico agudo, dá-se, possivelmente, pela alta atividade da xantina oxidase e da oxidação de NADPH (BLOOMER; GOLDFARB, 2004). Para Powers et al. (2011), ao mesmo tempo que os níveis fisiológicos de ROS são importantes para gerar força muscular, quando em excesso, eles acarretam em disfunções na contração muscular, resultando em fadiga. O aumento do consumo de oxigênio pode desestabilizar o equilíbrio entre ROS e antioxidantes, e, através do aumento da produção de ROS, gerar estresse oxidativo (MIRANDA-VILELA et al., 2010), que afeta não só a função muscular, mas também a sua adaptação (ZUO et al., 2013). As ROS, quando atuam de forma prejudicial, promovem alterações na expressão gênica, na resposta imunológica, no estresse oxidativo mitocondrial, na atrofia muscular e também nas distrofias neuromusculares (ZUO; PANNEL, 2015). Níveis excessivos de ROS e de nitrogênio são capazes de alterar

aguda ou cronicamente o músculo, principalmente através de marcadores inflamatórios, que podem comprometer a função contrátil, causar fadiga. (VOLLAARD; SHEARMAN; COOPER, 2005) e se tornar um fator prejudicial da performance, levando a consequências de longo prazo (SAHLIN et al., 2010).

Para King, Clanton e Laitano (2016), existem múltiplos mecanismos envolvidos nas mudanças do status oxidante/antioxidante durante o exercício, dentre eles, a produção de ROS, que está associada a mudanças a nível osmótico e mudanças de temperatura, que levam à hipertermia e à redução no volume de circulação sanguíneos. Os sintomas do estresse fisiológico induzido por exercício físico intenso são queda de desempenho, dor e diminuição da função muscular. Estes sintomas estão associados à depleção dos substratos energéticos, a lesões musculares, à hipertermia, à inflamação, à fadiga do sistema nervoso e ao estresse oxidativo (MCHUGH et al., 1999).

1.2 A IMERSÃO EM ÁGUA GELADA COMO TRATAMENTO AO DANO MUSCULAR INDUZIDO POR UMA SESSÃO EXTENUANTE DE EXERCÍCIO FÍSICO

O exercício físico induz ao dano muscular e este, por sua vez, gera uma resposta inflamatória, que é seguida pela fase de recuperação muscular (SMITH et al., 2008). São muitos os métodos, utilizados pela medicina esportiva, que visam acelerar o processo de recuperação muscular e melhorar a performance, dentre eles, a exposição ao frio ocupa papel de destaque. A crioterapia tem sido amplamente estudada e apresenta resultados positivos. Em estudo realizado por Yanagisawa et al. (2003a), concluiu-se que o esfriamento muscular tanto previne o edema, como diminui a extensão do dano na célula causados pelo exercício físico. O estudo mostrou também que o esfriamento aparenta ter efeito positivo no pH intracelular pós-exercício. Poppendieck et al. (2013) mostram, em metanálise, que, apesar de os efeitos do tratamento com frio na recuperação de atletas serem pequenos, quando utilizados apropriadamente, parecem ter efeitos relevantes. Os mesmos autores constataram, ainda, que a imersão em água gelada, quando feita em todo o corpo, tem resultados superiores à imersão parcial ou ao uso de bolsas térmicas.

A imersão em água gelada está entre os métodos mais utilizados de crioterapia e é conhecida por suas propriedades anti-inflamatórias e analgésicas, obtidas através de temperatura moderada ou extremamente baixa (BLEAKLEY et al., 2014). Este método, que é

definido como “imersão de segmentos corporais em água com temperatura menor que 15ºC”, tem mostrado resultados encorajadores acerca de sua eficácia no tratamento da dor muscular pós-exercício (BLEAKLEY et al., 2012). A imersão em água gelada é uma proposta de recuperação do exercício físico por atenuar a inflamação (BLEAKLEY; GLASGOW; WEBB, 2012), que, quando reduzida, pode estar relacionada com a diminuição da dor muscular tardia e com a melhora na função muscular (SWENSON; SWÄRD; KARLSSON, 1996).

O uso da imersão em água gelada, por períodos de 10 a 20 minutos em indivíduos submetidos a sessões de exercício físico intenso, previne o edema, a inflamação e a dor muscular tardia. Isso se deve não só a temperatura, mas também à pressão hidrostática na qual os indivíduos são submetidos (WILCOCK; CRONIN; HING, 2006). A vasoconstrição local, causada pelo uso de baixas temperaturas, reduz a propagação do líquido no espaço intersticial, o que favorece a redução do dano muscular e da inflamação aguda (DEGROOT et al., 2013). A redução da temperatura intramuscular reduz o metabolismo e limita a sinalização inflamatória e o edema. Outro fator importante é a redução da condução nervosa devido à baixa temperatura, o que leva a diminuição da percepção de dor (HOHENAUER et. al, 2015). Segundo revisão sistemática com meta-análise feita por Machado et al. (2015), a imersão em água gelada se mostra ligeiramente melhor na recuperação da dor muscular quando comparado à recuperação passiva. Existe uma relação entre dose-efeito que indica que a temperatura ideal para atingir melhores resultados na recuperação da dor muscular é de 11 a 15ºC, já o tempo de imersão varia entre 11 e 15 minutos. Em estudo de Mila-Kierzenkowska et al. (2009), foi demonstrado menor estresse oxidativo em sujeitos que associaram o exercício físico à imersão em água gelada, do que naqueles que realizaram apenas o exercício físico. Segundo os mesmos autores, o fato pode ser explicado pelo conceito de dose-resposta hormético, pois a crioterapia atua como um agente estressor, que, quando administrado em pequena dose, ativa respostas adaptativas para alcançar a homeostase celular e fisiológica.

Montgomery et al. (2008) afirma que repetidas séries de imersão em água gelada geram pequenos a moderados ganhos na performance, quando comparados com outras formas de recuperação, como roupas de compressão, suplementação de carboidrato e alongamentos em atletas de basquete competitivo. Ingram et al. (2009) mostraram que a imersão em água gelada obteve resultados melhores no retorno à performance basal de atletas do sexo masculino, quando comparado com contraste e grupo controle. Em um estudo conduzido por Bailey et al. (2007), foram analisados os resultados na recuperação pós-treinamento de corrida intermitente, utilizando a imersão em água gelada, durante 10 minutos a 10ºC, ou recuperação

passiva. A imersão em água gelada se mostrou superior na diminuição da dor muscular tardia e na manutenção da performance em 24 e 48h pós-treinamento.

A imersão também reduz a temperatura do tecido muscular, a saturação venosa de O_2, a concentração de mioglobina plasmática e o inchaço em maior quantidade quando comparada à recuperação ativa. (ROBERTS et al., 2014). Ihsan et. al (2013) mostraram que a imersão atenua o aumento da hemoglobina total durante a reperfusão pós-exercício e diminui a atividade metabólica muscular. A redução da demanda energética minimiza o desequilíbrio entre o fornecimento e a demanda de O_2 e isso leva a atenuação do estresse metabólico que a fibra muscular foi submetida (DEBOLD, 2012) Yanagisawa et al. (2003b) alegam que o resfriamento pós-exercício leva à diminuição da lesão muscular induzida por exercício e que os resultados nem sempre são demonstrados imediatamente, pois ocorre, na fase subaguda, tanto a redução do acúmulo de água intramuscular como a redução da extensão do dano na musculatura envolvida, causadas pela fase aguda. Em uma metanálise feita por Leeder et al. (2012), que visou investigar a imersão em água gelada como forma de recuperação de exercício físico vigoroso, foi constatado que, apesar dos mecanismos não estarem bem explicados, a imersão é uma estratégia positiva para reduzir os sintomas de dor muscular tardia após 24, 48, 72 e 96 horas pós exercício.

Apesar de a crioterapia ser um método frequentemente utilizado para recuperação, existem controvérsias sobre os benefícios desta alternativa de tratamento, pois, de um lado, há evidências de que indivíduos submetidos a este tipo de tratamento possuem maior nível de antioxidantes (BLEAKLEY; DAVISON, 2010), prevenção do edema e redução da extensão do dano celular (YANAGISAWA, 2003), redução do estresse oxidativo causado por exercício (MILA-KIERZENKOWSKA et al., 2009), redução do dano muscular e da disfunção mitocondrial (PUNTEL et al., 2011), e, por outro, em alguns estudos, o método não mostrou ser eficaz nos marcadores de lesão muscular e inflamação (ROWSELL et al., 2009), tampouco atuou na redução do estresse oxidativo causado pela lesão (SILVA et al., 2016). Costello et al. (2015) conclui que as evidências são insuficientes para afirmar que a imersão em água gelada reduz a dor e favorece a recuperação muscular.

Os mecanismos fisiológicos e bioquímicos desencadeados pelo uso da imersão em água gelada não estão claros, por isso, torna-se imprescindível que mais estudos sejam realizados, para que seja possível desenvolver orientações baseadas em evidências (BLEAKLEY; DAVISON, 2010). Apesar de amplamente estudados, os resultados se mostram controversos e

não definitivos, fato que pode ser explicado pela grande variação de protocolos utilizados pelos pesquisadores. Por esses motivos, o presente estudo se justifica na necessidade de preencher lacunas metodológicas sobre os efeitos bioquímicos da imersão nas alterações causadas por dano muscular induzido por exercício no decorrer do tempo de tratamento e, com isso, contribuir para que esta alternativa de tratamento seja feita de forma mais efetiva e embasada cientificamente.

1.3 OBJETIVOS

1.3.1 Objetivo Geral

Analisar os efeitos da imersão em água gelada sobre as alterações bioquímicas induzidas por exercício físico exaustivo.

1.3.2 Objetivos Específicos

- Analisar os efeitos da imersão em água gelada sobre as alterações bioquímicas induzidas por exercício físico exaustivo, a partir da avaliação dos seguintes parâmetros:
  - Os níveis de Diclorofluoresceína Oxidada (DCF-RS) no tecido muscular;
  - Os níveis de redução de Metil Tetrazólio (MTT) no tecido muscular;
  - Os níveis de Grupos Tióis (-SH) não proteicos no tecido muscular;
  - A atividade da enzima Acetilcolinesterase (AChE) no tecido muscular.
  - A atividade da enzima Creatina Quinase (CK) no plasma;

2. DESENVOLVIMENTO

O desenvolvimento desta dissertação está apresentado sob a forma de artigo científico. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no próprio artigo. O artigo encontra-se na formatação para publicação da revista científica Plos One.

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2.1 ARTIGO CIENTÍFICO

Full title: Cryotherapy enhances anti-inflammatory response, decreases reactive species production, increases cell viability and promotes redox balance induced by exhaustive exercise in mice

Short title: Cryotherapy decreases muscle damage induced by exhaustive exercise in mice

Andrezza B.V. Furtado, Diane D. Hartmann, Rodrigo P. Martins, Ingrid K. da Silva, Bárbara Duarte, Luis U. Signori, Felix A. Soares, Gustavo O. Puntel.

Cryotherapy decreases muscle damage induced by exhaustive exercise in mice

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Abstract

When exercise is performed intensively or exhaustively, it generates excessive inflammatory response that may lead to muscle damage. Cryotherapy using cold-water immersion is known for its anti-inflammatory and analgesic properties, and it is largely used to accelerate the muscular recovery process. The present study aimed to establish which effects are promoted by continuous cold-water immersion treatment in mice submitted to exercise-induced exhaustion after muscle damage. *Swiss albino* mice were divided into 4 treatment groups: Control, Cold-water Immersion (CWI), Swimming Exhaustive Protocol (SEP), and SEP+CWI. Treatment groups were subdivided in times of analysis: 0, 1, 3 and 5 days. Exhaustion groups were submitted to one SEP session, and the CWI groups submitted to one CWI session every 24 h (12 minutes at 12°C) every 24 h. Reactive species production, inflammatory and damage biomarkers, cell viability and antioxidant status were assessed. SEP+CWI group showed a decrease in the levels of inflammatory and damage biomarkers and reactive species production, and presented increased cell viability when compared to the SEP group. Additionally, strong correlations between non-protein –SH levels and oxidized dichlorofluorescein, acetylcholinesterase, creatine kinase and methyl-tetrazolium reduction levels were found. Furthermore, CWI increased acetylcholinesterase activity in the first two sessions. This study aimed to monitor the evolution of biochemical changes caused by exhaustive exercise and CWI treatment in time. The present study reports that CWI is an effective treatment after exercise-induced muscle damage. It is able to enhance anti-inflammatory response, decrease reactive species production, increase cell viability and promote redox balance, which shorten the recovery process.

Introduction

Chronic adaptations generated by regular physical exercise are well known for their capability to improve health and quality of life. In the long-term perspective, physical exercise causes regulation of metabolism and antioxidant status (1). However, when exercises are done in intense or exhaustive modes, several acute effects are triggered, including excessive inflammation, hormonal changes, and high production of reactive oxygen species, which may lead to oxidative stress, tissue damage, protein oxidation, lipid peroxidation, and DNA damage (1-3). These imbalances in the oxidative process during muscle contraction can contribute to decreases in contractile force, leading to exercise-induced exhaustion and consequently increased susceptibility to muscle damage (4).

It is well known that exercise-induced muscle damage generates inflammatory response, which is followed by the muscle recovery phase (5). There are several methods used by sports medicine that aim to accelerate the muscle recovery process and improve performance, such as cryotherapy, which plays a prominent role. Although it is frequently used in the recovery method, some controversies orbit around the real benefits of this alternative treatment. On one hand, researchers have shown that individuals submitted to cryotherapy have higher levels of antioxidants (6), decreased oxidative stress caused by physical exercise (7), lower inflammation levels and mitochondrial dysfunction (8). On the other hand, other studies have proved that cryotherapy is not effective in muscle injury or inflammation biomarkers (9), neither in decrease oxidative stress induced by lesion (10).

One of the most popular methods of cryotherapy is cold-water immersion (CWI) which is known for its anti-inflammatory and analgesic effects obtained through extreme or moderate exposure of body segments in water below 15ºC (11, 12). Additionally, recent studies have shown that CWI was more efficient than other forms of recovery because it

causes local vasoconstriction that leads to the reduction of the fluid propagation in the interstitial space. Hence, this method favors the reduction of muscle damage, acute inflammation (13), muscle tissue temperature, venous O2 saturation, plasma myoglobin concentration, and swelling (14).

Despite extensive research on CWI, results are controversial and indefinite, which is explained by protocol diversity in the already published studies. Such diversity makes physiological and biochemical mechanisms triggered by CWI not clearly described until now. In view of this, further studies are important, and then evidence-based guidelines can be developed (6). Our hypothesis is that CWI reduces the inflammatory process and reactive species formation, and increases antioxidant status and cellular viability after exercise-induced muscle damage in comparison to passive recovery. Considering this, the aim of this study is to establish which biochemical changes are induced by continuous CWI treatment in mice submitted to exercise-induced exhaustion after muscle damage.

Materials and methods

Animals and reagents

Adult male *Swiss albino* mice weighing 30-50g were used in this study. During the experimental protocol, animals were kept in cages of 10 animals each, with food and water *ad libitum*. Mice were maintained in a room with controlled temperature and photoperiod of 12-h light/dark. All the procedure were in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil (UFSM; 4185290915). Assay reagents were purchased from Sigma (St. Louis, MO, USA) and biochemical kits were obtained from the standard commercial supplier Labtest (Lagoa Santa, Brazil).

Experimental groups

The animals (n=80) were randomized and divided into four main homogeneous groups:

1) Control: animals were not submitted to either protocol of muscle damage (swimming exhaustion protocol - SEP) or treatment (CWI).
2) CWI: animals were submitted only to the CWI protocol.
3) Exhaustion: animals were submitted only to SEP.
4) SEP+CWI: animals were submitted to SEP and CWI protocols

Each group was subdivided into four groups, according to the different times of analysis: 0, 1, 3 and 5 days. The aim was to observe the evaluation of biomarkers in different periods after exhaustion protocol and singular or repeated sessions of CWI treatment. The subgroup sample sizes were calculated by a power analysis based on Puntel et al. (8) and determined that four animals would provide a statistical power of 95% at an alpha level of 5%.

Water adaptation

All animals were adapted to the water before the beginning of the experiment. The adaptation consisted of keeping the animals walking in shallow water at 31ºC for 20 min for 7 days. This protocol were adapted from Lima et. al (15) which goal was to adapt the animals to the water environment without promoting physical training.

Swimming exhaustion protocol

The SEP and SEP+CWI groups were submitted to the SEP according to the method proposed by Huang (16) with some modifications. It consisted of a swimming exercise in a tank with controlled temperature (31ºC) carrying constant loads of 10% of the body weight

(17) that were fixed on the mice tails. The exhaustion was characterized when the animal lost the coordinated movements and could not return to the surface within 7 seconds. The animals were submitted to the exercise only once in day 0.

Cold-Water Immersion

After the exhaustion protocol, the animals of SEP+CWI were immediately put in a tank with controlled temperature (12 ºC) for 12 min following the guidance previously described by Machado et al. (18). The animals of the CWI group were submitted to the same protocol. Both groups repeated the protocol every 24 h for 5 days. (Fig 1.)

Fig 1. Timeline of the experiments.

Tissue sampling

The animals were euthanized immediately after the protocols and blood was collected by heart puncture in previously heparinized syringe and both gastrocnemius muscles were quickly removed, weighted and placed on ice. Skeletal muscle tissue samples were homogenized within 10 min in 10 volumes of cold Tris 10mM (pH 7.4) and were centrifuged at 4000×*g* for 10 min to yield the low-speed supernatant fraction that was used for different biochemical assays in all trials. Whole blood samples were centrifuged at 1500×*g* for 10 min for plasma separation which was used for biochemical analysis.

Plasma assays

Creatine Kinase (CK)

CK activity was measured spectrophotometrically in plasma samples by standard commercially biological kits (Labtest, Lagoa Santa, Brazil).

Skeletal muscle homogenate assays

Measurement of metiltetrazolium (MTT) reduction levels

Aliquots of skeletal muscle homogenate (90 µL) were added to a medium containing 1 mg/mL of MTT and were incubated in the dark for 60 min at 37 °C. Then, 900 µL of DMSO were added. Formazan levels were measured spectrophotometrically at 570 nm and 630nm and results were corrected by the protein content as proposed by Mosmann (19).

Oxidized diclorofluoresceine (DCF-RS) levels

DCF-RS levels were measured according to Pérez-Severiano (20) with some modifications. Aliquots of skeletal muscle homogenate (50 µL) were added to a medium containing Tris-HCl buffer (10 mM; pH 7.4) and DCFH-DA (1µM). After DCFH-DA addition, the medium was incubated in the dark for 1h until fluorescence measurement procedure (excitation at 488nm and emission at 525 nm and both slit widths used were at 1,5 nm). DCF-RS levels were determined using a standard curve of DCF and the results were corrected by mg of protein.

Non-protein thiol (–SH) levels

Non-protein -SH levels were determined according to the method proposed by Ellman (21) with some modifications. Samples of skeletal muscle homogenate (500 µL) were precipitated with Trichloroacetic acid (5%) (250 µL) and subsequently centrifuged at 1800xg for 10 min. After the centrifugation, the supernatant fraction (300µL) was added to a reaction medium containing TFK (0.5 mM, pH 7) and DTNB (20 mM). Non-protein –SH levels were measured spectrophotometrically at 412nm. Results were calculated in relation to a standard curve constructed with GSH at known concentrations and also corrected by the protein content.

Acetylcholinesterase (AChE) activity

The AChE activity was estimated in skeletal muscle by the Ellman method (22). In a plate containing acetyltiocholine iodide (ATC), used as substrate, and etopropazine as butyrylcholinesterase (BChE) inhibitor. AChE were measured spectrophotometrically at 412 nm every 30 s for 2 min. Data were corrected by protein content.

Protein quantification

The protein content was estimated by the Bradford method (23) using bovine serum albumin (BSA) as the standard.

Statistical analyses

The Graphpad Prism 6.0 (San Diego, CA, USA) was used for all analyses. Data were expressed as mean and standard deviation (SD). Significance was assessed by Two-Way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. Statistical significance was set at $p < 0.05$. The main effects (Effect Size) were tested to reveal the side of the effect and complement P value (24) and are presented only when interactions between SEP and SEP+CWI were significant (Es. >0.8, positive or negative). Pearson's correlation coefficient was also calculated to assess the relationship between –SH non-protein levels and the other biomarkers measured.

Results

Injury markers in skeletal muscle and plasma

DCF-RS levels in skeletal muscle tissue are shown in figure 2. The SEP increased DCF-RS levels significantly immediately after the exercise ($p<0.05$) when compared to control group. The increase did not occur in the CWI or SEP+CWI group. In effect size

159 analysis there were differences between SEP and SEP+CWI groups in days 0 (Es. -1.74) and
160 5 (Es. -1.16).

161 **Fig 2. Effects of CWI on DCF-RS levels.** Data are expressed as mean ± SD (n=4) and were
162 analyzed by Two-Way ANOVA, followed by Tukey *post hoc* test. Differences were
163 considerate significant when p <0.05. Different letters means significant difference between
164 groups (p<0.05). Es.: effect size

165 The AChE activity in skeletal muscle tissue (Fig 3.) increased in CWI group only in
166 days 0 (p <0.05) and 1 (p<0.001). In effect size analyzes there were differences between SEP
167 and SPE+CWI groups on all days, however, in immediate time and in day 1, AChE increased
168 on SEP+CWI group (Es. 1.70; 3.16), and in days 3 and 5 the opposite occurred, AChE was
169 increased in SEP group (Es. -0.89; -1.64).

170 **Fig 3. Effects of CWI on AChE activity**. Data are expressed as mean ± SD (n=4) and were
171 analyzed by Two-Way ANOVA, followed by Tukey *post hoc* test. Differences were
172 considerate significant when p<0.05. Different letters means significant difference between
173 groups (p<0.05). Es.: effect size

174 In figure 4 the effect of CWI on CK enzyme modulation can be observed in SEP+
175 CWI. In days 0 (p < 0.05), 3 (p <0.001) and 5 (p < 0.001) occurred an increase in the CK
176 activity in SEP group. In effect size analysis the effects caused by CWI can be observed in
177 days 0 (Es. -1.06), 1 (Es. -1.15), 3 (Es. -1.97) and 5 (Es. -1.64).

178 **Fig 4**. **Effects of CWI on CK activity.** Data are expressed as mean ± SD (n=5) and were
179 analyzed by Two-Way ANOVA, followed by Tukey *post hoc* test. Differences were
180 considerate significant when p<0.05. Different letters means significant difference between
181 groups (p<0.05). Es.: effect size

182

Cell viability and antioxidant markers in skeletal muscle

After 5 days of CWI, the MTT levels (fig 5.) presented a significant value in time (p= 0.001). Nevertheless, no significant interaction between the groups was accessed by Two-Way ANOVA. In the Effectsize analysis, MTT levels were increased in SEP+CWI group in days 0 and 1 (Es. 2.90; 1.75).

Fig 5. Effects of CWI on MTT levels. Data are expressed as mean ± SD (n=4) and were analyzed by Two-Way ANOVA, followed by Tukey *post hoc* test. Differences were considerate significant when $p<0.05$. Es.: effect size

There was no difference in non-protein –SH levels (Fig 6.), although there were strong correlations between it and DCF-RS, CK and AChE (Table 1). In day 0, non-protein –SH levels were negative correlated with DCF-RS levels on SEP group (r= -0.8683) but positive correlated in SEP+CWI group (r= 0.9031). When correlated with CK, non-protein –SH levels presented, in day 1, negative correlation in group SEP (r= -0.8475) and positive correlation in group SEP+CWI (r= 0.7577).

Fig 6. Effects of CWI on non-protein –SH levels. Data are expressed as mean ± SD (n=4) and were analyzed by Two-Way ANOVA, followed by Tukey *post hoc* test. Differences were considerate significant when $p<0.05$.

Table 1. Pearson's correlation between non-protein –SH levels and other biomarkers

Day	Group	Biomarker	R	p
0	SEP	-SH x DCF-RS	-0.8683	0.131
0	SEP+CWI	-SH x DCF-RS	0.9031	0.096
0	SEP	-SH x CK	-0.9722	0.027
0	SEP+CWI	-SH x CK	-0.0075	0.992
1	SEP	-SH x AChE	0.9657	0.034
1	SEP+CWI	-SH x AChE	0.7098	0.290
1	SEP	-SH x CK	-0.8475	0.152

1	SEP+CWI	-SH x CK	0.7577	0.242
3	SEP	-SH x DCF-RS	0.9931	0.006
3	SEP+CWI	-SH x DCF-RS	0.8767	0.123

Pearson's correlation between non-protein –SH levels and DCF-RS levels, CK and AChE activity. Correlations were considered strong when $r = \geq \pm 0.7$.

Discussion

In the current study, biochemical changes induced by cold-water immersion treatment in mice submitted to exercise-induced exhaustion were evaluated. To the best of our knowledge, this is the first study to demonstrate the use of CWI for five days consecutively after a single exhaustive exercise session. It is also the first to show that AChE activity increases in the first two sessions of CWI treatment and decreases afterwards. In addition, it correlates antioxidants with oxidants or damage markers after exhaustive exercise with or without CWI treatment. When groups were treated with CWI after SEP, there was a positive correlation between them.

The most popular reason for using CWI is that it induces vasoconstriction, which leads to venous return increase, metabolite removal (25), restricting the infiltration of inflammatory cells into the muscle (26). These mechanisms protect the uninjured tissue near the damaged area for enzymatic reactions triggered by exercise induced damage (27). The proposed exhaustive protocol was able to change biochemical markers that prove muscle damage, such as increasing CK activity and DCF-RS production. On the other hand, the protocol does not lead to excessive damage, which may be equivalent to real sportive situations. In this study, CWI treatment was capable of decreasing reactive species (RS) formation and damage biomarkers, as well as increasing cellular viability after SEP. The results presented here support the hypothesis that CWI is better than passive recovery after exhaustive exercise and

corroborate with other study that suggest that CWI treatment is an effective intervention after exhaustive exercise (28).

There are multiple mechanisms involved in oxidant/antioxidant status during exercise (29). Excessive levels of ROS may acutely or chronically alter the muscle, mainly through inflammatory markers (30). Although reactive oxygen species formation was not directly assessed in this study, DCF-RS levels (8) were measured. In immediate time levels of DCF-RS were increased in the SEP group; however, the SEP+CWI group did not present an increment, which suggests that CWI may prevent DCF-RS increase caused by exercise-induced exhaustion. This finding demonstrates clearly that CWI was effective in limiting RS production just after exercise.

Considering that acetylcholine (ACh) is well known to inhibit TNF-α, IL-1β and macrophage migration (31), the increased AChE activity may indicate an impairment of ACh ability in regulating the inflammatory processes (32). In the present study, AChE activity was increased in the CWI group. This unexpected fact may be explained by the thermodynamic characteristics of AChE. Klichkhanov and Meilanov (33) revealed that when rat erythrocyte membranes are exposed to hypothermia, there is an increase in the degree of substrate inhibition for AChE, the maximum rate, and the Michaelis Menten constant. On the other hand, when exposed to situations of hyperthermia, such as 42-48°C, 60% of AChE is inactivated (34). Regarding thermodynamics, AChE was increased in CWI, although not in the same magnitude as the exercise situations (SEP and SEP+CWI groups), as a result of exercise-induced hyperthermia. A possible explanation is that the cold promotes isometric contractions as an adaption strategy in the first two applications of CWI, and after that the animals are adapted, therefore these contractions and AChE activity was decreased. Since the increase was easily observed in the groups that were only submitted to CWI, this finding leads us to believe that exhaustive exercise partially inhibits adaptive effects such as isometric

247 contractions in consequence of the hyperthermia caused by exercise. Furthermore, regarding
248 only the SEP and SEP+CWI groups, CWI was capable of decreasing AChE activity 24h after
249 damage, which shows that the muscle damage was likely decreased.

250 Cryotherapy has already shown that it can weaken or delay the infiltration of
251 inflammatory cells (8, 35-38), which is explained by vasoconstriction caused by exposure to
252 the cold. This causes the reduction of cellular permeability in lymphatic and capillary
253 vessels, and consequently reduces fluid propagation into the interstitial space (39). In this
254 study, CK levels increased on all days in the SEP group; however, the same increase was not
255 observed in the SEP+CWI group. This indicates that SEP was able to cause some degree of
256 muscle damage, which is limited by the CWI treatment for every 24 h during 5 days.
257 Furthermore, the difference between treated and non-treated groups also remained in 24–48 h,
258 which is the peak of inflammatory process (40). This result demonstrates that CWI may be an
259 effective intervention after exercise-induced damage even in the most acute phases of
260 inflammation.

261 Mitochondria plays a key role in energy supply, and it operates as an indicator of cell
262 viability (41). MTT reduction depends on oxidoreductase enzyme family activity, such as
263 dehydrogenase enzymes, and it is mainly located in mitochondria (42). In light of this, MTT
264 reduction assay can be used as an indicator of cell viability. In our experiments, CWI
265 improved cell viability through the increase of MTT reduction levels immediately and during
266 the first 24 h. This result proved that CWI treatment may act by preserving skeletal muscle
267 cell structure and improving cell viability even after exhaustive exercise, such as in the
268 present study.

269 The GSH is the most abundant thiol (-SH) present in the intracellular medium (43).
270 The thiol groups are an established redox sensor because of the moiety on the side chain of

the amino acid cysteine, which is particularly sensitive to redox reactions (44). Furthermore, other studies have shown that cryotherapy has positive effects on oxidant/antioxidant balance (45). Herein, a strong correlation between non-protein –SH and DCF-RS levels was found. This correlation was inversely proportional immediately after SEP, although this relation is proportional when exposed to CWI intervention. These results suggest that CWI intervention after exhaustive exercise prevents DCF-RS production by limiting oxidation of the non-protein –SH groups, which promotes redox balance. In the same way, non-protein –SH levels are inversely correlated with CK levels immediately and 24 h after exhaustion, suggesting that lower antioxidant levels might lead to damage.

Conclusions

Here we aimed to fill some gaps and limitations of previous research using muscle damage and cryotherapy. One of the points was the protocol of exercise-induced muscle damage that was planned to be more reliable with sportive situations that leads to exhaustive effort like professional sports matches. Another point was to monitor biochemical changes on time, and not only in the first 24-48h or only with one CWI session, as the majorities of studies performs. To subdivide groups in four different times of analysis, we end up getting a small sample size, despite this, our experiments was successful in report biochemical changes caused by our protocol of CWI intervention associated with SEP. We can conclude that CWI is an effective option of treatment after exercise-induced muscle damage and able to modulate anti-inflammatory response, decrease of RS production, increase cell viability and promote redox balance, which produces an improvement in the recovery process when compared with passive recovery.

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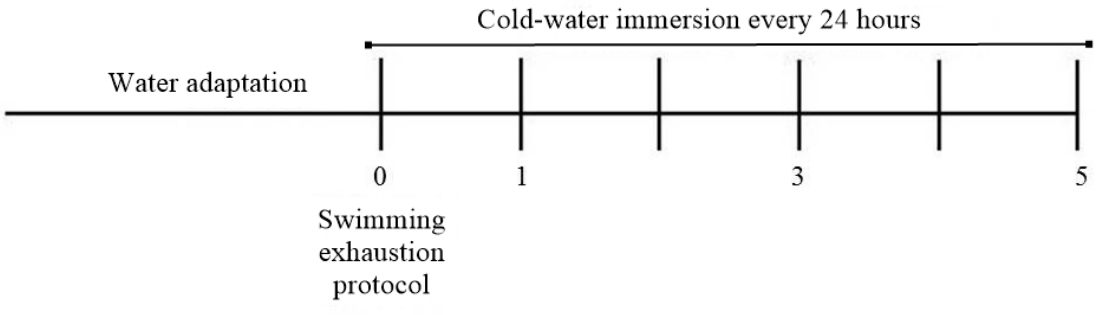
423 Fig 1.



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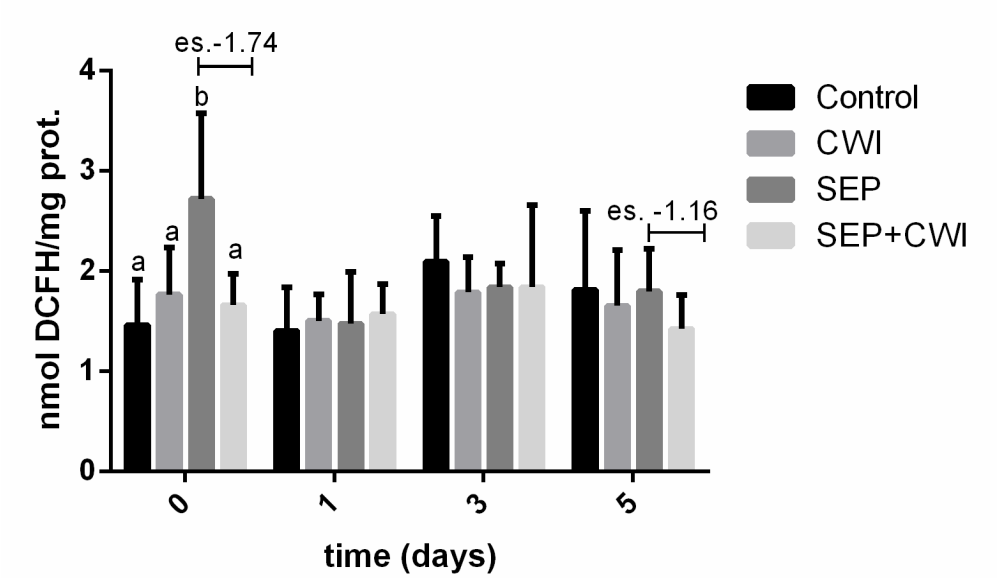

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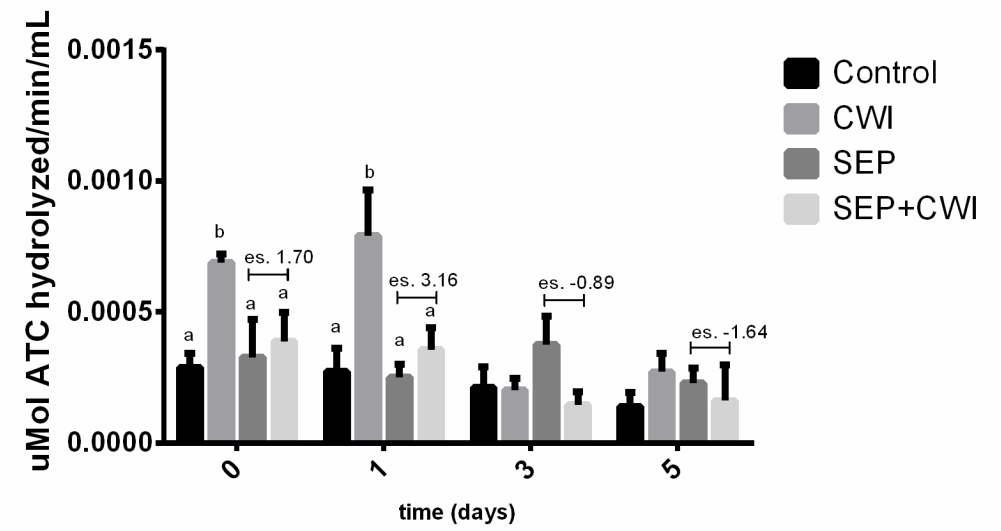

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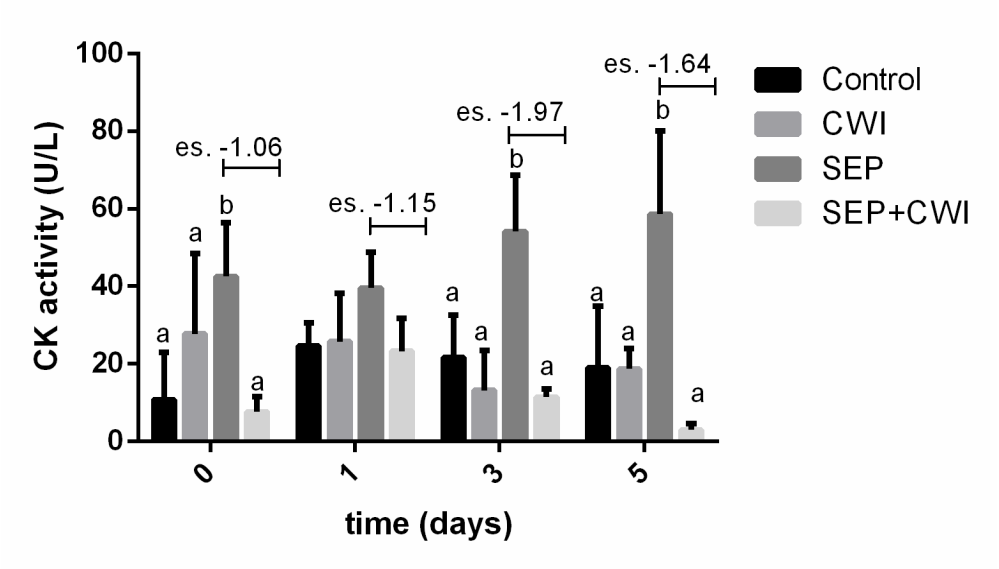

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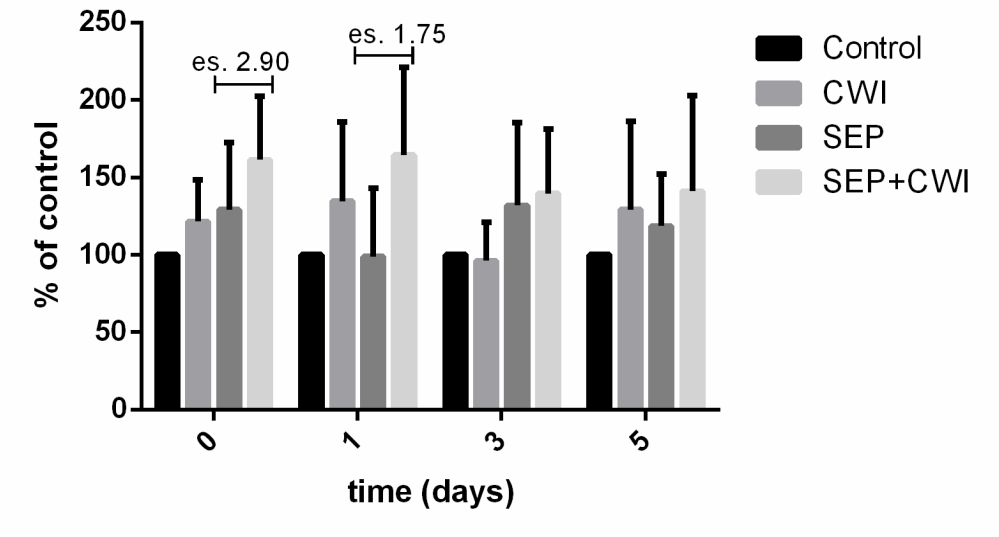

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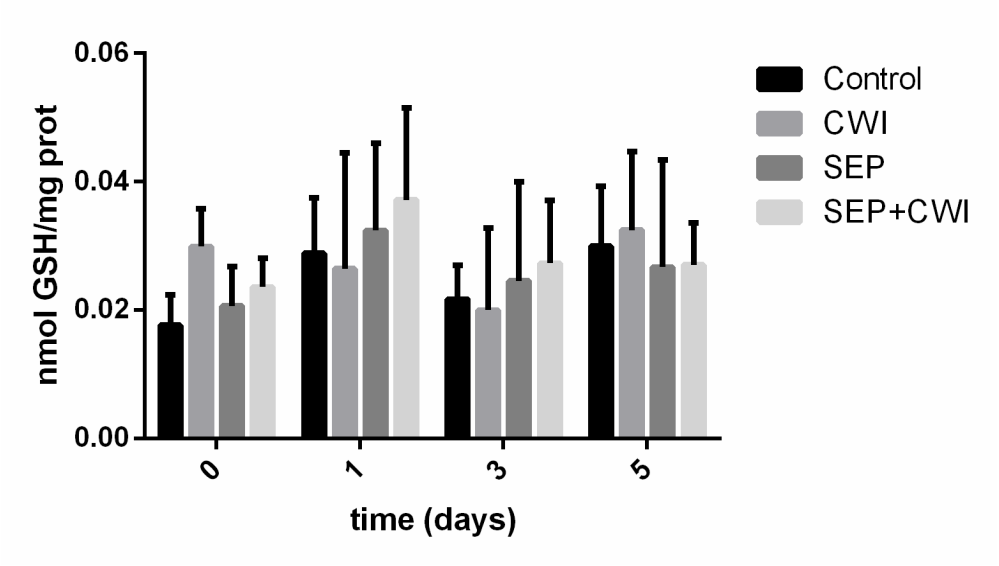
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time (days)
Control
CWI
SEP
SEP+CWI

434

3. CONCLUSÃO

Ao planejar este estudo, objetivou-se preencher lacunas deixadas por pesquisas previamente realizadas. O protocolo de exaustão por nado foi planejado para se assimilar com situações esportivas, nas quais ocorre dano muscular causado por fadiga. O uso da crioterapia e, mais especificamente, da imersão em água gelada, é amplamente utilizado imediatamente após a prática de esportes, como forma de acelerar o processo de recuperação. A maioria dos estudos que utilizam a imersão em água gelada como alternativa de tratamento avaliam, no máximo, 48h após a indução da lesão. Este estudo foi o primeiro a monitorar as alterações bioquímicas causadas pela imersão nos cinco dias posteriores a indução do dano muscular, desde as alterações mais agudas, causadas imediatamente após o dano até 120h pós-lesão.

Com os dados apresentados, pode-se concluir que a imersão em água gelada é uma alternativa de tratamento eficiente após dano muscular induzido por exercício, pois é capaz de modular a resposta anti-inflamatória, diminuir a produção de espécies reativas, aumentar a viabilidade celular e reestabelecer o equilíbrio redox, o que torna o processo de recuperação mais rápido quando comparado à recuperação passiva. Esta comprovada redução do dano muscular causada pela imersão em água gelada é relevante e pode servir de embasamento tanto para futuras pesquisas, como para a prática clínica baseada em evidências.

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